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Nuclear calcium signalling by individual cytoplasmic calcium puffs

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It is known that the nucleoplasmic ionised calcium concentration (Ca_n) controls nuclear functions such as transcription, although the source and nature of the signals which modulate Ca_n are unclear. Using confocal imaging, we investigated the subcellular origin of Ca_n signals in Fluo-3-loaded HeLa cells. Our data indicate that all signals which increased Ca_n were of cytoplasmic origin. Ca_n was elevated during the propagation of global Ca waves within cells. More strikingly, we found that individual cytoplasmic elementary release events e.g. Ca puffs, evoked by physiological levels of stimulation, caused transient Ca_n increases. Significantly, >70% of all Ca puffs originated within a 2–3 μm perinuclear zone and propagated anisotropically across the entire nucleus. Due to the relatively slow relaxation of Ca_n transients compared with those in the cytoplasm, repetitive perinuclear Ca puffs were integrated into a 'staircase' of increasing Ca_n. Due to the effective diffusion of Ca in the nucleoplasm, the nucleus served as a 'Ca tunnel', distributing Ca to parts of the cytosol which were otherwise not within the cytoplasmic diffusion radii of Ca puffs. Given the close proximity of the majority of puff sites to the nucleus, it seems that the elementary Ca release system is designed to facilitate nuclear Ca signalling. Consequently, Ca-dependent regulation of nuclear function must be considered at the microscopic elementary level.

Keywords: calcium/inositol 1,4,5-trisphosphate/nucleus/puff/wave

Introduction

It is well established that many nuclear activities are modulated by Ca_n (reviewed in Clapham, 1995; Santella, 1996). However, the mechanism(s) leading to Ca_n changes have provoked intense debate. Recent experiments have suggested that the nuclear envelope (NE) is freely permeable to Ca and other ions (Gerasimenko *et al.*, 1996a; Santella, 1996). Nuclei can therefore obtain Ca via passive diffusion from Ca_{cyt} signals, particularly when global regenerative Ca waves traverse the cytoplasm (Al-Mohanna *et al.*, 1994; Carroll *et al.*, 1994; Shirakawa and Miyazaki, 1996).

In addition, it has been shown that the NE is a functional Ca store (Gerasimenko *et al.*, 1995; Stehno-Bittel *et al.*, 1995), and the inner NE membrane expresses both types of intracellular Ca release channel, i.e. inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$) and ryanodine receptors (RyRs) (Humbert *et al.*, 1996; Santella and Kyozuka, 1997). These observations, coupled with the intrinsic ability of nuclei to generate the Ca-releasing messenger inositol 1,4,5-trisphosphate ($InsP_3$) (Divecha *et al.*, 1991), suggest that they may be able to regulate Ca_n independently of Ca_{cyt}. In support of this independent nuclear Ca signalling scheme, it has been shown that introduction of Ca-mobilizing messengers into either the nucleus of intact cells or isolated nuclei, evokes Ca_n signals in the absence of Ca_{cyt} elevation (Gerasimenko *et al.*, 1995; Hennager *et al.*, 1995; Santella and Kyozuka, 1997).

Since nuclear functions may be differentially governed by Ca signals of either nuclear or cytoplasmic origin (Hardingham *et al.*, 1997), it is important to know the precise origin of such signals. In addition to the lack of clarity with respect to the source of Ca for nuclear signalling, the spatial and temporal aspects of Ca_n signals are unclear. Several studies have shown that Ca_n either rapidly follows Ca_{cyt}, or increases after a temporal delay presumably imposed by the NE. The relaxation of Ca_n parallels the recovery of Ca_{cyt} in some (Allbritton *et al.*, 1994), but not all cells (Al-Mohanna *et al.*, 1994).

Cytoplasmic Ca signals result from spatially and temporally co-ordinated recruitment of subcellular Ca release units, such as 'Ca puffs' (Bootman and Berridge, 1995; Lipp and Niggli, 1996; Berridge, 1997). These units represent the elementary building-blocks of Ca signalling, and arise from the opening of either single $InsP_3Rs$ or RyRs, or clusters of these channels. Such elementary Ca release events are short-duration highly localized signals (Parker and Yao, 1991; Bootman and Berridge, 1995; Lipp and Niggli, 1996), which dissipate rapidly due to diffusion in the cytoplasm and sequestration into the intracellular stores.

In the present study, we used laser-scanning confocal microscopy of Fluo-3-loaded HeLa cells, in conjunction with stimuli below the threshold for triggering global regenerativity, to investigate the origin of Ca signals that influenced Ca_n. In particular, we examined whether elementary Ca release signals, such as puffs, affected Ca_n, and whether these elementary signals were triggered in the nucleus, thus providing evidence for Ca_n releasing units similar to those in the cytoplasm. Furthermore, we investigated the characteristics of Ca_n signals relative to those in the cytoplasm, and the interplay between nucleoplasmic and cytoplasmic Ca signals.

Results

Signalling into the nucleus via global Ca waves

HeLa cells produce a graded response to different levels of hormonal stimulation. With supra-threshold histamine

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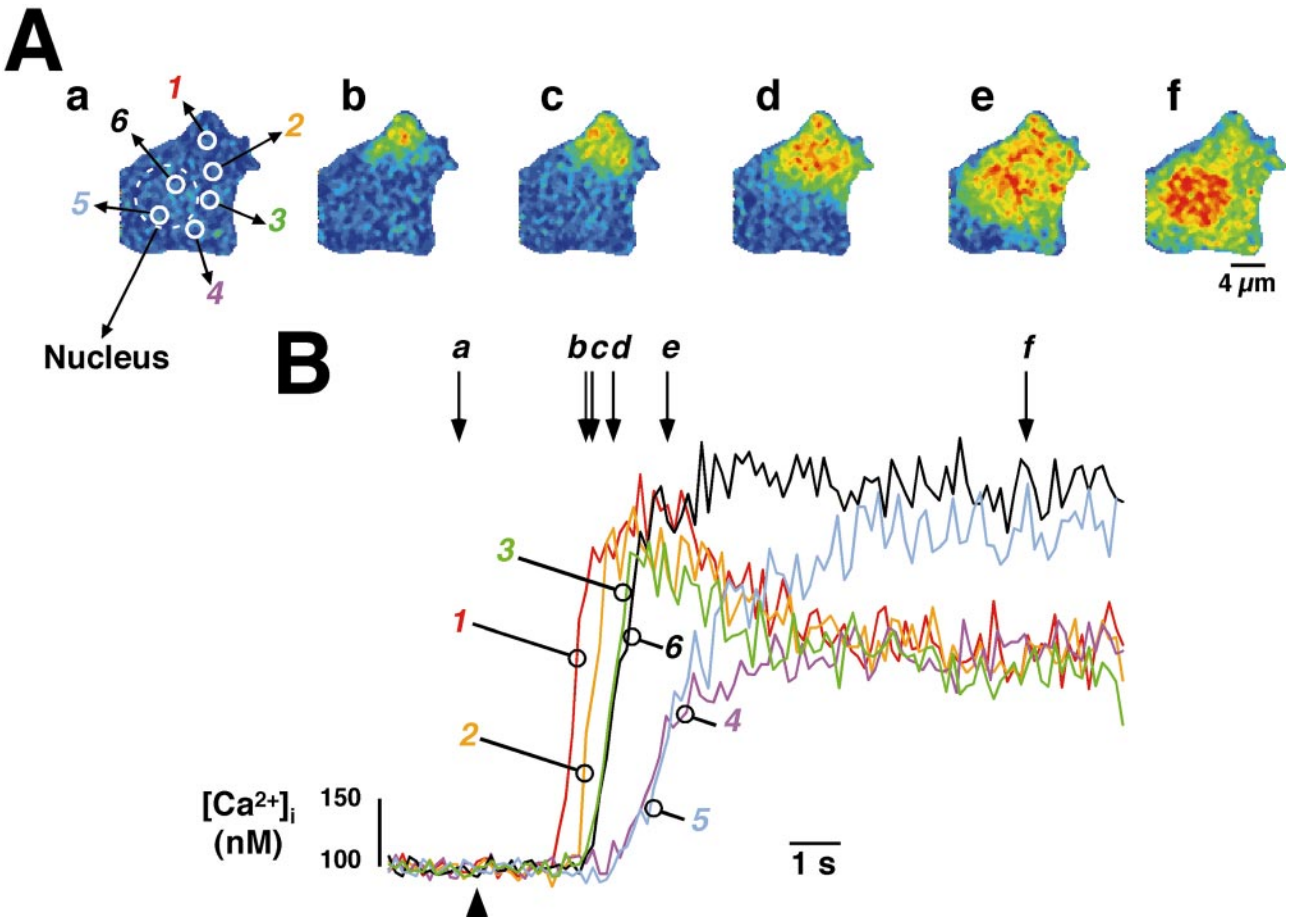

Fig. 1. Global Ca signals increase Ca_n. (**A**) and (**B**) show the spatial and temporal characteristics of a HeLa cell Ca wave triggered by 10 μM histamine (applied at the arrowhead in B). The sequence of confocal images in (A) shows the spatial spread of the Ca wave through nuclear (dashed white circle in Aa) and cytoplasmic compartments. The Ca signal in the regions outlined in (Aa) are plotted in (B), with corresponding colours and numbers. The traces show that Ca_n in the nuclear regions (5 and 6) increases with similar kinetics to the adjacent cytoplasmic regions (3 and 4), but Ca_n showed a more prolonged plateau. The times at which the cell images in (A) were taken are shown by the vertical arrows in (B).

concentrations, regenerative Ca waves are evoked, which originate from a consistent cytoplasmic locus and propagate throughout the cells (Bootman and Berridge, 1996; Bootman *et al.*, 1997). Such global signals invariably induce a rise of Ca_n, with kinetics resembling the spreading of Ca_{cyt} (Figure 1). Our previous analysis of Ca wave spreading, using a more rapid line-scanning approach, did not reveal any lag of the Ca wave in the nuclear region (Bootman *et al.*, 1997).

Signalling into the nucleus via elementary signals

With sub-threshold stimulation of HeLa cells, only abortive responses, comprising elementary events such as Ca puffs are observed (Bootman *et al.*, 1997a,b). We investigated the subcellular location of hormone-stimulated Ca puffs in the HeLa cells. Ca puff activity was largely observed around the nucleus (Figure 2A), and did not correlate with other cellular membranes, such as the plasma membrane. The data indicate that Ca puffs are distributed in a Gaussian manner within the perinuclear space; over 70% of all Ca puffs (n = 233 puff sites from 110 cells, with each site counted only once) originated within 2–3 μm of the NE.

Since the average distance of Ca puffs from the NE (~2–3 μm) is less than the spatial spread of such elementary signals (~4–6 μm), the close apposition of Ca puffs to the nucleus could allow them to influence Ca_n. We therefore examined the effect of such perinuclear Ca puffs on Ca_n. The sequences of confocal images in Figure 2B shows the spatial properties of Ca puff that originated either remote from the nucleus (Figure 2Ba) or in the perinuclear region (Figure 2Bb). The Ca puff remote from the nucleus displayed spatial and temporal characteristics similar to those previously observed (Yao *et al.*, 1995; Bootman *et al.*, 1997a,b). However, the perinuclear Ca puff spread rapidly into the entire nucleus and, as a consequence, the Ca signal originating from the initial Ca puff grew from ~3% to 30% of the cell volume.

Using the fluorescent stain $DiOC_6$, we investigated the morphology of the intracellular membranes within HeLa cells, to see if the clustering of elementary Ca release sites around the nucleus was due to a differential distribution of intracellular organelles. $DiOC_6$ stained various intracellular membranes including endoplasmic reticulum (ER), NE and mitochondria (Figure 3A; see Terasaki *et al.*, 1984, 1986). Although the very outermost parts of some cells did not take up the stain, suggesting that these areas were devoid of significant lipid membrane, the extent of $DiOC_6$ staining observed in thin confocal cell sections was

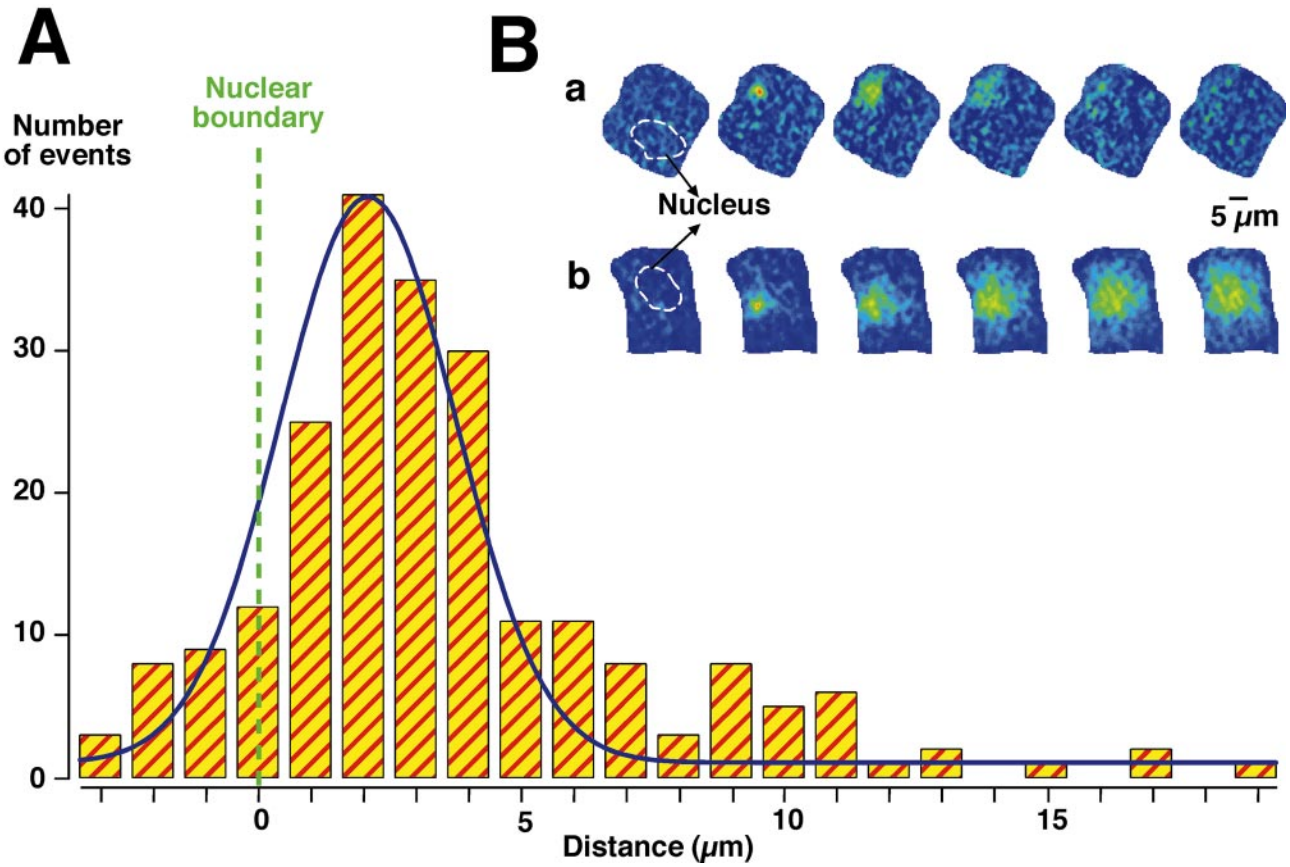

Fig. 2. HeLa cell Ca puffs are distributed around the nuclear envelope. HeLa cells were stimulated with threshold histamine concentrations (0.5–1 μM) to evoke elementary Ca release events. The histogram in (**A**) describes the occurrence of such release signals (n = 233) in 1 μm wide sections either side of the nuclear envelope. The dashed vertical line indicates the relative position of the nuclear envelope. The curve was fitted assuming a Gaussian distribution (full-width at half maximal amplitude ~3.8 μm; peak at 2 μm from the nuclear envelope). (**B**) depicts a time-series of confocal images (acquisition rate 7.5 Hz) showing a remote Ca puff (Ba, ~6 μm from nucleus) and a perinuclear Ca puff (Bb, ~2 μm from nucleus). The Ca signal resulting from the perinuclear Ca puff displayed a longer time course and occupied a larger volume in comparison with the remote release event.

qualitatively comparable in the perinuclear region (Figure 3B) and the cell periphery (Figure 3C). Since the ER is believed to be the major storage site for Ca used in signalling, these observations suggest that the close proximity of Ca puffs to the nucleus in HeLa cells is not due to a differential distribution of the intracellular Ca stores. Normalizing the Ca puff distribution for the thickness of the HeLa cells, indicated that the perinuclear localization of the release sites was not due simply to the thickness of the cytoplasm around the nucleus (data not shown). The $DiOC_6$ staining also revealed that mitochondria were often closely grouped around the nucleus, and were only sparsely distributed around the cell periphery (Figures 3A and B).

Diffusion of Ca from puff sites into the nucleus increases their duration and disperses Ca around the perinuclear area

Typical time-courses for perinuclear (Figure 4a) and remote Ca puffs (>6 μm distant; Figure 4b) from two different cells are displayed in Figure 4. The perinuclear Ca puffs persisted approximately ten times longer than the remote signals (compare the perinuclear Ca puffs in Figure 4A, trace 1 with the remote events in Figure 4B, trace 1). The rising phases of the perinuclear and remote puffs were similar. Only the relaxation of the signal was affected by the close proximity to the nucleus. Subtraction of the Ca_n signal (Figure 4A, trace 3) from that at the Ca puff site (Figure 4A, trace 1), restores the time-course of the Ca_{cyt} signal to that of a remote Ca puff (trace marked '1–3' in Figure 4A).

Although the time-courses of the perinuclear Ca puffs were exaggerated in comparison with the remote events, the most striking difference was in the relative contribution of the puffs to Ca_n. Whilst the remote Ca puffs failed to affect Ca_n (Figure 4B, trace 2), the perinuclear Ca puffs rapidly elevated Ca_n (Figure 4A, traces 2 and 3). The rate of rise and amplitude of the Ca_n signal was greatest in the nucleoplasmic region immediately adjacent to the Ca puff site. Ca_n was also elevated on the opposite side of the nucleus (Figure 4A, trace 3), but the response was smaller, with a slower rise time and occurred with a lag. This Ca_n gradient is consistent with the simple diffusion of Ca from one side of the nucleus to the other.

The spreading of the Ca puff into the nucleus also provoked a general perinuclear Ca increase. For example, Figure 4A, trace 4 shows that there was a modest elevation of Ca_{cyt} in the perinuclear space on the opposite side of the nucleus ~10 μm away from the original Ca puff. Monitoring Ca_{cyt} in a region ~10 μm from the perinuclear Ca puff but in the opposite direction to the nucleus (Figure 4A, trace 5), revealed that the Ca puff could not diffuse

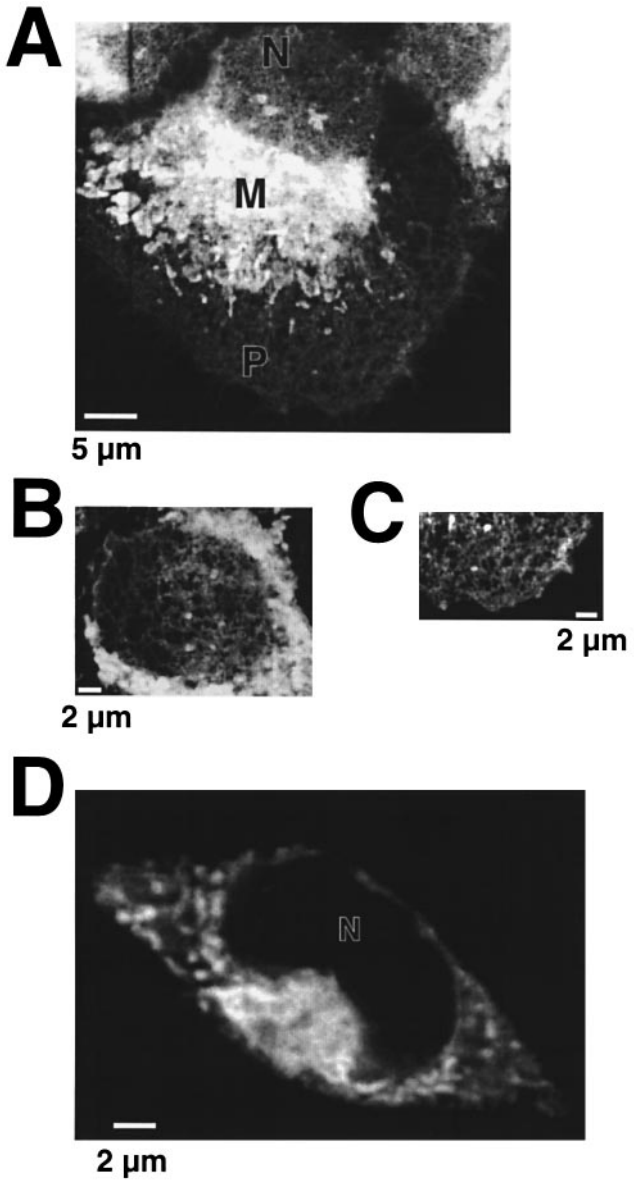

Fig. 3. Confocal images of $DiOC_6$-stained HeLa cells. (**A**) shows the membranous structures stained by $DiOC_6$. The nuclear region (N), mitochondria (M) and cell periphery (P) are distinctively stained regions. Higher magnification of the ER in perinuclear or peripheral areas (**B** and **C** respectively), reveals that the ER has a similar density and structure in such a thin (~1 μm) confocal section. Note that in (B), the nucleus is surrounded by many strongly-fluorescent mitochondria. (**D**) illustrates a typical nuclear invagination. The nucleus (N) appears dark since the confocal plane was approximately mid-way through the nucleus. The cytosolic region of the nuclear invagination contained numerous mitochondria.

the same distance through the cytoplasm. The long time-course and greater volume occupied by the perinuclear puffs consequently caused them to have a significant effect on the averaged 'global' signal (Figure 4A, trace 7), whereas the remote puffs had an almost negligible effect on the global signal (Figure 4B, trace 4).

'Mode-switching' of Ca puff diffusion into the nucleus

Although elementary Ca release sites had to be within a few micrometers of the NE to modulate Ca_n, nuclear proximity was not the sole determinant of their ability to enhance Ca_n. The cell response illustrated in Figure 5A, trace 1, for example, shows a cell responding to histamine stimulation with a train of Ca puffs originating from a perinuclear location ~3 μm from the NE. The time-course of each Ca puff varied substantially (Figure 5A, trace 1). The first two Ca puffs displayed a duration of $<$500 ms at half-maximal amplitude, typical of purely cytoplasmic events. The subsequent Ca puffs originating from the same site persisted for several seconds. Even though the amplitude of the Ca puffs changed only slightly, the two short-lived Ca puffs had a modest effect on Ca_n (Figure 5A, trace 2), and the global Ca signal (Figure 5A, trace 4), compared with the latter Ca puffs. The relative contribution of the cytosolic Ca puffs to Ca_n is depicted in Figure 5B, which shows that the ratio of Ca_n to Ca_{cyt} for each of the four Ca puffs shown in Figure 5A, trace 1, increased more than 2-fold.

The spatially restricted spreading of the Ca puffs (Figure 5A, trace 3) shows that none of them evoked a global Ca wave. Instead, the diffusibility of Ca from the perinuclear Ca puff site into the nucleus altered during the response, so that for the same increase of Ca_{cyt} a higher Ca_n was attained. Analysis of the rising phase of the Ca_n signals resulting from the second and third Ca puffs (marked by the black and red stars in Figure 5A, trace 2) revealed that the Ca_n signal resulting from the third Ca puff displayed an inflexion in its upstroke (Figure 5C), consistent with a sudden change in the permeability of the NE to Ca. Such an inflexion was not present in the rising phase of the cytoplasmic Ca puff (Figure 5A, trace 1, puff c).

Integration of elementary Ca release events in the nucleus

Between low frequency Ca puffs, both Ca_n and Ca_{cyt} returned to their resting levels (e.g. Figure 4A). A consequence of the long relaxation time for Ca_n was that when a train of perinuclear Ca puffs occurred with sufficient frequency (Figure 6, trace 1), their individual contributions were progressively summated, producing a 'staircase' of increasing Ca_n (Figure 6, trace 2). In a cytoplasmic region on the opposite side of the nucleus, Ca_{cyt} was slowly elevated without step-like increases (Figure 6, trace 3). As the response proceeded, both Ca_n and Ca_{cyt} increased following each Ca puff, until the cell reached threshold to trigger a regenerative Ca wave. Subtraction of the Ca_n signal (Figure 6, trace 2) from that at the puff site (Figure 6, trace 1) reduces the Ca_{cyt} response to a series of rapid release transients (Figure 6, trace marked '1–2'), with time-courses similar to remote Ca puffs.

Discussion

The aims of the present study were to examine the subcellular source of Ca used for nuclear signalling, and in particular whether Ca_n was influenced by elementary Ca release events originating either in the nucleus or cytoplasm. Global cytoplasmic Ca waves penetrated into the nucleus without a detectable delay (Figure 1). A similar rapid transfer of Ca_{cyt} to Ca_n has been reported in previous studies (Burnier *et al.*, 1994; O'Malley, 1994),

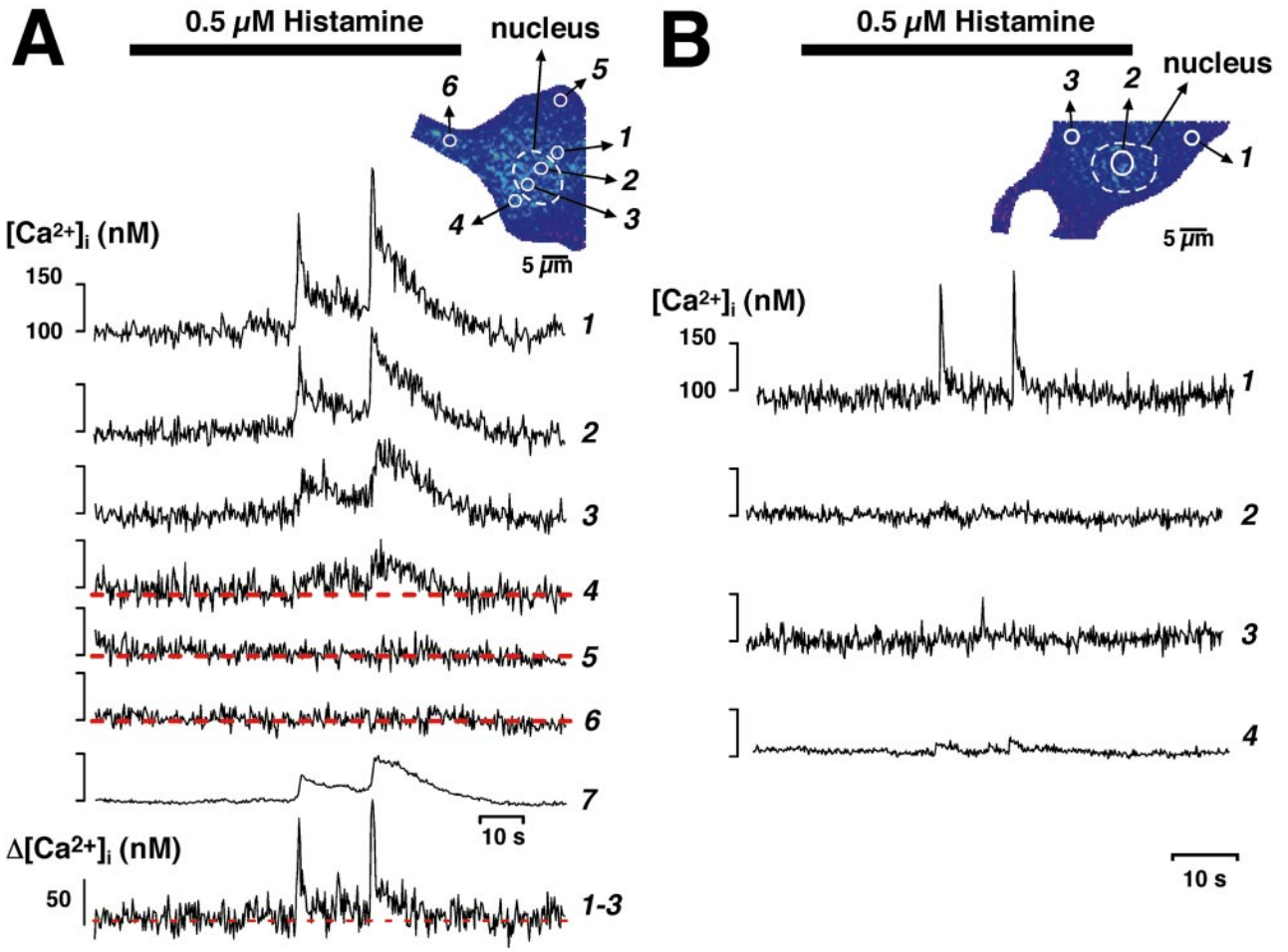

Fig. 4. Comparison of perinuclear and remote Ca^{2+} puffs**.** Stimulation of HeLa cells with 0.5 μM histamine evoked elementary release events from either perinuclear (**A**) or remote (**B**) locations. (A), The individual traces show the time-course of Ca at (1) the Ca puff site, (2) an adjacent region inside the nuclear envelope, (3) opposite side of the nucleus, (4) cytoplasmic region on the far side of the nucleus, (5) and (6) remote cytoplasmic sites and (7) the 'global' level obtained by averaging across the entire confocal section. (1–3) shows a trace resulting from a subtraction of the nucleoplasmic signal (trace 3) from that at the puff site (trace 1). The distances from regions (1 to 4), and (1 to 5) were the same. (B), Analysis of Ca puffs from a cytoplasmic release site remote from the nucleus. The traces represent the time-course of Ca at (1) the Ca puff site, (2) a nucleoplasmic location, (3) a remote cytoplasmic site and (4) the 'global' level. Note that (trace 3) shows a small elementary Ca release event, which was independent of the signals shown in (trace 1), but which also failed to influence Ca_n due to its remote location.

although not all nuclei are equally responsive to Ca_{cyt}, since in some cells the Ca_n signal can be lower in magnitude, or lag behind the cytoplasmic response (Al-Mohanna *et al.*, 1994). The differential velocity of Ca waves in different cells may underlie these apparently discrepant observations. If the NE induces a delay in Ca diffusion from the cytoplasm to the nucleoplasm it will be less pronounced, with slowly propagating Ca waves (e.g. HeLa cells; wave velocity ~17 μm/s, Bootman *et al.*, 1997), compared with those cells with more rapidly-propagating Ca waves (e.g. cardiac myocytes; wave velocity ~70 μm/s, Lipp and Niggli, 1993).

More striking than the effectiveness of global Ca waves on Ca_n, was the ability of elementary Ca release events, which are the least Ca signals evoked during physiological stimulation of intact cells (Bootman and Berridge, 1995; Lipp and Niggli, 1996), to trigger Ca_n increases. When such Ca puffs occur in the remote cytoplasm they are highly spatially confined (Figure 2Ba) and rapid events (Figure 4B). However, when they occur in a perinuclear region they anisotropically diffuse into the nucleus, and their volume and duration can increase ~10-fold (Figures 2Bb and 4A). This substantial prolongation of Ca_n, in comparison with the cytoplasmic source is probably due to the low buffering capacity of the nucleus (Fox *et al.*, 1997), together with the absence of Ca-ATPases on the inner NE (Humbert *et al.*, 1996). Consequently, Ca_n signals can only decay by diffusion through the nuclear pores. Similarly slow relaxation of Ca_n signals has been seen following global Ca waves (Figure 1; Lipp and Niggli, 1993; Al-Mohanna *et al.*, 1994). The lack of active Ca uptake mechanisms in the nucleus allows the Ca to diffuse over a longer distance than in the cytoplasm, indicating that the nucleus acts as a 'Ca tunnel', in a similar way to that suggested for the lumen of the ER (Mogami *et al.*, 1997). A consequence of the nuclear tunnelling is that any cytoplasmic Ca signal crossing the NE will invade the entire organelle. In support of this, we never observed a partial spreading of a Ca_n signal within the nucleus.

The preferential spreading of perinuclear Ca puffs into the nucleus may account for previous observations that Ca_n transients triggered by Ca-mobilizing hormones were faster and had a greater amplitude than those Ca_n signals evoked either by Ca entry or a slow leak of Ca from the

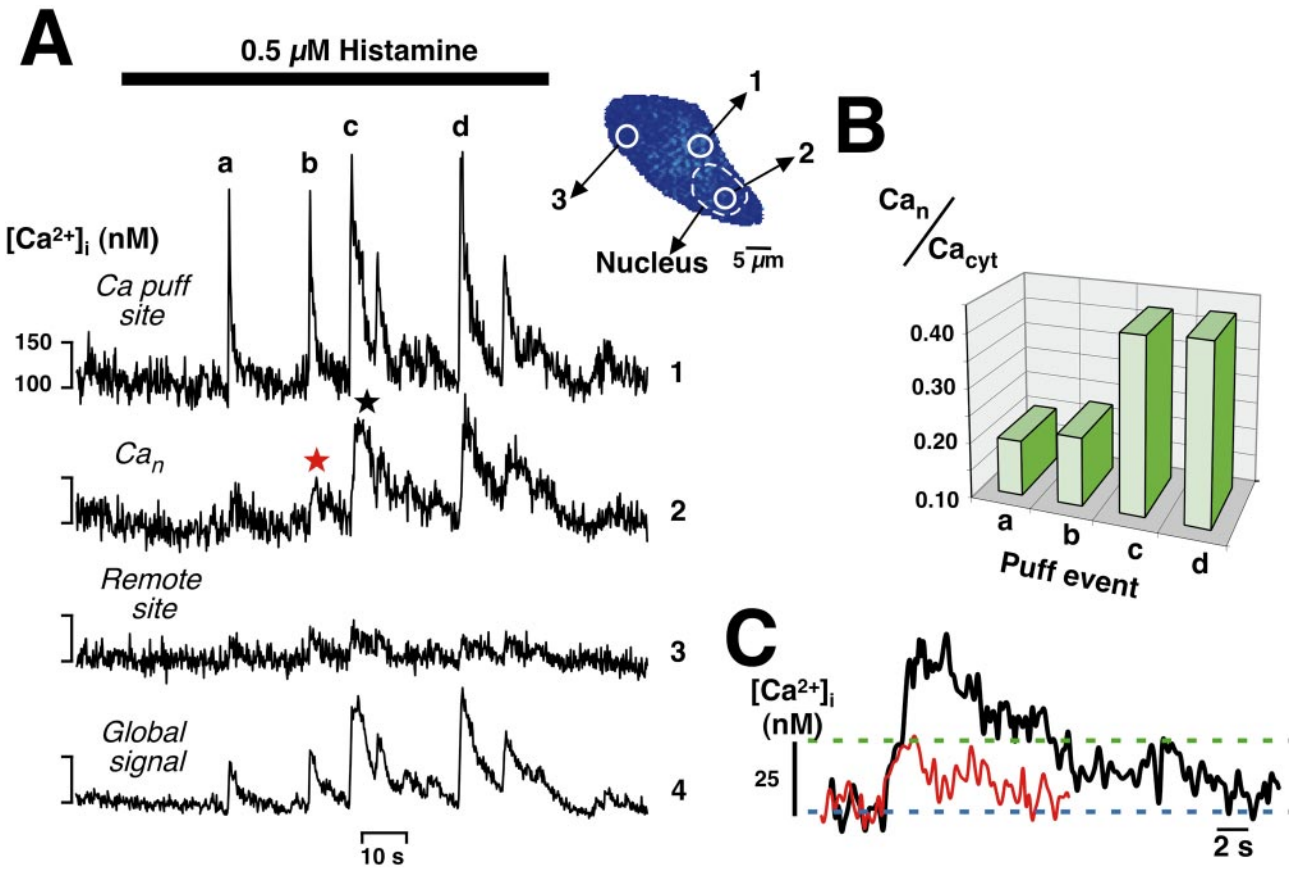

Fig. 5. Mode-switching of Ca diffusion into the nucleus by an individual Ca puff site. (**A**), mode-switching from a weak to a relatively stronger Ca_n contribution by an individual Ca puff site. The traces show the time-course of Ca signals stimulated by 0.5 μM histamine at (1) a Ca puff site located ~3 μm from the NE, (2) a nucleoplasmic region, (3) a remote cytoplasmic site and (4) global Ca. (**B**) shows the relative effect of the Ca puffs labelled a–d in (trace 1) on Ca_n. In (**C**), the two Ca_n transients marked with stars in (trace 2) were superimposed to allow comparison of their rising phases. The Ca_n signal evoked by Ca puff c (black trace) shows an inflexion in its rising phase, which was not present in the Ca_n signal following puff b (red trace).

intracellular stores (Brini *et al.*, 1993; Badminton *et al.*, 1996). In addition, a significant proportion of the mitochondrial population will also be exposed to Ca puffs (Figures 3A and B). It has been shown that mitochondria accumulate Ca during hormone-evoked responses (Rizzuto *et al.*, 1993), although the affinity of mitochondrial Ca uptake is too low for them to detect the average global Ca increase (usually $\leqslant$1 μM; see Figure 1). It was suggested that they must be located close to elementary release sites (Rizzuto *et al.*, 1993), where Ca_{cyt} is sufficiently high. The Ca puffs may therefore serve multiple signalling functions of increasing Ca_n, increasing mitochondrial Ca and triggering/propagating global Ca_{cyt} waves.

The small proportion of Ca puffs that were apparent inside the NE (Figure 2A) raises the possibility that such signals occur within the nucleus. However, further analysis shows that these signals were not of nuclear origin. Although all the events counted in the histogram (Figure 2) were transient, spatially discrete signals, the slower rise time of the Ca puffs that appeared within the nucleus (data not shown) suggests that they diffused into the confocal plane from either above or below. In addition, the nucleus in HeLa cells is usually invaginated (Figure 3D), and a Ca puff arising in such an invagination above or below the confocal plane, could apparently appear to be within the NE. This probably underlies the observation of Ca puffs up to 3 μm inside the NE (Figure 2A), i.e. in the middle of the on-average ~8 μm wide nuclei. Furthermore, if a significant number of Ca puffs originated at the inner NE, the anisotropic diffusion of Ca puffs within the nucleus would be expected to skew the distribution towards the nuclear compartment. This is not apparent (Figure 2A).

Although we cannot absolutely rule out intranuclear Ca release, our evidence supports the idea that Ca_n signals are derived solely from the diffusion of Ca from the cytoplasm. In >700 cells investigated, we never observed an elementary Ca release event that was clearly identifiable as originating in the nucleus. Nor did we ever observe the initiation of a Ca wave by an intranuclear Ca signal. These observations are consistent with other reports showing that Ca_n signals result solely from diffusion of cytoplasmic signals (Al-Mohanna *et al.*, 1994; Shirakawa and Miyazaki, 1996). In light of this, the inflexion in the rising phase of the intranuclear Ca transient described in Figure 5C most likely arose from a change in the NE Ca permeability and not from additional Ca-induced Ca release from within the nucleus.

Relative to global Ca signals, elementary events provide an economical method for controlling Ca_n. The relatively high diffusibility and low buffering of Ca in the nucleus compared with the cytoplasm (Fox *et al.*, 1997), enables

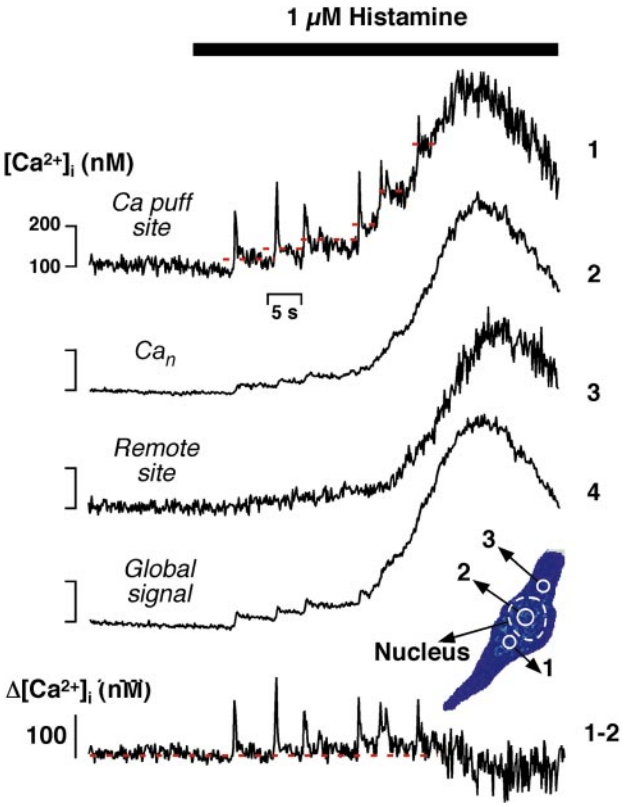

Fig. 6. Integration of cytosolic Ca puffs in the nucleus. Stimulation of a HeLa cell with 1 μM histamine resulted in a series of Ca puffs from a release site ~4 μm distant from the nucleus, which increased Ca_n in a step-wise manner. The traces show the time course of Ca signals at (1) the release site, (2) a nucleoplasmic region, (3) a remote cytosolic area and (4) global Ca. (1–2) shows a trace resulting from subtraction of the Ca_n signal (trace 2) from that at the Ca puff site (trace 1).

cells to generate substantial Ca_n signals with much less Ca (e.g. Figure 4A). Consequently, Ca_n can be elevated independently of bulk Ca_{cyt} changes. Secondly, the amplitude of the Ca_n signal evoked by perinuclear Ca puffs may trigger responses such as transcription, without the deleterious effects associated with the higher Ca concentrations that may occur during global Ca waves, e.g. nuclear calpain activation and apoptosis. In addition, each Ca puff provides a digital pulse of Ca, which at low frequencies gives rise to only transient Ca_n responses (Figure 5). At intermediate frequencies, such events would yield a sustained Ca_n level, and at still higher frequencies the Ca pulses are summated into a progressively increasing Ca_n value (Figure 6). This frequency-dependent integration of the elementary signals, provides a mechanism for differential control of nuclear activities possessing varying sensitivities to Ca.

Even though Ca signals may not originate in the nucleus, the integration of Ca puffs in the nucleoplasm has a considerable consequence for the initiation of regenerative global responses. For Ca puffs to activate such global Ca signals, they must occur with sufficient frequency to drive the 'ambient' Ca_{cyt} to a threshold where regenerativity ensues (manuscript in preparation). The integration (Figure 6) and tunnelling (Figure 4) of Ca signals in the nucleus increases the effectiveness of elementary release events in attaining the regenerative threshold. An example of this is shown in Figure 6, where the Ca signal became regenerative after a series of Ca puffs with an average frequency of ~0.18 Hz (Figure 6, trace 1). If similar events with the same low frequency occurred in the remote cytoplasm, the rapid recovery of such remote puffs (Figure 4B) would have prevented them from evoking a regenerative response.

The control of Ca_n by elementary Ca signals may not be a universal system for controlling nuclear function, since the influence of elementary Ca signals on Ca_n critically depends upon their perinuclear location. In some cell types, such as pancreatic acinar cells, localised Ca_{cyt} increases most often occur remote from the nucleus and do not affect Ca_n (Gerasimenko *et al.*, 1996b). In this cell type, only global Ca waves have the potential to invade the nucleoplasm. In larger cells, such as oocytes, the elementary Ca signals are clearly not clustered around the nucleus, but instead appear to be more homogeneously distributed (Parker *et al.*, 1996). In these cells very few of the elementary Ca signals would affect Ca_n. However, although the influence of elementary Ca release events on Ca_n may be less pronounced in some polarized or large cell types, the size and morphology of HeLa cells is similar to many other small mammalian cells. In such cells the control of Ca_n by elementary release signals could also be due to digital microscopic Ca signals, rather than bulk Ca_{cyt} changes.

Materials and methods

Cell culture

HeLa cells were grown on 40 mm glass coverslips as described elsewhere (Bootman *et al.*, 1997). Prior to the experiments, coverslips were mounted in a sandwich chamber and the cells were loaded with 3 μM Fluo-3AM (Molecular Probes, Eugene, OR) for 30 min at room temperature (20–22°C). Thereafter, the Fluo-3AM was washed off and exchanged with an extracellular solution containing (in mM): NaCl 121, KCl 5.4, $MgCl_2$ 0.8, $CaCl_2$ 1.8, $NaHCO_3$ 6, glucose 5.5, HEPES 25 adjusted to pH 7.3 with NaOH. The cells were allowed to de-esterify for an additional 30 min. Histamine was applied ($t_{1/2}$ of solution exchange <500 ms) by means of a solenoid-driven rapid switching device. $DiOC_6$ (Molecular Probes, Eugene, OR) was applied at 1 μg/ml for 30s. All experiments were performed at 20–22°C.

Ca^{2+} measurements

Confocal imaging of the Fluo-3 loaded HeLa cells was performed with NORAN *Odyssey* and *Oz* confocal microscopes (NORAN Instruments, Milton Keynes, UK). The slit apertures of the instruments were adjusted to give a confocal *z*-section depth of ~1.5 μm for Ca imaging, and ~1 μm for $DiOC_6$-stained samples. Confocal recordings were performed as a time series of confocal images (320×240 pixels, at 7.5–15 Hz). Data analysis and processing was performed as described previously (Bootman *et al.*, 1997).

Ca_{cyt} and Ca_n were calculated using a self-ratio method. The K_d of Fluo-3 for Ca was assumed to be 500 nM in both compartments. Since the K_d and dynamic range of Fluo-3 can be different in the cytoplasm and nucleoplasm (Al-Mohanna *et al.*, 1994), the Ca_n and Ca_{cyt} concentrations presented in this study may not be exactly comparable. However, the qualitative observation of nuclear Ca signals is not compromised by changes in the properties of Fluo-3.

Acknowledgements

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