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All three IP₃ receptor isoforms generate Ca²⁺ puffs that display similar characteristics*

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Abstract

Inositol trisphosphate (IP₃) evokes Ca²⁺ release through IP₃ receptors (IP₃Rs) to generate both local Ca²⁺ puffs arising from concerted openings of clustered IP₃Rs and cell-wide Ca²⁺ waves. Imaging Ca²⁺ puffs with single-channel resolution yields information on the localization and properties of native IP₃Rs in intact cells, but interpretation has been complicated because cells express varying proportions of three, structurally and functionally distinct isoforms of IP₃Rs. Here, we used TIRF and light-sheet microscopy to image Ca²⁺ puffs in HEK-293 cell lines generated by CRISPR/Cas9 technology to express exclusively IP₃R types 1, 2 or 3. Photorelease of the IP₃ analog i-IP₃ in all three cell lines evoked puffs with largely similar mean amplitudes, temporal characteristics and spatial extents. Moreover, the single-channel Ca²⁺ flux was similar among isoforms, indicating that clusters of different IP₃R isoforms contain comparable numbers of active channels. Our results show that all three IP₃R isoforms cluster to generate local Ca²⁺ puffs and, contrary to findings of divergent properties from in vitro electrophysiological studies, display similar conductances and gating kinetics in intact cells.

One sentence summary:

The three IP₃R isoforms produce Ca²⁺ puffs with largely indistinguishable features.

Editor's summary:

Different isoforms, similar Ca²⁺ puffs

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Author contributions:

Conception and design of the study by J.T.L. and I.P. Imaging data were collected and analyzed by J.T.L. and I.P. Generation and western blot characterization of KO cell lines were performed by K.J.A. and D.I.Y. The manuscript was written by J.T.L. and I.P., with input from K.J.A. and D.I.Y. All Authors have read and approve the published manuscript.

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Raw data are freely available to academic researchers on request. Custom software ("flika") used for data analysis is available at <http://flika-org.github.io>

Cells express three different isoforms of the inositol trisphosphate receptor (IP₃R), which underlie Ca²⁺ signals ranging from local puffs to global waves. Lock *et al.* used CRISPR/Cas9 gene editing to create HEK293 cell lines that expressed individual IP₃R isoforms. Despite their reported divergent functional properties, each isoform produced Ca²⁺ puffs with similar characteristics. Future work is required to determine how these conserved Ca²⁺ puffs give rise to different global Ca²⁺ signals.

Introduction

Cytosolic Ca²⁺ signals are utilized by all cells of the body to regulate cellular processes as diverse as gene transcription, secretion, mitochondrial energetics, electrical excitability and fertilization; indeed, often more than one process in the same cell (1, 2). The capacity to precisely and specifically regulate cellular events is largely attributable to an exquisite control of the spatial and temporal patterning of cytosolic free [Ca²⁺] transients (2). This control is exemplified by the second messenger pathway mediated by inositol 1,4,5-trisphosphate (IP₃). IP₃ is generated in response to activation of cell surface G-protein coupled receptors, and diffuses in the cytosol to bind to IP₃ receptors (IP₃Rs) in the membrane of the endoplasmic reticulum (ER), causing them to open and release Ca²⁺ ions sequestered in the ER lumen (3). The resulting cytosolic Ca²⁺ signals constitute a hierarchy of events, with increasing amounts of IP₃ progressively evoking Ca²⁺ liberation from individual IP₃Rs (4) (Ca²⁺ blips), local Ca²⁺ signals arising from clusters of several IP₃Rs (4–7) (Ca²⁺ puffs), and global Ca²⁺ waves that propagate through the cell (7–10).

The patterning of IP₃-mediated Ca²⁺ signals is determined both by the functional properties of IP₃Rs and by their spatial arrangement in the ER membrane. Crucially, the opening of IP₃R channels requires binding of cytosolic Ca²⁺ in addition to IP₃, leading to a phenomenon of Ca²⁺-induced Ca²⁺ release (CICR) (11, 12), such that Ca²⁺ diffusing from one open channel may trigger the opening of adjacent channels. The clustered distribution of IP₃Rs further shapes the extent of this regenerative process. CICR may remain restricted to a single cluster containing from a few to a few tens of functional IP₃Rs to produce a puff; or a global Ca²⁺ wave may be generated by successive cycles of CICR and Ca²⁺ diffusion between clusters (7, 9). The transition between these modes depends on factors including IP₃ concentration and the presence of cytoplasmic Ca²⁺ buffers that restrict the diffusion of Ca²⁺ ions (13, 14). Ca²⁺ puffs thus serve both as local signals in their own right, and as the building blocks of global cellular Ca²⁺ signals.

In vertebrates, three different genes encode three main types of IP₃Rs - IP₃R1 (15), IP₃R2 (16) and IP₃R3 (17) - that co-translationally oligomerize to form tetrameric Ca²⁺ release channels. The three isoforms have a similar monomeric molecular mass of ~ 300 kDa, but share only 60-80% amino acid homology (18). Concordant with this diversity, different isoforms are reported to exhibit distinct functional properties. For example, their binding affinities for IP₃ follow a rank order with IP₃R2>IP₃R1>IP₃R3 (19–21), and their differential modulation by cytosolic Ca²⁺ (20, 22–24), ATP (21, 25), binding proteins (26, 27), and posttranslational modifications (28, 29) further shape IP₃R behavior in a subtype-specific manner. Additional complexity arises from splice variants (30–32), and because

most cell types express two or three different isoforms (33–36) that may assemble into heterotetramers (33, 37, 38) with properties that can resemble a blend of their constituents or that are dominated by an individual isoform, depending upon cellular conditions (39, 40). It has thus been proposed that each IP₃R isoform functions as a specific hub to determine different trajectories of cell signaling, and that different cell types express and localize a particular complement of IP₃R isoforms to suit their particular needs (41).

Because of the complex and poorly determined mix of IP₃R isoforms in native cells, most studies of the biochemical and functional properties of specific IP₃R isoforms have utilized experimental systems predominantly or exclusively expressing a single isoform. These approaches include the use of cell types that predominantly express a particular isoform (11, 42, 43); overexpression of recombinant IP₃R isoforms in cell types that express low levels of endogenous IP₃Rs (21, 24, 44), and in DT40-3KO cells where all three IP₃R types have been disrupted (45); and by overexpression in an IP₃R-null human cell line (HEK-3KO) generated by CRISPR/Cas9 technology (46).

Information regarding the functional, single-channel properties of IP₃Rs derived by these approaches has primarily been obtained by electrophysiological recordings in reduced experimental systems - including isolated cell nuclei (43, 47–49) and IP₃Rs reconstituted into lipid bilayers (11, 12, 21, 24) – where the native cellular environment and spatial organization are disrupted. In contrast, Ca²⁺ imaging by total internal reflection fluorescent (TIRF) microscopy can resolve the contributions of Ca²⁺ flux through individual IP₃Rs, providing information on their single-channel properties and their localization in intact cells with minimal perturbation (50). However, to date, all intact-cell imaging studies of local Ca²⁺ signals have been done in native cells expressing their endogenous complement of IP₃Rs. Even though many studies utilized cell types (such as *Xenopus* oocytes (4, 5, 51–53) and SH-SY5Y neuroblastoma cells (50, 54–58) that predominantly express IP₃R1, the actual composition of the IP₃R channels that open to generate Ca²⁺ puffs cannot readily be inferred. For example, only about 3% of all the IP₃Rs in SH-SY5Y cells appear to be involved in generating puffs (59, 60), so those events could arise through a minor population of type 2 or 3 IP₃Rs, while the more numerous type 1 IP₃Rs remain silent.

Here we capitalize on the availability of HEK-KO cell lines in which pairs of IP₃R isoforms were knocked out by CRISPR/Cas9 technology to generate cells natively expressing exclusively type 1, 2, or 3 IP₃Rs (46). Utilizing TIRF Ca²⁺ imaging, we found that all three isoforms independently produced Ca²⁺ puffs in response to UV flash photorelease of the IP₃ analogue i-IP₃. Puffs evoked in cells expressing each isoform remained at fixed, stationary subcellular sites, as do puffs in native, wild-type cells (58). Thus, all three IP₃R isoforms can bind to some, as yet unidentified, cytoskeletal anchoring sites that define the location and composition of functional IP₃R clusters (58, 61). Moreover, we found substantial redundancy in the amplitudes, single-channel Ca²⁺ fluxes and spatial distribution of local signals mediated by the three IP₃R isoforms, although subtle differences in kinetics were apparent that may contribute to shaping the spatiotemporal patterning of cellular Ca²⁺ signals in native cells. A preliminary report of this study has been published in abstract form (62).

Results

IP₃R expression in WT and KO HEK-293 cell lines

To investigate the role of individual IP₃R isoforms in local Ca²⁺ signaling processes in mammalian cells, we utilized HEK-293 wild-type (WT) cells that were engineered by CRISPR/Cas9 technology to establish lines that endogenously express only a single IP₃R type - either IP₃R1, IP₃R2, or IP₃R3 - by knocking out pairs of isoforms from WT cells that regularly express all three (46). As a control, we used a triple-knockout cell line in which no IP₃Rs were expressed (HEK-3KO). Loss of protein expression was confirmed by Western blot using isoform-specific antibodies previously shown to effectively discriminate between IP₃R types (40). All three isoforms were detected by their respective type-specific antibody in WT cells, and none of three antibodies revealed immunoreactivities in the 3KO lane, confirming the complete disruption of the three IP₃R genes (Fig. 1A). In contrast, cells expressing types 1, 2 or 3 IP₃Rs displayed immunoreactivity to only the appropriate isoform-specific antibody and were devoid of signal from the other two antibodies (Fig. 1A). Densitometry measurements indicated the relative abundances of individual IP₃R isoforms in single isoform-expressing cells were not appreciably different from their respective expression in WT cells (Fig. 1A). These findings agree with previous results demonstrating the disruption of specific IP₃R alleles by corresponding sgRNAs, and collectively affirm that these CRISPR/Cas9 modified HEK-293 cell lines natively express single IP₃R isoforms (46).

Basal Ca²⁺ homeostasis in HEK-293 cell lines

We first investigated basal Ca²⁺ homeostasis by imaging whole-cell Ca²⁺ fluorescence signals in the HEK-293 cell lines expressing different IP₃R isoforms, and in WT and 3KO cells. Cells were loaded with cytosolic Ca²⁺ indicators (63) and imaged by wide-field (WF) epifluorescence microscopy at low magnification. Basal cytosolic free [Ca²⁺], as determined by the mean resting fluorescence (F₀) in cells loaded with the high-affinity Ca²⁺ indicator Cal-520 (fig. S1A) and by the mean resting fluorescence excitation ratio (340 /380 nm) in cells loaded with fura-2 (fig. S1B), was similar in the different cell lines. Intracellular Ca²⁺ store content, as assessed by the peak ratiometric (F/F₀) signals of the low affinity Ca²⁺ indicator Fluo-8L in cells treated with ionomycin to permeabilize intracellular organelles, was also similar between the cell lines (fig. S1C). Based on these data, we concluded that the flux of Ca²⁺ through open IP₃Rs would not be appreciably affected by any differences between the cell lines in the concentration gradient driving Ca²⁺ efflux from the ER, or by differences in basal cytosolic [Ca²⁺] that might affect IP₃R gating. Moreover, calculation of Ca²⁺ signals in terms of fluorescence ratio (F/F₀) would be substantially unaffected by any differences in basal fluorescence (F₀) among the cell lines.

Global Ca²⁺ signals evoked by carbachol and photoreleased i-IP₃

Next, we measured global, whole-cell Ca²⁺ signals evoked using bath application of a high concentration of the muscarinic receptor agonist carbachol (CCH) to maximally stimulate the endogenous IP₃ signaling pathway. To exclude signals arising from entry of Ca²⁺ across the plasma membrane, cells were bathed in a solution containing no added Ca²⁺ and EGTA 5 min before and during imaging. Almost all (>98%) of WT and single isoform-expressing

cells showed abrupt Ca^{2+} responses (Fig. 1B-E), whereas triple-knockout (3KO) cells devoid of IP_3Rs showed no discernable responses. The peak responses were greatest in cells expressing type 2 and 3 IP_3Rs , slightly smaller in WT cells, and substantially smaller in cells expressing type 1 IP_3Rs (Fig. 1F). To obtain a surrogate estimate of the relative numbers of active IP_3R /channels contributing to the initial release of ER Ca^{2+} in response to CCH, we measured the maximal rates of rise of fluorescence signals ($\Delta F/F_0$) during the rising phase of global Ca^{2+} signals in WT cells and in cells expressing single IP_3R isoforms. The rates of rise were fastest for cells expressing $\text{IP}_3\text{R2}$, intermediate for WT and $\text{IP}_3\text{R3}$ -expressing cells, and slowest for $\text{IP}_3\text{R1}$ -expressing cells (Fig. 1G).

We then applied flashes of UV light to photorelease a poorly-metabolized IP_3 analog, i- IP_3 , in cells loaded with a caged precursor together with Cal-520 (57, 64). Photoreleased i- IP_3 evoked intracellular Ca^{2+} liberation in WT and all three isoform-expressing cells (Fig. 1H-K), whereas 3KO cells were unresponsive. We attributed these signals to arise exclusively from Ca^{2+} liberation through IP_3Rs , given that recordings were made in Ca^{2+} -free bathing solution and that, in agreement with reports that HEK-293 cells lack ryanodine receptors (65, 66), i- IP_3 -evoked Ca^{2+} signals were not diminished in the presence of ryanodine (fig. S2A).

The mean amplitudes of i- IP_3 -evoked Ca^{2+} signals in WT cells showed a graded dependence on flash duration, with 100 ms flashes evoking little or no response, and flash durations 700 ms evoking apparently maximal signals, with peak $\Delta F/F_0$ of 7.6 (Fig. 1L). Mean responses in cells expressing exclusively $\text{IP}_3\text{R2}$ resembled those of WT cells; with a similar dependence on photolysis flash duration and slightly greater maximal amplitude (9.3 $\Delta F/F_0$) (Fig. 1L). In contrast, cells expressing type 1 (Fig. 1I) or type 3 (Fig. 1K) IP_3Rs were less responsive; respectively, 73% and 76% of cells responded to flash durations of 1000 ms. Moreover, the fluorescence signals mediated by type 1 and 3 IP_3Rs were smaller in amplitude (Fig. 1L), and their rise and fall kinetics were slower than in WT and $\text{IP}_3\text{R2}$ -expressing cells (Fig. 1I,K). The maximum rates of rise of Ca^{2+} signals evoked by 1000 ms photolysis flashes in the different cell lines mirrored their peak response sizes, with rates of rise being much slower in $\text{IP}_3\text{R1}$ - and $\text{IP}_3\text{R3}$ -expressing cells (Fig. 1M).

Ca^{2+} puffs in cell lines expressing single IP_3R isoforms

To better visualize local Ca^{2+} puffs, we additionally loaded cells with the slow Ca^{2+} buffer EGTA to inhibit the formation of Ca^{2+} waves and sharpen the spatiotemporal profile of puffs (14, 57). Imaging was performed by TIRF microscopy at a fast frame rate from a field that generally encompassed only a part of a single cell. Transient, highly localized increases in Cal-520 fluorescence arose following photorelease of i- IP_3 in both WT cells (Fig. 2A) and in cells expressing each single IP_3R isoform (Fig. 2, B-D). In all cell lines, puffs occurred throughout the 30s recording period (Fig. 2A-D), consistent with previous findings (57, 67) indicating that photoreleased i- IP_3 is not appreciably degraded during this time. The properties of Ca^{2+} puffs were not altered by the presence of ryanodine (fig. S2B). To avoid potential depletion of intracellular Ca^{2+} stores over time, we imaged puffs in a bath solution containing 2 mM Ca^{2+} rather than zero Ca^{2+} . In separate experiments, we did not detect

appreciable differences in puffs when imaged in a zero Ca^{2+} , EGTA-containing solution (fig. S3A-C).

We recorded fluorescence traces ($\Delta F/F_0$) from regions of interest centered on discrete subcellular sites and show representative records from sites exhibiting low and high frequencies of puffs in WT cells (Fig. 2A), and cells exclusively expressing type 1 (Fig. 2B), 2 (Fig. 2C), and 3 (Fig. 2D) IP_3Rs . The numbers of puffs varied markedly from one site to another, even in the same cell (Fig. 2A-D). Histograms plotting the numbers of puffs observed at a site (Fig. 2E-H) displayed skewed distributions, with many sites showing only a single puff during the recording period. The mean numbers of puff sites detected per imaging field were similar in WT cells and cells expressing IP_3R types 1 and 2, but were slightly fewer in cells expressing $\text{IP}_3\text{R3}$ (Fig. 2I). Under matched conditions, flash durations of 500-700 ms evoked similar mean total numbers of puffs following photorelease of $i\text{-IP}_3$ in cells expressing $\text{IP}_3\text{R1}$ and $\text{IP}_3\text{R2}$, and a greater mean number of puffs in $\text{IP}_3\text{R3}$ -expressing cells (Fig. 2J). WT cells were more responsive, such that weaker stimuli (250-300 ms flash durations) evoked similar or greater numbers of events than evoked by 500-700 ms flashes in single isoform-expressing cells (Fig. 2J).

To further examine the sensitivities of cells expressing single IP_3R isoforms to different concentrations of $i\text{-IP}_3$, in a separate set of experiments we counted the total numbers of puffs that arose throughout the imaging field in 10 s following photolysis flashes with varying durations ranging up to 1000 ms (Fig. 2K). We could not explore stronger stimuli because, even with cell loading of EGTA, they evoked global Ca^{2+} rises that obscured puffs. Brief UV flashes (durations ≤ 100 ms) were ineffective in evoking puffs in all single IP_3R -expressing cell lines, with mean numbers of puffs detected (≈ 4.5) little greater than spontaneous events occurring during the same time without stimulation (≈ 2.25) (Fig. 2K). Longer flash durations (≥ 300 ms) evoked progressively more puffs in all cell lines, and the mean numbers of puffs evoked by any given flash duration were similar for cells expressing each IP_3R isoform (Fig. 2K). We thus conclude that the clusters of IP_3Rs that generate local Ca^{2+} puffs display a similar dependence on $i\text{-IP}_3$ concentration, irrespective of the IP_3R isoform from which they are composed.

Amplitudes of puffs mediated by different IP_3R isoforms

To facilitate visual comparison of the magnitudes and time courses of puffs in WT cells and in cells expressing individual IP_3R isoforms we overlaid representative fluorescence traces ($\Delta F/F_0$) on an expanded timescale after aligning puffs to the times of their peak amplitude (Fig. 3A-D; traces). The amplitude distributions of puffs in WT cells and cells expressing each IP_3R isoform followed skewed Gaussian functions, with most puffs ranging between 0.3-0.6 ($\Delta F/F_0$) in peak amplitude (Fig. 3A-D; histograms). Plots of the cumulative distributions of puff amplitudes (Fig. 3E) further demonstrated the similarity in puff amplitudes between WT cells and those expressing specific IP_3R isoforms. Mean peak puff amplitudes were not significantly different between the different isoforms or WT cells (Fig. 3F).

Quantal analysis of Ca^{2+} flux through single channels formed by IP_3R isoforms

The TIRF signal during puffs closely tracks the underlying flux of Ca^{2+} into the cytosol (50, 68), which in turn is a function of the instantaneous number of channels open and the Ca^{2+} flux through each channel. The similarity in mean puff amplitudes among different IP_3R isoforms thus suggested that they likely involved the openings of similar numbers of channels with equivalent Ca^{2+} permeability, but it could also be due to differing numbers of active channels if the Ca^{2+} flux varied between isoforms. To discriminate between these possibilities, we performed a quantal analysis by exploiting the observation that many puffs exhibit step-wise transitions in fluorescence levels that we interpreted to reflect the openings and closings of individual IP_3R channels (50, 55).

Numerous puffs in WT cells and in cells expressing each individual IP_3R isoform exhibited step-wise fluorescence dwell states during both their rising and falling phases, and we also observed small events with a 'square' appearance that abruptly rose and fell from the baseline (Fig. 4A-D; traces). Measurements of the amplitudes ($\Delta F/F_0$) of these fluorescence dwell-state levels in WT cells, and in cells expressing single isoforms, displayed multimodal distributions (Fig. 4A-D; histograms). These distributions were fit well by a sum of four Gaussian functions, with successive means of approximately 0.13, 0.26, 0.38, and $0.5 \Delta F/F_0$. The mean amplitude of the first Gaussian corresponded closely with our prior estimate ($\Delta F/F_0 \sim 0.11$) of the fluorescence signal resulting from opening of a single IP_3R channel in a neuroblastoma cell line (50, 55), and the means of successive Gaussian fits recurred at near-integer multiples. The peaks of these successive Gaussians incremented linearly and almost identically for all cell lines, with a regression fit giving a mean slope of $0.126 \Delta F/F_0$ per quantal step for WT and each IP_3R isoform (Fig. 4E).

These results indicated that homomeric channels formed by all three IP_3R isoforms mediated similar Ca^{2+} fluxes, which were also indistinguishable from the unitary Ca^{2+} flux in WT cells. Because we found no differences in Ca^{2+} store content or basal cytosolic $[\text{Ca}^{2+}]$ among the cell lines (fig. S1A-C), this further implies that the Ca^{2+} permeabilities of channels formed by each IP_3R isoforms are almost identical. Moreover, the close similarity in mean puff amplitudes across the different cell lines (Fig. 3B-F) indicated that puffs mediated by each isoform involved the openings, on average, of 4 channels (mean puff amplitude / unitary channel amplitude, $0.51/0.126 \Delta F/F_0$).

Puff kinetics

The distributions of puff rise, fall, and full duration at half maximal amplitude (FDHM) times were similar in WT cells (Fig. 5A) and cells expressing exclusively type 1 (Fig. 5B) or type 2 IP_3Rs (Fig. 5C). In contrast, puffs in cells expressing type 3 IP_3Rs displayed appreciably faster rise and fall times, and shorter durations (Fig. 5D). Overlaid cumulative distribution curves of puff kinetics further highlight these observations (Fig. 5E). Quantification of mean puff rise, fall and duration times demonstrated that the kinetics of IP_3 -mediated Ca^{2+} puffs in WT cells and type 1 and 2 IP_3R -expressing cells were similar and not significantly different from one another, whereas all parameters were significantly faster for puffs mediated by type 3 IP_3Rs (Fig. 5F).

Spatial extent of fluorescence signals during puffs

The algorithm we used to detect and analyze puffs also determined a measure of the spatial extent of the fluorescence signal (full width at half-maximal amplitude; FWHM) by fitting a 2D Gaussian function to a temporally filtered image of the puff (Fig. 6A). Histograms (Fig. 6B-E) and cumulative curves (Fig. 6F) plot the distributions of FWHM values for WT and individual isoform-expressing cells, with a hard cut-off at $\sim 2.4 \mu\text{m}$ imposed by a requirement to fit a minimum of 2 pixels. Comparison of mean FWHM values (Fig. 6G) demonstrated that the spatial spread of puffs mediated WT cells and cells expressing single IP₃R isoforms were generally similar, except that IP₃R3-mediated puffs were slightly more restricted than puffs in IP₃R1-expressing cells, possibly as a consequence of their briefer kinetics.

Spatial organization of IP₃R clusters as reflected by the localizations of Ca²⁺ signals

The center of mass of the fluorescence signal during a puff is localized with a precision of several tens of nm by our algorithm that determines the centroids of 2D Gaussian fits to the fluorescence signals, and provides a weighted estimate of the centroid of those individual IP₃R channels open during that event (69). To derive information on the functional architecture of clusters of homomeric IP₃Rs of different isoforms we mapped the x, y coordinates of individual puffs recurring at given sites in cells expressing different IP₃R isoforms (Fig. 7A-D, left panels).

For each cell line, we used an all-neighbor distance analysis routine (56) to measure the distance of the centroid location of each puff to that of every other puff within a $5 \mu\text{m}$ radius (Fig. 7A-D, center panels). If puffs arose at randomly and homogeneously distributed sites, the distribution of neighbor-neighbor distances is expected to show a linear increase with increasing distance (70). In contrast, the histograms for all cell types showed a prominent peak at distances $< 0.5 \mu\text{m}$, consistent with successive puffs occurring repetitively in close proximity, at fixed locations. The black lines overlaid on the all-neighbor distance distributions were drawn to approximate the profiles expected from a random distribution of sites. By subtracting these predicted random distributions from the observed all-neighbor distance distributions, we obtained a measure of the clustering of puffs at specific sites. These distributions showed peaks at distances of a few hundred nm, declining towards that expected from a random distribution at distances approaching $2 \mu\text{m}$ (Fig. 7A-D, right panels). To better compare the extent of clustering of puffs among the different IP₃R isoforms, we replotted and superimposed the clustering data (Fig. 7A-D, right panels) after normalizing the counts to the same peak value (Fig. 7E). The clustering data for WT cells and cells expressing type 2 and 3 IP₃Rs showed similar profiles, whereas the broader profile for puffs in cells expressing the type 1 IP₃R (Fig. 7E, red curve) suggests that puffs in these cells were less tightly clustered.

Cellular locations of puff sites

TIRF microscopy provides information only on those local Ca²⁺ signals that arise in close proximity to the plasma membrane, adjacent to the cover-glass. To determine the distribution of puff sites throughout the volume of the cell we utilized lattice light-sheet microscopy to visualize Ca²⁺ signals with high temporal resolution in diagonal 'slices' cutting through the

cell interior (71). Puffs in SH-SY5Y neuroblastoma cells (57, 71) and HeLa cells (61) arise almost exclusively at sites close to the plasma membrane. However, in HEK-293 cells we observed puffs arising at sites both close to the membrane (Fig. 8A) and deeper into the cell (Fig. 8B). Puffs in cells expressing each individual IP₃R isoform and in WT cells arose primarily around the cell membrane, but in each cell line appreciable numbers (~20%) of puffs were observed in the cell interior (Fig. 8C).

Discussion

Ca²⁺ puffs are localized cytosolic Ca²⁺ signals that arise through liberation of Ca²⁺ stored in the ER lumen through tight clusters comprising small numbers of IP₃ receptor/channels. Puffs function as basic building blocks of cellular Ca²⁺ signaling (52), and serve to trigger global, cell-wide Ca²⁺ waves (10). The ability to image Ca²⁺ signals with a single-channel level of resolution enables us to determine the properties of individual IP₃Rs in an intact cellular environment. However, studies to date have been hampered because native cells express various proportions of the three IP₃R isoforms (33–36), so the composition of the IP₃R channels underlying Ca²⁺ puffs could not be inferred. Although DT40 cell lines derived from avian B cells by homologous recombination to express the entire complement of IP₃R types (45) have been utilized to investigate the behavior of select IP₃R isoforms in their native environment (20), we find that the small size and limited TIRF footprint of these cells render them unsuitable for studying Ca²⁺ puffs. Here, we circumvented these problems by high resolution imaging of Ca²⁺ puffs in HEK-293 cell lines genetically engineered to each express only a single IP₃R isoform (46).

Our most obvious finding is that cells expressing exclusively type 1, 2 or 3 IP₃Rs all generated puffs in response to photorelease of i-IP₃. As in WT HEK-293 cells and many other native cell types, these puffs recurred at stationary subcellular sites and displayed amplitudes and properties consistent with their arising through the concerted opening of a small number of closely adjacent IP₃R channels (6, 57, 61, 72, 73). Although a majority of IP₃Rs (of all subtypes) are mobile within the ER membrane (61, 70, 74–76), the immobility of puff sites, together with observations of stationary clusters of fluorescently-tagged IP₃Rs (61, 70), support the hypothesis that puffs arise from a distinct pool of IP₃Rs that are tethered to as yet unidentified cytoskeletal anchoring sites, where they are ‘licensed’ to preferentially respond to IP₃ (56, 58, 61, 77).

We thus conclude that all three IP₃R isoforms express the binding site(s) that enable their scaffolding into small clusters. Because we found that the mean number of channels that opened during a puff was similar in cells expressing each isoform of IP₃R, this finding further suggests that the stoichiometry of the IP₃R cluster is determined by the architecture of the underlying scaffolding structure and is independent of the specific IP₃R isoform. Finally, we observed that the distribution of puff sites throughout the cell volume, primarily in a cortical shell adjacent to the plasma membrane, was similar for cells expressing each isoform. This again is consistent with the notion that each IP₃R isoform binds to a preexisting cellular scaffold.

A further observation was that the properties of puffs generated by each isoform of IP₃R were generally similar. Photorelease of equivalent amounts of i-IP₃ in cell lines exclusively expressing individual isoforms evoked puffs at similar frequencies and with similar amplitudes. Moreover, the single-channel Ca²⁺ fluxes through all three IP₃R isoforms were indistinguishable, indicating that they had similar permeabilities, given that ER Ca²⁺ store content and basal cytosolic [Ca²⁺] did not differ between the cell lines. This result agrees with a majority of studies that report similar single-channel conductances as determined from single-channel electrophysiological recordings of monovalent ion currents through different IP₃R isoforms. However, there are reports of differences between different cell types, suggesting that factors such as membrane environment may affect the conductance (48).

We observed the greatest difference between puffs generated by the different IP₃R isoforms in terms of their kinetics. Puffs mediated by type 3 IP₃Rs were briefer than those mediated by the other isoforms, implying that in terms of cumulative cellular Ca²⁺ liberation they 'pack less of a punch'. The molecular basis for this kinetic difference of the type 3 IP₃R remains unclear. Indeed, the mechanisms underlying the termination of Ca²⁺ liberation during puffs are unresolved, likely involving feedback by Ca²⁺-dependent inhibition of IP₃Rs, as well as coupled gating analogous to that seen with ryanodine receptors (78). Both of these factors may potentially differ between IP₃R isoforms.

In contrast to our observations in minimally perturbed intact cells that the three IP₃R isoforms mediate closely similar local Ca²⁺ signals, electrophysiological studies utilizing reduced, in vitro experimental settings have shown marked differences between the single-channel gating properties of IP₃R isoforms (for reviews see (18, 48)). Systematic comparisons between the electrophysiological data and our findings in the intact cell are confounded by several factors, which include differences in the environments to which the IP₃Rs are exposed, as exemplified by the wide range of Ca²⁺ dependencies reported for the same recombinant IP₃R expressed in different cell types (48). In light of this issue, the most straightforward data for comparison derive from analysis of homotetrameric channels of each of the three IP₃R isoforms expressed in DT40-3KO cells (25, 48, 79). Those experiments showed complex differences between the isoforms in the Ca²⁺-dependence of their mean open probability (Po), with optimal Ca²⁺ concentrations over a roughly 10-fold range. In terms of IP₃ sensitivity, the sequence was found to be IP₃R2 > IP₃R1 > IP₃R3, based on observed Po values at optimal Ca²⁺ concentrations and respective sub-saturating IP₃ concentrations of 0.51, 0.27 and 0.17 (48). These findings differ from our observations that the frequencies of puffs evoked by the same photorelease of i-IP₃ were similar among cells expressing each IP₃R isoform.

A further problem in comparing the activity of IP₃Rs during puffs with their properties as determined from electrophysiological measurements is that the latter generally report steady-state properties, whereas puffs are highly dynamic, with rapid and large changes in Ca²⁺ concentrations. The cytosolic Ca²⁺ nanodomain at a puff site likely rises to tens or even hundreds of μM once the first channel opens (80), so that IP₃Rs in the cluster experience high concentrations that promote maximal activation and subsequent maximal inhibition. Rapid saturation of Ca²⁺ binding to sites on the IP₃R may explain why puffs

mediated by the three isoforms appear so similar, despite differences that are apparent at lower Ca^{2+} concentrations in steady-state electrophysiological recordings. Indeed, the gating of IP_3Rs during puffs likely hinges on the rapid kinetics of their modulation by Ca^{2+} . To date, however, the dynamic responses of single IP_3Rs to abrupt changes in ligand have been studied only for the single insect IP_3R isoform and no data are available for the mammalian isoforms (47).

Our results reveal intriguing discrepancies between the local (puffs) and global Ca^{2+} signals mediated by the different IP_3R isoforms. Although the amplitudes and frequencies of puffs evoked by a given photolysis flash were closely similar among cells expressing each IP_3R isoform, the global cellular Ca^{2+} responses evoked by strong photorelease of i-IP_3 in cells not loaded with EGTA were smaller in cells expressing $\text{IP}_3\text{R1}$ and $\text{IP}_3\text{R3}$ than in those expressing $\text{IP}_3\text{R2}$. We speculate these differences may arise from differences in the functional properties of the greater fraction of IP_3Rs that underlie the global Ca^{2+} response, given that stimulation with the receptor agonist CCH elicited much larger whole-cell Ca^{2+} signals and more rapid rates of rise, when compared to i-IP_3 , in cells expressing solely types 1 and 3 IP_3Rs . Puffs are generated by the small sub-population of IP_3Rs that are anchored at discrete cluster sites, and which respond preferentially at lower $[\text{IP}_3]$ than do the greater bulk of mobile IP_3Rs that underlie the global cellular Ca^{2+} response (56, 57, 61, 77). Thus, the sensitivity to IP_3 and other properties of the bulk population of IP_3Rs may differ more widely between the different isoforms than is apparent for the IP_3Rs clustered at puff sites. Also, it remains possible that differences in IP_3R expression levels impacted the whole-cell Ca^{2+} responses, because assembly of IP_3Rs at the cytoskeletal structures defining puff sites might remain saturated even if the expression levels of the various isoforms differed appreciably, whereas the numbers of IP_3Rs available to mediate the much larger global signals would be diminished at low expression levels.

In summary, we utilized ‘optical patch-clamp’ imaging of Ca^{2+} flux with single-channel resolution (81) to study the physiological functioning of IP_3Rs in intact, minimally perturbed cells (50, 57, 58). Our results in HEK-293 cells showed that IP_3Rs composed solely of type 1, 2 or 3 isoforms were equally capable of producing Ca^{2+} puffs that recurred at fixed sites, indicating that each isoform is able to bind to some, as yet unidentified, cytoskeletal anchoring sites that define the location and composition of functional IP_3R clusters (58, 61). The mean numbers of functional IP_3R channels clustered at each site and their Ca^{2+} permeabilities were similar for homomeric channels formed by individual isoforms, as well as for channels in WT cells that are likely heteromers comprised from 2 or 3 different isoforms (33, 37, 38). In contrast to proposals that the properties of native heteromeric IP_3Rs may be determined by the mix of their constituent isoforms (39, 40), and that cell types express a particular complement of IP_3R isoforms to suit their particular needs (41), our results indicate substantial redundancy among the IP_3R isoforms in regard to their function and spatial localization at puff sites. While this manuscript was under review, another group, using the same commercially available HEK KO cell lines that we generated, published a study detailing similar findings to ours (82).

Materials and Methods

Generation of HEK-293 cells expressing single IP₃R isoforms

Single guide RNAs (sgRNAs) targeting the fourth exon in IP₃R1, IP₃R2 or IP₃R3 genes were designed utilizing <http://crispr.mit.edu/> (83) as described previously (46).

Oligonucleotides corresponding to these sgRNAs were synthesized by Integrated DNA Technologies (IDT) and cloned into a pX458 vector encoding Cas9 nuclease and EGFP (obtained from Addgene). sgRNA constructs in various combinations were transfected into HEK-293 wild-type (WT) cells using Lipofectamine 2000 following the manufacturer's instructions. 48 hrs post-transfection, flow cytometry was used to sort EGFP-expressing cells and these sorted cells were grown as single cells in 96-well plates. Clones were propagated and expanded for three weeks and then harvested for characterization by Western blot. Potential clones were subsequently genotyped as described in (46), which confirmed the introduction of base pair insertions and deletions in both alleles.

Characterization of IP₃R isoform expression by Western Blot

Cells were washed with ice-cold PBS and solubilized for 30 min in lysis buffer composed of 10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1 mM NaF, 20 mM Na₄P₂O₇, 2 mM Na₃VO₄, 1% Triton X-100 (v/v), 0.5% sodium deoxycholate (w/v), and 10% glycerol with a mixture of protease inhibitors (Roche). Lysates were centrifuged at 16,000 × g for 10 min at 4°C and supernatants were then collected, and protein concentrations determined using Dc protein assay kit (Bio-Rad). Equal amounts of lysate (10 µg) were fractionated by SDS-PAGE and electrotransferred to nitrocellulose membranes. Blots were probed with antibodies to IP₃R type 1 (rabbit polyclonal antibodies against the C-terminal 19 aa of rat IP₃R1), type 2 (rabbit polyclonal antibodies against 320-338 aa of rat IP₃R2), or type 3 (mouse monoclonal antibodies against 22-230 aa of human IP₃R3), as well as antibody to calreticulin to serve as a loading control. Following incubation with horseradish peroxidase conjugated secondary antibodies (Bio-Rad), blots were imaged using enhanced chemiluminescent substrate (Thermo Scientific). Antibodies against type 1 and 2 IP₃R were from Pocono Rabbit Farms and Laboratories, and antibodies against type 3 IP₃R were from BD Transduction Laboratories. Antibodies against calreticulin were from BD biosciences (#612136). Band intensities were measured on a LiCor Odyssey imaging system. For densitometry measurements, each band stained with isoform-specific IP₃R antibodies was first normalized to its corresponding loading control band stained with calreticulin antibodies, before normalizing the expression of individual IP₃R isoforms in single isoform-expressing cells to their respective expression in WT cells.

Cell Culture and loading

CRISPR/Cas9 modified HEK-293 cells lines endogenously expressing single IP₃R isoforms and an IP₃R null cell line (3KO) were generated and characterized by the Yule lab and distributed through Kerafast (<http://www.kerafast.com/>). For Ca²⁺ imaging experiments by the Parker lab, these cells were purchased from Kerafast: IP₃R type 1 (#EUR031), type 2 (#EUR032), type 3 (#EUR033) and 3KO (#EUR030). The parental HEK-293 WT cells used to establish the genetically engineered cells was provided directly by the Yule lab. To

confirm the identity of HEK KO cell lines obtained through Kerafast, protein lysates of all cell lines used for imaging were prepared by the Parker lab and sent to the Yule lab for characterization by Western blot. All cell lines were cultured on plastic 75 cm flasks in Eagle's Minimum Essential Medium (ATCC #30-2003), supplemented with 10% fetal bovine serum (Omega Scientific #FB-11), and maintained at 37°C in a humidified incubator gassed with 95% air and 5% CO₂. For imaging, cells were collected using 0.25% Trypsin-EDTA (Gibco #25200-056) and grown on poly-D-lysine coated (500 µg/ml; Sigma #P0899) 35-mm glass-bottom imaging dishes (MatTek #P35-1.5-14-C) or 12 mm glass coverslips for 2-3 days.

Immediately before imaging, cells were incubated with membrane-permeable esters of the fluorescent Ca²⁺ dye Cal-520/AM (5 µM; AAT Bioquest #21130) and the caged IP₃ analogue ci-IP₃/PM [D-2,3,-O-Isopropylidene-6-O-(2-nitro-4,5 dimethoxy) benzyl-myo-Inositol 1,4,5,-trisphosphate Hexakis (propionoxymethyl) ester] (1 µM; SiChem #cag-iso-2-145-10) for 1 hr at room temperature (RT) in a Ca²⁺-containing HEPES buffered salt solution (Ca²⁺-HBSS). In experiments with ionomycin, cells were instead loaded with 5 µM Fluo-8L/AM (AAT Bioquest # 21097) for 1 hr at RT. In experiments using fura-2, cells were loaded with 5 µM fura-2/AM for 1 hr at RT. Where indicated, cells were loaded for an additional hour with EGTA/AM (5 µM; ThermoFisher #E1219). Ca²⁺ indicators, caged i-IP₃, and EGTA/AM were all solubilized with DMSO/20% pluronic F127 (ThermoFisher #P3000MP). Ca²⁺-HBSS contained (in mM) 135 NaCl, 5.4 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose (pH=7.4). Zero Ca²⁺/EGTA-HBSS consisted of the same formulation as Ca²⁺-HBSS, except CaCl₂ was omitted and 300 µM EGTA was included. Carbachol was from Sigma-Aldrich (#C4382) and ryanodine was from Enzo Life Sciences (#ALX-630-062-M001). For lattice light-sheet imaging, the plasma membrane was stained by adding Cell Mask Deep Red (Thermo Fisher #C10046) to the bathing solution in the imaging chamber (1/50,000 dilution).

Ca²⁺ imaging

TIRF and WF imaging of Ca²⁺ signals was accomplished using a custom-built system, based around an Olympus IX50 microscope equipped with either an Olympus 10× objective (NA 0.4) for WF microscopy or with an Olympus 60× objective (NA 1.45) for TIRF mode. Fluorescence images were acquired with an Evolve EMCCD camera (Photometrics), utilizing 488 nm laser fluorescence excitation and a 510 long pass emission filter. WF images were captured from a 278 × 243 pixel field (1 pixel = 2.2 µm) at a rate of 10 frames s⁻¹. TIRF images were captured using 2×2 pixel binning for a final field of 70 × 35 pixels (1 pixel = 0.53µm) at a rate of ~ 333 frames s⁻¹. Where indicated, some TIRF imaging was performed with a larger field of view (100 × 100 or 128 × 128 pixels) at ~ 151 or ~ 125 frames s⁻¹, respectively. To photorelease i-IP₃, UV light from a xenon arc lamp was filtered through a 350-400 nm bandpass filter and introduced by a UV-reflecting dichroic in the light path to uniformly illuminate the field of view. The amount of i-IP₃ released was controlled by varying the flash duration, set by an electronically controlled shutter (UniBlitz). All image data were streamed to computer memory using Metamorph v7.7 (Universal Imaging/Molecular Devices) and stored on hard disc for offline analysis. WF ratiometric imaging of cells loaded with fura-2 was performed with a Nikon Eclipse Ti microscope equipped with a

40X objective and a Hamamatsu EMCCD camera. Light from an arc lamp was filtered through 340 nm and 380 nm filters and collected through a 510 nm filter. Alternating frames with 340 and 380 nm excitation (respectively, 400 ms and 100 ms exposure times) were captured using NIS Elements software (Nikon) and stored on hard disc for offline analysis.

Lattice light-sheet imaging was performed using a custom-built system as described (71). Images were acquired with an Andor Zyla 4.2 sCMOS camera from a single, diagonal light-sheet slice (512×256 pixels, 1 pixel = 0.11 μm) at 250 frames s^{-1} . Ca^{2+} puffs were imaged in Cal-520- and EGTA-loaded cells for several seconds following photorelease of i-IP_3 by a flash from a 405 nm laser diode, utilizing 473 nm laser fluorescence excitation and a 510-560 nm bandpass emission filter. A 562 nm laser and 590 nm long-pass filter were then used to image the plasma membrane stained with Deep Red Dye. The locations of puff were marked by visual inspection, and overlaid on the plasma membrane image to classify puffs as near-membrane (within $\sim 2 \mu\text{m}$) or arising deeper within the cell.

Image Analysis

Image data in MetaMorph stk or TIF formats were processed using Flika (<http://flika-org.github.io>), a freely available open-source image processing and analysis software written in the Python programming language. The raw image stack was first processed to create a ratio image stack (F/F_0), where the fluorescence intensity of each pixel in a given frame was calculated as that intensity (F) divided by the mean resting fluorescence intensity at the pixel averaged over 500 frames prior to photorelease of i-IP_3 (F_0). A custom plug-in (“detect puffs”) was then applied for automated detection and analysis of local Ca^{2+} signals (84). All Ca^{2+} puffs identified by the algorithm were verified by visual inspection prior to further analysis. Measurements of peak puff amplitudes (F/F_0) and kinetics were performed by the algorithm on a 3×3 pixel region of interest centered over the centroid of each event and were exported to EXCEL spreadsheets for further analysis. The spatial spread of each puff was calculated from a 2D Gaussian fitted to the mean peak signal derived from a temporally filtered image stack. Dwell state amplitudes (F/F_0) of step-wise transitions during individual puffs were manually measured as the difference in fluorescence from the mean baseline preceding the puff to the mean amplitude of a fluorescence level that remained stable for 10 ms; or as the difference between successive steps (55). Distances between puff centroids were determined from the x-y coordinates of each puff to that of every other puff in the same cell using a nearest-neighbor algorithm written in Python. The amplitudes of whole-cell Ca^{2+} signals were determined from regions of interest encompassing entire individual cells from the F/F_0 image stack and exported to EXCEL spreadsheets for analysis. The maximum rate of rise of global cellular Ca^{2+} signals in individual cells was determined from the steepest slope during the rising phase of the F/F_0 fluorescence signal. All additional analysis and graphing was performed in Microcal Origin v6.0 (OriginLab).

Statistics

Mean values are expressed throughout as standardized mean ± 1 s.e.m, where n = the number of imaging fields analyzed. Comparison of mean values between two groups was assessed by both Student’s T-test (parametric) and Mann-Whitney test (non-parametric). Comparisons of mean values between three or more groups were assessed by both one-way

ANOVA with Tukey post hoc tests (parametric) and Kruskal-Wallis with Mann-Whitney post hoc tests (non-parametric). Differences were considered significant when indicated by both parametric and non-parametric approaches; the larger of the two P values are presented throughout. Statistical significance in figures is denoted as * = $P < 0.05$ and ** = $P < 0.01$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1:

Protein expression of IP₃ receptor isoforms and global carbachol- and i-IP₃-evoked Ca²⁺ signals in HEK WT cells and CRISPR/Cas9 modified HEK-293 cells that endogenously express exclusively type 1, 2, or 3 IP₃Rs. (A) Equivalent amounts of lysate proteins from HEK wild-type (WT), triple IP₃R-knockout (3KO) and single IP₃R isoform-expressing cells were immunoblotted with isoform-specific antibodies as indicated; data are representative of 3 independent experiments. Bar graphs show the relative expression of each IP₃R isoform in single-isoform expressing cells normalized to their respective expression in WT cells. (B-M)

Fluorescence records and analysis were made from regions of interest encompassing individual cells loaded with Ca^{2+} -indicator Cal-520. (**B-E**) Traces show representative, superimposed records of global fluorescence ratio signals (F/F_0) evoked by bath application of 100 μM carbachol (CCH; denoted by line) in WT cells (**B**; $n=131$) and in cells expressing solely types 1 (**C**; $n=130$), 2 (**D**; $n=186$), or 3 (**E**, $n=148$) IP_3Rs . (**F**) Bars show the mean peak amplitudes of the fluorescence signals (F/F_0) evoked by 100 μM CCH in WT and single IP_3R -expressing cells; $n = 3$ imaging fields for each cell line with cell totals of 392 (WT), 383 ($\text{IP}_3\text{R1}$), 508 ($\text{IP}_3\text{R2}$), and 494 ($\text{IP}_3\text{R3}$). (**G**) Bars show the mean maximum rate of rise of the fluorescence signal ($F/F_0 \text{ s}^{-1}$), determined from 100 randomly chosen cells for each cell line with $n = 3$ imaging fields. (**H-K**) Superimposed traces of IP_3 -mediated global fluorescence signals (F/F_0) evoked by a 1000 ms duration photolysis flash (marked by an arrow) in WT cells (**G**; $n=134$) and in cells expressing solely type 1 (**H**; $n=117$), 2 (**I**; $n=140$), and 3 (**J**; $n=112$) IP_3Rs . The delayed individual-cell signals in **H** and **J** are long-latency initial responses, rather than recurrent signals in cells that had already responded. (**L**) Dose response curves of the mean peak amplitudes of the fluorescence signals (F/F_0) following photolysis flashes of different durations for WT cells and cells expressing single isoforms (depicted by different symbols and colors as indicated). For all cell lines, data points are means ± 1 s.e.m of $n=3$ imaging fields with totals of 336–465 cells. Statistical comparisons between cell lines for a given UV flash duration were determined by ANOVA with Tukey post hoc tests: (300 ms) yielded P values of <0.01 for WT vs. R1, WT vs. R2, WT vs. R3; (700 ms) $P<0.01$ for WT vs. R1, WT vs. R3, R1 vs. R2, R2 vs. R3; (1000 ms) $P<0.01$ for WT vs. R1, WT vs. R3, R1 vs. R2, R2 vs. R3; (2000 ms) $P<0.01$ for WT vs. R1, WT vs. R3, R1 vs. R2, R2 vs. R3. (**M**) Bars show the mean maximum rate of rise ($F/F_0 \text{ s}^{-1}$) of fluorescence signals following a 1000 ms photolysis flash, determined from 100 randomly chosen cells for each cell line with $n = 3$ imaging fields. Traces in **B-E** and **H-K** are shown on identical magnitude (F/F_0), but different time scales. Statistically significant differences in panels **F**, **G** and **M** were determined by ANOVA with Tukey post hoc tests; * $P<0.05$ and ** $P<0.01$.

Fig. 2:

Local, subcellular Ca^{2+} puffs evoked by photoreleased i-P_3 in WT cells and CRISPR/Cas9 modified HEK-293 cells expressing exclusively types 1, 2, or 3 IP_3Rs . (**A-D**), Traces show Cal-520 fluorescence ratio measurements ($\Delta F/F_0$) from $1.5 \times 1.5 \mu\text{m}$ regions of interest centered on puff sites in WT cells (**A**), and cells expressing exclusively $\text{IP}_3\text{R1}$ (**B**), $\text{IP}_3\text{R2}$ (**C**), and $\text{IP}_3\text{R3}$ (**D**). Each panel shows records that are representative of puff sites exhibiting low and high frequencies of Ca^{2+} puffs; arrows and gaps in the traces mark the time of the photolysis flash. Traces in **A** were obtained using a 250 ms photolysis flash duration, and

those in **B-D** with a flash duration of 500 ms. (**E-H**) Histograms depict the distributions of Ca^{2+} puff frequencies (number of puffs per site per 30s recording) occurring at discrete puff sites in WT cells and cells expressing individual IP_3R isoforms. (**I**) Mean numbers of puff sites per imaging field ($37 \times 19 \mu\text{m}$) for WT cells and cells expressing individual IP_3R isoforms; * $P < 0.05$ when assessed by ANOVA with Tukey post hoc tests. (**J**) Mean numbers of puffs per imaging field for WT cells and cells expressing individual IP_3R isoforms; ** $P < 0.01$ when assessed by ANOVA with Tukey post hoc tests. Data in **E-J** are from totals of 480 (WT), 272 ($\text{IP}_3\text{R1}$), 338 ($\text{IP}_3\text{R2}$), and 304 ($\text{IP}_3\text{R3}$) puff sites, from 13, 8, 9, and 11 cells, respectively. Error bars in **I,J** depict 1 s.e.m. (**K**) Dose/response relationship for puffs generated by different IP_3R isoforms. The data are from experiments in which records were acquired at a slower frame rate ($151 \text{ frames s}^{-1}$) than in panels **A-J** and from larger imaging fields ($53 \times 53 \mu\text{m}$) that typically contained 2-3 cells. The plot shows the numbers of puffs detected throughout the imaging field in 10 s following photolysis flashes of various durations in cells expressing solely type 1, 2 or 3 IP_3Rs (depicted by different symbols and colors, as indicated). Data points are means $\pm$ 1 s.e.m from $n = 4-7$ imaging fields.

Fig. 3:

Amplitudes of Ca^{2+} puffs evoked in WT HEK cells and cells exclusively expressing IP_3R types 1, 2, and 3. (A) The fluorescence traces (F/F_0) on the left illustrate representative Ca^{2+} puffs evoked by photoreleased i- IP_3 in WT cells. Each panel shows several puffs, superimposed after alignment of the times of peak amplitude. The histogram plots the distribution of peak puff amplitudes (F/F_0) measured from 1841 events in 13 cells. (B-D) Panels show corresponding examples of puff traces and amplitude distributions in cells exclusively expressing $\text{IP}_3\text{R1}$ (B; histogram is from 810 events from 8 cells), $\text{IP}_3\text{R2}$ (C; 796

events from 9 cells), and IP₃R3 (**D**; 1438 events from 11 cells). (**E**) Cumulative frequency curves showing the percentage of all detected events as a cumulative function of puff amplitude for WT cells and cells expressing each individual isoform (depicted by different colored symbols as indicated). (**F**) Bars show mean peak puff amplitudes (F/F_0) in WT cells and cells expressing each IP₃R isoform, with n = the number of cells examined as listed above; error bars denote 1 s.e.m. Mean peak puff amplitudes were not significantly different between cell lines when assessed by either ANOVA or Kruskal-Wallis tests.

Fig. 4:

Quantal analysis of single-channel IP₃R Ca²⁺ flux during Ca²⁺ puffs. (A) The traces on the left show representative puffs in a WT cell, presented on an expanded timescale to illustrate stepwise increments and decrements of fluorescence ($\Delta F/F_0$) resulting from the successive openings and closings of individual IP₃R channels. Fluorescence measurements were made from $1.5 \times 1.5 \mu\text{m}$ regions of interest centered on puff sites. Horizontal lines drawn at integer multiples of fluorescence amplitude indicate the numbers of open channels during dwell times at different step amplitudes. The histogram shows the distribution of amplitudes

(F/F_0) of visually identified mean dwell levels in WT cells. The curves show individual components of a multi (4) Gaussian fit to the distribution. Data are from 415 WT dwell states. (**B-D**) Corresponding examples of stepwise puffs and of step dwell-state amplitude distributions and multi-Gaussian fits to data from cells expressing exclusively IP₃R1 (**B**), IP₃R2 (**C**) and IP₃R3 (**D**). Data are from 665 (IP₃R1), 659 (IP₃R2), and 696 (IP₃R3) dwell states. (**E**) The graph shows the center values of the four Gaussian distributions against their ordinate number (1-4). Different symbols represent data from the different cell lines, as indicated. Symbols overlap where not visible. The line is a regression fit to data from all cell types, constrained to pass through the origin, with a slope of $F/F_0 = 0.126$ per unitary step level.

Fig. 5:

Temporal characteristics of Ca^{2+} puffs in WT cells and cells expressing exclusively IP₃R types 1, 2 or 3. **(A-D)** Histograms show the distributions of Ca^{2+} puff rise times (from 20% to 80% of peak value; left), fall times (fall from 80% to 20% of peak; center), and full duration at half maximal amplitude (FDHM; right) for the different cell lines, as indicated. **(E)** Cumulative frequency curves show the percentage of all detected events as a function of puff rise time (left), fall time (center), and duration (right) for WT cells and cells expressing each isoform (depicted as indicated by different colored symbols). **(F)** Bars show mean puff

rise times (left), fall times (center), and durations (right); error bars denote 1 s.e.m, with n = the number of cells examined. * $P < 0.05$, and ** $P < 0.01$ when determined by ANOVA with Tukey post hoc tests. Data in this figure were derived from the same cells and recordings as in Fig. 3: WT, 1841 events from 13 cells; IP₃R1, 810 events from 8 cells; IP₃R2, 796 events from 9 cells; and IP₃R3, 1438 events from 11 cells.

Fig. 6: Spatial spread of fluorescence signal during Ca^{2+} puffs evoked in WT cells and cell lines exclusively expressing specific IP₃R isoforms. (A) The left panel shows a representative, temporally filtered image of a Ca^{2+} puff, with increasing fluorescence (F/F_0) depicted on a pseudocolor scale and by increasing height. The right panel shows a 2D Gaussian fit to this puff; scale bar is 5 μm . (B-E) Plots showing the distributions of puff spatial widths (FWHM; full-width at half-maximal amplitude of the Gaussian fit) measured, respectively, in WT cells and cells expressing type 1, 2 and 3 IP₃Rs. The algorithm used to fit the image data

truncated measurements at widths $<2.4\ \mu\text{m}$. **(F)** Cumulative frequency curves show the cumulative percentage of all detected events as a function of increasing spatial width (FWHM) of puff fluorescence signals for WT cells and cells expressing each isoform (depicted by different colored symbols). **(G)** Bars show mean spatial widths; error bars denote 1 s.e.m, with n = the number of cells. $**P<0.01$ when determined by ANOVA with Tukey post hoc tests. Data in this figure were derived from the same cells and recordings as in Fig. 3: WT, 1841 events from 13 cells; IP₃R1, 810 events from 8 cells; IP₃R2, 796 events from 9 cells; and IP₃R3, 1438 events from 11 cells.

Fig. 7: Subcellular localizations of Ca^{2+} puffs in WT cells and cells exclusively expressing types 1, 2 or 3 IP_3R . (A) The left panel shows a representative example of the locations of Ca^{2+} puffs recurring at an individual site. Circles mark the centroid localizations of Ca^{2+} fluorescence evoked by puffs within a $1 \times 1 \mu\text{m}$ region of interest (outlined by the box) that was centered on the puff site. The middle panel shows a distribution plot of neighbor-to-neighbor distances between every puff localization and every other localization. Distances were calculated for all puff localizations within the $37 \times 19 \mu\text{m}$ imaging field, out to a radius of 5

μm around each localization. The black line, generated by a linear fit to the counts between 1 to 5 μm and constrained through zero, approximates the distribution expected if puff locations were randomly distributed. The right panel plots the difference between the observed and predicted random distribution of neighbor-neighbor distances, to provide a measure of the clustering of puff locations. **(B-D)** Corresponding examples of puff localizations and neighbor-neighbor distance distributions for cells expressing exclusively IP₃R1, IP₃R2 and IP₃R3. **(E)** Neighbor-neighbor distance distributions for WT and each isoform, replotted and overlaid from the right panels in **A-D**. Data from the different cell lines are depicted by different colored symbols and lines, and are shown after normalizing to their respective peaks and plotting on an expanded scale to better visualize differences in the spatial localization of puffs mediated by the different IP₃R isoforms. Data in this figure were derived from the same cells and recordings as in Fig. 3: WT, 1841 events from 13 cells; IP₃R1, 810 events from 8 cells; IP₃R2, 796 events from 9 cells; and IP₃R3, 1438 events from 11 cells.

Fig. 8:

Distribution of puff sites throughout the interior of WT cells and cells expressing individual IP₃R isoforms, visualized by lattice light-sheet microscopy. (A,B) The images illustrate a single, diagonal light-sheet section through a cell expressing type 3 IP₃Rs. The plasma membrane is depicted in red, and local Ca²⁺ puffs (F/F_0) in green. Images of puffs arising at different times and locations are superimposed on the membrane image. Representative examples are shown of puffs arising immediately adjacent to the plasma membrane (A) and deep in the interior of the same cell (B); scale bar is 5 μ m. (C) Bar graph summarizing the

percentages of puffs arising near the cell edge or deeper ($>2\ \mu\text{m}$) within the cell interior in WT ($n = 42$), IP₃R1 (8), IP₃R2 (12), and IP₃R3 (9) cells.

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