Microscopic properties of elementary Ca\textsuperscript{2+} release sites in non-excitatory cells

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Background: Elementary Ca\textsuperscript{2+} signals, such as Ca\textsuperscript{2+} puffs, that arise from the activation of clusters of inositol 1,4,5-trisphosphate (InsP\textsubscript{3}) receptors are the building blocks for local and global Ca\textsuperscript{2+} signalling. We previously found that one, or a few, Ca\textsuperscript{2+} puff sites within agonist-stimulated cells act as 'pacemakers' to initiate global Ca\textsuperscript{2+} waves. The factors that distinguish those pacemaker Ca\textsuperscript{2+} puff sites from the other Ca\textsuperscript{2+} release sites that simply participate in Ca\textsuperscript{2+} wave propagation are unknown.

Results: The spatiotemporal properties of Ca\textsuperscript{2+} puffs were investigated using confocal microscopy of fluo3-loaded HeLa cells. The same pacemaker Ca\textsuperscript{2+} puff sites were activated during stimulation of cells with different agonists. The majority of agonist-stimulated pacemaker Ca\textsuperscript{2+} puffs originated in a perinuclear location. The positions of such Ca\textsuperscript{2+} puff sites were stable for up to 2 hours, and were not affected by disruption of the actin cytoskeleton. A similar perinuclear distribution of Ca\textsuperscript{2+} puff sites was also observed when InsP\textsubscript{3} receptors were directly stimulated with thimerosal or membrane-permeant InsP\textsubscript{3} esters. Immunostaining indicated that the perinuclear position of pacemaker Ca\textsuperscript{2+} puff sites was not due to the localised expression of InsP\textsubscript{3} receptors.

Conclusions: The pacemaker Ca\textsuperscript{2+} puff sites that initiate Ca\textsuperscript{2+} responses are temporally and spatially stable within cells. Those Ca\textsuperscript{2+} release sites are distinguished from their neighbours by an intrinsically higher InsP\textsubscript{3} sensitivity.

Background

Stimulation of cells with hormones that activate the enzyme phospholipase C often evokes spatially and temporally complex intracellular calcium signals [1–5]. The link between phospholipase C and Ca\textsuperscript{2+} signalling is the intracellular messenger inositol 1,4,5-trisphosphate (InsP\textsubscript{3}), which diffuses from its site of production into the cytosol and binds to specific Ca\textsuperscript{2+}-mobilising channels (InsP\textsubscript{3} receptors) [2,6]. Hormone-evoked Ca\textsuperscript{2+} signals are commonly observed as Ca\textsuperscript{2+} waves, where an initial Ca\textsuperscript{2+} puff is followed by a wave that spreads throughout the cell in a saltatoric manner, reflecting the sequential activation of elementary Ca\textsuperscript{2+} release sites spaced ~6 µm apart [12,28].

When a cell is stimulated with a Ca\textsuperscript{2+}-mobilising hormone, there is usually a period of several seconds (the 'latency') before a global Ca\textsuperscript{2+} wave is observed. We previously found that the recruitment of Ca\textsuperscript{2+} puffs occurs during this latency, and that the cumulative activity of Ca\textsuperscript{2+} puffs provides the pacemaker Ca\textsuperscript{2+} rise necessary to trigger an ensuing regenerative response [13,27]. Once triggered, the Ca\textsuperscript{2+} wave spreads throughout the cell in a saltatoric manner, reflecting the sequential activation of elementary Ca\textsuperscript{2+} release sites spaced ~6 µm apart [12,28].

Surprisingly, in the majority of HeLa cells only one or a few 'pacemaker' Ca\textsuperscript{2+} puff sites are active during the latency, and the activity of these few individual sites determines whether a global Ca\textsuperscript{2+} wave or an abortive
response is evoked. Repetitive stimulation of a cell consistently recruits the same elementary Ca\textsuperscript{2+} release sites [13]. The factors that determine which of the elementary Ca\textsuperscript{2+} release sites act as the pacemaker are unknown. The consistent recruitment of the same pacemaker puff sites by repetitive stimulation is in accordance with earlier video imaging studies of Ca\textsuperscript{2+} signals in several cell types, which indicated that InsP\textsubscript{3}-dependent Ca\textsuperscript{2+} waves usually arise from a conserved cellular region [29–32]

In the present study, we examined the characteristics of Ca\textsuperscript{2+} puffs triggered by different Ca\textsuperscript{2+}-mobilising agonists and the spatiotemporal stability of the pacemaker Ca\textsuperscript{2+} puff sites in HeLa cells. Our data indicate that the same pacemaker sites were common to all Ca\textsuperscript{2+}-mobilising agonists, membrane-permeant InsP\textsubscript{3} esters and thimerosal, suggesting that the pacemaker puff sites have an intrinsic enhancement in sensitivity to InsP\textsubscript{3} compared to the other InsP\textsubscript{3} receptors, which simply participate in Ca\textsuperscript{2+} wave propagation.

**Results**

**Characteristics of elementary Ca\textsuperscript{2+} signals evoked by different Ca\textsuperscript{2+}-mobilising agonists**

HeLa cells express receptors for multiple agonists that mobilise Ca\textsuperscript{2+} from intracellular InsP\textsubscript{3}-sensitive stores. We examined the elementary Ca\textsuperscript{2+} signals evoked by three such agonists—histamine, acetylcholine and ATP. By empirically matching concentrations, we found that all three agonists evoked a similar response in individual cells and recruited the same pacemaker Ca\textsuperscript{2+} puff sites (Figures 1 and 2a–c). The cell shown in Figure 1, there was a single pacemaker Ca\textsuperscript{2+} puff site (Figure 1a), which responded to histamine and then subsequently to ATP. The similarity in spatial organisation of the elementary Ca\textsuperscript{2+} signals evoked by histamine or ATP is apparent in the line scans (Figure 1b) and the surface plots (Figure 1c). In the cell shown in Figure 2a–c, two pacemaker Ca\textsuperscript{2+} puff sites were activated in response to either acetylcholine or histamine.

**Recruitment of pacemaker Ca\textsuperscript{2+} puffs by direct stimulation of InsP\textsubscript{3} receptors**

The recruitment of Ca\textsuperscript{2+} puffs by ATP, acetylcholine or histamine from the same location and with identical spatiotemporal properties (Figures 1 and 2a–c), suggests that the characteristics of the elementary events are not determined by the stimulating agonist. To further examine the properties of Ca\textsuperscript{2+} puffs independently of receptor stimulation, we utilised a membrane-permeant InsP\textsubscript{3} ester (InsP\textsubscript{3}BM) [33]. Application of 10 μM InsP\textsubscript{3}BM ester (see Materials and methods) resulted in the activation of Ca\textsuperscript{2+} puffs in 53% of cells (n = 57) after a variable latency of usually 5–10 minutes (Figures 2d–f and 3a). The InsP\textsubscript{3}BM-induced Ca\textsuperscript{2+} release activity reversed within 30 minutes after washout of the ester (data not shown). The Ca\textsuperscript{2+} puffs triggered by InsP\textsubscript{3}BM occurred in exactly the same locations (Figure 2e) as those evoked after a 10 min recovery period. Similar results were obtained when the order of agonist addition was switched.
by a prior histamine application (Figure 2d), and with the same spatiotemporal characteristics (Figure 2f).

Another form of membrane-permeant InsP₃ ester, D-InsP₃PM (50 µM), also activated typical Ca²⁺ puffs (Figure 3b). In addition, incubation of the cells with either AlF₄⁻ (50 µM AlCl₃ + 50 mM NaF) to directly stimulate G-proteins, or the thiol-alkylating reagent thimerosal (1 µM), which sensitises InsP₃ receptors to the basal level of InsP₃ [34], triggered Ca²⁺ puffs and eventually led to regenerative global Ca²⁺ responses in all cells (Figure 3c,d). The inability of InsP₃BM and D-InsP₃PM to activate regenerative cytosolic Ca²⁺ signals (Figure 3a,b) was probably due to the fact that they could only modestly increase the steady-state levels of intracellular InsP₃ at the concentrations at which they were applied. In contrast, continuous stimulation with AlF₄⁻ or thimerosal would progressively enhance either the intracellular InsP₃ concentration or the sensitivity of InsP₃ receptors to the point at which regenerativity ensued.

Subcellular distribution of pacemaker Ca²⁺ puff sites and InsP₃ receptors

Previous studies revealed that the majority of histamine-induced Ca²⁺ puffs occur within 3 µm of the nuclear envelope [35]. A similar perinuclear localisation of Ca²⁺ puffs was observed following stimulation with AlF₄⁻, InsP₃BM or thimerosal (Figure 4a). There was no significant difference in the average distance of the Ca²⁺ puffs from the nuclear envelope in cells stimulated with these three reagents (Figure 4b). The average distance of Ca²⁺ puffs from the nucleus was statistically greater for thimerosal compared with all the other agonists. The similar perinuclear distribution of Ca²⁺ puffs evoked by InsP₃ esters, histamine or AlF₄⁻ suggests that localised InsP₃ production does not determine which elementary Ca²⁺ release sites will act as pacemakers.

We investigated the distribution of InsP₃ receptors in HeLa cells using isoform-specific antibodies, to examine the possibility that the localisation of pacemaker Ca²⁺ puffs around the nucleus arose because of an enhanced perinuclear expression of a particular type of InsP₃ receptor. Cells were stained with antibodies raised against InsP₃ receptor type 1 or type 3, which are the most abundant isoforms in HeLa cells (> 90% of total InsP₃ receptor mRNA; H. DeSmedt, J.B. Parys and L. Missiaen, personal communication). With each antibody, a decreasing gradient of staining was observed from the nuclear envelope to the plasma membrane (Figure 5a), indicating that the InsP₃ receptor density was highest in the perinuclear regions, and least at the cell periphery. The pattern of staining with both antibodies was generally continuous.
around the perinuclear and peripheral regions (Figure 5a). No distinctive patches of staining were observed within the cells. Comparison of the distributions of InsP3 receptor staining and histamine-evoked Ca2+ puffs (Figure 5b) indicated that the location of the pacemaker Ca2+ puffs did not correlate with the profile of InsP3 receptor staining. Although InsP3 receptor staining was highest in the region where the pacemaker Ca2+ puffs were observed, its profile with both anti-type-1 and anti-type-3 antibodies was significantly broader than the distribution of pacemaker Ca2+ puffs during histamine stimulation. Interestingly, the distribution of pacemaker Ca2+ puffs activated by thimerosal more closely resembled the profile of InsP3 receptor staining (Figure 5b).

**Effect of cytoskeletal disruption on the spatial stability of pacemaker Ca2+ puff sites**

Given that several studies have shown that the cytoskeleton can influence InsP3-mediated Ca2+ signalling [36,37], we investigated whether an interaction between cytoskeletal components and InsP3 receptors is responsible for the spatial stability of the pacemaker Ca2+ puff sites in HeLa cells. Treatment of cells with cytochalasin D (5 µM; 30 minute incubation) effectively depolymerised the microfilament network in HeLa cells. No cytosolic Ca2+ changes were associated with cytochalasin D treatment on its own (data not shown). Although prolonged treatment with cytochalasin D caused pronounced blebbing of the plasma membrane, and eventually led to the cells rounding-up and detaching from the glass coverslips (data not shown), the shape of the cells did not alter appreciably for 30 minutes after incubation with cytochalasin D. We therefore compared the location of elementary Ca2+ events in cells before and after cytochalasin D treatment.

Control cells, which were incubated for 30 minutes with vehicle alone, displayed repetitive Ca2+ puffs in response to pulsatile applications of histamine (Figure 6a). The location of the Ca2+ puffs was exactly the same for the first and fourth histamine stimulation (Figures 6c), indicating that these sites were spatially stable for a period of 90 minutes. In separate experiments, we continued histamine pulses for up to 2 hours and observed pacemaker Ca2+ puffs in the same locations (data not shown).

Cells treated with cytochalasin D showed similar responses to control cells (Figure 6b). All cells displayed Ca2+ puffs in response to histamine application and, most significantly, the location of the pacemaker Ca2+ puffs and their spatiotemporal characteristics remained the same (Figure 6c). These data indicate that the perinuclear localisation of the pacemaker Ca2+ puff sites is not dependent on an intact microfilament network. In addition, cytochalasin D treatment did not affect the sensitivity of the cells to histamine, refilling of the Ca2+ stores or the characteristics of global cytosolic Ca2+ signals (data not shown).

**Discussion**

The initiation of regenerative Ca2+ signals in agonist-stimulated cells depends on the progressive recruitment of Ca2+ puffs [13,38]. For many HeLa cells, a single Ca2+ puff site acts as the sole pacemaker for the initiation of global Ca2+ signals [13]. We previously observed that the pacemaker Ca2+ puff sites were largely distributed around the nucleus [35], but the mechanism causing the perinuclear positioning of such pacemaker Ca2+ puffs was unclear.

An obvious explanation for the perinuclear localisation of pacemaker Ca2+ puff sites would be the clustering of hormone receptors and/or phospholipase C in the proximity of the nucleus, leading to gradients of InsP3 concentration. Although InsP3 is highly diffusible inside...
cells [39], evidence has been presented that spatially restricted InsP3 concentrations are more than ten times higher than those in the bulk cytoplasm [40]. As histamine, AlF4−, thimerosal and two forms of membrane-permeant InsP2 ester all triggered Ca2+ puffs with largely perinuclear distributions (Figure 4), it is unlikely that localised InsP3 production causes pacemaker Ca2+ puffs to occur around the nucleus.

The non-decremental propagation of Ca2+ waves within HeLa cells (Figures 1 and 2a–c) [12] indicates that InsP3 receptors are expressed throughout the cytoplasm of these cells. The perinuclear localisation of pacemaker Ca2+ puffs could plausibly be due to clustering of InsP3 receptors around the nucleus. Immunostaining HeLa cells for InsP3 receptor types 1 and 3 did not reveal any distinct spots of InsP3 receptor expression around the nucleus (Figure 5a). The immunostaining did, however, reveal that the density of InsP3 receptor expression decreased with distance from the nucleus to the cell periphery (Figure 5b). If all elementary Ca2+ release sites had an equal chance of activation, such an increased density of InsP3 receptors around the nucleus could explain why pacemaker Ca2+ puffs are largely perinuclear.

The distribution of pacemaker Ca2+ puffs was spatially narrower than the profile of InsP3 receptors, however (Figure 5b). Furthermore, although the peak InsP3 receptor immunofluorescence was observed in the perinuclear region (Figure 5a), it was generally evenly distributed around the nucleus. As most cells employ only one single pacemaker Ca2+ puff site, and not multiple Ca2+ puff sites around the circumference of the nucleus, these data suggest that something other than InsP3 receptor density determines the perinuclear position of the initiation sites.

Our data indicate that neither InsP3 concentration gradients, nor localised expression of InsP3 receptors determines the perinuclear localisation of pacemaker Ca2+ puff sites. Our favoured explanation is that the perinuclear location of pacemaker Ca2+ puffs is due to an intrinsic difference in the sensitivity of the InsP3 receptors at those sites. Assuming that the pacemaker Ca2+ puff sites have a significantly higher sensitivity to InsP3 than their neighbours, it is obvious that such sites will be the first to respond during stimulation with any InsP3 receptor agonist.

Of all the Ca2+-mobilising agents used, only thimerosal triggered pacemaker Ca2+ puffs with a profile that matched the InsP3 receptor distribution (Figure 5b). The fact that thimerosal evoked a significantly broader distribution of Ca2+ puffs than the other stimuli is consistent with the suggestion that differences in sensitivity to InsP3 distinguish the pacemaker Ca2+ puffs from the rest of the InsP3 receptor clusters. Thimerosal causes Ca2+ release by sensitising InsP3 receptors to the basal level of InsP3 inside cells [34]. It is unlikely that thimerosal will discriminate between InsP3 receptors in any particular location, rather it will probably sensitize InsP3 receptors as it encounters them. The broad distribution of Ca2+ puffs observed with thimerosal indicates that elementary Ca2+ release sites other than those activated by hormones can act as pacemakers, but it is necessary to increase their sensitivity to InsP3 so that they respond.

By repetitively stimulating HeLa cells at regular intervals, we have found that the pacemaker Ca2+ puff sites have a fixed position for at least 2 hours. The spatial stability of these sites is not dependent upon an intact cytoskeleton. Furthermore, in contrast to earlier studies using other cell types in which cytochalasin D was found to either block Ca2+ entry [37] or Ca2+ release [36], depolymerisation of
the actin microfilaments in HeLa cells had no effect on the ability of hormones to release Ca²⁺ or activate Ca²⁺ entry (data not shown; see also [41]).

The consistent initiation of Ca²⁺ waves at a fixed subcellular region was first described in hepatocytes by Thomas and colleagues [9,29,42]. Their video-imaging studies revealed that different phospholipase C agonists and the oxidising agent tert-butyl hydroperoxide activated Ca²⁺ waves starting from a near-subplasmalemmal region. Similar observations of conserved initiation sites have since been made using other cell types [31,43]. In oligodendrocyte processes, Ca²⁺ signals were found to originate in specialised regions where calreticulin-containing endoplasmic reticulum, InsP₃ receptors and mitochondria are co-localised [44,45]. Interestingly, the presence of energised mitochondria is crucial for the activation of Ca²⁺ release at these sites [45]. In HeLa cells, mitochondria are unlikely to determine the pacemaker Ca²⁺ puff sites because they are distributed within a larger cellular area than that occupied by the pacemaker Ca²⁺ puff sites [35].

In polarised cells, such as pancreatic acinar cells or submandibular gland cells, Ca²⁺ signals have been observed to consistently originate in the apical regions away from the nucleus [46–48] where type 2 InsP₃ receptors are abundantly expressed [47,48]. Similar to the pacemaker Ca²⁺ puffs in HeLa cells, the apical initiation sites in pancreatic acinar cells or submandibular gland cells are distinguished by an intrinsically higher sensitivity to InsP₃. Differences in sensitivity of InsP₃ receptor clusters might therefore be a ubiquitous mechanism of determining which cellular regions behave as initiators. It should be pointed out, however, that the apical Ca²⁺ spikes observed in polarised cells are very different in kinetics and spatial extent [46–48] to the Ca²⁺ puffs in HeLa cells, indicating that the arrangement of InsP₃ receptors underlying these two types of initiation signal must be rather different.

**Conclusions**

Our data indicate that global Ca²⁺ signals are initiated through the activity of one or a few pacemaker Ca²⁺ puff sites. These pacemaker Ca²⁺ puff sites are temporally and spatially stable, and are distinguished by an enhanced sensitivity to InsP₃ in comparison to the remaining InsP₃ receptor clusters, which simply participate in Ca²⁺ wave propagation. The reason why such initiation sites are located around the nucleus is unclear, but it is not due to clustering of InsP₃ receptors or interactions of InsP₃ receptors with the actin cytoskeleton.

**Materials and methods**

**Imaging**

HeLa cell culture and preparation for imaging was performed as described previously [34]. The culture medium was replaced with an extracellular medium (EM) containing: 121 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, 1.8 mM CaCl₂, 6 mM NaHCO₃, 5.5 mM glucose and 25 mM HEPES pH 7.3. Cells were loaded with fluo-3 by incubation with 2 µM fluo-3 acetoxyethyl ester (Molecular Probes Inc.) for 30 min, followed by a 30 min de-esterification period. All incubations and experiments were carried out at room temperature (20–22°C). Confocal cell imaging was performed as described elsewhere [13]. Briefly, a single glass coverslip was mounted on the stage of a Nikon Diaphot inverted microscope attached to a Noran Odyssey or Oz laser-scanning confocal microscope, equipped with a standard argon-ion laser for illumination. Fluo-3 was excited using the 488 nm laser line, and the emitted fluorescence was collected at wavelengths >505 nm. Images were acquired using the confocal microscopes in image mode at frequencies between 7.5 and 30 Hz (frame size 256 x 256 pixels). Absolute values for Ca²⁺ were calculated according to the equation

\[ [Ca^{2+}] = K_d \frac{(f-f_{min})}{(f_{max}-f)} \]

where \( f_{min} \) and \( f_{max} \) were determined by permeabilising the cells with A23187 in the presence of 10 mM EGTA or 10 mM CaCl₂, respectively. The \( K_d \) of fluo-3 for Ca²⁺ inside HeLa cells was determined empirically to be 810 nM [49].
Spatial stability of pacemaker Ca\textsuperscript{2+} puff sites. (a) Activity of single pacemaker Ca\textsuperscript{2+} puff sites in cells repeatedly stimulated with histamine (a) without or (b) with cytochalasin D treatment. The upper traces in (a,b) illustrate the temporal profile of the Ca\textsuperscript{2+} puffs observed at the sites indicated by small circles in the cell images shown on the left. The large dashed circles indicate the position of the nucleus in each cell. The panels at the bottom of the Ca\textsuperscript{2+} traces in (a,b) denote the periods of stimulation with histamine and incubation with cytochalasin D. The spatial profile of the Ca\textsuperscript{2+} puffs denoted by 1 and 2 in (a) and by 1 and 3 in (b) are represented by the corresponding surface plots in 6b.

Cell stimulation

Cells were stimulated by continuous superfusion with EM supplemented with the hormones and chemicals described in the figures. Solutions were applied using a home-made solenoid-controlled perfusion system (ideal time = 0.5 s). The membrane-permeant InsP\textsubscript{3} esters were used as described earlier [33]. Essentially, the cells were continuously stimulated with 10 \textmu M of either racemic myo-inositol 1,4,5-trisphosphate (InsP\textsubscript{3}BM), D-myosin-1,4,5-trisphosphate (InsP\textsubscript{3}PM) or L-myosin-1,4,5-trisphosphate (InsP\textsubscript{3}PM). Both InsP\textsubscript{3}BM and D-InsP\textsubscript{3}PM enhanced Ca\textsuperscript{2+} release, whereas L-InsP\textsubscript{3}PM was ineffective (data not shown).

Immunocytochemistry

The polyclonal antibody used for detection of InsP\textsubscript{3} receptor type 1, which was raised against 15 amino acids in the carboxyl terminus [90], was donated by J.B. Payne (KUL, Leuven, Belgium). The monoclonal antibody against InsP\textsubscript{3} receptor type 3 was purchased from Transduction Laboratories.

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References


