CMLS, Cell. Mol. Life Sci. 57 (2000) 371–378
1420-682X/00/030371-08 $ 1.50 + 0.20/0
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CMLS Cellular and Molecular Life Sciences

Nuclear calcium signalling

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Abstract. The topic of nuclear Ca^{2+} signalling is beset by discrepant observations of substantial nuclear/cytoplasmic gradients. The reasons why some labs have recorded such gradients, whilst other workers see equilibration of Ca^{2+}_{cyt} and Ca^{2+}_{nuc} using the same cells and techniques, is unexplained. Furthermore, how such gradients could arise across the NE that possesses many highly-conductive NPCs is a mystery. Although nuclei may have the capacity to be autonomous signalling entities, with functional Ca^{2+} release channels and an inositide cycle, the balance of evidence suggests that Ca^{2+} release on the inner NE does not occur during physiological stimulation. Our work suggests that elementary Ca^{2+} release events originating in the cytoplasm can give rise to Ca^{2+}_{nuc} signals without causing elevation of the bulk cytoplasm. Clearly, the many Ca^{2+} signalling mechanisms that may impinge on Ca^{2+}_{nuc} will remain a topic of controversy and debate for some time.

Introduction

Of all the diverse areas of calcium research, probably as many words have been written on the subject of nuclear Ca^{2+} signalling as any other topic. Although investigations into the regulation of nuclear Ca^{2+} have been pursued for well over a decade by many laboratories using various technical approaches, there is still controversy concerning the source and nature of the Ca^{2+} signals that occur in the nucleoplasm. Essentially, the debate centers on observations that Ca^{2+} signals in nuclear and cytoplasmic compartments have been found to occur independently in several different cell types. The paradox is that the nucleoplasmic Ca^{2+} (Ca^{2+}_{nuc}) and cytoplasmic Ca^{2+} (Ca^{2+}_{cyt}) should be in equilibrium for all ions, including Ca^{2+}, due to the numerous nuclear pore complexes (NPCs) that span the inner and outer nuclear envelope (NE). Since the NPCs have a channel diameter of ~ 9 nm [1, 2], they should not prevent Ca^{2+} diffusion unless they become almost fully occluded, a situation that has not yet been convincingly reported. Therefore, the discrepant observations of nuclear/cytosolic Ca^{2+} gradients cannot be easily explained. The interest in understanding how nuclear Ca^{2+} signals arise and whether they can be dissociated from cytosolic Ca^{2+} increases is compounded by observations that Ca^{2+}_{nuc} and Ca^{2+}_{cyt} can have different functions, such as regulation of gene transcription [3] and maturation of oocytes [4]. This article focuses on the mechanisms and sources of nuclear Ca^{2+} signalling. Several previous reviews have also discussed this issue in depth [1, 2, 5–8]. The functions of Ca^{2+}_{nuc} will not be dealt with in this article, and the reader is directed to other chapters in this issue and to recent reviews [9–12].

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Nuclear-cytosolic Ca^{2+} gradients: the evidence

A survey of the literature reveals that almost half of the studies that have examined nuclear Ca^{2+} signalling suggest that cells display nuclear-cytosolic Ca^{2+} gradients of some sort. These observations have been made using various cell types and different Ca^{2+} reporting techniques. Conversely, many other studies have not found evidence of nuclear-cytosolic Ca^{2+} gradients, sometimes with the same cells and using techniques similar to labs where gradients were detected.

The nuclear-cytosolic Ca^{2+} gradients that have been observed can be loosely grouped into three types. First, in cells where fast Ca^{2+} waves can propagate, e.g.

neurons and cardiac myocytes, transient Ca^{2+}_{nuc} and Ca^{2+}_{cyt} gradients have been observed [13, 14]. Second, in some cell types, Ca^{2+}_{nuc} and Ca^{2+}_{cyt} have been found to increase codependently but to deviate from each other by up to several hundred nanomolar for many minutes [15]. Third, persistent steep Ca^{2+} concentration gradients across the NE have been observed where Ca^{2+} clearly does not equilibrate between the nuclear and cytosolic compartments. Whereas the short-lived Ca^{2+} gradients across the NE can be easily understood as a simple kinetic delay in equilibration between Ca^{2+}_{cyt} and Ca^{2+}_{nuc} whilst Ca^{2+} slowly diffuses through the NPCs, the mechanisms underlying the many observations of sustained gradients are not yet known.

One of the most often cited examples of a nuclear-cytoplasmic gradient was observed by Bolsover and colleagues [13]. They studied Ca^{2+}_{cyt} and Ca^{2+}_{nuc} changes that occurred following depolarisation of primary cultured neuronal cells, and found that the nuclear and cytosolic compartments were in equilibrium only up to Ca^{2+} concentrations ~ 300 nM. After this point, Ca^{2+}_{nuc} increased proportionally with Ca^{2+}_{cyt}, but to considerably lower levels. The validity of these observations critically depends on the accuracy of the calibration of Ca^{2+}_{nuc} and Ca^{2+}_{cyt}. The authors did obtain calibration curves for Fluo3, the Ca^{2+} indicator that they used, in both cytosolic and nuclear compartments. However, it has been noted by O'Malley and colleagues [16] that the technique of osmotically shocking the cells to isolate the nuclei, as used by Al-Mohanna and colleagues [13], would most likely invalidate the nuclear calibration. The observed deviation of Ca^{2+}_{nuc} and Ca^{2+}_{cyt} may therefore not be entirely reliable.

An intriguing example of specific nuclear and cytoplasmic Ca^{2+} signalling has been presented by Santella and colleagues when observing the responses of starfish oocytes to the maturating hormone 1-methyladenine [17]. Stimulation of oocytes with this hormone reproducibly elicits three distinguishable Ca^{2+} spikes in a precise temporal sequence. The first and third spikes occur largely in the cytoplasm with little change of Ca^{2+}_{nuc}. Conversely, the second spike occurs in the nucleus, and does not measurably alter Ca^{2+}_{cyt}.

Ca^{2+} permeability of the NE

The most crucial factor for long-lived cytosolic-nuclear Ca^{2+} gradients to be generated is that the nucleus must somehow become impermeable to the passage of ions. This essentially means that the NPC must become substantially occluded. The NPC is a supramolecular assembly with a molecular mass of $\sim 125{,}000$ kDa. It contains aqueous channels with functional diameters of ~ 9 nm, which allow solutes and macromolecules < 20–40 kDa to pass easily by passive diffusion [18]. Larger molecules need nuclear localisation signals (NLSs) and can be transported by energy-dependent translocation through the NPC.

It has been suggested that NLS-mediated transport and passive diffusion of small molecules (10 kDa dextran) through NPCs can be inhibited by depletion of the NE Ca^{2+} store with ionophores or thapsigargin [18; but see 19]. A nuclear pore protein, designated Gp210, contains multiple Ca^{2+} binding domains predicted to reside within the NE lumen, and has been suggested to mediate the effects of Ca^{2+} store depletion. Visualisation of the three-dimensional structure using atomic force microscopy has suggested that the NPC can adopt different conformational states correlating with the Ca^{2+} load of the NE [20]. When the Ca^{2+} stores are depleted, the inner pore of the NPCs appear to contract, and a central 'plug' becomes obvious. The nature of this central plug is controversial. On their nucleoplasmic face NPCs have a basket-like assembly, and it has been suggested that the plug represents this structure projecting back through the pore. Another possibility is that the plug simply represents cargo trapped in the process of transport across the NPC [21], since the presence of the plug was ATP-dependent. In addition to the action of Ca^{2+} within the lumen of the NE, Ca^{2+} outside the stores may also regulate NPC conformation. Micromolar Ca^{2+} concentrations were found to appreciably open the distal cytoplasmic end of NPCs [21].

Despite the observations of alterations in NPC conformation, there is still little evidence that these large pores become sufficiently occluded to block Ca^{2+} diffusion. Stehno-Bittel and colleagues [22] found that diffusion of Mn^{2+} (acting as a Ca^{2+} surrogate) was unaffected by depletion of NE Ca^{2+} stores, even when using conditions that caused narrowing of the NPC channel, enhanced the number of channels bearing a plug and inhibited passage of 10-kDa dextran-Ca^{2+} green. Consistent with this, Gerasimenko and colleagues [23] found that dextran-linked Ca^{2+} indicators loaded into the nucleoplasm were rapidly responsive to changes in extranuclear Ca^{2+} concentration.

One of the most surprising findings with regard to ionic movement across NPCs is the observation of electrophysiological recordings with giga-ohm resistances from nucleus-attached patches. Such studies have found conductances ranging from 200 to 3000 pS, and have suggested that the conductance of NPCs can be modulated (for discussion, see [6]). For example, La^{3+}, Zn^{2+} and guanosine 5′-O-(3-thiotriphosphate) (GTPγS) depressed ionic movement, whereas adenosine 5′-triphosphate (ATP) increased current flow [24].

It is not surprising that the NE may act to delay the diffusion of Ca^{2+} between the cytosol and the nucleus, and that the extent of the lag would be proportional to the number of NPCs and perhaps also their ongoing

transport activities. However, the delay in transmission of Ca^{2+} signals from cytosol to the nucleus seems to differ considerably in different cell types. Injection of germinal vesicle-stage hamster oocytes with Ca^{2+} into either the nucleus or cytoplasm caused Ca^{2+} rises in both compartments with a delay of ~ 1.5 s, indicating a modest diffusional barrier [25]. Depolarisation of primary rat sensory neurons evoked a radially diffusing Ca^{2+} wave that stopped at the NE for ~ 3 s before equilibration. In the same study, depolarisation of neuroblastoma cells caused a Ca^{2+} wave that passed unhindered through the NE [13]. A similar lack of delay in the equilibration of Ca^{2+}_{cyt} and Ca^{2+}_{nuc} signals was observed in another neuroblastoma cell line [26].

Nuclear Ca^{2+} signalling machinery

In addition to requiring NPC occlusion, nuclear-specific Ca^{2+} signals need to be initiated by Ca^{2+} release channels in the inner membrane of the NE. To date, a large body of evidence has been gathered to suggest that nuclei do indeed express functional Ca^{2+} release channels directed towards the nucleoplasm. By carefully separating inner and outer rat hepatocyte NE membranes, Humbert and colleagues [27] showed that $InsP_3Rs$ were localised on the inner NE at similar density and with the same affinity as for those on the endoplasmic reticulum (ER). $InsP_3$ rapidly released Ca^{2+} from these channels in a heparin-sensitive manner. In addition, Ca^{2+} mobilisation caused by photolysis of caged $InsP_3$ in the nucleus [17] or addition of $InsP_3$ to isolated nuclei [23] has demonstrated functional $InsP_3Rs$ that are vectorially oriented towards the nucleoplasm.

Ryanodine receptors (RyRs) sensitive to cyclic adenosine diphosphate ribose (cADPR) have also been identified on the inner NE, using RyR-specific antibodies and Ca^{2+} release by cADPR [17, 23]. The inner NE therefore appears to express both of the major Ca^{2+} releasing channels.

The outer NE membrane is distinct from the inner NE, but is physically and functionally continuous with the ER in that it expresses $InsP_3Rs$ and RyRs. In addition, the outer NE has been suggested to express inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) receptors [27], which may control an unusual ATP-independent Ca^{2+} uptake mechanism [27, 28].

Another piece of evidence that has been used to vindicate autonomous nuclear Ca^{2+} signalling was the identification of a nuclear phosphoinositide cycle that was under plasma membrane receptor control [29, 30]. Before this can be accepted, however, it has to be determined whether this cycle operates within the nucleus at all, or whether it is responsive to Ca^{2+}-releasing receptors other than those activated by growth factors. However, the ability to make $InsP_3$ (or perhaps cADPR) within the nucleus coupled with the presence of functional Ca^{2+} release channels endows nuclei with the possibility of an autonomous Ca^{2+} regulation system. One aspect of Ca^{2+} regulation that cannot take place within the nucleus is Ca^{2+} sequestration, since there are as yet no identified Ca^{2+}ATPases or exchangers on the inner NE [27]. Although the NE can accumulate Ca^{2+} [23], it has to occur via Ca^{2+}ATPases located on the outer NE. Therefore, apart from the simple buffering of Ca^{2+} perhaps by oligonucleotides and proteins within the nuclear matrix, the decay of Ca^{2+} signals within the nucleus can only occur due to the diffusion of Ca^{2+} out of the NPC. Since all Ca^{2+}_{nuc} signals eventually relax, this could indicate that translocation of Ca^{2+} between the nucleus and cytoplasm is never occluded.

The rate-limiting diffusion of Ca^{2+} from the nucleoplasm via the NPCs is most likely responsible for the slower recovery of Ca^{2+}_{nuc} compared with Ca^{2+}_{cyt}, which has been observed in a few cell types. In neuronal cells, for example, Ca^{2+}_{nuc} fell more slowly than Ca^{2+}_{cyt} (time constants of ~ 21 and 11 s, respectively; [13]). A similar phenomenon can be seen in electrically stimulated cardiac myocytes [14]. A surprisingly slow recovery of Ca^{2+}_{nuc} signals was observed in hippocampal neurones activated by depolarisation [31]. The Ca^{2+}_{nuc} transients increased in proportion to Ca^{2+}_{cyt} signals, but they persisted for many minutes after Ca^{2+}_{cyt} had recovered.

A finding that may have interesting consequences for the generation of nuclear-specific Ca^{2+} signals is the observation of NE tubules and invaginations protruding across or some distance into the nucleus [32, 33]. These NE invaginations are present in many cell types, and appear to have a complex, dynamically changing morphology. Although there is presently little evidence for the involvement of such structures in Ca^{2+} signalling, they do appear to possess an NPC-bearing double membrane that is continuous with the NE [32], and they may therefore also be sites where Ca^{2+} release channels are located. Such NE tubules could provide a source for Ca^{2+} signals deep inside the nucleus.

Do nuclear $InsP_3Rs$ and RyRs respond during cell stimulation?

As described earlier, nuclei do express functional $InsP_3Rs$ and RyRs that can be activated to release Ca^{2+} in either isolated nuclei [23] or intact cells [17, 34]. Given that nuclei may be able to make their own $InsP_3$, and that NPCs have a pore diameter sufficient to allow the passage of both $InsP_3$ and cADPR, Ca^{2+} release from the inner NE could be expected to occur every time a cell is stimulated. In support of this, injection of heparin into the cytoplasm or nucleus of agonist-stimulated HeLa cells was found to inhibit Ca^{2+} signals only

in the injected compartment [35]. Although this study did not distinguish between nuclear generation of $InsP_3$ or cytoplasmically generated $InsP_3$ that diffused across the NPCs, it did indicate that the nucleus was able to generate Ca^{2+} signals independently of the cytoplasm. However, observations that nuclear Ca^{2+} release can occur independently of the cytoplasm are outnumbered by studies that suggest a minor role for Ca^{2+} release channels on the inner NE during physiological stimulation.

DeLisle and colleagues found that $InsP_3$ injected into the cytoplasm of *Xenopus* oocytes increased both Ca^{2+}_{nuc} and Ca^{2+}_{cyt}. The elevation of Ca^{2+}_{nuc} could be abolished by a prior injection of heparin into the cytoplasm only. The nuclear $InsP_3Rs$ were not blocked by the cytosolic heparin injection, because subsequent nuclear injection of $InsP_3$ elicited a Ca^{2+}_{nuc} rise. These data indicate that the nuclear $InsP_3Rs$ were functional but did not respond to cytoplasmic injection of $InsP_3$ [34]. Rat basophilic leukemia (RBL) cells respond to antigen stimulation with a series of Ca^{2+} oscillations that appear as simultaneous increases of Ca^{2+}_{nuc} and Ca^{2+}_{cyt}. Introduction of a non-NPC-permeant 70-kDa heparin conjugate into the cytosol blocked both the nuclear and cytosolic Ca^{2+} signals [36], suggesting that $InsP_3$ produced in the cytosol does not open nuclear $InsP_3Rs$. Furthermore, when caged $InsP_3$ was photoreleased inside the nucleus of a hamster oocyte, the resultant Ca^{2+} signal did not arise from within the nucleoplasmic space but rather initiated from a cortical region beside the nucleus [25]. The surprising conclusion from these studies is that $InsP_3$ may not pass across the NPC to open $InsP_3Rs$ on the inner NE, or that the nuclear $InsP_3Rs$ are more resistant to opening than their cytosolic counterparts.

The studies in starfish oocytes, where distinctive Ca^{2+}_{nuc} and Ca^{2+}_{cyt} signals occur in a temporally consistent manner after stimulation with maturation hormone (see above; [17]), do not necessarily indicate that nuclear $InsP_3Rs$ or RyRs are opening. The Ca^{2+}_{nuc} signals could arise from Ca^{2+} stores located outside the nucleus. Indeed, the fact that nuclear injection of heparin and a cADPR antagonist inhibited both nuclear and the second cytosolic Ca^{2+} elevations indicates overlap of the Ca^{2+} stores from which these signals arise.

Nuclear-cytosolic Ca^{2+} gradients: facts and artifacts

Some of the early studies of nuclear/cytosolic Ca^{2+} gradients are now known to be compromised by the fact that the fluorescence output of Ca^{2+} indicator dyes is altered by their cytoplasmic or nucleoplasmic environment. Our laboratory has found that many of the currently used fluorescent Ca^{2+} indicators are brighter in the nucleoplasm than in the cytoplasm of many cell types at all Ca^{2+} concentrations (manuscript in preparation; see also [26, 37–39]). In the case of Fluo3, for example, this is due to the indicator having a lower K_d for Ca^{2+} in the nucleus compared with the cytoplasm (620 ± 45 vs. 810 ± 50nM; [40]). Similar observations were made by Bolsover and colleagues [13]. For other indicators, their dynamic range can also differ between nuclear and cytosolic compartments. This must be taken into account during the calculation of actual Ca^{2+} concentrations [16].

In addition to changes in the affinity of the indicators, their fluorescent properties may also change between the nucleus and cytoplasm. To quantitate such effects, Clapham and colleagues examined Fluo3 fluorescence in isolated crude *Xenopus* oocyte nuclear and cytosolic homogenates [39]. Essentially, they found that the absorbance and fluorescence intensity of Fluo3 was higher in the nucleoplasm, despite setting identical pH and Ca^{2+} concentrations. The consequence of these observations is that without proper independent calibration of an indicator in the nucleus or cytoplasm, apparent differences in Ca^{2+}_{nuc} and Ca^{2+}_{cyt} would be expected. Such artifactual gradients have been demonstrated in many cell types, both before and after stimulation.

It is also important to note that fluorescent Ca^{2+} indicators seem to bind to nucleoplasmic contents, so that a gradual accumulation of indicator occurs. The indicators are released by the nucleoplasm very slowly, suggesting a high affinity binding or covalent attachment [22, 23; but see [41]). O'Malley [42] suggested that such an accumulation of Fluo3 in the nucleus was responsible for the nuclear/cytoplasmic Ca^{2+} gradient observed in neuronal cells. With correction for the different indicator concentrations, the amplitude of the Ca^{2+}_{nuc} transient was the same as that in the cytosol.

Further problems can arise from the partitioning or sequestration of Ca^{2+} indicators into intracellular stores, such as the ER. For example, using Fluo3-loaded neuronal cells Bolsover and colleagues [13] observed an apparent amplification of the Ca^{2+}_{nuc} transient, which was not obvious when the cells were injected with dextran-linked Calcium Green. The artifactual amplification was due to sequestration of Fluo3 into organelles. Essentially, the sequestered indicator was unresponsive to cytoplasmic Ca^{2+} levels, so that the true change of Ca^{2+}_{cyt} following stimulation was underestimated, whereas the Ca^{2+}_{nuc} change was not. Similar problems were reported using Indo1-loaded pancreatic β cells [41].

Although the problems associated with the use of fluorescent indicators can be circumvented with careful calibration of fluorescent signals, there is still no consensus on the existence of Ca^{2+} gradients across the NE. Furthermore, other techniques that have been brought to bear on the question have exacerbated the

argument. In particular, the Ca^{2+}-sensitive photoprotein aequorin has been used to investigate the issue of nuclear/cytosolic Ca^{2+} gradients and has provided discrepant results. Aequorin provides distinct advantages in that it can be precisely localised to one cellular compartment and that its response is less sensitive to its environment than fluorescent indicators. The major drawback of this technique is that the light output from aequorin largely limits its use to cell populations. Using photoproteins to simultaneously monitor Ca^{2+}_{nuc} and Ca^{2+}_{cyt}, Campbell and colleagues observed that Ca^{2+} signals evoked by stimulation of HeLa cells with histamine were always lower in the nucleus compared with the cytoplasm [15]. In contrast, Rizzuto and colleagues [43] found that histamine-stimulated Ca^{2+}_{nuc} and Ca^{2+}_{cyt} signals recorded with aequorin were comparable in amplitude and kinetics. Although these two studies did not undertake exactly the same experiments, there is a marked difference in the interpretation of their results. Therefore, the contradictions concerning the occurrence of nuclear/cytosolic Ca^{2+} gradients cannot be simply attributed to problems in calibrating fluorescent indicators.

Elementary Ca^{2+} signals can give rise to long-lasting Ca^{2+}_{nuc} elevations

In many cell types, global cytoplasmic Ca signals result from spatially and temporally coordinated recruitment of subcellular Ca^{2+} release events, such as 'Ca^{2+} puffs' and 'Ca^{2+} sparks' [44, 45]. These events represent the elementary building blocks of Ca^{2+} signalling, and arise from the opening of either single $InsP_3Rs$ or RyRs, or clusters of these channels. Such elementary Ca^{2+} release events are short-duration highly localised signals [46, 47], which dissipate rapidly due to diffusion in the cytoplasm and sequestration into the intracellular stores. Unless these Ca^{2+} release units become functionally coupled, such elementary release events always remain spatially restricted.

Using rapid confocal microscopy of Fluo3-loaded HeLa cells responding to threshold concentrations of agonist, we found that many of the independently firing Ca^{2+} puff sites are in a perinuclear location, usually within ~4 μm of the NE [48] (fig. 1). Although Ca^{2+} puffs usually only spread ~4–6 μm [47], their perinuclear positioning means that they can reach the NE, diffuse through the NPCs and elevate Ca^{2+}_{nuc} (fig. 2B). Because

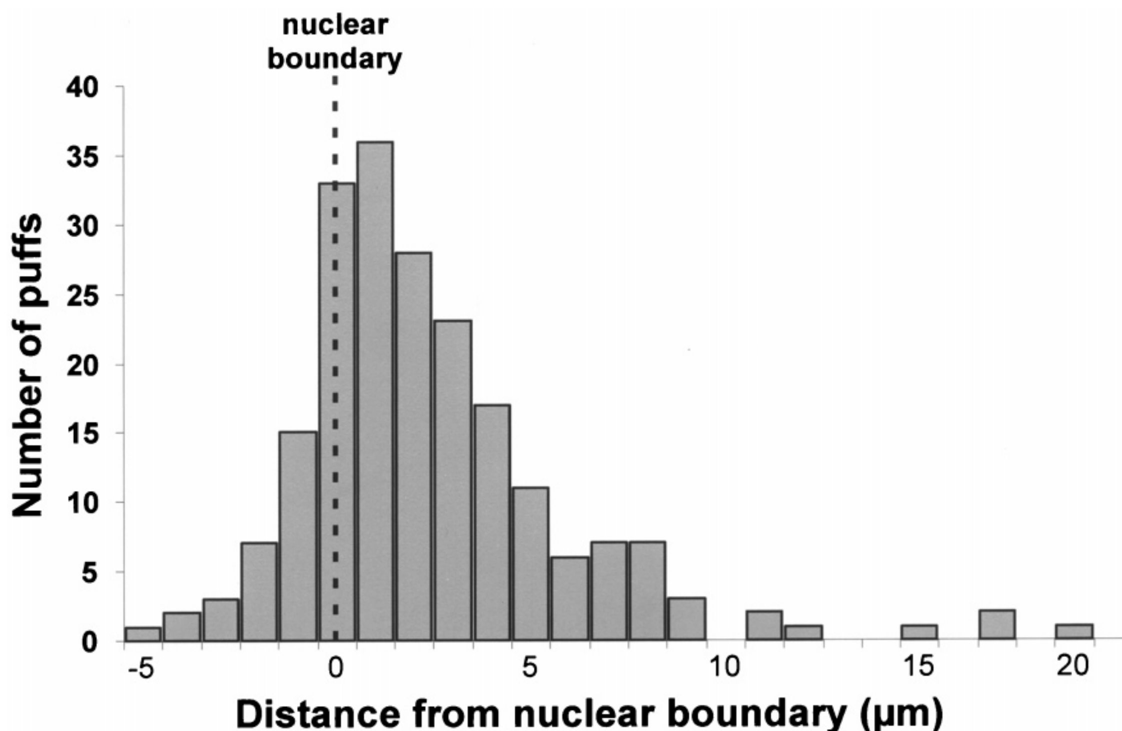

Figure 1. HeLa cell Ca^{2+} puffs are distributed around the nuclear envelope. HeLa cells were stimulated with threshold histamine concentrations (0.5–1 μM) to evoke nonregenerative elementary Ca^{2+} release events. The histogram describes the occurrence of such release signals ($n = 206$) in 1 μm-wide regions on either side of the nuclear envelope. The dashed vertical line indicates the relative position of the nuclear envelope.

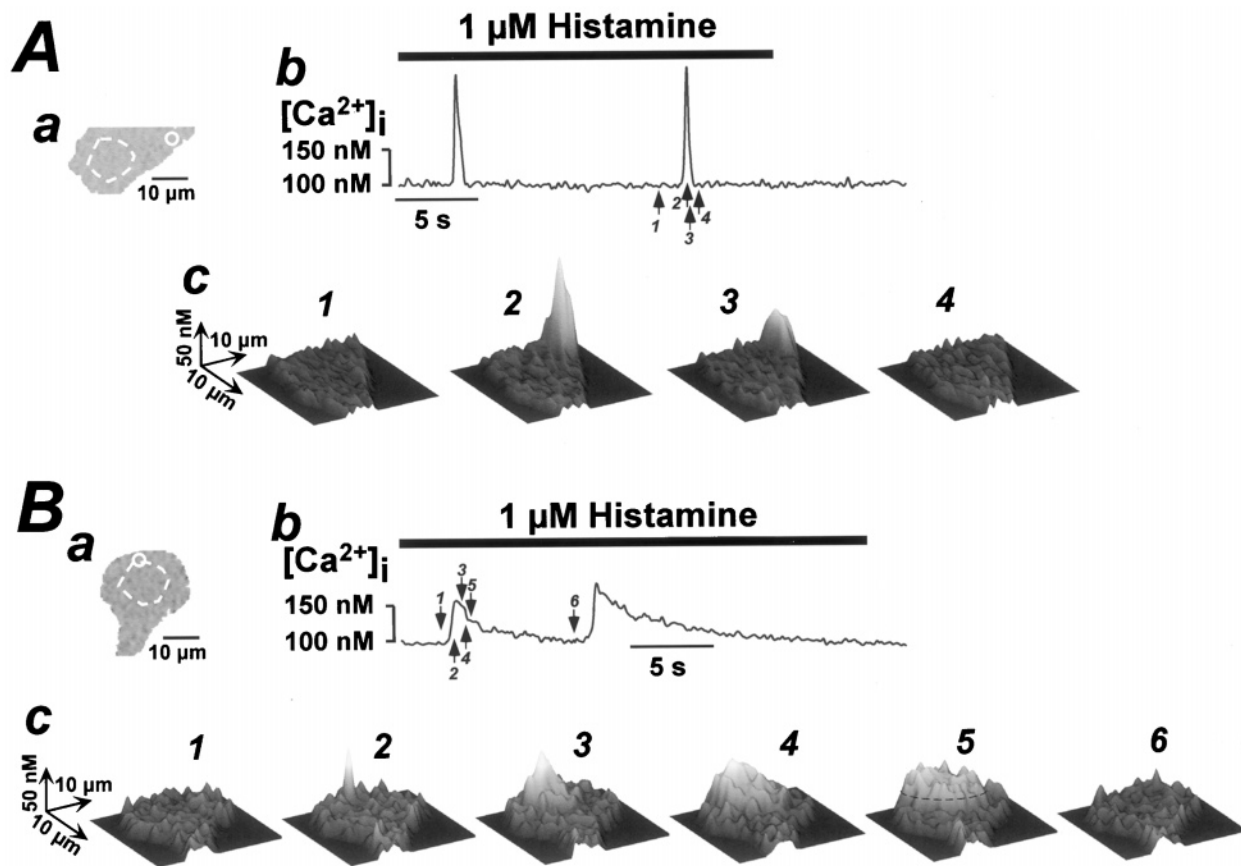

Figure 2. Diffusion of Ca^{2+} from perinuclear Ca^{2+} puff sites across the nuclear envelope. *A* and *B* illustrate the typical time course and spatial spread of Ca^{2+} puffs arising at a location remote from the nucleus (*A*) or just outside the NE (*B*) in two different histamine-stimulated HeLa cells. The locations of the Ca^{2+} puff sites (continuous circles) and the nuclei (dashed circles) are depicted in *A*a and *B*a. Both sites showed two Ca^{2+} puffs during the period of stimulation. The time course of the Ca^{2+} signals arising at the Ca^{2+} puff site remote from the nucleus (*A*b) was much more rapid in decay than those occurring in the perinuclear location (*B*b). This increased delay in the recovery of the perinuclear Ca^{2+} puff site was due to the spread of Ca^{2+} into the nucleus, which was not observed with the remote Ca^{2+} puff site. To illustrate the spatial spread of the Ca^{2+} signals more clearly, montages of images are shown in *A*c and *B*c, where the amplitude of Ca^{2+} signals is indicated by the height of the cell surface. The times at which the images in *A*c and *B*c were taken are marked by the corresponding numbers on the line plots in *A*b and *B*b, respectively. The complete invasion of the nucleus by the Ca^{2+} signal arising at the perinuclear Ca^{2+} puff site can be seen in *B*c (image 5), where the position of the nucleus is marked with a dashed line. The images were captured using confocal imaging of fluo-3-loaded cells [48].

the cytoplasm possesses Ca^{2+}ATPases, whereas the inner NE does not, the diffusion of Ca^{2+} from the puff sites appears to be anisotropically directed into the nucleus. Furthermore, due to the lack of Ca^{2+} buffering/sequestration in the nucleoplasm [49], the Ca^{2+} diffusing through the NPCs eventually fills the entire nucleus, so that the Ca^{2+} puffs that were originally only a few micrometres in diameter subsequently occupy a much larger volume [48] (fig. 2). Indeed, the Ca^{2+} signal was observed to tunnel completely through the nucleus and exit from the NE on the opposite side of the nucleus to where the original event was recorded. Significantly, the Ca^{2+}_{nuc} signal decreases in amplitude and velocity as it passes across the nucleus [49]. This suggests that simple diffusion is responsible for invasion of Ca^{2+} signals throughout the nucleus, and that if any regenerative Ca^{2+} release occurs inside the nucleus, it is negligible.

A further consequence of invading the nucleus is that the duration of the signal arising from each Ca^{2+} puff persists for much longer. When they are located remotely from the NE, Ca^{2+} puffs usually reach peak within 50 ms and decay within 1 s [47]. The perinuclear Ca^{2+} puffs have the same rise time, but Ca^{2+}_{nuc} signal evoked by these events can persist for >10 s [48] (fig. 2). If perinuclear Ca^{2+} puffs are activated with sufficient frequency, Ca^{2+}_{nuc} can remain consistently elevated. Strikingly, this persistent elevation of Ca^{2+}_{nuc} occurs with

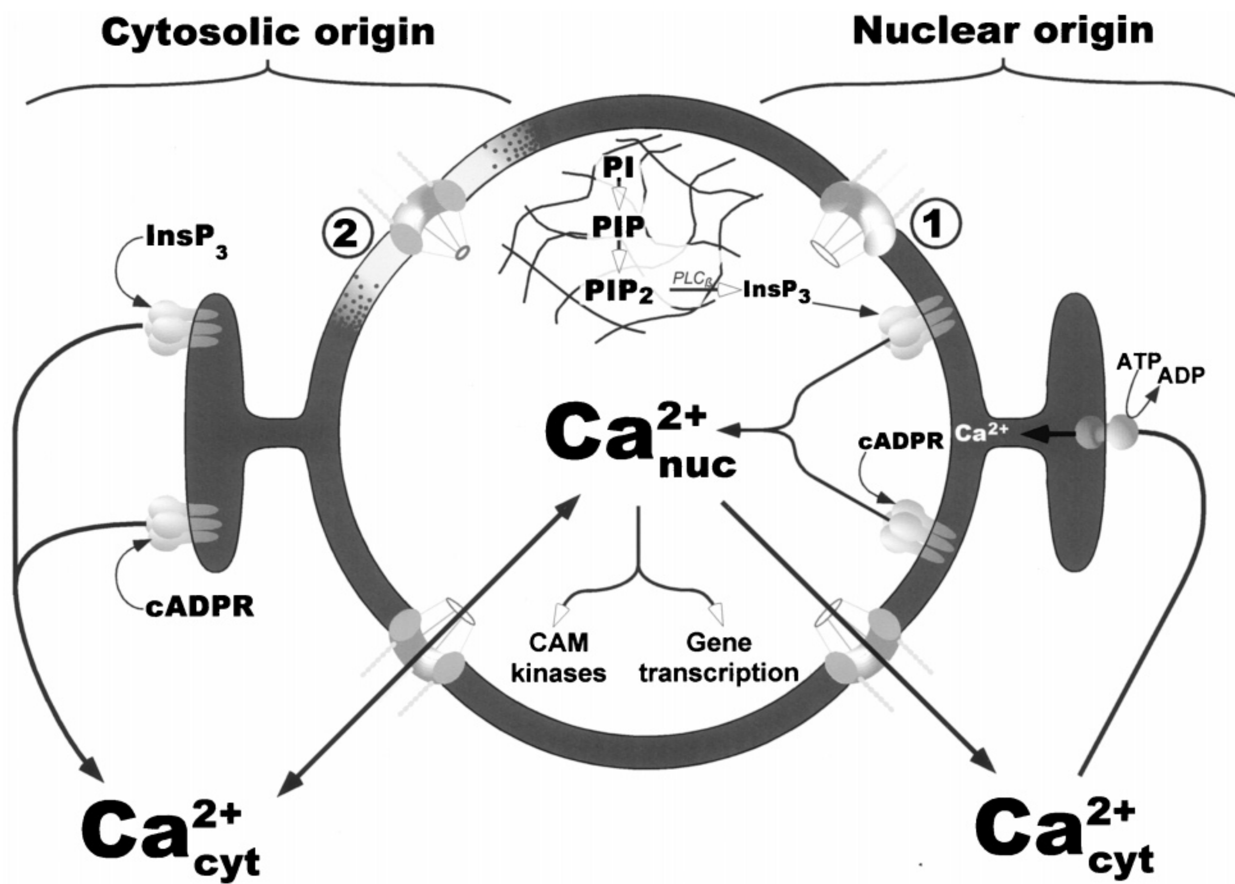

Figure 3. Summary of nuclear Ca^{2+} transport mechanisms. This figure depicts the routes by which Ca^{2+}_{nuc} can increase or exit from the nucleus. Essentially, Ca^{2+}_{nuc} signals may originate in the cytoplasm or in the nucleus. Relaxation of Ca^{2+}_{nuc} signals only occurs by Ca^{2+} diffusing through the NPCs. Depletion of the NE Ca^{2+} store changes NPC structure (marked as 1 and 2), for example so that the nucleoplasmic basket is contracted [21]. However, this does not appear to alter the entry or exit of Ca^{2+} through the NPCs.

no detectable change of Ca^{2+}_{cyt}, except at the cytoplasmic Ca^{2+} puff sites itself. Using such elementary Ca^{2+} release events arising from sites positioned around the nucleus, cells can apparently attain a nuclear-specific Ca^{2+} signal, even though the response actually originates within the cytoplasm. Each Ca^{2+} puff can be considered as a digital pulse of Ca^{2+}, which at low frequencies gives rise to only transient Ca^{2+}_{nuc} responses. At intermediate frequencies, such events would yield a sustained Ca^{2+}_{nuc} level, and at still higher frequencies the Ca^{2+} pulses can summate into a progressively increasing Ca^{2+}_{nuc}. This frequency-dependent integration of the elementary signals may provide a mechanism for differential control of nuclear activities possessing varying sensitivities to Ca^{2+}. (fig. 3)

Although we cannot see a delay in the transmission of Ca^{2+} signals from the cytoplasm to the nucleus, others, also using confocal microscopy, have observed that Ca^{2+}_{nuc} changes can precede those in the cytoplasm [49]. However, when using confocal microscopes it is important to remember that Ca^{2+} signals may enter the plane of focus from above or below, and can give misleading information concerning the apparent origin of a Ca^{2+} response. Using strict criteria for the expected rise time of in-focus events, we have not yet observed Ca^{2+} release events that have convincingly initiated in the nucleus. We would therefore suggest that the population of Ca^{2+} puffs that appear to originate in the nucleus (fig. 1) represents out-of-focus events originating above or below the nucleus.

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