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Functional determination of calcium-binding sites required for the activation of inositol 1,4,5-trisphosphate receptors

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Inositol 1,4,5-trisphosphate receptors (IP₃Rs) initiate a diverse array of physiological responses by carefully orchestrating intracellular calcium (Ca²⁺) signals in response to various external cues. Notably, IP₃R channel activity is determined by several obligatory factors, including IP₃, Ca²⁺, and ATP. The critical basic amino acid residues in the N-terminal IP₃-binding core (IBC) region that facilitate IP₃ binding are well characterized. In contrast, the residues conferring regulation by Ca²⁺ have yet to be ascertained. Using comparative structural analysis of Ca²⁺-binding sites identified in two main families of intracellular Ca²⁺-release channels, ryanodine receptors (RyRs) and IP₃Rs, we identified putative acidic residues coordinating Ca²⁺ in the cytosolic calcium sensor region in IP₃Rs. We determined the consequences of substituting putative Ca²⁺ binding, acidic residues in IP₃R family members. We show that the agonist-induced Ca²⁺ release, single-channel open probability (P₀), and Ca²⁺ sensitivities are markedly altered when the negative charge on the conserved acidic side chain residues is neutralized. Remarkably, neutralizing the negatively charged side chain on two of the residues individually in the putative Ca²⁺-binding pocket shifted the Ca²⁺ required to activate IP₃R to higher concentrations, indicating that these residues likely are a component of the Ca²⁺ activation site in IP₃R. Taken together, our findings indicate that Ca²⁺ binding to a well-conserved activation site is a common underlying mechanism resulting in increased channel activity shared by IP₃Rs and RyRs.

inositol 1,4,5 triphosphate receptor | calcium signaling | ion channel regulation

An increase in the intracellular calcium concentration ([Ca²⁺]_i) is essential for innumerable fundamental biological processes. Following stimulation, the concerted action of a diverse array of proteins, collectively termed the Ca²⁺ signaling “toolkit,” functions to increase and sequester Ca²⁺ to precisely define the spatiotemporal characteristics of Ca²⁺ signals necessary to control diverse physiological endpoints with specificity and fidelity (1, 2). As a function of exquisite regulation of their activity by a multitude of diverse modulatory inputs, the inositol 1,4,5-trisphosphate receptor family (IP₃R) of Ca²⁺ release channels are a central component of this cellular machinery. In vertebrates, there are three genes that code for IP₃R, *ITPR1–3*, which result in three distinct IP₃R subtypes: IP₃R type 1 (IP₃R1), IP₃R type 2 (IP₃R2), and IP₃R type 3 (IP₃R3), which share approximately 60 to 70% amino acid sequence identity (3–7). IP₃Rs are ubiquitously expressed and predominantly localized to the endoplasmic reticulum (ER) membrane, where functionally they are assembled as either homo- or heterotetramers. While binding of IP₃, generated as a result of the activation of phospholipase C is necessary to gate the channel, it is also clear that the binding of Ca²⁺ itself is obligatory to activate the receptor (8). Thus, IP₃ and Ca²⁺ are considered coagonists of IP₃R (9).

Early work using truncated IP₃R1 established that the N-terminal 788 amino acid residues were required to bind IP₃. Subsequently, mutagenesis approaches established the minimal IP₃-binding core (IBC, residues 224 to 557) and identified the positively charged residues required for coordinating the phosphate moieties in IP₃ (10). More recent X-ray crystallography of the isolated IBC domains (11, 12) and single-particle cryoelectron microscopy (cryo-EM) studies (13–16) have revealed the conserved three-dimensional architecture of the ligand-binding domains (LBDs) that includes β-trefoil domains (β-TF1) (residues 1 to 225) and βTF2 (residues 226 to 435), and a helical armadillo repeat, ARM1 domain (residues 436 to 665), providing structural determinants for the coordination of IP₃ and adenophostin A, a structural mimetic of IP₃, in the IP₃-binding pocket. These studies showed that ligand binding is accompanied by conformational changes in the LBDs (11, 12, 14, 15) and also involves global conformational changes in the cytoplasmic scaffold connected to the channel pore (14, 15). Critically, the functional relevance of these residues has also been confirmed by mutagenesis (17). Indeed, one of the residues (R269) important for IP₃ binding is the locus

Significance

The inositol 1,4,5-trisphosphate receptor (IP₃R) Ca²⁺ release channel is a key component of the Ca²⁺ signaling cellular machinery. Regulation of IP₃R activity is fundamental for defining the spatiotemporal characteristics of Ca²⁺ signals important for the appropriate activation of effectors. The most important regulator of IP₃R activity is an increase in [Ca²⁺] itself, which initially activates and then, at higher concentrations, inhibits channel activity. While the residues in IP₃R important for IP₃ binding are established, the motifs that coordinate Ca²⁺ have not been functionally determined. Based on predictions from recently published cryo-EM structures of IP₃Rs, we used imaging techniques and electrophysiology to experimentally define the identity of the evolutionarily well-conserved residues in IP₃Rs essential for Ca²⁺ binding and channel activation.

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The authors declare no competing interest.

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of a point mutation (R269W) that results in spino-cerebellar ataxia in patients as a result of a poorly functional IP₃R1 (18). Furthermore, the mutagenesis of these residues, in increasing numbers of IP₃R monomers within a concatenated tetramer, has led to the demonstration that IP₃ binding to all four subunits in an IP₃R tetramer is an absolute prerequisite for channel opening (19).

Extensive functional characterization at the level of Ca²⁺ release and at the single-channel level have demonstrated that Ca²⁺ modulates IP₃R channel opening in a complex manner (8, 20–25). In the presence of IP₃, low concentrations of Ca²⁺ (<300 nM) facilitate channel opening; however, at higher concentrations, Ca²⁺ promotes channel closure to result in a bell-shaped regulation of IP₃R activity (8). These data are consistent with Ca²⁺ interacting at two distinct sites, one that results in activation and a further site that attenuates activity (26). Models of this regulation have postulated that IP₃ binding modulates the affinity of the Ca²⁺-binding sites for Ca²⁺ to favor increased or decreased IP₃R channel activity (24, 27–30). This control of IP₃R activity by the interplay between coagonists is widely considered to mechanistically underlie the hierarchy of Ca²⁺ signaling events observed in cells, including localized Ca²⁺ blips, Ca²⁺ puffs, propagating Ca²⁺ waves, and Ca²⁺ oscillations (21, 31–33).

While the determinants of IP₃ binding are firmly established (10), elucidating the motifs that coordinate Ca²⁺ in IP₃R have proved elusive and have yet to be functionally determined (34). Notably, using gel overlay assays, several linear fragments of IP₃R across various domains were shown to bind ⁴⁵Ca²⁺ (35, 36). Nevertheless, point mutations at conserved acidic amino acids in regions identified in the IBC were without any effect on Ca²⁺-dependent activation of IP₃R, ruling out the possibility of Ca²⁺-binding site(s) in this domain (37). A further series of studies proposed glutamate (E) 2101 (Fig. 1*B*) as a key residue in IP₃R for activation by Ca²⁺. Mutating this residue diminished Ca²⁺ sensitivity for activation of IP₃R1 several-fold, but it also altered the IP₃R sensitivity to inhibition by Ca²⁺ (38, 39). Nevertheless, there is little structural evidence supporting direct binding of this residue to Ca²⁺, indicating that this site perhaps has an allosteric role in stabilizing a conformation favoring Ca²⁺ binding to bona fide Ca²⁺-binding site(s) (21, 27).

Perhaps the best clues to potential Ca²⁺-binding sites in IP₃R come from structural studies of the ryanodine receptor (RyR) (38–40). IP₃Rs share structural and functional features with RyRs; both are intracellular Ca²⁺ release channels with tetrameric architecture and are regulated in a biphasic manner by Ca²⁺ (8). The cryo-EM structure of RyR determined in the presence and absence of its activating ligands (Ca²⁺/adenosine triphosphate [ATP]/caffeine) revealed several regions that coordinate Ca²⁺ ions in RyRs. These include paired EF-hand domains in the central region of RyR and a pocket of negatively charged residues in the core solenoid and C-terminal domain (40). While mutation of the EF hands did not alter Ca²⁺ activation of RyR2, mutation of the putative binding pocket in RyR1/2 altered Ca²⁺-dependent activation of RyRs (41, 42). Of note, an analogous Ca²⁺-binding site is present in the juxta membrane domain of IP₃R between the third armadillo repeat (ARM3) and linker (LNK) domains and is conserved in IP₃Rs across species and through IP₃R subtypes (43). Two cryo-EM studies of human IP₃R3 in the presence of IP₃ and Ca²⁺ have suggested two Ca²⁺-binding sites, including a site within the putative Ca²⁺ sensor regions located at the

interface between the ARM3 (residues 1,587 to 2,119) and LNK (residues 2,538 to 2,608) domains (15, 44). The first structure (15, 44) was solved in supra-physiological [Ca²⁺], but the limited resolution of the reconstruction precludes the identification of any structural determinants within the Ca²⁺-binding pockets. Moreover, the channel pore was not engaged under the aforementioned conditions; therefore, it is unclear whether either site relates to stimulation or inhibition of the channel. A second recent human IP₃R3 (hIP₃R3) structure solved at 3.6 Å resolution under more physiological [Ca²⁺] reported a Ca²⁺ density in the putative Ca²⁺ sensor region in two structures extracted from the same cryo-EM dataset (44). In one of their reported structures, the ion conduction pore was dilated to allow the passage of Ca²⁺. However, the functional role of this Ca²⁺ binding site remains to be elucidated.

In this study, based on our single-particle cryo-EM structure of IP₃R1 determined at ~3.26 Å resolution (45), in which a clear interaction between Ca²⁺ and the putative Ca²⁺ sensor region is documented, we performed a series of experiments to examine the consequences of substituting putative Ca²⁺ binding-conserved residues in IP₃R subtypes. We generated stable cell lines expressing wild-type (WT) IP₃Rs and compared agonist-evoked Ca²⁺ release, Ca²⁺ puffs, and single-channel properties with their counterparts harboring substitutions at putative Ca²⁺ coordinating sites. Neutralizing the negative charge on side chain residues at these conserved Ca²⁺ coordinating residues decimated both Ca²⁺ release and Ca²⁺ puff activity evoked by IP₃-generating agonists. Remarkably, substitution with a smaller but similarly charged residue markedly diminished agonist-induced Ca²⁺ release and Ca²⁺ puffs compared to their WT counterparts. Of note, comparison of single-channel properties revealed that the Ca²⁺ sensitivity was dramatically right shifted by charge neutralization, indicating that this site is likely the Ca²⁺ activation site. In contrast, retaining charge on the side chain residue had little effect on Ca²⁺ sensitivity; however, open probability of channel was significantly diminished. Overall, our investigations demonstrate a critical role for the negative charge on side chains of conserved Ca²⁺ coordinating residues in IP₃Rs for electrostatic interactions with Ca²⁺ to facilitate channel opening and subsequent activation of agonist-induced Ca²⁺ release.

Results

Identification of Putative Ca²⁺ Coordinating Residues in IP₃Rs.

Our high-resolution cryo-EM structures of rat IP₃R1 (rIP₃R1) in physiologically relevant Ca²⁺-bound states in the presence or absence of IP₃ and ATP clearly identifies Ca²⁺ bound in the putative Ca²⁺ sensor site formed by E1978, E2042 (in the ARM3 domain, chartreuse), and T2657 residue (in the LNK domain, orange), corresponding to E1938, E2002, and T2614 in the human sequence (Fig. 1*A*), respectively (45). A comparative structural alignment strongly indicates that a putative Ca²⁺ sensor site in rIP₃R1 is analogous to the Ca²⁺-binding pockets in IP₃R3 (tan, PDB ID: 6DR2) and RyR1 (gray, PDB ID: 5T15) (Fig. 1*B*). Moreover, the three aforementioned residues constituting the putative Ca²⁺ sensor site in human IP₃R1 (hIP₃R1) are well conserved across all hIP₃R (15) and RyR subtypes (40, 41, 43) (Fig. 1*C*). Strikingly, these residues are also highly conserved in the evolution of IP₃R and RyR proteins across various organisms (*SI Appendix, Fig. S1A*). In contrast, the putative Ca²⁺-binding pocket located between the α-helical domain (HD) and the ARM2 in hIP₃R3 (15) is not conserved between RyRs and IP₃Rs (*SI Appendix, Fig. S1B*). In addition,

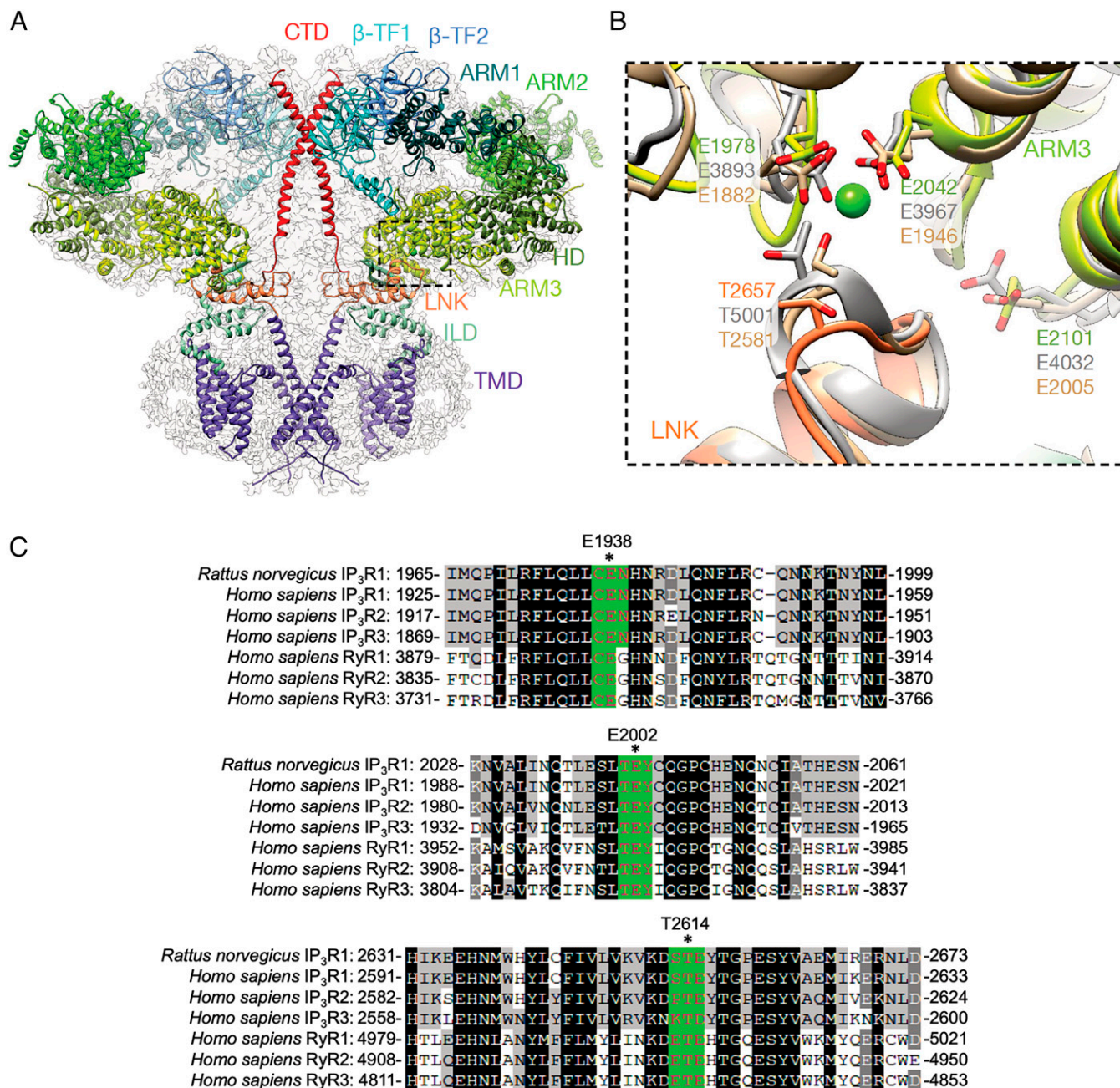


Fig. 1. Structural and sequence conservation of the Ca²⁺-binding site across IP₃R and RyR families of Ca²⁺ release channels. (A) Two opposite subunits in the rat IP₃R1 are color-coded by domains and overlaid with cryo-EM density. (B) Structural alignment of IP₃R1 Ca²⁺-binding sensor domain (indicated with dashed line in (A); PDB ID: 8EAQ; EMDB: EMD-27982 in (45) with IP₃R3 (tan, PDB ID: 6UQK) and RyR1 (gray, PDB ID: 5T15) Ca²⁺-binding pockets with identical residues labeled. The equivalent human residues in IP₃R1 that form the Ca²⁺ binding pocket are hE1938/rE1978, hE2002/rE2042, hT2614/rT2657, and hE2109/rE2101. (C) Sequence alignment for the Ca²⁺-sensor region across all three IP₃R and RyR subtypes. The conserved E1938, E2002, and T2614 residues in IP₃R1 are highlighted in green.

we could not detect any Ca²⁺ densities in this region in our cryo-EM structure of IP₃R1 (45). These observations indicate that conserved residues forming the Ca²⁺-binding site in the two main families of intracellular Ca²⁺-release channels are good candidates to mediate the regulation of IP₃R by Ca²⁺.

Substitutions at the Putative Ca²⁺ Coordinating Residues in hIP₃R1 Diminished Agonist-Induced Ca²⁺ Release. IP₃R1 is the best-studied subtype in terms of regulation by Ca²⁺ (13, 46, 47). Therefore, we stably overexpressed WT hIP₃R1 or hIP₃R1 with substitutions at the 2002 glutamic acid (E) residue (Fig. 1C) to aspartate (D), alanine (A), or glutamine (Q) in HEK-3KO cells previously generated in our laboratory by CRISPR-Cas9

technology to lack native IP₃R (19). The rationale for the substitutions chosen being that the E-D residue change retained the side chain charge; the E-Q substitution resulted in a similar-size side chain that does not preserve the side chain charge but is still hydrophilic; and the E-A substitution maintains the β carbon moiety but has no other side chain chemistry.

When expressed in HEK-3KO cells, IP₃R1 protein levels of exogenously (exo) expressed WT hIP₃R1, hIP₃R1 E2002D, hIP₃R1 E2002A, and hIP₃R1 E2002Q stable cell lines were higher than WT HEK293 and endogenous (endo) hIP₃R1 cells previously generated in our laboratory by CRISPR-Cas9 technology to lack the other two native IP₃R (19) (Fig. 2 A and B). When stably expressed, all of the mutants were properly targeted

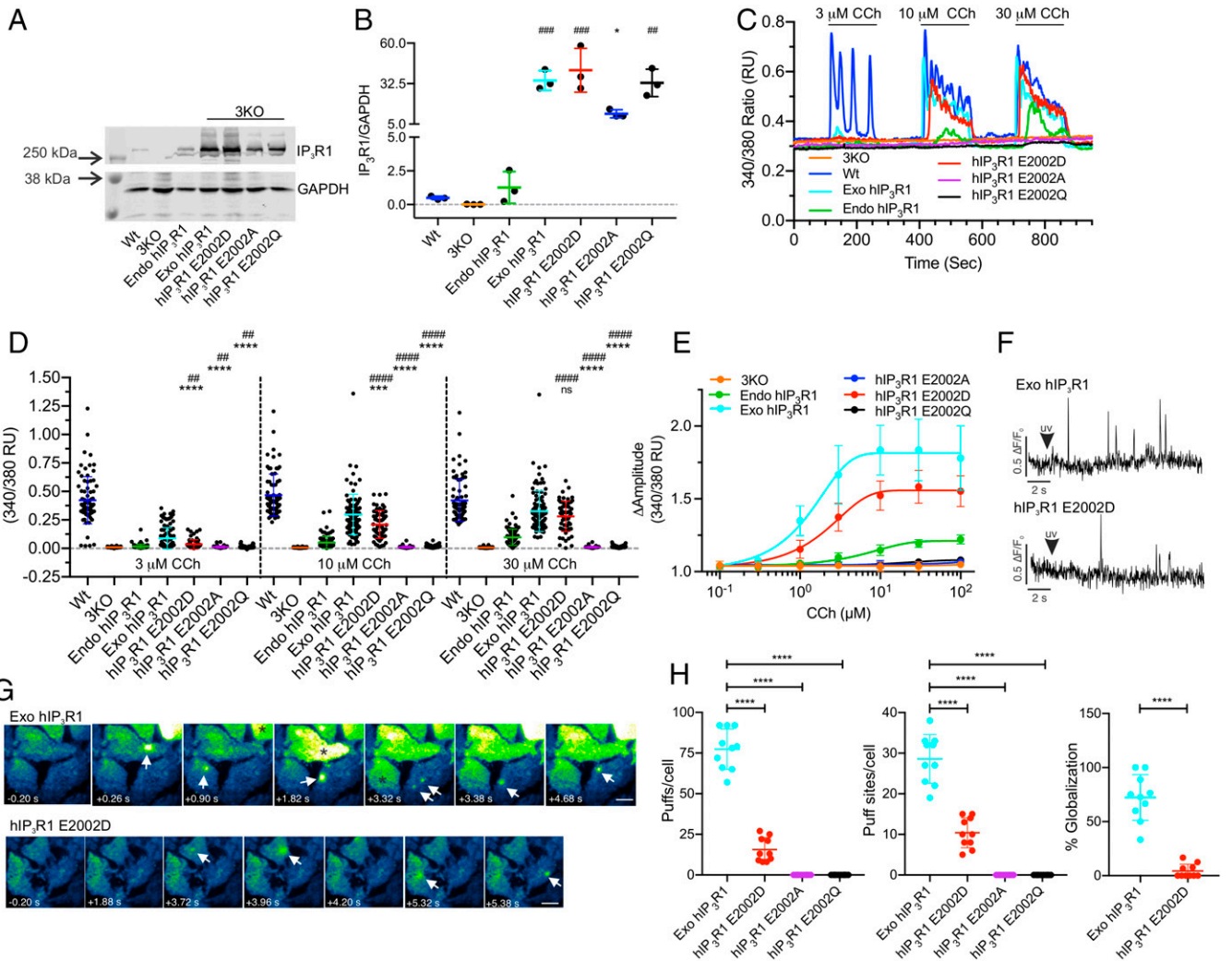


Fig. 2. Substituting E2002 with D, A, or Q residue in hIP₃R1 significantly diminished agonist-induced Ca²⁺ signals when stably expressed in HEK-3KO cells. Stable hIP₃R1 cell lines with a substitution at E2002 position to D, A, or Q were generated in HEK-3KO cells. (A) A representative western blot depicting IP₃R1 and GAPDH protein levels in WT HEK293 (Wt), HEK-3KO (3KO), HEK-2KO (lacking native IP₃R2 and IP₃R3 subtypes, endo hIP₃R1), 3KO cells overexpressing WT IP₃R1 (exo hIP₃R1), and E2002D/A/Q substitution (hr1 E2002D/A/Q). (B) Scatterplot depicting quantification of IP₃R1 protein level normalized to GAPDH from three independent western blots. Data are means ± SDs of three (*n* = 3) independent experiments. ###*P* < 0.01, ####*P* < 0.001 when compared to endo hIP₃R1 cell line and **P* < 0.05 when compared to exo hIP₃R1 cell line; one-way ANOVA with Tukey's test was performed. (C) Representative traces showing CCh-induced (3, 10, and 30 μM) Ca²⁺ release from 3KO (orange), WT (blue), endo hIP₃R1 (green), exo hIP₃R1 (cyan), hIP₃R1 E2002D (red), hIP₃R1 E2002A (purple), and hIP₃R1 E2002Q (black) cells in single-cell assays. (D) Scatterplot summarizing change in amplitude (peak ratio - basal ratio: average of 20 ratio points immediately preceding addition) to increasing doses of CCh (3, 10, and 30 μM) for experiments similar to those shown in (C). Data are means ± SDs of at least three independent experiments. ###*P* < 0.01, ####*P* < 0.0001 when compared to endo hIP₃R1 cell line and ****P* < 0.001, *****P* < 0.0001 when compared to exo hIP₃R1 cell line; Mann-Whitney *U* test was performed. ns: not significant. (E) Dose-response curve showing maximum amplitude from Fura-2/AM loaded 3KO (orange), endo hIP₃R1 (green), exo hIP₃R1 (cyan), hIP₃R1 E2002D (red), hIP₃R1 E2002A (purple), and hIP₃R1 E2002Q (black) cells in population-based assays when treated with increasing concentrations (100 nM, 300 nM, 1 μM, 3 μM, 10 μM, 30 μM, and 100 μM) of CCh. (F) Representative traces of Cal-520 fluorescence ratios (ΔF/F₀) from the center of a single puff site (~1 × 1 μm) evoked by photolysis of ci-IP₃ at 2 s (indicated by arrow) in exo hIP₃R1 (upper trace) and hIP₃R1 E2002D (lower trace) cells using a TIRF microscope. (G) TIRFM images from exo hIP₃R1 (Upper panel) and hIP₃R1 E2002D (Lower panel) showing Ca²⁺ puffs at indicated time point. Typical of at least 10 independent experiments. Arrows indicate Ca²⁺ puffs/puff sites, and asterisks indicate globalization of Ca²⁺ signals. (H) Scatterplots summarizing number of puffs/cell, puff sites/cell, and % globalization in exo hIP₃R1 cells (cyan), hIP₃R1 E2002D (red), hIP₃R1 E2002A (purple), and hIP₃R1 E2002Q (black) cells (*n* = 10 cells). Data are means ± SDs of 10 (*n* = 10) independent experiments. *****P* < 0.0001 when compared to exo hIP₃R1 cell line; Mann-Whitney *U* test was performed. Unless otherwise stated, all of the data above comes from at least *n* = 3 experiments.

to the ER, exhibiting a characteristic reticular expression pattern (SI Appendix, Fig. S2A). In addition, each mutant receptor ran exclusively as a tetramer on native nondenaturing gels (SI Appendix, Fig. S2B). To investigate the functional consequences of these substitutions on hIP₃R1 channel activity, we performed single-cell imaging assays to assess IP₃-induced Ca²⁺ release using carbachol (CCh) and trypsin as agonists acting via the G_αq-coupled M3 muscarinic receptor and protease-activated receptor 2, respectively. CCh-induced Ca²⁺ release was markedly diminished in the hIP₃R1 E2002D cells when compared to exo

hIP₃R1 cells at lower doses of CCh (3, 10 μM); however, such differences disappeared at a higher dose of CCh (30 μM), presumably due to the recruitment of additional IP₃Rs caused by Ca²⁺-induced Ca²⁺ release at higher concentrations of CCh (Fig. 2 C and D). CCh-induced Ca²⁺ release was significantly attenuated in hIP₃R1 E2002A and hIP₃R1 E2002Q cells as compared to exo and endo hIP₃R1 cells at all doses of CCh (3, 10, and 30 μM) (Fig. 2 C and D). Moreover, the percentage of responding cells was also significantly lower in stable cells with substitutions at the E2002 residue compared to WT cells

(SI Appendix, Fig. S3A). Similarly, trypsin-evoked Ca^{2+} release was also significantly attenuated in cells with substitutions at the E2002 site when compared to both endo and exo hIP₃R1 cells (SI Appendix, Fig. S3 C and D). The HEK-3KO cells, as previously demonstrated, failed to respond to stimulation with either trypsin (SI Appendix, Fig. S3 C and D) (18, 19) or CCh (Fig. 2 C–E). Furthermore, in a population-based assay performed on a FlexStation3 plate reader with microfluidics, CCh-induced Ca^{2+} release as reflected by both the maximum amplitude changes (Fig. 2E) and area under the curve (AUC) (SI Appendix, Fig. S3B) were markedly attenuated in E2002D and decimated in E2002A and E2002Q cells when compared to both exo and endo IP₃R1 cells. These results suggest that the negative charge on the side chain residue at the 2002 position in hIP₃R1 is critical for binding to Ca^{2+} , and neutralizing this charge prevented IP₃-induced Ca^{2+} release and IP₃R1 activation.

Next, to measure the activity of the hIP₃R more directly and without the global effects on neighboring IP₃R by increasing Ca^{2+} , we investigated the effect of substitutions at the E2002 site on the fundamental Ca^{2+} signals mediated by IP₃Rs (called Ca^{2+} puffs) upon uncaging caged inositol triphosphate (ci-IP₃) using total internal reflection microscopy (TIRFM) (31, 33, 48–50). As shown in the representative traces (Fig. 2F) and images (Fig. 2G), the number of fundamental Ca^{2+} signals obtained from the hIP₃R1 E2002D cells were significantly diminished when compared to WT exo hIP₃R1 cells following the uncaging of ci-IP₃ (Movies S1 and S2). The number of puffs per cell, puff sites per cell, and the percentage of cells in which Ca^{2+} signals globalized were significantly attenuated in hIP₃R1 E2002D cells as compared to WT hIP₃R1 cells (Fig. 2H). The rise and fall times did not differ between these two cells (SI Appendix, Fig. S3E). We failed to detect any puffs in cells expressing hIP₃R1 with either E2002A or E2002Q, again reinforcing the importance of the negative charge at this residue in the Ca^{2+} activation site (Fig. 2H).

Substituting the Putative Ca^{2+} Coordinating Residue in hIP₃R3 Diminished Agonist-Induced Ca^{2+} Release. Given that this residue is identical in all human IP₃R subtypes, we postulated that if E2002 indeed represented an important charged residue for the coordination of Ca^{2+} and subsequent activity of hIP₃R1, then the functional effect of mutating this residue should be conserved in other hIP₃R subtypes. Therefore, we generated stable cell lines in HEK-3KO with substitution at the analogous E1946 residue in hIP₃R3 (Fig. 1C). The IP₃R3 protein levels of the hIP₃R3 E1946Q stable cell line were slightly more than the WT HEK293 and endogenous hIP₃R3 cells previously generated in our laboratory by CRISPR-Cas9 technology to lack the other two native IP₃Rs (Fig. 3 A and B). When stably expressed, hIP₃R3 E1946Q was properly targeted to the ER, exhibiting a characteristic reticular expression pattern (SI Appendix, Fig. S4A). In addition, the mutant receptor, like hIP₃R3, ran exclusively as a tetramer on native nondenaturing gels (SI Appendix, Fig. S4B). To determine the functional consequence of this substitution, as before, we performed single-cell imaging assays to assess IP₃-induced Ca^{2+} release using CCh and trypsin as agonists. While the exo hIP₃R3, endo hIP₃R3 and WT HEK293 cells responded to agonist stimulation, the hIP₃R3 E1946Q cells, like 3KO cells, did not respond to various doses of CCh (3, 10, and 30 μM) (Fig. 3 C and D). The percentage of responding cells were also significantly lower in stable cells with the E1946Q substitution as compared to WT cells (SI Appendix, Fig. S5A).

To further substantiate these results, we performed single-cell imaging experiments using trypsin (500 nM) as an agonist.

A consistent lack of trypsin-induced Ca^{2+} release in hIP₃R3 E1946Q cells was observed when compared to WT stable cells (SI Appendix, Fig. S5 C and D). Interestingly, both the WT and endo hIP₃R3 cells, which express IP₃R3 protein levels comparable to those of hIP₃R3 E1946Q cells, responded to trypsin (SI Appendix, Fig. S5 C and D) and various doses of CCh (Fig. 3 C and D). Furthermore, we also performed population-based assays to determine CCh-induced Ca^{2+} release in hIP₃R3 E1946Q cells. Similar to single-cell imaging assays, CCh-induced Ca^{2+} release, as represented by the maximum amplitude changes (Fig. 3E) and AUC (SI Appendix, Fig. S5B), was abolished in hIP₃R3 E1946Q cells when compared to both exo and endo hIP₃R3 cells. Overall, these results indicate that the negative charge on the conserved side chain residues in both the hIP₃R1 and hIP₃R3 subtypes is critical for coordinating Ca^{2+} and the activity of hIP₃R subtypes.

Is the negative charge on the conserved side chain residues in IP₃R3 essential for Ca^{2+} binding and activity across species? To address this question, we next generated stable cell lines expressing rat IP₃R3 (rIP₃R3) WT or rIP₃R3 with E1945Q substitution in HEK-3KO cells (SI Appendix, Fig. S6 A and B). We performed single-cell imaging and population-based assays to determine agonist-induced Ca^{2+} release using these stable cells. Remarkably, both the CCh- (SI Appendix, Fig. S6 C–E) and trypsin-induced (SI Appendix, Fig. S6 H and I) Ca^{2+} release was decreased in rIP₃R3 E1945Q cells as compared to rIP₃R3 WT cells. A similar decrease in CCh-induced Ca^{2+} release was observed in population-based assays (SI Appendix, Fig. S6 F and G). To summarize, these observations indicate that the negative charge on the conserved side chain residues in IP₃Rs across multiple organisms is critical for binding to Ca^{2+} and the ensuing agonist-induced Ca^{2+} release.

Loss of Negative Charge in the Putative Ca^{2+} -Binding Pocket Shifts the Ca^{2+} Sensitivity for Activation of hIP₃R1. The previous experiments suggest strongly that negatively charged residues in a putative Ca^{2+} -binding pocket centered around E2002 are necessary for the full activity of hIP₃R, but they do not provide biophysical, mechanistic insight into how Ca^{2+} binding controls IP₃R activity. We therefore generated stable cell lines expressing WT hIP₃R1 or hIP₃R1 with substitutions at the E2002 and E1938 residues in DT40-3KO chicken lymphocytes, IP₃R null cells (SI Appendix, Fig. S7 A and B). Interestingly, consistent with HEK-3KO cells, neutralizing the negative charge on side chain of either of these residues significantly abrogated Ca^{2+} signals evoked by trypsin in population-based assays in DT40 cells (SI Appendix, Fig. S7 C and D).

Next, we performed “on-nucleus” single-channel recordings of IP₃R1 activity after exposure to IP₃ and various [Ca^{2+}]. As described previously, by our laboratory (51–53) and others (24, 54), at an optimum [ATP] (5 mM) and at a saturating [IP₃] (10 μM), the open probability (P_o) of the hIP₃R increased with increasing [Ca^{2+}]; channel activity was barely evident at 10 nM Ca^{2+} and increased to reach a maximum P_o of ~ 0.7 at 200 nM Ca^{2+} . Increasing [IP₃] to 100 μM , failed to augment activity further. At higher [Ca^{2+}], hIP₃R1 activity decreased and was essentially absent at 100 μM Ca^{2+} (Fig. 4 A and G). Similar experiments were then performed in cells expressing hIP₃R1 harboring the conservative charge mutation E2002D. While single-channel activity was reduced in cells expressing this mutation (maximal P_o ~ 0.45 at 200 nM Ca^{2+}), a similar dependency on Ca^{2+} for activation and inhibition compared to WT hIP₃R1 was retained by this mutation. The decrease in overall channel activity was not due to a reduction in the

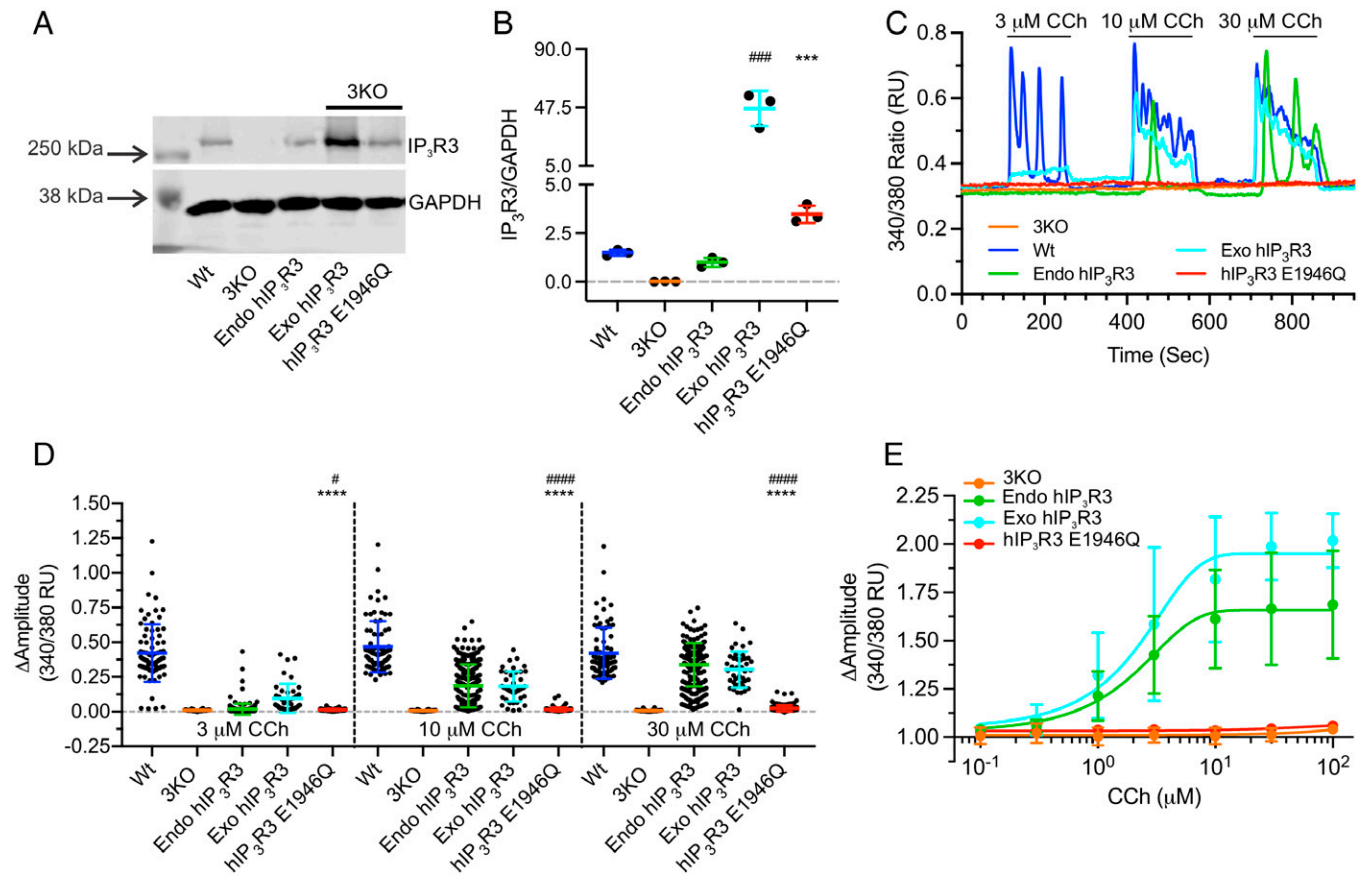


Fig. 3. Substituting E1946 with Q residue in hIP₃R3 significantly diminished agonist-induced Ca²⁺ release when stably expressed in HEK-3KO cells. Stable hIP₃R3 cells with substitution at the E1946 position to Q were generated in HEK-3KO cells. (A) A representative western blot depicting IP₃R3 and GAPDH protein levels in HEK293 (Wt), HEK-3KO (3KO), HEK-2KO (lacking native IP₃R1 and IP₃R2 subtypes), endo hIP₃R3, 3KO cells overexpressing WT hIP₃R3 (exo hIP₃R3), and 3KO cells overexpressing hIP₃R3 with E1946Q substitution (hIP₃R3 E1946Q). (B) Scatterplot depicting quantification of IP₃R3 protein level normalized to GAPDH from three independent western blots. Data are means ± SDs of three (*n* = 3) independent experiments. ###*P* < 0.001 when compared to endo hIP₃R3 cell line and ****P* < 0.001 when compared to exo hIP₃R3 cell line; one-way ANOVA with Tukey's test was performed. (C) Representative traces showing CCh-induced (3, 10, and 30 μM) Ca²⁺ release from 3KO (orange), Wt (blue), endo hIP₃R3 (green), exo hIP₃R3 (cyan), and hIP₃R3 E1946Q (red) cells in single-cell assays. (D) Scatterplot summarizing change in amplitude (peak ratio – basal ratio: average of 20 ratio points immediately preceding addition) to increasing doses of CCh for experiments similar to those shown in (C). WT and 3KO data from Fig. 2D is shown for comparison. Data are means ± SDs of three (*n* = 3) independent experiments. #*P* < 0.05, #####*P* < 0.0001 when compared to endo hIP₃R3 cell line and *****P* < 0.0001 when compared to exo hIP₃R3 cell line; Mann-Whitney *U* test was performed. (E) Dose-response curve showing change in maximum amplitude from Fura-2/AM loaded 3KO (orange), endo hIP₃R3 (green), exo hIP₃R3 (cyan), and hIP₃R3 E1946Q (red) cells in population-based assays when treated with increasing concentrations (100 nM, 300 nM, 1 μM, 3 μM, 10 μM, 30 μM, and 100 μM) of CCh. Unless otherwise stated, all of the data above comes from at least *n* = 3 experiments.

sensitivity to IP₃, as increasing IP₃ to 100 μM had no further effect on hIP₃R1 P_o (Fig. 4 B and G).

Next, we investigated the consequences of substitution of E2002 to alanine. Strikingly, no IP₃R activity was evident at [Ca²⁺] in a range that resulted in enhanced P_o in WT hIP₃R1 (10 to 200 nM Ca²⁺). IP₃R activity, albeit reduced in comparison to the hIP₃R1 retaining negative charge at this residue, was observed at 1 μM, which was further enhanced by increasing [Ca²⁺] to 3 μM and then subsequently reduced as [Ca²⁺] was increased further (Fig. 4 C and G). Again, increasing the [IP₃] to 100 μM failed to increase overall activity at optimum [Ca²⁺] for this mutation. The decreased sensitivity to activation by Ca²⁺ can be readily observed in the pooled data presented in Fig. 4G and insert. Notably, the [Ca²⁺] required for the inhibition of activity was largely unaltered by this substitution. We failed to record any single-channel activity when E2002 was mutated to glutamine. Importantly, the current-voltage relationship for each mutant was essentially identical, indicating that the single-channel conductance of each mutant was unchanged (Fig. 4F).

As previously reported, the IP₃R1 exhibits modal gating; increased activity is associated with increased bursting activity (51).

An increase in Ca²⁺ flux is not associated with a change in open or closed times within the burst, but an increase in the duration of bursts (*SI Appendix, Fig. S8 A–C*). We observed that fundamental gating characteristics were not altered in any of the mutations. An increase in [Ca²⁺] both increased the burst length and concomitantly decreased the interburst interval. These data indicate that an increase in IP₃R1 activity by Ca²⁺ is primarily associated with a destabilization of a long-closed state, while the attenuation of IP₃R1 activity by Ca²⁺ is associated with a transition to and stabilization of this long-closed state (*SI Appendix, Fig. S8 C–G*).

We also generated DT40-3KO cell lines stably expressing similar mutations at E1938 (*SI Appendix, Fig. S7 A and B*), which is predicted to be an additional negatively charged residue essential for coordinating Ca²⁺ in the Ca²⁺-sensing site (Fig. 1). In a fashion similar to that of mutations in E2002, conservation of charge by the substitution of E1938 for D in hIP₃R1 resulted in a decrease in the maximum overall channel activity, with no change in the absolute sensitivity to IP₃, but the relationship for activation and inhibition of channel activity by Ca²⁺ was retained; a maximal P_o was attained at 200 nM Ca²⁺, which subsequently decreased at a [Ca²⁺] > 1 μM (Fig. 4 D and G). IP₃R1 activity in

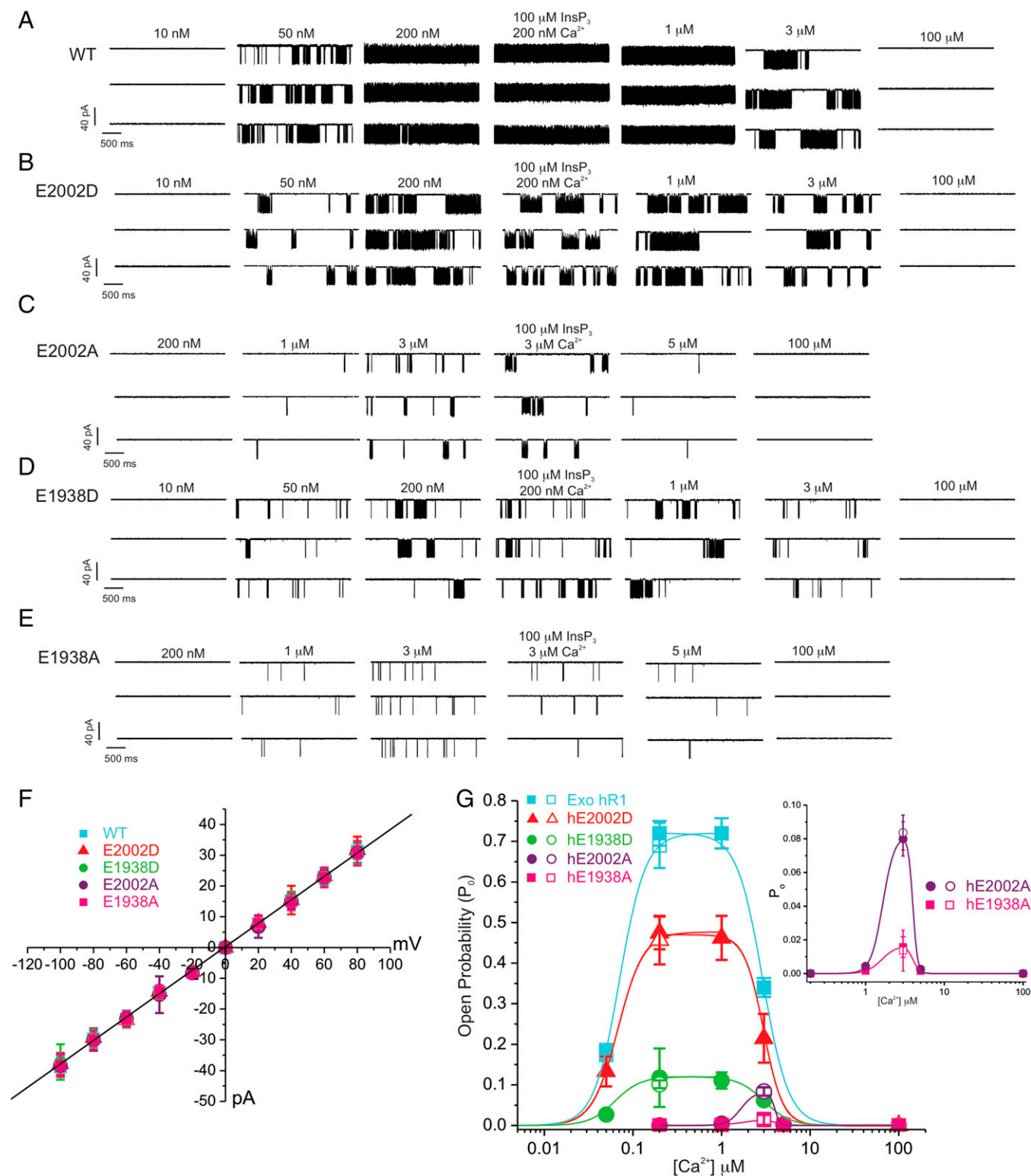


Fig. 4. Importance of negative charges in Ca^{2+} sensor site for regulation of hIP_3R_1 by Ca^{2+} . The activity of WT hIP_3R_1 and mutants was monitored in the “on-nucleus” configuration in DT40-3KO cells stably expressing the indicated constructs as detailed in *Materials and Methods*. (A) Representative sweeps are shown at the indicated $[\text{Ca}^{2+}]$ stimulated by $10 \mu\text{M}$ IP_3 (unless stated otherwise) in the presence of optimal ATP for WT hIP_3R_1 . Maximal activity is observed at $200 \text{ nM}^{-1} \mu\text{M}$ Ca^{2+} and then subsequently decreases. Maximal P_o was not increased by increasing $[\text{IP}_3]$ to $100 \mu\text{M}$. (B) Representative activity is shown for E2002D hIP_3R_1 . The maximal P_o at each $[\text{Ca}^{2+}]$ is reduced compared with WT hIP_3R_1 , but the regulation by increasing Ca^{2+} mirrors that seen with WT hIP_3R_1 . (C) Representative activity is shown for E2002A hIP_3R_1 . Maximal P_o is markedly reduced and the $[\text{Ca}^{2+}]$ where maximum activity is observed is right shifted to higher $[\text{Ca}^{2+}]$. Inhibition of IP_3R activity occurs at similar $[\text{Ca}^{2+}]$ to WT hIP_3R_1 . (D) Representative activity is shown for E1938D hIP_3R_1 . In a fashion similar to that of E2002D, the maximal P_o at each $[\text{Ca}^{2+}]$ is reduced compared with WT hIP_3R_1 , but the regulation by increasing Ca^{2+} mirrors that seen with WT hIP_3R_1 . (E) Representative activity is shown for E1938A hIP_3R_1 . In a fashion similar to that of E2002A, maximal P_o is markedly reduced and the $[\text{Ca}^{2+}]$ where maximum activity is observed is right shifted to higher $[\text{Ca}^{2+}]$. Inhibition of IP_3R activity occurs at similar $[\text{Ca}^{2+}]$ to WT hIP_3R_1 . (F) Current versus voltage relationship for the WT (Exo hR1) and mutant hIP_3R_1 channels generated at 10 mM and optimal $[\text{Ca}^{2+}]$ for each construct. (G) Pooled data. Solid symbols: recordings obtained using $10 \mu\text{M}$ IP_3 at indicated Ca^{2+} concentrations from Exo hR1 (Exo hIP_3R_1 : cyan), hE2002D (red), hE1938D (green), hE2002A (purple), and hE1938A (pink). Open symbols: recordings obtained using $100 \mu\text{M}$ IP_3 at an optimal Ca^{2+} concentration (200 nM). Inset shows pooled data for alanine mutations with an expanded y axis.

cells expressing hIP₃R1 E1938A again displayed a right-shifted, decreased sensitivity to Ca²⁺, similar to E2002A, with maximum activity observed at 3 μM Ca²⁺ before subsequently decreasing (Fig. 4 E and G). Similar to E2002D, the inhibition of activity characteristic of higher [Ca²⁺] was largely unaltered when compared to WT hIP₃R1. In total, these data are consistent with E2002 and E1938 comprising key Ca²⁺-binding residues responsible for the activation phase of the bell-shaped Ca²⁺ relationship underlying IP₃R activity.

Discussion

The regulation of IP₃R induced Ca²⁺ release by Ca²⁺ was recognized early. Initial studies reported that IP₃-induced Ca²⁺ release from the ER was inhibited by Ca²⁺ (55, 56). Subsequently, a series of seminal studies reported that increasing [Ca²⁺] first positively potentiates IP₃R activity and then at higher concentrations reduces channel activity, resulting in the characteristic bell-shaped regulation of IP₃-induced Ca²⁺ release and channel activity, which is thought to be pivotally important for the spatiotemporal control of Ca²⁺ release (8, 20, 22, 24, 57–60). Ongoing studies have since investigated whether this fundamental regulation is directly through the interaction with motifs in the IP₃R protein itself, or through an accessory protein. For example, single-channel experiments in which [Ca²⁺] can be rigorously controlled on both faces of the channel indicate that IP₃R activity appears to be regulated by changes in [Ca²⁺]_i close to the open channel pore, as opposed to the [Ca²⁺] in the ER lumen (29). These data make it improbable that potential Ca²⁺ binding site(s) on the luminal face of IP₃R contribute to activation. Moreover, the failure to detect any interactions between Ca²⁺-binding proteins and IP₃R as a function of changes in the [Ca²⁺] makes it unlikely that Ca²⁺ sensitivity is conferred through an interaction with a binding partner (61). Taken together, these findings strongly indicate that the Ca²⁺ binding site(s) lie within the receptor.

In terms of known Ca²⁺-binding motifs, IP₃R lacks a canonical EF hand, C2 domain, or other conventional Ca²⁺-binding motifs (27); however, Ca²⁺ binds to several linear fragments spread across various domains (35, 36). Of note, rIP₃R1 (residues 1,961 to 2,219) and rIP₃R2 (residues 1,914 to 2,173) coupling domain fragments expressed as recombinant proteins in bacteria are strongly bound to ⁴⁵Ca²⁺ (61). Nevertheless, residues coordinating Ca²⁺ in the IP₃R that are responsible for modulating activity have not been functionally described. Recently, by comparing cryo-EM structures of RyR obtained in the presence and absence of its activating ligands (Ca²⁺/ATP/caffeine), putative amino acid residues forming the Ca²⁺-activating site were identified (40). Moreover, mutational analysis experimentally confirmed that these residues confer Ca²⁺ sensitivity to the RyR channel (41). Cryo-EM structures of RyR resolved in the presence of Ca²⁺ revealed that the pore remained open, consequently permitting the flow of Ca²⁺ ions from the ER to the cytoplasm (40). As noted, the key residues forming the putative Ca²⁺ sensor site in IP₃R1 (45), hIP₃R2 and hIP₃R3, are perfectly conserved and similar to the Ca²⁺-activating site identified in human RyRs (Fig. 1). Moreover, Ca²⁺-binding residues at this activating site in RyR and IP₃R are identical in a number of species representing mammals, fish, and birds and also highly conserved in some lower order organisms (Fig. 1 and *SI Appendix, Fig. S1A*). This conservation, together with our mutagenesis in IP₃R subtypes from two species, makes it highly likely that this Ca²⁺ sensor region represents the Ca²⁺ activation site in at least all mammalian IP₃R and RyR subtypes. Nevertheless, even though these

residues are evolutionarily well conserved, IP₃R in *Capsaspora owczarzewski*, paradoxically, appears not to be regulated by Ca²⁺ in a manner similar to mammalian IP₃R (62) and thus, this may indicate that other structural elements required for allosteric regulation of activity by Ca²⁺ are absent in this species. Interestingly, in *Drosophila melanogaster*, a point mutation (wc703, G2117E; G2123 in variant used in *SI Appendix, Fig. S1A*) in the calcium sensor region of IP₃R caused an increase in the Ca²⁺-binding affinity/sensitivity and open probability of the channel (63, 64). The glutamate residue in this gain-of-function mutation may stabilize Ca²⁺ coordination in the Ca²⁺-activating site, thereby augmenting the Ca²⁺ sensitivity of IP₃R. In the hIP₃R3 structure, the HD/ARM2 putative Ca²⁺ binding site was poorly resolved (15). This site, while conserved in IP₃R subtypes, is not present in RyRs (*SI Appendix, Fig. S1B*), and we could not detect any Ca²⁺ densities at this site in our cryo-EM structures (45). Any role for the site in the regulation of IP₃R activity should be the subject of further investigation.

In the present study, based on striking similarities between RyRs and IP₃R structures at the putative Ca²⁺ sensor site, we generated several stable cell lines in HEK-3KO and DT40-3KO cells (lacking all three native IP₃R) expressing hIP₃R1, hIP₃R3, and rIP₃R3 with substitutions at conserved amino acid residues forming the Ca²⁺ sensor site (Figs. 2 and 3 and *SI Appendix, Figs. S6 and S7*). We then interrogated the channel properties of these mutants when compared to their WT counterparts. A common concern associated with taking a mutagenesis approach to studying structure-function relationships is that mutations may grossly alter the structure of the protein in a manner unrelated to the targeted disruption. While this cannot be unequivocally ruled out without a high-resolution structure of each mutant, the observation that the mutants formed tetrameric structures that were properly localized to the ER argues against a major disruption in the protein structure (*SI Appendix, Figs. S2 and S4*). This idea is further reinforced by data demonstrating that the single-channel conductance (Fig. 4F) and gating characteristics of the mutants (*SI Appendix, Fig. S8*), indicative of the structure of the pore and the ability of conformational changes in the protein to open the pore, were identical to WT IP₃R. Agonist-induced Ca²⁺ release was significantly reduced when the glutamic acid residue at position 2002 in hIP₃R1 was substituted for the native aspartate residue (Fig. 2 and *SI Appendix, Fig. S3*) at this position, indicating that disruption of the structure of the putative binding pocket following reduction in the size of the residue, without altering charge, disrupted Ca²⁺ regulation of IP₃R activity. Furthermore, agonist-induced Ca²⁺ release was completely abrogated upon neutralizing the negative charge on the glutamic acid side chain residue at the 2002 position by substitution with either glutamine or alanine (Fig. 2 and *SI Appendix, Fig. S3*), indicating that the negative charge of this residue is pivotal for activity. Similar data were obtained in DT40 cells upon neutralizing negative charge on side chain residue at either E2002 or E1938 residue in hIP₃R1 (*SI Appendix, Fig. S7*).

Recent technological advances, using high-speed TIRFM, allow near-electrophysiological measurement of the biophysical characteristics of fundamental Ca²⁺ signals arising from a cluster of IP₃R called Ca²⁺ puffs (31, 48, 65–68). Consistent with the effect on global Ca²⁺ signals, Ca²⁺ puffs were also significantly diminished in E2002D cells or completely abrogated in E2002A and E2002Q cells (Fig. 2 F–H). Notably, while diminished in number, Ca²⁺ puffs in cells expressing E2002D retained the kinetics of WT IP₃R1 consistent with this mutant

retaining its bell-shaped modulation by Ca^{2+} (Fig. 4G). Similarly, the substitution of glutamine for glutamic acid at the analogous 1946 position in hIP₃R3 also decimated agonist-induced Ca^{2+} release (Fig. 3 and *SI Appendix*, Fig. S5). A similar result was obtained upon mutating the analogous residue in rIP₃R3 (*SI Appendix*, Fig. S6). We did not consider substitution of multiple Ca^{2+} coordinating residues in a site to avoid excessive perturbations in the native structure of IP₃Rs.

Our single-channel data provide unparalleled mechanistic insight into how the binding of Ca^{2+} to the Ca^{2+} sensor site influences the activity of the IP₃R. Mutation of either E2002 or E1938 by substitution with an aspartate acid residue (Fig. 4B and D) or an alanine residue (Fig. 4C and E) did not alter the single-channel conductance (Fig. 4F) or the modal gating of the channel in bursts (*SI Appendix*, Fig. S8). Notably, however, mutation of these residues with either substitution had marked effects on channel P_{on} , and, most strikingly, the loss of negative charge markedly altered the activation of channel activity by Ca^{2+} without altering the inhibition at higher $[\text{Ca}^{2+}]$ (Fig. 4A–E). This is most clearly appreciated by comparing the pooled activity relationships for WT hIP₃R1 with the charge-conserved aspartic acid substitutions at either site with the mutations to alanine (Fig. 4G). Clearly, while the activity of the aspartic acid (Fig. 4B and D)–substituted mutants are reduced compared to WT hIP₃R1 (Fig. 4A), the activity is regulated with an identical bell-shaped relationship (Fig. 4G). In contrast, the alanine mutants have further reduced maximum activity, but peak activation is achieved at higher $[\text{Ca}^{2+}]$ when compared to WT or glutamic acid–harboring mutants (Fig. 4C and E). The reduction in overall activity of each mutant compared to WT is consistent with disruption of the overall integrity of the Ca^{2+} -binding pocket by alteration in the size and charge of side chains resulting in disordered transduction of conformational changes in Ca^{2+} binding to gating of the pore. Primarily, these data further reinforce that the negative charge on both amino acid residues E2002 and E1938 are critically important for coordinating Ca^{2+} in the Ca^{2+} sensor site, which leads to the activation of IP₃R.

Given that each protomer contributes one Ca^{2+} binding site in the tetrameric IP₃R assembly, a remaining important question relates to the stoichiometry of Ca^{2+} binding necessary for activation. Ca^{2+} activation of IP₃R single-channel activity is generally reported to be modestly positively cooperative (21), which may suggest that binding of more than a single Ca^{2+} activates the channel. We envision that a concatenated receptor approach with increasing numbers of subunits harboring Ca^{2+} binding site mutants will be used to elucidate how many Ca^{2+} are necessary to open the channel (19). In addition, as IP₃R subtypes are differentially modulated by IP₃, ATP, and activation by Ca^{2+} and overall activity is determined by the interplay between these regulatory inputs (21, 25, 52, 53, 69), whether the effects of Ca^{2+} binding are distinct in the context of particular IP₃R subtypes or heterotetrametric assemblies should be determined. Further work is also necessary to elucidate the structural basis of inhibition of IP₃R activity by Ca^{2+} .

Materials and Methods

Plasmid Constructs. Substitutions to amino acids D, A, or Q at the desired site/residue in rIP₃R3, hIP₃R3, and hIP₃R1 were generated using *Pfu* Ultra II Hot-start 2X Master Mix (Agilent Technologies) and appropriate primers obtained from Integrated DNA Technologies (*SI Appendix*, Table S1). A QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies #210518) was used to introduce the desired substitution in cDNAs encoding the rIP₃R3 (NP_037270),

hIP₃R3 (NP_002215.2), and hIP₃R1 (NP_001093422.2) in pDNA3.1 expression plasmid using mutagenic primers. The introduction of desired substitution and coding regions for all of the constructs was confirmed by Sanger sequencing.

Alignment of IP₃R Protein Sequences from Various Organisms. Alignment of IP₃R protein sequences from *Rattus norvegicus*–short IP₃R1 (used in Fig. 1C) (P29994.2), IP₃R1 (NP_001257525.1) (used in *SI Appendix*, Fig. S1), IP₃R2 (NP_112308.1), and IP₃R3 (NP_037270.2); *Homo sapiens*–IP₃R1 (NP_001093422.2), IP₃R2 (NP_002214.2), IP₃R3 (NP_002215.2), RyR1 (NP_000531.2), RyR2 (NP_001026.2), and RyR3 (NP_001027.3); *Capsaspora owczaraki*–IP₃RA (XP_004347577.1); *Caenorhabditis elegans*–IP₃R1 (NP_001023170.1); *Drosophila melanogaster*–IP₃R (NP_730941.1); *Danio rerio*–IP₃R1 (XP_021335554.1); *Gallus gallus*–IP₃R1 (NP_001167530.1); *Mus musculus*–IP₃R1 (NP_034715.3); *Bos taurus*–IP₃R1 (NP_777266.1); *Canis familiaris*–IP₃R1 (XP_005632286.1); *Macaca mulatta*–IP₃R1 (NP_034715.3); and *Pan troglodytes*–IP₃R1 (XP_009443057.1) were generated using GeneDoc.

Cell Culture, Transfection, and Generation of Stable Cell Lines. DT40-3KO, chicken B lymphocyte cells engineered through homologous recombination for the deletion of all of the three-native endogenous IP₃R isoforms (70), were cultured in RPMI 1640 media supplemented with 1% chicken serum, 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin in an incubator set to 39 °C with 5% CO₂. Transfections in DT40-3KO were performed as previously described (71). In brief, 5 million cells were washed with phosphate-buffered saline (PBS) and electroporated with 4 to 6 µg of appropriate plasmid construct using an Amaxa cell nucleofactor (Lonza Laboratories) and nucleofection reagent (362.88 mM ATP-disodium salt, 590.26 mM MgCl₂ 6H₂O, 146.97 mM KH₂PO₄, 23.81 mM NaHCO₃, and 3.7 mM glucose at pH 7.4). Following transfection, the cells were allowed to recover for 24 h and were subsequently transferred into 96-well plates containing media supplemented with 2 mg/mL G418. Next, 10 to 14 d after transfection, clones expressing the desired construct were expanded and subsequently screened by western blotting. Cell lines stably expressing the construct were used in further experiments.

HEK-3KO and HEK293 cells engineered in our laboratory using CRISPR-Cas technology for the deletion of all of the three-native endogenous IP₃R isoforms (19) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin in an incubator set to 37 °C with 5% CO₂. Transfections of the appropriate plasmid construct were performed using a previously described protocol (19). In brief, 1 million cells were washed with PBS and electroporated with 5 to 10 µg appropriate plasmid using Amaxa cell nucleofactor kit T (Lonza Laboratories). Cells were allowed to recover for 48 h, and subsequently subcultured into new 10 cm² plates containing media supplemented with 1.5 to 2 mg/mL G418. Following 7 d of selection, individual colonies of cells were picked and transferred to new 24-well plates containing media supplemented with 1.5 to 2 mg/mL G418. Clonal lines were expanded and those expressing the desired constructs were confirmed by western blotting (18). All of the experiments using HEK cell lines were approved by the University of Rochester Institutional Biosafety Committee (GNT-Yule-15-007 rev0821).

Western Blotting. For western blotting, total protein was isolated from indicated control and stable cell lines using membrane-bound extraction buffer (10 mM Tris-HCl, 10 mM NaCl, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM ethylenediaminetetraacetate, 1 mM NaF, 20 mM Na₄P₂O₇, 2 mM Na₃VO₄, 1% Triton X-100 (vol/vol), 0.5% sodium deoxycholate [wt/vol], and 10% glycerol) supplemented with protease inhibitors (Roche). Briefly, for protein isolation, following the addition of an appropriate amount of lysis buffer, cells were harvested in 1.5-mL tubes and placed on ice for 30 min. To disrupt the pellet, the tubes were vortexed for 10 s every 10 min and returned on ice. Following incubation on ice, the cell lysates were centrifuged at 13,000 rpm at 4 °C for 10 min. The supernatant was transferred to new labeled tubes. Protein concentration in the lysates was estimated using the D_c protein assay kit (Bio-Rad). Equal amounts of lysates were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membranes were incubated with the indicated primary antibodies and appropriate secondary antibodies before imaging with an Odyssey infrared imaging system (LICOR Biosciences). Band intensities were quantified using Image Studio Lite version 5.2 and presented as ratios of IP₃R to glyceraldehyde 3-phosphate dehydrogenase

(GAPDH). The IP₃R1 antibody (#ARC154, Antibody Research Corporation) was used at a 1:1,000 dilution, IP₃R3 antibody (#610313, BD Transduction Laboratories) was used at a 1:1,000 dilution, GAPDH (#AM4300, Invitrogen) was used at a 1:75,000 dilution, and secondary goat anti-rabbit (SA535571, Invitrogen) and secondary goat anti-mouse (SA535521, Invitrogen) antibodies were used at a 1:10,000 dilution (67).

Native Blue PAGE Analysis. HEK-3KO and stable cell lines expressing WT or mutant IP₃R1/3 constructs were lysed in CHAPS lysis buffer supplemented with protease inhibitors (Roche). After 15 min on ice, cell lysates were cleared by centrifugation at 13,000 rpm for 10 min at 4 °C. Next, 15 µg cleared lysates were mixed with 4X sample buffer (BN2003, Invitrogen) and 5% G-250 sample additive (BN2004, Invitrogen). Samples were separated at 4 °C on a 3 to 12% Native PAGE Novex (BN2011BX10, Invitrogen) at 150 V for 1.5 h using dark cathode buffer and then at 250 V for 1 h using light cathode buffer (BN2007, Invitrogen) in the inner chamber with anode buffer in the outer chamber. Separated proteins were transferred onto polyvinylidene difluoride membrane using NuPAGE Transfer buffer (NP0006-1, Invitrogen) overnight at 4 °C. Appropriate primary and secondary antibodies were used to detect IP₃R1/3 complexes, as indicated for western blotting. Molecular weight markers are based on the mobility of unstained protein standard (LC075, Invitrogen).

Immunocytochemistry and Confocal Microscopy. HEK-3KO and HEK-3KO cells stably expressing WT and mutant IP₃R1/3 constructs were plated on poly-D-lysine (100 µg/mL)-coated coverslips. At roughly 50% confluent, cells were fixed using 4% paraformaldehyde at room temperature for 10 min. Subsequently, coverslips were washed with PBS, and cells were blocked in 10% bovine serum albumin (BSA) for 1 h. Following blocking, cells were incubated in primary antibody against IP₃R3 (BD Transduction) and IP₃R1 (#ARC154, Antibody Research Corporation) overnight at 4 °C. The following day, the primary antibody was removed, and coverslips were washed 3 times with PBS for 10 min with gentle rocking. Subsequently, the appropriate secondary antibody conjugated to Alexa Fluor 488 (Invitrogen) was incubated for 1 h at room temperature with gentle rocking. After incubation, coverslips were washed with PBS and mounted on slides. After allowing slides to dry, coverslips were sealed onto slides and imaged using confocal microscopy using an Olympus Fluoview 1000 microscope.

Measurement of Cytosolic Ca²⁺ in Intact Cells. Population-based Ca²⁺ imaging in the indicated cell lines was performed as described previously (18, 67). Briefly, adherent cells were plated in 10 cm² cell culture dishes. Upon attaining 90 to 100% confluency, the cells were loaded with 4 µM Fura-2/AM in cell culture media and incubated at 37 °C in the dark for 1 h. The cells were subsequently washed 3 times with imaging buffer (10 mM HEPES, 1.26 mM Ca²⁺, 137 mM NaCl, 4.7 mM KCl, 5.5 mM glucose, 1 mM Na₂HPO₄, and 0.56 mM MgCl₂, at pH 7.4). An equal number (300,000 cells/well) of cells were seeded into each well of a black-walled 96-well plate. In contrast, suspended cells were cultured in 75 cm² flasks, pelleted and washed once with imaging buffer before counting. An equal number (500,000 cells/well) of cells were incubated with 4 µM Fura-2/AM in imaging buffer at room temperature for 1 h with constant rocking. Subsequently, the cells were pelleted and washed twice before being seeded into wells of a black-walled 96-well plate. The cells were centrifuged at 200 × *g* for 2 min to plate the bottom of the wells and incubated at appropriate temperature for 30 min before imaging. Fura-2/AM imaging was carried out by alternatively exciting the loaded cells between 340 and 380 nm; emission was monitored at 510 nm using FlexStation 3 (Molecular Devices). Data were exported to Microsoft Excel, where the peak response to increasing concentrations of agonist (0.1 to 100 µM CCh or 0.01 to 3 µM trypsin) was determined by calculating the 340/380 ratio and normalizing it to the average of the first 5 data points of the experiment (the basal Ca²⁺ value). AUC was calculated in GraphPad Prism 8 as increases above baseline that are >10% of the distance from the minimum to the maximum *Y* using the 5 data points before agonist addition as the baseline. Curve fitting was performed using a logistic dose-response equation in GraphPad Prism 8. Data were reported as triplicates from at least three individual plates.

Single-cell Ca²⁺ imaging in indicated cell lines was performed as described previously. Briefly, cells were seeded on 15-mm glass coverslips in 12-well plates and left undisturbed overnight. Once attached to the coverslip, the cells were washed with imaging buffer before attachment of the glass coverslip

to a Warner perfusion chamber using vacuum grease. Subsequently, the cells were loaded with 2 µM Fura-2/AM for 25 min in the dark at room temperature. Cells were then perfused with imaging buffer and stimulated with indicated concentrations of CCh or trypsin. Ca²⁺ imaging was performed using an inverted epifluorescence Nikon microscope equipped with a 40× oil immersion objective. Fura-2/AM imaging was carried out by alternatively exciting the loaded cells between 340 nm and 380 nm; emission was monitored at 505 nm. Images were captured every second with an exposure of 20 ms and 4 × 4 binning using a digital camera driven by TILL Photonics software as previously described (18). Image acquisition was performed using TILLvisiON software and data were exported to Microsoft Excel, where data were analyzed for change in peak amplitude and percentage of cells with predefined peak amplitudes. Each experiment was repeated at least three times.

Detection and Analysis of Ca²⁺ Puffs Using TIRFM. Stable hIP₃R1 exogenous WT cells or cells with substitutions at the E2002 site were cultured on 15-mm glass coverslips coated with poly-D-lysine (100 µg/mL) in a 35-mm dish for 36 h. Before imaging, the cells were washed three times with imaging buffer. The cells were subsequently incubated with Cal520-AM (5 µM; AAT Bioquest #21130) and ci-IP₃/PM (1 µM, Tocris #6210) in imaging buffer with 0.01% BSA in the dark at room temperature. After 1-h incubation, the cells were washed 3 times with imaging buffer and incubated in imaging buffer containing EGTA-AM (5 µM, Invitrogen #E1219). After 45 min of incubation, the media was replaced with fresh imaging buffer and incubated for an additional 30 min at room temperature to allow for the de-esterification of loaded reagents.

Following loading, the coverslip was mounted in a chamber and imaged using an Olympus IX81 inverted TIRFM equipped with oil-immersion PLAPO OTIRFM 60× objective lens/1.45 numerical aperture. Olympus CellSens Dimensions 2.3 (Build 189987) software was used for imaging. The cells were illuminated using a 488-nm laser to excite Cal-520 and the emitted fluorescence was collected through a band-pass filter by a Hamamatsu ORCA-Fusion complementary metal oxide semiconductor camera. The angle of the excitation beam was adjusted to achieve TIRF with a penetration depth of ~140 nm. Images were captured from a final field of 86.7 × 86.7 µm (400 × 400 pixels, 1 pixel = 216 nm) at a rate of ~50 frames/second (binning 2 × 2) by directly streaming into random access memory. To photorelease IP₃, ultraviolet (UV) light from a laser was introduced to uniformly illuminate the field of view. Both the intensity of the UV flash and the duration (1 s) for uncaging IP₃ were optimized to prevent spontaneous puffs in the absence of loaded ci-IP₃. Images were exported as .vsi files. Images, 5 s before and 60 s after flash photolysis of ci-IP₃, were captured, as described previously (67).

The .vsi files were converted to .tif files using Fiji and further processed using FLIKA, a Python programming-based tool for image processing (72). From each recording, ~100 frames (~2 s) before photolysis of ci-IP₃ were averaged to obtain a ratio image stack (F/F₀) and standard deviation for each pixel for recording up to 13 s following photolysis. The image stack was Gaussian filtered, and pixels that exceeded a critical value (1.0 for our analysis) were located. The "Detect-puffs" plug-in was used to detect the number of clusters (puff sites), number of events (number of puffs), and the amplitudes and durations of localized Ca²⁺ signals from individual cells. All of the puffs identified automatically by the algorithm were manually confirmed before analysis. The results from FLIKA were saved to Microsoft Excel and graphs were plotted using GraphPad Prism 8 (73).

Preparation of DT40 Cell Nuclei. Isolated DT40 nuclei were prepared using homogenization. The homogenization buffer (HB) contained 250 mM sucrose, 150 mM KCl, 3 mM 2-mercaptoethanol (β-ME), 10 mM Tris, 1 mM phenylmethanesulphonyl fluoride, pH 7.5, with a complete protease inhibitor tablet (Roche). Cells were washed and resuspended in HB before nuclear isolation using an RZR 2021 homogenizer (Heidolph Instruments) with 15 strokes at 1,200 rpm. A 3-µL aliquot of nuclear suspension was placed in a 3-mL bath solution, which contained 140 mM KCl, 10 mM HEPES, 500 µM BAPTA (1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid), and 246 nM free Ca²⁺, pH 7.1. Nuclei were allowed to adhere to a plastic culture dish for 10 min before patching.

On-Nuclei Patch-Clamp Experiments. Single IP₃R channel potassium currents (*i_k*) were measured in the on-nucleus patch-clamp configuration using pCLAMP 9 and an Axopatch 200B amplifier (Molecular Devices). Pipette solution contained 140 mM KCl, 10 mM HEPES, 5 mM ATP, with varying concentrations of IP₃, BAPTA, and free Ca²⁺. Free [Ca²⁺] was calculated using Max Chelator freeware and verified fluorometrically. 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. The data are representative of between three and five experiments for each condition presented. Pipette resistances were typically 20 MΩ and seal resistances were >5 GΩ (51).

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. Only patches with one apparent channel were considered for analyses. Probability of opening (P_o), unitary current (*i_k*), open and closed times, and burst analyses were calculated using Clampfit 9 and Origin 6 software (OriginLab). 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. The threshold for an open event was set at 50% of the maximum open current and events shorter than 0.1 ms were ignored. A “burst” was defined as a period of channel opening following a period of no channel activity, which was >5 times the mean closed time (0.2 ms) within a burst. Ca²⁺ dependency curves were fitted separately for activation and inhibition with the logistic equation:

$$Y = [(A1 - A2)/(1 + (X/X_0)^p)] + A2,$$

where A1 and A2 are asymptotes, X is the concentration of Ca²⁺, X₀ is the half-maximal concentration, and p is the slope related to the Hill coefficient. Equation parameters were estimated using a nonlinear, least-squares algorithm.

Statistical Analysis. All of the statistical tests were conducted in GraphPad Prism 9 and data are presented as the mean ± SD. Statistical significance was determined using one-way ANOVA with Tukey’s test or the Mann-Whitney U test as indicated in the figure legends.

Data, Materials, and Software Availability. All of the study data are included in the article and/or *SI Appendix*.

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