Microscopic properties of elementary Ca^{2+} release sites in non-excitable cells

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Background: Elementary Ca^{2+} signals, such as 'Ca^{2+} puffs', that arise from the activation of clusters of inositol 1,4,5,-trisphosphate ($InsP_3$) receptors are the building blocks for local and global Ca^{2+} signalling. We previously found that one, or a few, Ca^{2+} puff sites within agonist-stimulated cells act as 'pacemakers' to initiate global Ca^{2+} waves. The factors that distinguish these pacemaker Ca^{2+} puff sites from the other Ca^{2+} release sites that simply participate in Ca^{2+} wave propagation are unknown.

Results: The spatiotemporal properties of Ca^{2+} puffs were investigated using confocal microscopy of fluo3-loaded HeLa cells. The same pacemaker Ca^{2+} puff sites were activated during stimulation of cells with different agonists. The majority of agonist-stimulated pacemaker Ca^{2+} puffs originated in a perinuclear location. The positions of such Ca^{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^{2+} puff sites was also observed when $InsP_3$ receptors were directly stimulated with thimerosal or membrane-permeant $InsP_3$ esters. Immunostaining indicated that the perinuclear position of pacemaker Ca^{2+} puffs was not due to the localised expression of $InsP_3$ receptors.

Conclusions: The pacemaker Ca^{2+} puff sites that initiate Ca^{2+} responses are temporally and spatially stable within cells. These Ca^{2+} release sites are distinguished from their neighbours by an intrinsically higher $InsP_3$ sensitivity.

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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^{2+} signalling is the intracellular messenger inositol 1,4,5-trisphosphate ($InsP_3$), which diffuses from its site of production into the cytosol and binds to specific Ca^{2+}-releasing channels ($InsP_3$ receptors) [2,6]. Hormone-evoked Ca^{2+} signals are commonly observed as Ca^{2+} waves, where an initial Ca^{2+} increase in a subcellular region triggers a regenerative propagation of the Ca^{2+} signal throughout the cell; a 'global' response [5,7–11].

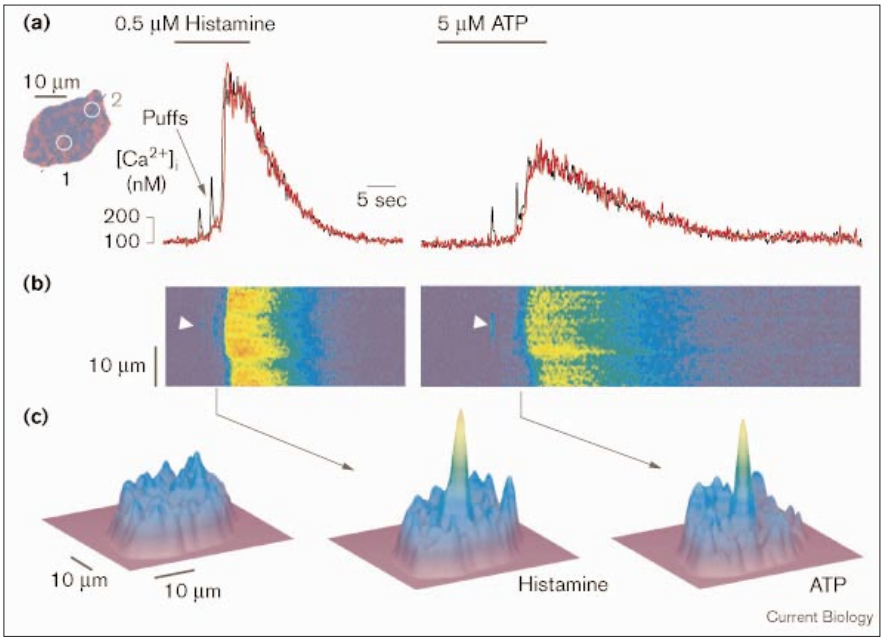
We previously found that the initiation and propagation of global Ca^{2+} signals in HeLa cells relies on the spatiotemporal recruitment of 'elementary' Ca^{2+} release events [12,13]. The amplitudes of these elementary Ca^{2+} signals typically range from ~15 to 600 nM, with a spatial spread of ~2–7 μm and a total duration of ~1 second [14]. Equivalent events, also arising from $InsP_3$ receptor activation, have been observed in *Xenopus* oocytes [15,16], PC12 cells [17,18] and endothelial cells [19]. Parker and colleagues denoted these localised $InsP_3$-receptor-dependent events as 'Ca^{2+} puffs' [15]. The non-stereotypic nature of Ca^{2+} puffs indicates that they arise from the activity of variable numbers of $InsP_3$ receptors [14,20].

Ca^{2+} signals analogous to Ca^{2+} puffs, but arising from ryanodine receptors and thereby denoted as 'Ca^{2+} sparks', have been observed in various muscle tissues [21–24]. Ca^{2+} sparks underlie excitation–contraction coupling in cardiac and skeletal muscle [25], and might play a role in controlling smooth muscle tone [26].

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

Surprisingly, in the majority of HeLa cells only one or a few 'pacemaker' Ca^{2+} puff sites are active during the latency, and the activity of these few individual sites determines whether a global Ca^{2+} wave or an abortive

Figure 1



Comparison of Ca^{2+} puffs stimulated by histamine or ATP in a single HeLa cell. **(a)** Traces show typical Ca^{2+} puffs and global Ca^{2+} transients recorded from two cellular locations following activation of the cell by histamine (left) or ATP (right). The Ca^{2+} puffs (arrow) can be seen at the onset of the global Ca^{2+} rise. The regions from which the traces were obtained are depicted in the inset cell image. The pacemaker Ca^{2+} puffs originate from position 1 in the inset. The black bars above mark the period of hormone addition. **(b)** Corresponding line-scan plots of the responses depicted in (a). The dashed line on the cell image in (a) shows the position of the scanned line. The Ca^{2+} puffs (white arrowhead) are visible at the front of the global Ca^{2+} responses, and were in the same positions for both ATP or histamine stimulation. **(c)** The spatial profiles of the Ca^{2+} puffs are depicted by surface plots. The events triggered by histamine and ATP are shown in the centre and right-hand images, respectively. The left-hand image shows the cell when it was unstimulated. The amplitude of the Ca^{2+} signals is coded in both the height and colour of the surface plots. The cell was stimulated with histamine first, and then by ATP after a 10 min recovery period. Similar results were obtained when the order of agonist addition was switched.

response is evoked. Repetitive stimulation of a cell consistently recruits the same pacemaker Ca^{2+} puff site [13]. The factors that determine which of the elementary Ca^{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^{2+} signals in several cell types, which indicated that $InsP_3$-dependent Ca^{2+} waves usually arise from a conserved cellular region [29–32]

In the present study, we examined the characteristics of Ca^{2+} puffs triggered by different Ca^{2+}-mobilising agonists and the spatiotemporal stability of the pacemaker Ca^{2+} puff sites in HeLa cells. Our data indicate that the same pacemaker sites were common to all Ca^{2+}-mobilising agonists, membrane-permeant $InsP_3$ esters and thimerosal, suggesting that the pacemaker puff sites have an intrinsic enhancement in sensitivity to $InsP_3$ compared to the other $InsP_3$ receptors, which simply participate in Ca^{2+} wave propagation.

Results

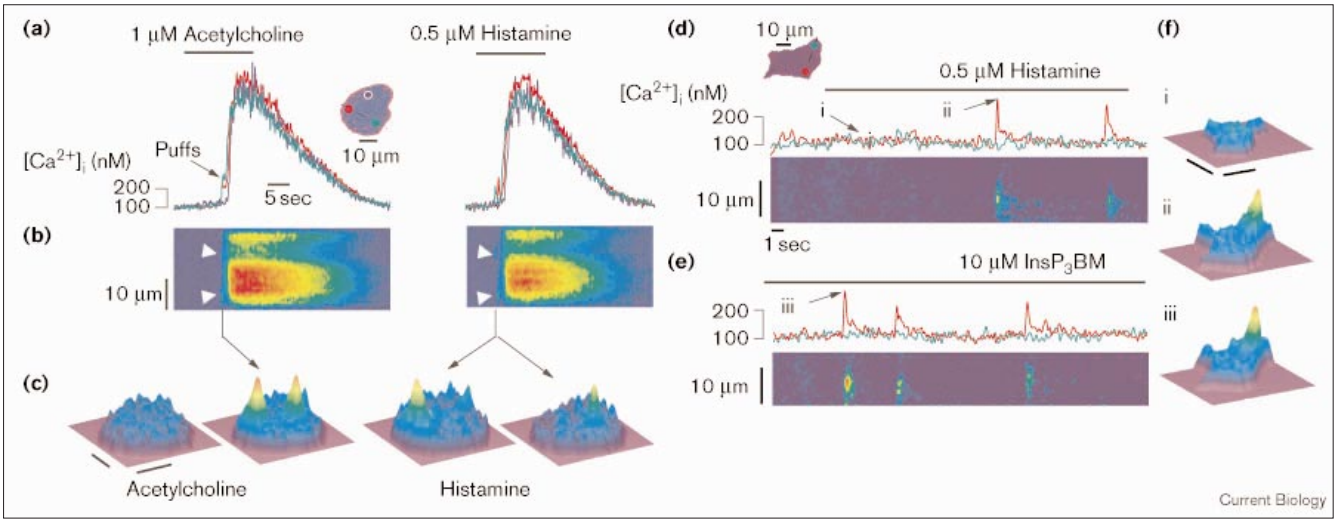
Characteristics of elementary Ca^{2+} signals evoked by different Ca^{2+}-mobilising agonists

HeLa cells express receptors for multiple agonists that mobilise Ca^{2+} from intracellular $InsP_3$-sensitive stores. We examined the elementary Ca^{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^{2+} puff sites (Figures 1 and 2a–c). In the cell shown in Figure 1, there was a single pacemaker Ca^{2+} puff site (Figure 1a), which responded to histamine and then subsequently to ATP. The similarity in spatial organisation of the elementary Ca^{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^{2+} puff sites were activated in response to either acetylcholine or histamine.

Recruitment of pacemaker Ca^{2+} puffs by direct stimulation of $InsP_3$ receptors

The recruitment of Ca^{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^{2+} puffs independently of receptor stimulation, we utilised a membrane-permeant $InsP_3$ ester ($InsP_3$BM) [33]. Application of 10 μM $InsP_3$BM ester (see Materials and methods) resulted in the activation of Ca^{2+} puffs in 53% of cells (n = 57) after a variable latency of usually 5–10 minutes (Figures 2d–f and 3a). The $InsP_3$BM-induced Ca^{2+} release activity reversed within 30 minutes after washout of the ester (data not shown). The Ca^{2+} puffs triggered by $InsP_3$BM occurred in exactly the same locations (Figure 2e) as those evoked

Figure 2



Spatiotemporal properties of Ca^{2+} puffs evoked by agonists and $InsP_3BM$. **(a–c)** A cell stimulated briefly with acetylcholine and then 10 min later with histamine has similar responses to those shown in Figure 1. The elementary Ca^{2+} release sites were activated simultaneously by acetylcholine (left-hand panels), but independently by histamine (right-hand panels). **(d–f)** The similarity in response of a single HeLa cell to histamine and $InsP_3BM$. Typical Ca^{2+} puffs activated either by histamine or $InsP_3BM$ in the same HeLa cell are shown. The traces were obtained by averaging the signals in the regions shown by the coloured circles on the cell image (inset in (d)), and line-scan plots were derived by scanning the region marked by the dashed line. The surface plots in (f) show the profiles of Ca^{2+} in the cell at rest (upper panel), and Ca^{2+} puffs triggered by histamine (middle panel) or $InsP_3BM$ (lower panel). The times at which cell images were captured to derive the surface plots in (f) are marked by the corresponding numerals in (d,e). The cell was first stimulated with histamine, and then allowed to recover for 15 min before application of $InsP_3BM$.

by a prior histamine application (Figure 2d), and with the same spatiotemporal characteristics (Figure 2f).

Another form of membrane-permeant $InsP_3$ ester, D-$InsP_3PM$ (50 μM), also activated typical Ca^{2+} puffs (Figure 3b). In addition, incubation of the cells with either AlF_4^- (50 μM $AlCl_3$ + 50 mM NaF) to directly stimulate G-proteins, or the thiol-alkylating reagent thimerosal (1 μM), which sensitises $InsP_3$ receptors to the basal level of $InsP_3$ [34], triggered Ca^{2+} puffs and eventually led to regenerative global Ca^{2+} responses in all cells (Figure 3c,d). The inability of $InsP_3BM$ and D-$InsP_3PM$ to activate regenerative cytosolic Ca^{2+} signals (Figure 3a,b) was probably due to the fact that they could only modestly increase the steady-state levels of intracellular $InsP_3$ at the concentrations at which they were applied. In contrast, continuous stimulation with AlF_4^- or thimerosal would progressively enhance either the intracellular $InsP_3$ concentration or the sensitivity of $InsP_3$ receptors to the point at which regenerativity ensued.

Subcellular distribution of pacemaker Ca^{2+} puff sites and $InsP_3$ receptors

Previous studies revealed that the majority of histamine-induced Ca^{2+} puffs occur within 3 μm of the nuclear envelope [35]. A similar perinuclear localisation of Ca^{2+} puffs was observed following stimulation with AlF_4^-, $InsP_3BM$ or thimerosal (Figure 4a). There was no significant difference in the average distance of the Ca^{2+} puffs from the nuclear envelope in cells stimulated with these three reagents (Figure 4b). In contrast, cells stimulated with thimerosal had a broader distribution of Ca^{2+} puffs (Figure 4b). The average distance of Ca^{2+} puffs from the nucleus was statistically greater for thimerosal compared with all the other agonists. The similar perinuclear distribution of Ca^{2+} puffs evoked by $InsP_3$ esters, histamine or AlF_4^- suggests that localised $InsP_3$ production does not determine which elementary Ca^{2+} release sites will act as pacemakers.

We investigated the distribution of $InsP_3$ receptors in HeLa cells using isoform-specific antibodies, to examine the possibility that the localisation of pacemaker Ca^{2+} puffs around the nucleus arose because of an enhanced perinuclear expression of a particular type of $InsP_3$ receptor. Cells were stained with antibodies raised against $InsP_3$ receptor type 1 or type 3, which are the most abundant isoforms in HeLa cells (> 90% of total $InsP_3$ 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 $InsP_3$ 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 $InsP_3$ receptor staining and histamine-evoked Ca^{2+} puffs (Figure 5b) indicated that the location of the pacemaker Ca^{2+} puffs did not correlate with the profile of $InsP_3$ receptor staining. Although $InsP_3$ receptor staining was highest in the region where the pacemaker Ca^{2+} puffs were observed, its profile with both anti-type-1 and anti-type-3 antibodies was significantly broader than the distribution of pacemaker Ca^{2+} puffs during histamine stimulation. Interestingly, the distribution of pacemaker Ca^{2+} puffs activated by thimerosal more closely resembled the profile of $InsP_3$ receptor staining (Figure 5b).

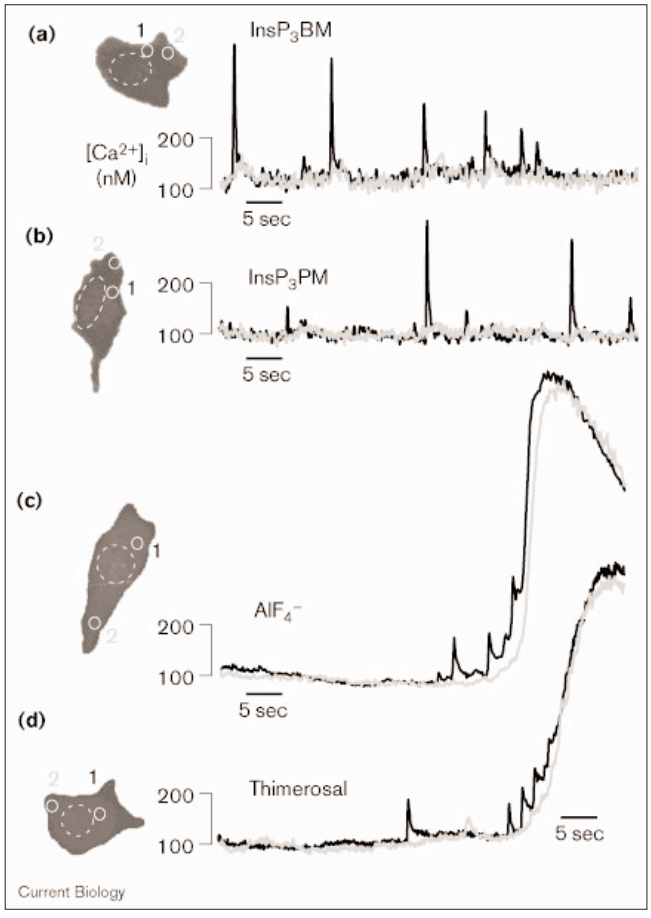
Effect of cytoskeletal disruption on the spatial stability of pacemaker Ca^{2+} puff sites

Given that several studies have shown that the cytoskeleton can influence $InsP_3$-mediated Ca^{2+} signalling [36,37], we investigated whether an interaction between cytoskeletal components and $InsP_3$ receptors is responsible for the spatial stability of the pacemaker Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} events in cells before and after cytochalasin D treatment.

Control cells, which were incubated for 30 minutes with vehicle alone, displayed repetitive Ca^{2+} puffs in response to pulsatile applications of histamine (Figure 6a). The location of the Ca^{2+} 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 Ca^{2+} puffs in the same locations (data not shown).

Cells treated with cytochalasin D showed similar responses to control cells (Figure 6b). All cells displayed Ca^{2+} puffs in response to histamine application and, most significantly, the location of the pacemaker Ca^{2+} puffs and their spatiotemporal characteristics remained the same (Figure 6c). These data indicate that the perinuclear localisation of the pacemaker Ca^{2+} 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 Ca^{2+} stores or the characteristics of global cytosolic Ca^{2+} signals (data not shown).

Figure 3



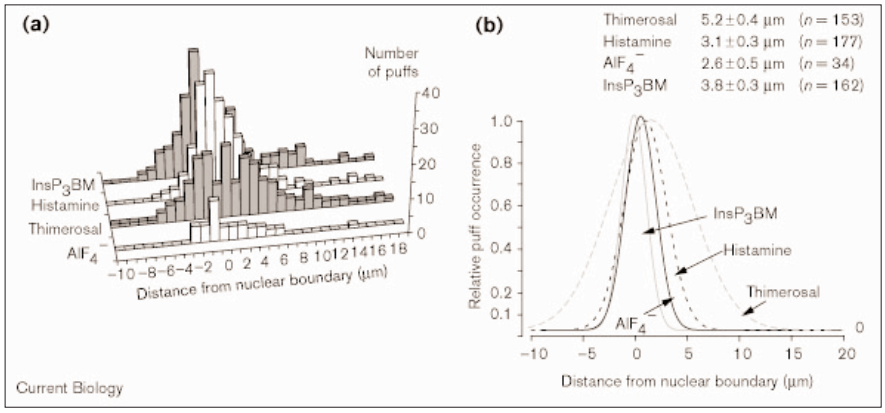
Ca^{2+} puffs and regenerative Ca^{2+} signals evoked by $InsP_3$ esters, AlF_4^- and thimerosal. The traces depict typical Ca^{2+} responses following stimulation of HeLa cells with **(a)** $InsP_3$BM, **(b)** D-$InsP_3$PM, **(c)** AlF_4^- or **(d)** thimerosal. The regions from which the traces were obtained are depicted in the cell images shown to the left. The large dashed circles in the cell images depict the position of the nuclei. The agonists were applied several minutes before the start of the recordings, and application was maintained for the duration of the traces.

Discussion

The initiation of regenerative Ca^{2+} signals in agonist-stimulated cells depends on the progressive recruitment of Ca^{2+} puffs [13,38]. For many HeLa cells, a single Ca^{2+} puff site acts as the sole pacemaker for the initiation of global Ca^{2+} signals [13]. We previously observed that the pacemaker Ca^{2+} puff sites were largely distributed around the nucleus [35], but the mechanism causing the perinuclear positioning of such pacemaker Ca^{2+} puffs was unclear.

An obvious explanation for the perinuclear localisation of pacemaker Ca^{2+} puff sites would be the clustering of hormone receptors and/or phospholipase C in the proximity of the nucleus, leading to gradients of $InsP_3$ concentration. Although $InsP_3$ is highly diffusible inside

Figure 4



Perinuclear position of pacemaker Ca^{2+} puffs stimulated by $InsP_3BM$, AlF_4^- or thimerosal. **(a)** Histograms show the distribution of pacemaker Ca^{2+} puffs relative to the nuclear envelope. In **(b)**, the data were normalised and fitted assuming a Gaussian distribution. The Ca^{2+} puffs that appeared to occur within the nucleus (that is, those with a negative distance from the nuclear envelope) probably arose from events occurring outside the nucleus above or below the plane of focus [35]. The averaged data (inset) were therefore calculated from only those events that had a positive distance from the nuclear envelope (data are mean ± SEM). Both the Gaussian fit to the entire data set and the averaged data indicate that thimerosal evoked a broader distribution of Ca^{2+} puffs than the other agonists. There was no statistically significant difference between the distributions of Ca^{2+} puffs triggered by histamine, $InsP_3BM$ or AlF_4^- (comparisons made using Student's *t*-test; GraphPad Instat). The distribution of thimerosal-evoked elementary events, however, was statistically different from all the other agonists ($p < 0.01$).

cells [39], evidence has been presented that spatially restricted $InsP_3$ generation can occur, leading to localised $InsP_3$ concentrations that are more than ten times higher than those in the bulk cytoplasm [40]. As histamine, AlF_4^-, thimerosal and two forms of membrane-permeant $InsP_3$ ester all triggered Ca^{2+} puffs with largely perinuclear distributions (Figure 4), it is unlikely that localised $InsP_3$ production causes pacemaker Ca^{2+} puffs to occur around the nucleus.

The non-decremental propagation of Ca^{2+} waves within HeLa cells (Figures 1 and 2a–c) [12] indicates that $InsP_3$ receptors are expressed throughout the cytoplasm of these cells. The perinuclear localisation of pacemaker Ca^{2+} puffs could plausibly be due to clustering of $InsP_3$ receptors around the nucleus. Immunostaining HeLa cells for $InsP_3$ receptor types 1 and 3 did not reveal any distinct spots of $InsP_3$ receptor expression around the nucleus (Figure 5a). The immunostaining did, however, reveal that the density of $InsP_3$ receptor expression decreased with distance from the nucleus to the cell periphery (Figure 5b). If all elementary Ca^{2+} release sites had an equal chance of activation, such an increased density of $InsP_3$ receptors around the nucleus could explain why pacemaker Ca^{2+} puffs are largely perinuclear. The distribution of pacemaker Ca^{2+} puffs was spatially narrower than the profile of $InsP_3$ receptors, however (Figure 5b). Furthermore, although the peak $InsP_3$ 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 Ca^{2+} puff site, and not multiple Ca^{2+} puff sites around the circumference of the nucleus, these data suggest that something other than $InsP_3$ receptor density determines the perinuclear position of the initiation sites

Our data indicate that neither $InsP_3$ concentration gradients, nor localised expression of $InsP_3$ receptors determines the perinuclear localisation of pacemaker Ca^{2+} puff sites. Our favoured explanation is that the perinuclear location of pacemaker Ca^{2+} puffs is due to an intrinsic difference in the sensitivity of the $InsP_3$ receptors at those sites. Assuming that the pacemaker Ca^{2+} puff sites have a significantly higher sensitivity to $InsP_3$ than their neighbours, it is obvious that such sites will be the first to respond during stimulation with any $InsP_3$ receptor agonist.

Of all the Ca^{2+}-mobilising agents used, only thimerosal triggered pacemaker Ca^{2+} puffs with a profile that matched the $InsP_3$ receptor distribution (Figure 5b). The fact that thimerosal evoked a significantly broader distribution of Ca^{2+} puffs than the other stimuli is consistent with the suggestion that differences in sensitivity to $InsP_3$ distinguish the pacemaker Ca^{2+} puffs from the rest of the $InsP_3$ receptor clusters. Thimerosal causes Ca^{2+} release by sensitising $InsP_3$ receptors to the basal level of $InsP_3$ inside cells [34]. It is unlikely that thimerosal will discriminate between $InsP_3$ receptors in any particular location, rather it will probably sensitise $InsP_3$ receptors as it encounters them. The broad distribution of Ca^{2+} puffs observed with thimerosal indicates that elementary Ca^{2+} release sites other than those activated by hormones can act as pacemakers, but it is necessary to increase their sensitivity to $InsP_3$ so that they respond.

By repetitively stimulating HeLa cells at regular intervals, we have found that the pacemaker Ca^{2+} 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 Ca^{2+} entry [37] or Ca^{2+} release [36], depolymerisation of

the actin microfilaments in HeLa cells had no effect on the ability of hormones to release Ca^{2+} or activate Ca^{2+} entry (data not shown; see also [41]).

The consistent initiation of Ca^{2+} 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 Ca^{2+} waves starting from a near-subplasmallemal region. Similar observations of conserved initiation sites have since been made using other cell types [31,43]. In oligodendrocyte processes, Ca^{2+} signals were found to originate in specialised regions where calreticulin-containing endoplasmic reticulum, $InsP_3$ receptors and mitochondria are co-localised [44,45]. Interestingly, the presence of energised mitochondria is crucial for the activation of Ca^{2+} release at these sites [45]. In HeLa cells, mitochondria are unlikely to determine the pacemaker Ca^{2+} puffs sites because they are distributed within a larger cellular area than that occupied by the pacemaker Ca^{2+} puff sites [35].

In polarised cells, such as pancreatic acinar cells or submandibular gland cells, Ca^{2+} signals have been observed to consistently originate in the apical regions away from the nucleus [46–48] where type 2 $InsP_3$ receptors are abundantly expressed [47,48]. Similar to the pacemaker Ca^{2+} puffs in HeLa cells, the apical initiation sites in pancreatic acinar cells or submandibular gland cells are distinguished by an intrinsically higher sensitivity to $InsP_3$. Differences in sensitivity of $InsP_3$ 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 Ca^{2+} spikes observed in polarised cells are very different in kinetics and spatial extent [46–48] to the Ca^{2+} puffs in HeLa cells, indicating that the arrangement of $InsP_3$ receptors underlying these two types of initiation signal must be rather different.

Conclusions

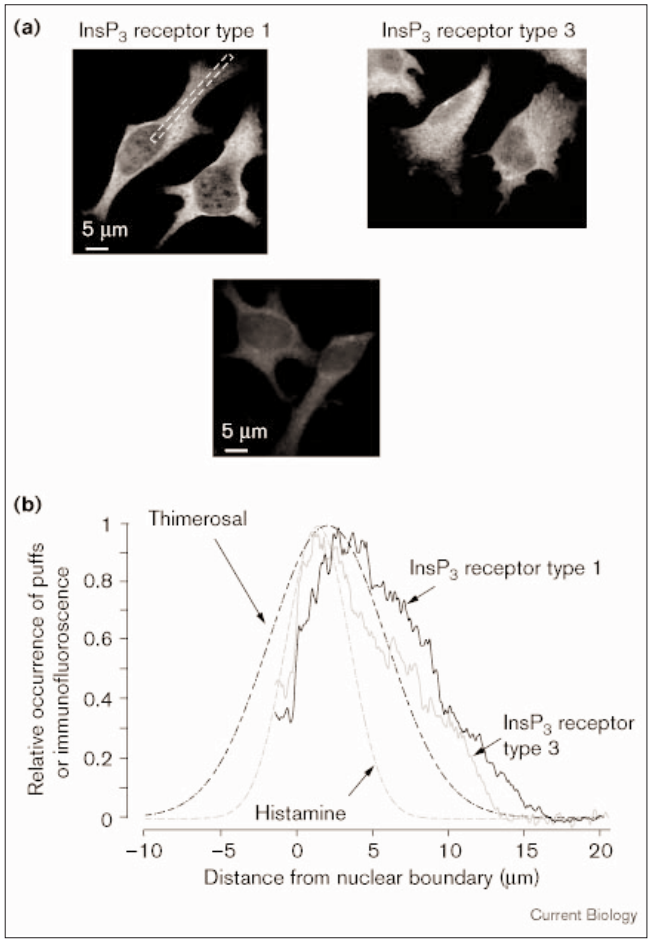
Our data indicate that global Ca^{2+} signals are initiated through the activity of one or a few pacemaker Ca^{2+} puff sites. These pacemaker Ca^{2+} puff sites are temporally and spatially stable, and are distinguished by an enhanced sensitivity to $InsP_3$ in comparison to the remaining $InsP_3$ receptor clusters, which simply participate in Ca^{2+} wave propagation. The reason why such initiation sites are located around the nucleus is unclear, but it is not due to clustering of $InsP_3$ receptors or interactions of $InsP_3$ receptors with the actin cytoskeleton.

Materials and methods

Imaging

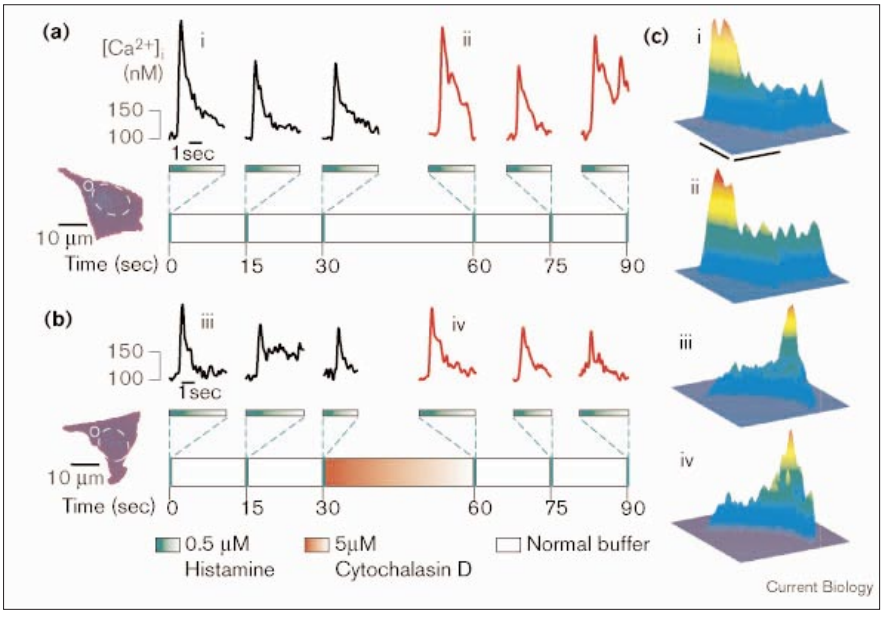
HeLa cell culture and preparation for imaging was performed as described previously [34]. The culture medium was replaced with an extracellular medium (EM) containing: 121 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 6 mM $NaHCO_3$, 5.5 mM glucose and 25 mM HEPES pH 7.3. Cells were loaded with fluo-3 by incubation with 2 μM fluo-3 acetoxymethyl 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 × 256 pixels). Absolute values for Ca^{2+}_i were calculated according to the equation $[Ca^{2+}] = K_d((f-f_{min})/(f_{max}-f))$, where f_{min} and f_{max} were determined by permeabilising the cells with A23187 in the presence of 10 mM EGTA or 10 mM $CaCl_2$, respectively. The K_d of fluo-3 for Ca^{2+} inside HeLa cells was determined empirically to be 810 nM [49].

Figure 5



Immunostaining and distribution profiles of $InsP_3$ receptors in HeLa cells. **(a)** Typical confocal images (z depth < 1 μm) of HeLa cells stained with an antibody against $InsP_3$ receptor type 1 (left) or type 3 (right). Typical background staining observed with the secondary antibody only is shown in the lower panel. To examine the profile of $InsP_3$ receptor distribution, the intensity of immunofluorescence staining in regions running perpendicular to the nuclear envelope (such as that shown by the dashed box in the left panel) were recorded. Averaged profiles for antibodies against $InsP_3$ receptor types 1 or 3 are shown in **(b)**. For comparison, the Gaussian fits to the distribution of the Ca^{2+} puffs evoked by histamine or thimerosal shown in Figure 4b are superimposed.

Figure 6



Spatial stability of pacemaker Ca^{2+} puff sites. **(a,b)** The activity of single pacemaker Ca^{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^{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^{2+} traces in (a,b) denote the periods of stimulation with histamine and incubation with cytochalasin D. The spatial profile of the Ca^{2+} puffs denoted by i and ii in (a), and by iii and iv in (b) are represented by the corresponding surface plots in **(c)**.

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 (dead time < 0.5 s). The membrane-permeant $InsP_3$ esters were used as described earlier [33]. Essentially, the cells were continually stimulated with 10 µM of either racemic *myo*-inositol 1,4,5-trisphosphate hexakis(butyryloxymethyl) ester ($InsP_3BM$), D-*myo*-inositol 1,4,5-trisphosphate hexakis(propionyloxymethyl) ester (D-$InsP_3PM$) or L-*myo*-inositol 1,4,5-trisphosphate hexakis(propionyloxymethyl) ester (L-$InsP_3PM$). Both $InsP_3BM$ and D-$InsP_3PM$ enhanced Ca^{2+} release, whereas L-$InsP_3PM$ was ineffective (data not shown).

Immunocytochemistry

The polyclonal antibody used for detection of $InsP_3$ receptor type 1, which was raised against 15 amino acids in the carboxyl terminus [50], was donated by J.B. Parys (KUL, Leuven, Belgium). The monoclonal antibody against $InsP_3$ receptor type 3 was purchased from Transduction Laboratories.

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References

1. Tsien RW, Tsien RY: **Calcium channels, stores, and oscillations.** *Annu Rev Cell Biol* 191, **6:**715-760.
2. Berridge MJ: **Inositol trisphosphate and calcium signalling.** *Nature* 1993, **361:**315-325.
3. Petersen OH, Petersen CCH, Kasai H: **Calcium and hormone action.** *Annu Rev Physiol* 1994, **56:**297-319.
4. Berridge MJ, Lipp P, Bootman MD: **Calcium signalling.** *Curr Biol* 1999, **9:**157-159.
5. Berridge MJ, Bootman MD, Lipp P: **Calcium — a life and death signal.** *Nature* 1998, **395:**645-648.
6. Taylor CW: **Inositol trisphosphate receptors: Ca^{2+}-modulated intracellular Ca^{2+} channels.** *Biochim Biophys Acta* 1998, **1436:**19-33
7. Bootman MD, Berridge MJ: **The elemental principles of calcium signalling.** *Cell* 1995, **83:**675-678.
8. Jacob R, Merritt JE, Hallam JE, Rink TJ: **Repetitive spikes in cytoplasmic calcium evoked by histamine in human endothelial cells.** *Nature* 1988, **335:**40-45.
9. Thomas AP, Renard DC, Rooney TA: **Spatial and temporal organisation of calcium signalling in hepatocytes.** *Cell Calcium* 1991, **12:**111-126.
10. Thomas AP, Bird GSJ, Hajnoczky G, RobbGaspers LD, Putney JW: **Spatial and temporal aspects of cellular calcium signaling.** *FASEB J* 1996, **10:**1505-1517.
11. Berridge MJ: **Elementary and global aspects of calcium signalling.** *J Physiol* 1997, **499:**291-306.
12. Bootman MD, Niggli E, Berridge MJ, Lipp P: **Imaging the hierarchical Ca^{2+} signalling system in HeLa cells.** *J Physiol* 1997, **499:**307-314.
13. Bootman MD, Berridge MJ, Lipp P: **Cooking with calcium; the recipes for composing global signals from elementary events.** *Cell* 1997, **91:**367-373.
14. Thomas D, Lipp P, Berridge MJ, Bootman MD: **Hormone-stimulated calcium puffs in non-excitable cells are not stereotypic, but reflect activation of different size channel clusters and variable recruitment of channels within a cluster.** *J Biol Chem* 1998, **273:**27130-27136.
15. Parker I, Yao Y: **Regenerative release of calcium from functionally discrete subcellular stores by inositol trisphosphate.** *Proc R Soc Lond* 1994, **246:**269-274.
16. Yao Y, Choi J, Parker I: **Quantal puffs of intracellular Ca^{2+} evoked by inositol trisphosphate in *Xenopus* oocytes.** *J Physiol* 1995, **482:**533-553.
17. Reber BFX, Schindelholz A: **Detection of a trigger zone of bradykinin-induced fast calcium waves in PC12 neurites.** *Pflügers Arch* 1996, **432:**893-903.
18. Koizumi S, Bootman MD, Bobanovic LK, Schell MJ, Berridge MJ, Lipp P: **Characterisation of elementary Ca^{2+} release signals in NGF-differentiated PC12 cells and hippocampal neurones.** *Neuron* 1999, **22:**125-137.
19. Hüser J, Blatter LA: **Elementary events of agonist-induced Ca^{2+} release in vascular endothelial cells.** *Am J Physiol* 1997, **42:**C1775-C1782.
20. Sun XP, Callamaras N, Marchant JS, Parker I: **A continuum of $InsP_3$-mediated elementary Ca^{2+} signalling events in *Xenopus* oocytes.** *J Physiol* 1998, **509:**67-80.
21. Cheng H, Lederer WJ, Cannell MB: **Calcium sparks — elementary events underlying excitation–contraction coupling in heart-muscle.** *Science* 1993, **262:**740-744.
22. Lipp P, Niggli E: **Modulation of Ca^{2+} release in cultured neonatal rat cardiac myocytes — insight from subcellular release patterns revealed by confocal microscopy.** *Circ Res* 1994, **74:**979-990.

23. Lopez-Lopez JR, Shacklock PS, Balke CW, Wier WG: **Local calcium transients triggered by single L-type calcium channel currents in cardiac cells.** *Science* 1995, **268:**1042-1045.
24. Arnaudeau S, Macrez-Lepretre N, Mironneau J: **Activation of calcium sparks by angiotensin II in vascular myocytes.** *Biochem Biophys Res Comm* 1996, **222:**809-815.
25. Lipp P, Niggli E: **A hierarchical concept of cellular and subcellular Ca^{2+} signalling.** *Prog Biophys Mol Biol* 1996, **65:**265-296.
26. Porter VA, Bonev AD, Knot HJ, Heppner TJ, Stevenson AS, Kleppisch T, *et al.*: **Frequency modulation of Ca^{2+} sparks is involved in regulation of arterial diameter by cyclic nucleotides.** *Am J Physiol* 1998, **43:**C1346-C1355.
27. Bobanovic F, Bootman MD, Berridge MJ, Parkinson NA, Lipp P: **Elementary $[Ca^{2+}]_i$ signals generated by electroporation functionally mimic those evoked by hormonal stimulation.** *FASEB J* 1999, **13:**365-376.
28. Callamaras N, Marchant JS, Sun XP, Parker I: **Activation and co-ordination of $InsP_3$-mediated elementary Ca^{2+} events during global Ca^{2+} signals in *Xenopus* oocytes.** *J Physiol* 1998, **509:**81-91.
29. Rooney TA, Sass EJ, Thomas AP: **Agonist-induced cytosolic calcium oscillations originate from a specific locus in single hepatocytes.** *J Biol Chem* 1990, **265:**10792-10796.
30. Bootman MD, Berridge MJ: **Subcellular Ca^{2+} signals underlying waves and graded responses in HeLa cells.** *Curr Biol* 1996, **6:**855-865.
31. Simpson PB, Mehotra S, Lange GD, Russell JT: **High density of endoplasmic reticulum proteins and mitochondria at specialised Ca^{2+} release sites in oligodendrocyte processes.** *J Biol Chem* 1997, **272:**22654-22661.
32. Morgan AJ, Jacob R: **Differential modulation of the phases of a Ca^{2+} spike by the store Ca^{2+}-ATPase in human umbilical vein endothelial cells.** *J Physiol* 1998, **513:**83-101.
33. Li W, Schultz C, Llopis J, Tsien RY: **Membrane-permeant esters of inositol polyphosphates, chemical syntheses and biological applications.** *Tetrahedron* 1997, **53:**12017-12040.
34. Bootman MD, Taylor CW, Berridge MJ: **The thiol reagent, thimerosal, evokes Ca^{2+} spikes by sensitizing the inositol 1,4,5-trisphosphate receptor.** *J Biol Chem* 1992, **267:**25113-25119.
35. Lipp P, Thomas D, Berridge MJ, Bootman MD: **Nuclear calcium signalling by individual cytoplasmic calcium puffs.** *EMBO J* 1997, **16:**7166-7173.
36. Ribeiro CMP, Reece J, Putney JW: **Role of the cytoskeleton in calcium signaling in NIH 3T3 cells — an intact cytoskeleton is required for agonist-induced $[Ca^{2+}]_i$ signaling, but not for capacitative calcium entry.** *J Biol Chem* 1997, **272:**26555-26561.
37. Holda JR, Blatter LA: **Capacitative calcium entry is inhibited in vascular endothelial cells by disruption of cytoskeletal microfilaments.** *FEBS Lett* 1997, **403:**191-196.
38. Marchant J, Callamaras N, Parker I: **Initiation of IP_3-mediated Ca^{2+} waves in *Xenopus* oocytes.** *EMBO J* 1999, **18:**5285-5299.
39. Allbritton NL, Meyer T, Stryer L: **Range of messenger action of calcium ion and inositol 1,4,5,-trisphosphate.** *Science* 1992, **258:**1812-1815.
40. Zhao H, Khademazad M, Muallem S: **Agonist-mediated Ca^{2+} release in permeabilised UMR-106-01 cells.** *J Biol Chem* 1990, **265:**14822-14827.
41. Patterson RL, van Rossum DB, Gill DL: **Store-operated Ca^{2+} entry: evidence for a secretion-like coupling model.** *Cell* 1999, **98:**487-499.
42. Rooney TA, Renard DC, Sass EJ, Thomas AP: **Oscillatory cytosolic calcium waves independent of stimulated inositol 1,4,5-trisphosphate formation in hepatocytes.** *J Biol Chem* 1991, **266:**12272-12282.
43. Yagodin SV, Holtzcalw L, Sheppard CA, Russell JT: **Nonlinear propagation of agonist-induced cytoplasmic calcium waves in single astrocytes.** *J Neurobiol* 1994, **25:**265-280.
44. Sheppard CA, Simpson PB, Nucifora FC, Ross CA, Lange GD, Russell JT: **Comparison of type 2 inositol 1,4,5-trisphosphate receptor distribution and subcellular Ca^{2+} release sites that support Ca^{2+} waves in cultured astrocytes.** *J Neurochem* 1997, **68:**2317-2327.
45. Simpson PB, Russell JT: **Mitochondria support inositol 1,4,5-trisphosphate-mediated Ca^{2+} waves in cultured oligodendrocytes.** *J Biol Chem* 1996, **271:**33493-33501.
46. Kasai H, Li YX, Miyashita Y: **Subcellular distribution of Ca^{2+} release channels underlying Ca^{2+} waves and oscillations in exocrine pancreas.** *Cell* 1993, **74:**669-677.
47. Yamamoto-Hino M, Miyawaki A, Segawa A, Adachi E, Yamashina S, Fujimoto T, *et al.*: **Apical vesicles bearing inositol 1,4,5-trisphosphate receptors in the Ca^{2+} initiation site of ductal epithelium of submandibular gland.** *J Cell Biol* 1998, **141:**135-142.
48. Lee MG, Xu X, Zeng W, Diaz J, Wojcikiewicz RJ, Kuo TH, *et al.*: **Polarised expression of Ca^{2+} channels in pancreatic and salivary gland cells. Correlation with initiation and propagation of $[Ca^{2+}]_i$ waves.** *J Biol Chem* 1997, **272:**15765-15770.
49. Tovey SC, Thomas D, Lipp P, Berridge MJ, Bootman MD: **Cytoplasmic and nucleoplasmic calibration of fluo-3 confirms the lack of nucleo-cytoplasmic Ca^{2+} gradients during stimulation of HeLa cells.** *J Physiol* 1998, **509P**.
50. Parys JB, de Smedt H, Missiaen L, Bootman MD, Sienaert I, Casteels R: **Rat basophilic leukemia cells as a model system for inositol 1,4,5-trisphosphate receptor IV, a receptor of the type II family: functional comparison and immunological detection.** *Cell Calcium* 1995, **17:**239-249.

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