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Comparison of Ca^{2+} puffs evoked by extracellular agonists and photoreleased IP_3

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Abstract

The inositol trisphosphate (IP_3) signaling pathway evokes local Ca^{2+} signals (Ca^{2+} puffs) that arise from the concerted openings of clustered IP_3 receptor/channels in the ER membrane. Physiological activation is triggered by binding of agonists to G-protein-coupled receptors (GPCRs) on the cell surface, leading to cleavage of phosphatidyl inositol bisphosphate and release of IP_3 into the cytosol. Photorelease of IP_3 from a caged precursor provides a convenient and widely employed means to study the final stage of IP_3-mediated Ca^{2+} liberation, bypassing upstream signaling events to enable more precise control of the timing and relative concentration of cytosolic IP_3. Here, we address whether Ca^{2+} puffs evoked by photoreleased IP_3 fully replicate those arising from physiological agonist stimulation. We imaged puffs in individual SH-SY5Y neuroblastoma cells that were sequentially stimulated by picospritzing extracellular agonist (carbachol, CCH or bradykinin, BK) followed by photorelease of a poorly-metabolized IP_3 analog, i-IP_3. The centroid localizations of fluorescence signals during puffs evoked in the same cells by agonists and photorelease substantially overlapped (within ~1 μm), suggesting that IP_3 from both sources accesses the same, or closely co-localized clusters of IP_3Rs. Moreover, the time course and spatial spread of puffs evoked by agonists and photorelease matched closely. Because photolysis generates IP_3 uniformly throughout the cytoplasm, our results imply that IP_3 generated in SH-SY5Y cells by activation of receptors to CCH and BK also exerts broadly distributed actions, rather than specifically activating a subpopulation of IP_3Rs that are scaffolded in close proximity to cell surface receptors to form a signaling nanodomain.

Graphical abstract

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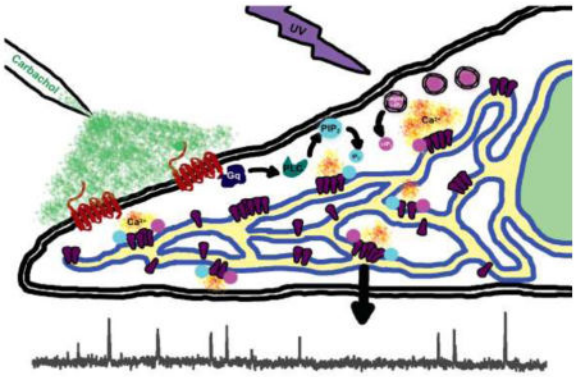
Disclosures

All authors declare they have no competing financial interests.

Author contributions

Conception and design of the study by J.T.L, I.F.S, and I.P. Data were collected and analyzed by J.T.L. I.P. and I.F.S. directed the study. Manuscript was written by J.T.L. and I.P. All authors have read and approve the published manuscript.



Keywords

Ca^{2+} puffs; IP_3; GPCRs; calcium imaging; signaling nanodomains

1. Introduction

Numerous G-protein-coupled cell surface receptors (GPCRs) function by activating phospholipase C to cleave membrane phosphatidyl inositol bisphosphate, generating inositol trisphosphate (IP_3) that then diffuses in the cytosol to bind to IP_3 receptor/channels (IP_3R) in the membrane of the endoplasmic reticulum (ER) and causes them to open and release Ca^{2+} ions sequestered in the ER lumen [1]. Experimental studies of IP_3-mediated Ca^{2+} release in intact cells may be facilitated by circumventing the upstream components of this signaling pathway, utilizing photorelease of IP_3 from a caged precursor to enable better control of the timing and magnitude of [IP_3] transients than is possible by application of extracellular agonists[2 4]. Here, we address the question of whether GPCRs access the same population of IP_3Rs that are activated by photoreleased IP_3 by comparing the localizations and properties of subcellular Ca^{2+} puffs evoked by these different means. Our results further bear on the broader topic of whether particular GPCRs specifically interact with a subset of IP_3Rs by virtue of spatial proximity[5–9].

IP_3 is a small, soluble molecule that, based on the spread of radiolabeled IP_3 in extracts of oocyte cytoplasm, has long been thought to diffuse in the cytosol at a rate (diffusion coefficient ~280 $\mu m^2\ s^{-1}$) nearly as fast as in free solution[10]. Consequently, it has been considered a ‘global’ messenger that would rapidly spread throughout a cell before being degraded[11]. However, we recently described that diffusion of IP_3 in neuroblastoma cells is much (30-fold) slower than this, so its range of action may be only a few μm[12]. At an even shorter scale, IP_3 has been reported to act as a highly localized signal in neurons and smooth muscle, where IP_3Rs may be tightly colocalized with cell surface GPCRs to generate specificity in receptor function[5–7].

To identify the sites where IP_3Rs are activated we imaged Ca^{2+} puffs in SH-SY5Y neuroblastoma cells. These transient, local events are generated by the concerted openings of small numbers of tightly clustered receptor/channels[13]. By determining the centroid locations of the fluorescence signals we could map Ca^{2+} puffs with sub-micron

precision[14], allowing us to compare the locations of IP_3R clusters activated in the same cell by sequential stimulation by GPCR agonists, followed by global photolysis of caged IP_3. SH-SY5Y cells endogenously express BK2 [15] and mACh3 [16]receptors, which we activated by extracellular application of bradykinin (BK) and carbachol (CCH).

2. Experimental methods

SH-SY5Y neuroblastoma cells were cultured and imaged as described previously[17]. In brief, cells were loaded by incubation with membrane-permeable esters of the Ca^{2+} indicator Cal-520; a caged precursor of a slowly metabolized IP_3 analog, i-IP_3 (SiChem; #cag-iso-2-145-10); and EGTA, a slow Ca^{2+} buffer introduced to inhibit propagation of cytosolic Ca^{2+} waves and sharpen the spatio-temporal profile of puffs[18,19]. Wide-field (WF) epi-fluorescence images of cells were acquired in a HEPES buffered salt solution containing 2 mM Ca^{2+} (HBSS) using a custom-built microscope equipped with a 60X objective (Olympus, NA 1.45) and electron-multiplied ccd camera (Photometrics Evolve; 220 frames s^{-1}, 2x2 binning for a final pixel size of 0.53 μm at the specimen). Ca^{2+} puffs were first evoked by picospritzing agonist (10 nM BK or 500 nM CCH) from a blunt (tip diameter ~1–2 μm) micropipette positioned above the cell, and subsequently by photoreleasing i-IP_3 by a flash of UV light (350–400 nm) from a Xenon arc lamp focused to evenly illuminate the same cell. Image stacks were analyzed using a custom algorithm[20] to locate the centroids of puffs with sub-pixel resolution and to derive traces of fluorescence over time from small regions centered on these sites. For a full description of the materials and procedures see Supplementary Methods online.

3. Results

Fig. 1 illustrates our experimental protocol. An SH-SY5Y cell was first stimulated by applying an agonist (500 nM CCH in this instance) from a micropipette positioned above the cell. The pressure, duration and timing of the pneumatic pulses were empirically adjusted to evoke local Ca^{2+} puffs without triggering global Ca^{2+} waves, and trials with fluorescein in the pipette solution showed that the plume of ejected solution engulfed the cell. Responses typically persisted for only a few seconds following a single pulse of agonist, so we delivered several pulses throughout a ~ 2 min recording period to generate repeated puffs that arose at several sites within the cell (Figs. 1A–C). After waiting ~ 5 min for agonist-evoked responses to fully cease, we then delivered a flash of UV light to uniformly photorelease i-IP_3 throughout the same cell, with the flash intensity and duration adjusted to again evoke puffs at multiple sites without triggering Ca^{2+} waves (Figs. 1E–G). Activity following a single flash persisted for several minutes (Fig. 1F) because the photoreleased i-IP_3 analog is poorly metabolized. For this reason switching the stimulant order, i.e. first stimulating with i-IP_3 followed by CCH, was impracticable. The same protocol was used to compare responses evoked by local application of bradykinin (10 nM) with those evoked by photoreleased i-IP_3.

3.1 Locations of puffs evoked by GPCR agonists and photoreleased IP_3

The stacks of raw fluorescence images were processed to form ratio ($\Delta F/F_0$) image sequences that were analyzed[20] to locate the centroid positions of all puffs evoked in a

given cell by agonist application and then by photoreleased i-IP_3, as illustrated in Figs. 1A,E. Fig 2A shows the same data, replotted to overlay the centroid locations of CCH and i-IP_3-evoked puffs, and Fig. 2B shows enlarged plots of puff localizations at sites where several repeated puffs were evoked by both stimuli. We had previously shown that localizations of IP_3-evoked puffs recorded over several minutes cluster tightly (within < 1 μm), indicating that the underlying cluster of IP_3Rs remains almost stationary[14]. Consistent with this, successive puffs evoked by CCH localized closely with one another (open circles, Fig. 2B), as did puffs evoked by photoreleased i-IP_3 (filled circles, Fig. 2B). Importantly, the locations of CCH-evoked puffs closely co-localized with those of i-IP_3-evoked puffs in the same cell (Fig. 2B).

To analyze these spatial distributions, we measured distances from the centroid localization of every puff to that of every other puff in that cell. Fig. 2C plots the pooled distribution of these all-neighbor distances for CCH-evoked puffs in 7 cells. The distribution showed a sharp peak at distances < 1μm (shown on an expanded scale in the inset plot), which represents repeated puffs arising at the same, fixed sites, whereas the long 'tail' of the distribution represents distances between different puff sites (IP_3R clusters). A similar sharp peak was evident for i-IP_3-evoked puffs (Fig. 2D). Finally, Fig. 2E plots the distribution of distances between puffs evoked in the same cells by CCH and i-IP_3. Again, a sharp peak was evident at short distances, suggesting that both stimuli evoke Ca^{2+} release from the same clusters of IP_3Rs. Although the co-localization (width of the initial peak in the distribution) was slightly wider than for the self localizations (CCH to CCH and i-IP_3 to i-IP_3), that may have arisen from slight movement during the ~5 min recovery interval between trials with agonist application and photorelease.

Figs. 2F–J present a corresponding analysis of localizations of puffs evoked sequentially by BK and photorelease of i-IP_3. Similar to the results with CCH, puffs evoked by BK recurred at fixed, tightly localized sites (Fig. 2H), and co-localized with i-IP_3-evoked puffs at these sites (Fig. 2J); albeit with a wider spread than observed for co-localization between CCH- and i-IP_3-evoked puffs.

3.2 Spatiotemporal characteristics of puffs evoked by GPCR agonists and photoreleased IP_3

For each puff, the analysis software determined the peak amplitude of the fluorescence ratio change ($\Delta F/F_0$), the full duration at half maximum amplitude (FDHM), and the spatial spread of the fluorescence signal as full width at half maximal amplitude (FWHM), at the time of the peak signal[20]. Histograms showing the distributions of these parameters are plotted in Fig. 3 for puffs evoked by CCH and BK, in each case accompanied by the distributions of puffs evoked by photoreleased i-IP_3 in the same cells.

Puff amplitudes largely reflect the number of IP_3R channels that open during an event; in turn a function of the number of channels clustered at a puff site and the fraction of those that are activated by a given concentration of IP_3[21]. The amplitudes of puffs evoked by CCH (Fig. 3A) and BK (Fig. 3B) were, on average significantly smaller than those evoked by i-IP_3 (mean ± SD $\Delta F/F_0$ values, 0.60 ± 0.25 for CCH vs. 0.82 ± 0.33 for i-IP_3; 0.77 ± 0.55 BK vs. 1.05 ± 0.52 i-IP_3). However, these differences may largely be attributable to

differences in the resulting concentrations of IP_3, as the observed frequencies of puffs – a parameter that varies steeply with IP_3 concentration – were appreciably higher with photoreleased i-IP_3 (0.83 puffs per cell per s for CCH vs. 1.35 for i-IP_3: 0.54 for BK vs. 1.16 for i-IP_3).

The temporal durations (FDHM) and spatial widths (FWHM) of Ca^{2+} puffs evoked by stimulation with the GPCR agonists CCH or BK or by photorelease of i-IP_3 were closely similar.

4. Summary and Discussion

Our results indicate that local Ca^{2+} puffs evoked by photorelease of i-IP_3 from a caged precursor in SH-SY5Y neuroblastoma cells resemble closely those evoked in the same cells by activation of cell surface receptors by CCH and BK in terms of their locations, duration and spatial extent. Although GPCR-mediated cleavage of membrane phosphatidyl inositol bisphosphate by phospholipase C results in formation of diacylglycerol in addition to IP_3, we did not observe any differences in puff properties that might be attributable to that second messenger. We thus conclude that caged IP_3 provides an experimental system that mimics Ca^{2+} release evoked via GPCR-coupled formation of IP_3, while greatly facilitating the precise and reproducible control of the magnitude, timing and spatial localization of cytosolic IP_3.

The concentration of i-IP_3 generated by photorelease in our experiments would have been almost uniform throughout the cytosol. Therefore, the finding that puff sites (clusters of IP_3Rs) responding to CCH and BK map onto those seen with photoreleased i-IP_3 suggests that IP_3 formed by GPCR stimulation also distributes widely throughout the cytoplasm, and that the receptors to CCH and BK both access the same population of IP_3Rs. This conclusion differs from the notion of signaling nanodomains that have been proposed in neurons and other cell types, wherein highly localized gradients of IP_3 concentration activate IP_3Rs scaffolded in close proximity to GPCRs to confer selectivity and specificity in responses to different agonists [5,6,8,9]. Nevertheless, interactions between cell surface receptors and IP_3Rs may still be constrained even in cells lacking such nanodomains, albeit on a coarser scale. Although IP_3 has widely been considered to diffuse freely in the cytosol [10,11], we recently reported that its diffusion is strongly hindered in SH-SY5Y cells, reducing its range of action to a few μm [12]. Thus, different receptors might differentially access spatially separated populations of IP_3Rs if they were inhomogeneously distributed across the cell membrane on a scale of a few μm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- GPCR agonists and photoreleased i-IP_3 evoke Ca^{2+} puffs at overlapping sites.
- Puffs evoked by GPCR agonists and photoreleased i-IP_3 have similar kinetics and spatial spread.
- Photoreleased i-IP_3 faithfully mimics puffs evoked by the physiological IP_3 pathway.
- Muscarinic and bradykinin receptors access the same IP_3 receptors.

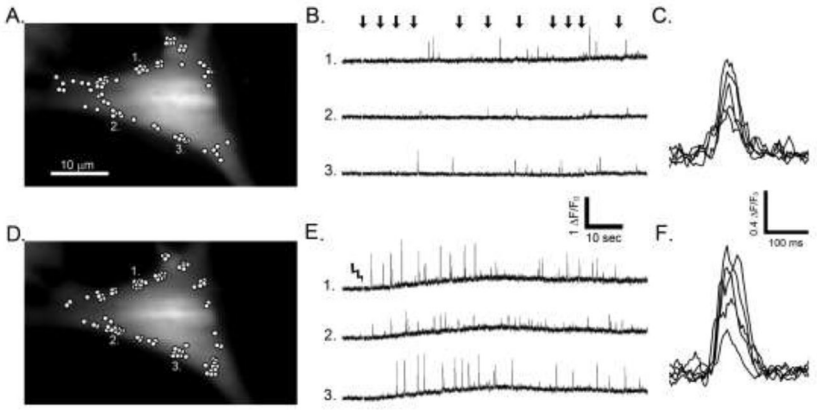

Fig. 1.

Experimental procedure. (**A**) The background-subtracted resting Cal-520 fluorescence of a single SH-SY5Y cell is shown overlaid with dots indicating the centroid localizations of puffs evoked by application of CCH. (**B)** Traces showing fluorescence ratio measurements ($\Delta F/F_0$) from regions of interest (~1 μm square) centered on the puff sites numbered in A. Arrows indicate times at which repeated applications of CCH were delivered by pneumatic pressure pulses. (**C**) Superimposed examples of puffs evoked by CCH, shown on an expanded timescale and aligned by their rising phases. (**E–F**) Corresponding records of puffs evoked in the same cell about 5 min later by flash photorelease of i-IP_3 using UV light uniformly illuminating the entire cell. The lightning symbol marks the time of the photolysis flash.

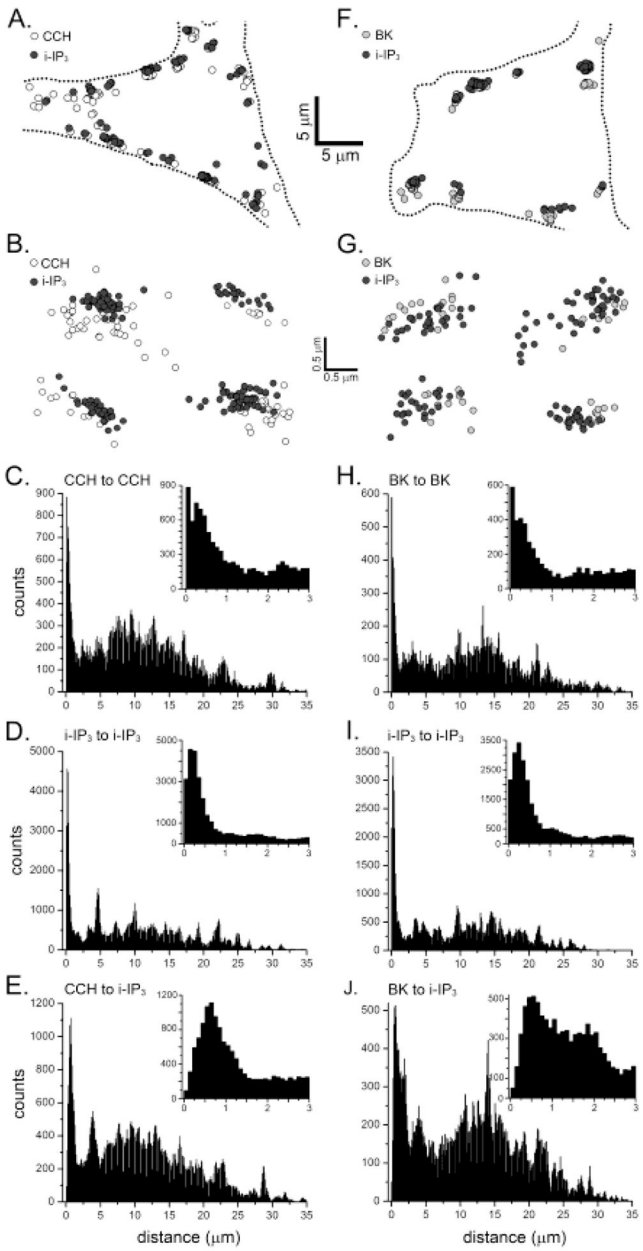

Fig. 2.

GPCR agonists and i-IP_3 evoke Ca^{2+} puffs at overlapping subcellular sites. (**A**) The dotted outline indicates the boundary of the same cell as in Fig. 1. Open circles mark the centroid localizations of puffs evoked by CCH, and filled circles mark localizations of puffs evoked by photoreleased i-IP_3. (**B**) Examples of localizations of CCH-evoked puffs (open circles) and i-IP_3-evoked puffs (filled circles) at four sites, shown on an expanded scale. (**C**) Distribution of all-neighbor distances between localizations of puffs evoked by CCH. The inset in this and other panels shows the same data re-plotted on an expanded distance scale. (**D**) Distribution of all-neighbor distances between localizations of puffs evoked by i-IP_3. (**E**) Distribution of all-neighbor distances between localizations of puffs evoked by CCH and

puffs evoked in the same cell by i-IP_3. Data in C–E are from 7 cells; 581 puffs evoked by CCH and 946 evoked by i-IP_3. (**F,G**) Localizations of puffs evoked by BK and i-IP_3 in a different cell. (**H–J**) Corresponding distributions of all-neighbor distances for puffs evoked by BK (**H**), i-IP_3 (**I**), and between BK- and i-IP_3-evoked puffs (**J**). Data are from 7 cells; 383 puffs evoked by BK and 809 evoked by i-IP_3.

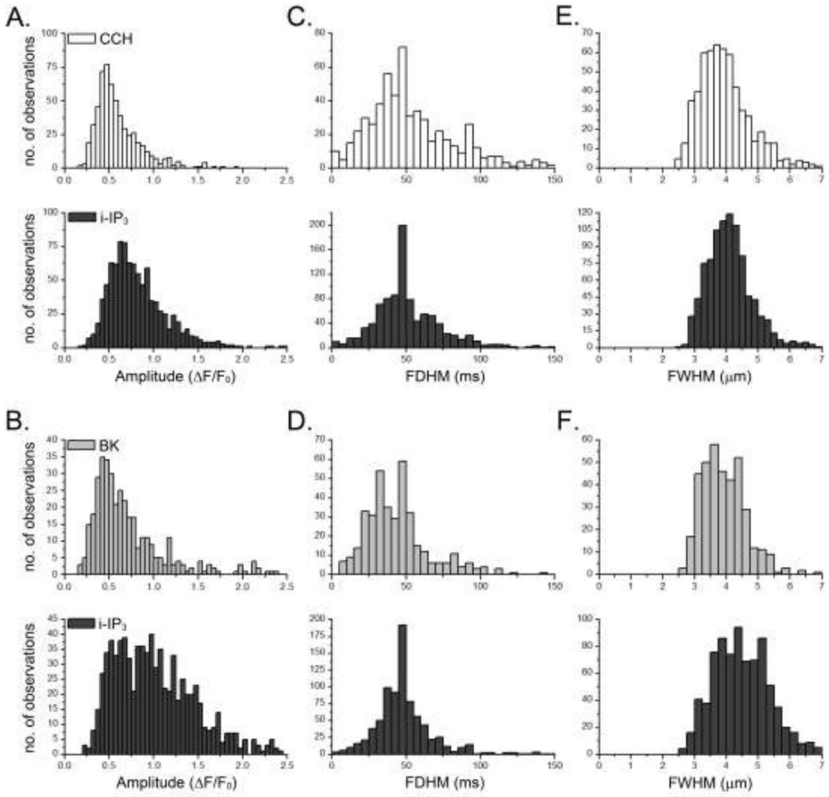

Fig. 3.

Comparison of Ca^{2+} puffs evoked by GPCR agonists with those evoked in the same cells by i-IP_3. Histograms show the distributions of puff amplitudes (peak $\Delta F/F_0$: **A,B**), full-duration at half-maximal amplitude (**C,D**), and full spatial width at half-maximal amplitude (**E,F**). In each panel, the upper histogram shows the distribution of puffs evoked by CCH (open bars) or BK (grey bars) and the lower histogram shows the distribution of puffs evoked in the same cells by photoreleased i-IP_3 (black bars). The numbers of cells and puffs analyzed are as stated in the legend to Fig. 2.