An update on nuclear calcium signalling

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An update on nuclear calcium signalling

Martin D. Bootman¹, Claire Fearnley¹, Ioannis Smyrnias¹, Fraser MacDonald² and H. Llewelyn Roderick¹,³,*

¹Laboratory of Molecular Signalling, The Babraham Institute, Babraham, Cambridge CB22 3AT, UK
²KCL Dental Institute, Department of Orthodontics, St Thomas Street, London SE1 9RT, UK
³Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PD, UK
*Authors for correspondence (e-mails: martin.bootman@bbsrc.ac.uk; llewelyn.roderick@bbsrc.ac.uk)

Summary
Over the past 15 years or so, numerous studies have sought to characterise how nuclear calcium (Ca²⁺) signals are generated and reversed, and to understand how events that occur in the nucleoplasm influence cellular Ca²⁺ activity, and vice versa. In this Commentary, we describe mechanisms of nuclear Ca²⁺ signalling and discuss what is known about the origin and physiological significance of nuclear Ca²⁺ transients. In particular, we focus on the idea that the nucleus has an autonomous Ca²⁺ signalling system that can generate its own Ca²⁺ transients that modulate processes such as gene transcription. We also discuss the role of nuclear pores and the nuclear envelope in controlling ion flux into the nucleoplasm.

Key words: Calcium, Gene transcription, InsP₃, Nucleus, Paclitaxel, Ryanodine, Signalling, Spindle checkpoint, Taxol

Introduction
Calcium (Ca²⁺) is a key player in signal transduction. It modulates diverse cellular activities ranging from fertilisation to cell death (Berridge et al., 2000). It is well known that different cell types use various elements from a broad Ca²⁺ signalling ‘toolkit’ (Box 1); consequently, the characteristics of global Ca²⁺ signals, and their physiological effects, can vary considerably (Berridge et al., 2000). This might also be the case for nuclear Ca²⁺ signalling, which is essentially a complex form of local Ca²⁺ signalling. As described below, the mechanisms of nuclear Ca²⁺ signalling are extensive, and it is likely that there is considerable flexibility in how Ca²⁺ signals are triggered in the nucleus and in the downstream targets that are affected. In this Commentary, we discuss what is known about the origin and physiological significance of nuclear Ca²⁺ transients. In particular, we focus on the idea that the nucleus has an autonomous Ca²⁺ signalling system that can generate its own Ca²⁺ transients, which modulate processes such as gene transcription. We also discuss the role of nuclear pores and the nuclear envelope in controlling ion flux into the nucleoplasm. It should be noted that nuclear Ca²⁺ signalling has been the subject of previous reviews, some of which have presented different points of view (Bootman et al., 2000; Gerasimenko and Gerasimenko, 2004; Gomes et al., 2006; Lee et al., 1998; Santella and Carafoli, 1997).

The physiological significance of nuclear Ca²⁺ signalling
It has been well established that Ca²⁺ signals occurring inside cells affect activities within the nucleus. For example, the amplitude and frequency of global cellular Ca²⁺ signals can regulate the transcription of various genes (Dolmetsch et al., 1998). But what is the significance of the nuclear component of these Ca²⁺ transients? Do patterns of nuclear Ca²⁺ signalling simply track cytosolic Ca²⁺ signalling, or are there independent nuclear Ca²⁺ signalling mechanisms that are necessary for proper cell function?

A precise method for analysing the function of nuclear Ca²⁺ is to specifically localise a buffering molecule within the nucleoplasm, which prevents changes in nuclear Ca²⁺ levels but does not affect cytosolic Ca²⁺ signals. Nathanson and colleagues employed this approach to examine the involvement of nuclear Ca²⁺ in regulating the proliferation of hepatocyte cell lines (Rodrigues et al., 2007). They observed that targeting the Ca²⁺-binding protein parvalbumin to the nucleus significantly reduced cell proliferation and altered the proportions of cells that were in the S and G₂-M phases of the cell cycle. By contrast, the expression of parvalbumin in the cytosol did not alter cell proliferation or cell cycle distribution. Most interestingly, the expression of the parvalbumin in the nucleus, but not in the cytosol, reduced the development of tumours when HepG2 cells were subcutaneously implanted into nude mice. Using a similar approach, the nuclear expression of parvalbumin was found to inhibit activation of the Elk1 transcription factor following stimulation of hepatoma cells with epidermal growth factor (EGF), whereas buffering cytosolic Ca²⁺ did not have the same effect (Pusl et al., 2002). Collectively, these data suggest that nuclear Ca²⁺ signals are important for regulating cell proliferation in some contexts.

Ca²⁺ is a central player in many cellular signal-transduction cascades that modulate gene transcription. Signalling cascades mediate changes in gene expression by stimulating the translocation of transcription factors from the cytosol to the nucleus, or by causing the translocation and/or activation of enzymes that regulate the activity of nuclear transcription factors or the structure of chromatin. Although an increase in the concentration of nuclear Ca²⁺ might not be the initiating signal for such events, it can be necessary for gene transcription to occur or to be sustained. For example, nuclear Ca²⁺ can activate gene transcription via the nuclear factor of activated T cells (NFAT) family of proteins. In quiescent cells, NFAT is mainly found in the cytosol, but in response to an increase in cytosolic Ca²⁺, it rapidly (~10 minutes) translocates to the nucleus (Tomida et al., 2003). The translocation of NFAT occurs following its dephosphorylation by the Ca²⁺-calmodulin-sensitive phosphatase calcineurin (Mellstrom et al., 2008), which exposes an intrinsic nuclear localisation sequence (NLS) in NFAT. NFAT and calcineurin associate in a Ca²⁺-dependent manner, and it has been reported that they translocate into the nucleus as a complex (Shibasaki et al., 1996). When Ca²⁺ is elevated in the nucleus, calcineurin remains active and associated with NFAT, and thereby sustains its transcriptional activity. Calcineurin mediates this effect by outcompeting kinases that promote NFAT re-phosphorylation.
Cytosolic Ca\(^{2+}\) signals can be evoked by chemical stimuli (such as hormones, growth factors and toxins), environmental changes (such as changes in pH or temperature shifts), mechanical deformation or depolarisation. Depending on the cell type, the nature of the stimulus and the extent of stimulation, Ca\(^{2+}\) signals can be transient, oscillatory or sustained (Thomas et al., 1997), and can occur globally or as subcellular events (Bootman et al., 2001). To generate such a diverse spectrum of signals, cells employ a range of messengers and mechanisms to evoke Ca\(^{2+}\) fluxes between various cellular compartments. For example, cells can access Ca\(^{2+}\) from a variety of intracellular stores (including the sarcoplasmic or endoplasmic reticulum, the Golgi, acidic vesicles and the NE) and via influx from the extracellular space. The figure depicts the basic Ca\(^{2+}\) cycle present in all mammalian cell types. The pathways that increase cytosolic Ca\(^{2+}\) levels are shown with red arrows and the mechanisms that reduce Ca\(^{2+}\) levels are depicted with blue arrows.

Ca\(^{2+}\) influx can occur via several different types of channels that have diverse activation mechanisms. The characteristics of the Ca\(^{2+}\) signals evoked by these channels depends on their biophysical properties, their expression levels and their location within the plasma membrane of the cells. Release of Ca\(^{2+}\) from intracellular stores is also mediated by several different types of Ca\(^{2+}\) channel, of which InsP\(_3\)Rs (see Box 2) and RyRs are the best characterised. The magnitude of Ca\(^{2+}\) signals can be limited by various buffers inside cells, such as Ca\(^{2+}\)-binding proteins and mitochondria. These buffers shape the spatial domain, timescale and amplitude of Ca\(^{2+}\) rises. Elevation of cytosolic Ca\(^{2+}\) concentration is transduced into functional changes in cellular activity by numerous sensors that bind Ca\(^{2+}\). Cellular Ca\(^{2+}\) sensors have varying affinities and locations, allowing them to specifically respond to different Ca\(^{2+}\) patterns.

An example of a nuclear-localised transcription factor that is regulated by both nuclear and cytosolic Ca\(^{2+}\) signals is cyclic AMP response element-binding protein (CREB). The signalling mechanisms that underlie CREB activation have been worked out particularly well in neurons, in which it has been established that depolarisation leads to the rapid phosphorylation of CREB on Ser133 by Ca\(^{2+}\)-calmodulin-dependent kinase IV, which provides a necessary, but not sufficient, signal for CREB-mediated gene transcription (Dolmetsch et al., 2001). The triggering event is an increase in cytosolic Ca\(^{2+}\) levels in the immediate vicinity of L-type Ca\(^{2+}\) channels or N-methyl-D-aspartate receptors that are found in the synapse, at a site that is distal to the nucleus (Shaywitz and Greenberg, 1999). As CREB is only found in the nucleus, the phosphorylation of Ser133 is mediated by one of several Ca\(^{2+}\)-sensitive signal transduction cascades that convey information from the remote synapses into the nucleus. In the nucleus, phosphorylated CREB binds additional proteins to form a transcriptionally active complex (Shaywitz and Greenberg, 1999). Although the synaptic Ca\(^{2+}\) signal does not need to propagate to the nucleus to induce phosphorylation of Ser133 on CREB, an increase in the level of nuclear Ca\(^{2+}\) is required for CREB-mediated gene transcription to occur. This is because additional Ca\(^{2+}\)-dependent phosphorylation of CREB and its co-activators is required (Chawla et al., 1998; Kornhauser et al., 2002). Indeed, nuclear injection of an artificial Ca\(^{2+}\) buffer prevents CREB-mediated gene transcription, but not transcription induced by the serum-response element, which is sensitive to cytosolic Ca\(^{2+}\) buffering (Hardingham et al., 1997).

Another well-known target of nuclear Ca\(^{2+}\) signalling is the transcriptional regulator downstream regulatory element antagonist modulator (DREAM). It is well established that DREAM represses the expression of prodynorphin, an opiate-receptor precursor, and that mice that lack DREAM have a constitutive analgesic condition (Cheng et al., 2002). DREAM contains four Ca\(^{2+}\)-binding EF hands (a widely expressed structural motif in proteins that bind Ca\(^{2+}\)), and is directly regulated by an increase in nuclear Ca\(^{2+}\) levels (Mellstrom et al., 2008). When the concentration of Ca\(^{2+}\) in the nucleus is low, DREAM represses gene transcription by binding to DNA and displacing other transcriptional activators such as CREB (Ledo et al., 2002). An increase in the concentration of nuclear Ca\(^{2+}\) causes DREAM to dissociate from its DNA binding sites and thereby allows the transcription of its target genes.

Space limitations prevent a comprehensive discussion of the downstream targets of Ca\(^{2+}\) signalling pathways in the nucleus. However, it is evident from the in vitro and in vivo experiments described above that nucleoplasmic and cytosolic Ca\(^{2+}\) signals can synergistically control important cellular events. Furthermore, the nuclear component of global Ca\(^{2+}\) signals – or an independent increase in nuclear Ca\(^{2+}\) alone – can regulate specific nuclear processes.

**Mechanisms that regulate the level or function of Ca\(^{2+}\) in the nucleus**

Changes in nuclear Ca\(^{2+}\) concentration clearly impact on many cellular functions, but what are the mechanisms involved in regulating nuclear Ca\(^{2+}\) levels, and how are they related to those that operate in the cytosol? In the following section, we describe the mechanisms by which Ca\(^{2+}\) moves into and out of the nucleus and how intranuclear Ca\(^{2+}\) stores are triggered. Together, these mechanisms contribute to the regulation of Ca\(^{2+}\)-dependent nuclear signalling pathways (Fig. 1).

**InsP\(_3\)Rs, RyRs and NAADPRs**

To date, the most widely implicated mechanism for nuclear Ca\(^{2+}\) release is via the activation of inositol(1,4,5)-trisphosphate receptors (InsP\(_3\)Rs) (see Box 2). Evidence in support of a role for nuclear InsP\(_3\)Rs in regulating nuclear Ca\(^{2+}\) release has been obtained by using InsP\(_3\)-binding assays (Humbert et al., 1996). InsP\(_3\)R-specific antibodies (Leite et al., 2003; Malviya, 1994; Steno-Bittel et al., 1995), electrophysiological recordings from nuclear envelope (NE) patches or from whole nuclei (Bustamante et al., 2000; Steno-Bittel...
et al., 1995), nuclear microinjection of InsP$_3$ (Hennager et al., 1995; Huh et al., 2007; Santella et al., 2003), direct visualisation of InsP$_3$-evoked nuclear Ca$^{2+}$ signals (Higazi et al., 2009), reconstitution of nuclear InsP$_3$Rs into lipid bilayers (Leite et al., 2003) and prevention of nuclear Ca$^{2+}$ signals by using InsP$_3$R antagonists (Kumar et al., 2008). Furthermore, the addition of InsP$_3$ to nuclei that were isolated from Xenopus oocytes, Aplysia neurons, mammalian epithelial cells or pancreatic acinar cells (Bezin et al., 2008a; Gerasimenko et al., 1995; Quesada and Verdugo, 2005; Stehno-Bittel et al., 1995) has been shown to induce nucleoplasmic Ca$^{2+}$ transients. Not all of these studies differentiated between expression of InsP$_3$Rs on the inner or outer NE. However, it is clear that InsP$_3$Rs are present on both the inner and outer NE, and that those channels expressed on the inner NE can trigger the release of Ca$^{2+}$ from the NE lumen directly into the nucleoplasm. The actual proportion of the total cellular InsP$_3$Rs that are found on the inner NE (or other putative nuclear stores; see below) is probably very small (<1%) (Laflamme et al., 2002). However, their privileged access to the nucleoplasm might confer them with an essential local signalling function.

It has also been proposed that InsP$_3$Rs are present within heterochromatin and euchromatin, where they are expressed on small vesicular Ca$^{2+}$ stores that also contain high-capacity Ca$^{2+}$-binding chromogranins (Yoo et al., 2007). The presence of small InsP$_3$-sensitive vesicular structures could help to explain the curious spotted distribution of nuclear InsP$_3$R staining that is sometimes observed when using InsP$_3$R-specific antibodies (particularly type-2-InsP$_3$R-specific antibodies) (Garcia et al., 2004; Laflamme et al., 2002; Leite et al., 2003). If this is the case, these vesicular structures could represent nuclear InsP$_3$-sensitive Ca$^{2+}$ stores that might operate independently of cytosolic Ca$^{2+}$ channels. Exactly how these vesicles are formed and how chromogranins and InsP$_3$Rs are trafficked to them is unclear. It is known that chromogranins can interact with InsP$_3$Rs and potentiate their opening (Thrower et al., 2003), and it has been proposed that the colocalisation of chromogranins with InsP$_3$Rs on nuclear vesicles underlies the increased sensitivity of nuclei to InsP$_3$-evoked Ca$^{2+}$ release (Huh et al., 2007). However, more work is needed to verify the existence and function of these nucleoplasmic Ca$^{2+}$ stores.

In addition to InsP$_3$Rs, it has been demonstrated that ryanodine receptors (RyRs) and nicotinic acid adenine dinucleotide phosphate receptors (NAADPRs) are expressed inside, or on the NE, of nuclei in various cell types, and that they regulate nuclear Ca$^{2+}$ signalling. For example, cyclic ADP ribose or NAADP have been shown to cause the release of Ca$^{2+}$ from the NE and to evoke Ca$^{2+}$ transients in nuclei isolated from Aplysia neurons (Bezin et al., 2008b), osteoblasts (Adebanjo et al., 2000) and pancreatic acinar cells (Gerasimenko et al., 2003).

The nucleoplasmic reticulum
Ca$^{2+}$-release channels have been found on nucleoplasm-facing invaginations of the NE, which are known as the nucleoplasic reticulum (or nuclear tubules) (Echevarria et al., 2003; Lee et al., 2006; Lui et al., 2003; Schermelleh et al., 2008). Although such structures have been observed in a wide range of cell types (Fricker et al., 1997), they might not be universal (Bezin et al., 2008b). For reasons that are unknown, the structure of the nucleoplasmic reticulum seems to vary from being made up of simple solitary projections (Fig. 2) to a complex branched network, and it is interesting that the tubules often colocalise with nucleoli (Fricker et al., 1997).
et al., 1997). The lumen in the centre of the nucleoplasmic reticulum tubules is contiguous with the cytosol and presumably allows cytosolic messengers to gain increased access to the deeper parts of the nucleus. There is evidence that functional Ca^{2+}-release channels localise on the nucleoplasmic reticulum (Echevarria et al., 2003; Marius et al., 2006). For example, photorelease of caged InsP_3 (Echevarria et al., 2003) or caged Ca^{2+} (Marius et al., 2006) in the vicinity of the nucleoplasmic reticulum initiated regenerative nuclear Ca^{2+} signals. These data indicate that InsP_3Rs and RyRs are present on the nucleoplasmic reticulum. It has been suggested that the presence of a nucleoplasmic reticulum increases the surface-to-volume ratio within the nucleus, thereby facilitating both the entry and exit of Ca^{2+}.

**Calmodulin**

As indicated above, cytosolic and nuclear activities can be coordinated by the spread of Ca^{2+} between these compartments. In
addition, the $\text{Ca}^{2+}$-dependent movement of enzymes (such as calcineurin) or other signalling mediators can convey signals into and out of the nucleus. One such moiety is the ubiquitous $\text{Ca}^{2+}$-binding protein calmodulin, which has been proposed to translocate into the nucleus following cellular stimulation. Although translocation of calmodulin from the cytosol into the nucleus has been observed in numerous cell types following elevation of cytosolic $\text{Ca}^{2+}$ (Mermelstein et al., 2001; Thorogate and Torok, 2004; Wu and Bers, 2007), the mechanism by which calmodulin enters the nucleus is not clear. Calmodulin does not contain an obvious motif for nuclear localisation, and might therefore ‘piggy-back’ on other proteins that are translocated into the nucleus (Thorogate and Torok, 2007).

Although calmodulin is constitutively present in the nucleus, translocation of additional calmodulin, together with an increase in $\text{Ca}^{2+}$ levels, could be crucial for nuclear signalling pathways. For example, calmodulin translocation in depolarised neurons occurred over the same time-course as CREB phosphorylation (Deisseroth et al., 1998). Buffering nuclear calmodulin, or inhibiting the nuclear influx of calmodulin, prevented the depolarisation-induced phosphorylation of CREB on Ser133. Furthermore, transgenic mice expressing a calmodulin-binding protein that was specifically expressed in the nuclei of forebrain neurons had a decreased level of CREB phosphorylation and impaired long-term memory consolidation (Limback-Stokin et al., 2004). Calmodulin is one example of many proteins that probably move into the nucleus to allow $\text{Ca}^{2+}$ signals to function. $\text{Ca}^{2+}$ causes its many functions by binding to proteins and altering their configurations to make them active or inactive. Therefore, the movement of signalling proteins concomitant with nucleoplasmic $\text{Ca}^{2+}$ changes is important for $\text{Ca}^{2+}$ to trigger downstream events.

Can NPCs inhibit $\text{Ca}^{2+}$ diffusion?

Nuclear pore complexes (NPCs) are the major gateway for ions and macromolecules to pass between the cytosol and nucleoplasm (Box 3). Measurements of the size of the NPC central channel indicate that particles smaller than 9 nm should pass through without restriction. In particular, $\text{Ca}^{2+}$ ions, which have a hydrated radius of ~4 Å, should pass through easily. Although the central NPC channel can become partially occluded during the transport of various cargos, which could plausibly affect $\text{Ca}^{2+}$ movement, ions and small molecules can pass unhindered through other channels that are thought to exist at the periphery of NPCs (Kramer et al., 2007). From these simple considerations, it would be expected that NPCs provide a constitutive conduit for $\text{Ca}^{2+}$ moving between the nucleoplasm and the cytosol.

However, it has been suggested that NPCs can attain a conformation in which they do not permit ion transport. Using patch-clamp techniques, it is possible to form giga-ohm seals on the outer NE for periods of many minutes without any evident ion flux (Bustamante et al., 2000). This is a striking finding, as even a conservative estimate of NPC density suggests that a typical patch-clamp experiment should encompass 10-100 NPCs. As the pores have a conductance of ~1 nanosiemens each, there should be a considerable current whenever a patch electrode is attached (Danker et al., 1997). However, electrophysiological recordings often detect only a few (about six per patch) operartive channels (Bustamante et al., 2000). These observations have led to the suggestion that NPCs mainly exist in a closed conformation in which they are not ion conductive.

An alternative viewpoint that has arisen from studies carried out using a different electrophysiological approach to monitor NPC activity.
Instead of using membrane-attached patches, Oberleithner and colleagues employed an ‘hourglass’ technique in which an isolated nucleus was sandwiched in a thin capillary tube. Using this method, it was observed that NPCs are constitutively open under physiological conditions (Danker et al., 2001). It was possible to modulate the NPC current recorded using the hourglass technique. For example, the addition of ATP or the depletion of the NE Ca\(^{2+}\) store increased the conductance of the NPC, but the NPCs always permitted cation flux (Shahin et al., 2001).

These latter findings arguably convey the physiological properties of NPCs most accurately. It is likely that patch-clamping the NE distorts the membrane and somehow causes the NPCs to become non-conductive, whereas, under native conditions that are maintained using the hourglass technique, NPCs allow the constitutive passage of Ca\(^{2+}\) and other ions. Therefore, NPCs cannot be considered a complete diffusion break for Ca\(^{2+}\). Rather, ions in the nuleoplasm or cytosol have permanent access to the other compartment (Eder and Bading, 2007). At most, NPCs might act as a diffusion filter and introduce a kinetic delay in the equilibration of nuclear-cytosolic Ca\(^{2+}\) concentrations. The extent of the kinetic delay might be subject to modulation: although NPCs do not close, their conductance can change in response to factors such as Ca\(^{2+}\) and ATP. Furthermore, the extent of NPC expression on the NE can vary from 1-50 NPCs per \(\mu\)m\(^2\), depending on cell type (Wang and Clapham, 1999). A greater expression of NPCs would allow a more rapid equilibration of cytosolic and nuclear Ca\(^{2+}\) concentrations.

**Regulation of NPCs – a role for Ca\(^{2+}\)?**

Numerous studies have proposed that NPCs are regulated by changes in Ca\(^{2+}\) concentration either within the lumen of the NE, or at the cytosolic-nucleoplasmic face. However, there is little consistency in the nature of the observed effect. A widely cited example is the demonstration that altering the Ca\(^{2+}\) concentration in the lumen of the NE regulates both passive diffusion and NLS-mediated protein transport through NPCs (Greber and Gerace, 1995): depletion of the NE Ca\(^{2+}\) store was found to attenuate the nuclear influx of proteins bearing a NLS and of a non-specific fluorescent dextran molecule. It was suggested that the Ca\(^{2+}\) sensor in the NE was the integral membrane protein gp210, a nucleoporin of 210 kDa that functions to anchor NPCs. gp210 interacts with the nucleoplasm or cytosol and thereby mediates changes in NPC structure (Greber et al., 1990).

In support of this idea, an antibody specific for the luminal domain of gp210 was found to inhibit both passive diffusion and signal-mediated transport into the nucleus (Greber and Gerace, 1992).

A similar finding was reported when the nuclear import of histone H1 (a ~21 kDa constitutively expressed nuclear protein) and two smaller fluorescently tagged dextran molecules (of 3 and 10 kDa) was compared in intact cardiomyocytes (Perez-Terzic et al., 1999). Reducing the Ca\(^{2+}\) concentration in the NE lumen by treating cells with a Ca\(^{2+}\) chelator, a Ca\(^{2+}\)-pump inhibitor or ionomycin prevented the energy-dependent import of the histone. The same treatments prevented the passive influx of 10 kDa dextran, but not the 3 kDa dextran species. These data indicate that NPC function is sensitive to the concentration of Ca\(^{2+}\) within the NE, and that altering luminal Ca\(^{2+}\) concentration can regulate both active and passive flux of large, but not small, molecules. However, a similar study examining the diffusion of GFP between the nucleoplasm and cytosol of intact Xenopus oocytes reached the opposite conclusion; the NE reduced diffusion of GFP by ~100-fold, but depletion of Ca\(^{2+}\) from the lumen of the NE made no difference (Wei et al., 2003). Yet a different finding was reported in a study of the movement of photo-activated GFP across the NE in hepatocytes (O’Brian et al., 2007). In this case, a rise in cytosolic Ca\(^{2+}\) levels, and not changes in NE Ca\(^{2+}\) content, increased the influx (but not the efflux) of photo-activated GFP across the NE. In this latter study, it was also demonstrated that Ca\(^{2+}\) signals evoked by InsP\(_3\)Rs regulated the influx of GFP into the nucleus, suggesting a putative link between cell activation by InsP\(_3\)-producing stimuli and the control of nuclear trafficking.

These discrepant studies highlight some of the controversy surrounding how NPC opening is regulated. As yet, there is little consensus about what effects cytosolic Ca\(^{2+}\), or Ca\(^{2+}\) within the NE, have on NPC structure. However, although the results of the various studies are not entirely consistent, they suggest that Ca\(^{2+}\) is somehow important in regulating nuclear traffic. The mechanism by which Ca\(^{2+}\) might regulate NPC permeability is unclear. One of the most commonly proposed theories is that the central ‘plug’ moves and blocks the channel in response to changes in the Ca\(^{2+}\) concentration in either the cytosol or the lumen of the NE (see Box 3). Consistent with this idea, several studies reported Ca\(^{2+}\)-dependent changes in the shape of NPCs (Stoffler et al., 1999) and, in particular, alterations in the number of NPCs bearing a central plug. A key technique in these investigations was atomic force microscopy (AFM), which can be used to scan the three-dimensional topography of the cytosolic face of NPCs. AFM can resolve changes in the surface of large macromolecules, and has been used to show that the depletion of Ca\(^{2+}\) from the lumen of the NE significantly and reversibly increased the proportion of NPCs with a central plug (Wang and Clapham, 1999). The observed change in NPC structure that is caused by luminal Ca\(^{2+}\) depletion is in line with the Ca\(^{2+}\)-dependent alterations in NPC transport described above (Perez-Terzic et al., 1999), and supports the concept of allosteric regulation of NPC gating by Ca\(^{2+}\). However, the reported changes in NPC structure are not the same in different studies (Erickson et al., 2006), and alteration in NPC structure following Ca\(^{2+}\) release from the NE is not universally observed (Stoffler et al., 2006).

Even if changes in NPC permeability do indeed occur under physiological conditions, it is not clear what difference this makes to cellular growth or activity. It has been argued that simply altering the permeability of the nucleus to species of different molecular masses is a rather crude mechanism for regulating nuclear-to-cytosolic flux (Torok, 2007). Much greater specificity for NPC transit can be achieved by cellular signalling mechanisms that either promote or diminish the exposure of molecular tags (such as the NLS and NES) that promote NPC transport.

**Nuclear Ca\(^{2+}\) signalling in intact cells**

It has been established that Ca\(^{2+}\) signals occur in the nucleus of intact cells. For example, confocal imaging studies have shown that as Ca\(^{2+}\) waves ‘sweep’ through the cytosol of hormone-stimulated cells, the nucleus becomes engulfed by the signal (Lipp et al., 1997). However, a major controversial issue concerning nuclear Ca\(^{2+}\) signalling is whether Ca\(^{2+}\) transients are generated autonomously in the nucleoplasm, and whether they can be distinct from cytosolic Ca\(^{2+}\) changes. Numerous studies have examined Ca\(^{2+}\) signalling in isolated nuclei (Adebajo et al., 2000; Bezin et al., 2008b; Gerasimenko et al., 1995). The advantage of studying nuclei in isolation is that changes in nucleoplasmic Ca\(^{2+}\) only occur if Ca\(^{2+}\) is released from channels within the inner NE. Although the NPCs are present in this type of preparation, it is unlikely that Ca\(^{2+}\) released...
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from channels on the outer NE (that face away from the nucleus), or from potential remnants of the ER, can diffuse into the nucleoplasm. Rather, any Ca\(^{2+}\) that is released outside of the NE is rapidly dissipated and diluted. Isolated nuclei therefore represent a system in which nucleoplasm-directed Ca\(^{2+}\)-release channels can be examined. However, although these studies are useful for examining the potential of nuclei to generate their own Ca\(^{2+}\) signals, they do not determine whether these signals are at all significant compared with the larger fluxes of Ca\(^{2+}\) that occur in the cytosol of intact cells. The key issue, therefore, is to establish how nuclear Ca\(^{2+}\) signals arise within the physiological context of intact cells, and whether Ca\(^{2+}\) channels facing the nucleoplasm have a significant role.

Measuring nuclear Ca\(^{2+}\) signals: key considerations

The idea that nuclear Ca\(^{2+}\) levels are regulated independently from those in the cytosol emerged as soon as imaging technology advanced to allow monitoring of Ca\(^{2+}\) changes simultaneously in different cellular compartments (Bkaily et al., 1997). Using both wide-field and confocal fluorescence microscopy of living cells, several groups began to report that nuclear Ca\(^{2+}\) concentration differed from that in the cytosol. The extent to which the Ca\(^{2+}\) signals in these two compartments differed was highly variable between studies. In some cases, the nuclear and cytosolic Ca\(^{2+}\) levels were found to be entirely independent, whereas others reported modest differences in the kinetics or amplitude of the nucleoplasmic and cytosolic responses (Leite et al., 2003; Santella et al., 2003). Although the examples of differential Ca\(^{2+}\) concentration in the nucleus and cytosol are too numerous to mention in detail, some are discussed below, and Table 1 lists some of the reported findings.

Owing to their brightness, Ca\(^{2+}\)-sensitive fluorescent indicators have been used in most studies that compared the properties of nuclear and cytosolic Ca\(^{2+}\) signals. However, it is certain that many of these studies are invalid as they failed to recognise that organic fluorochromes have different characteristics in the nuclear and cytosolic compartments (Birch et al., 1992; Bkaily et al., 2006). Indeed, one of the most prominent early studies of nuclear Ca\(^{2+}\) regulation – which suggested that Ca\(^{2+}\) concentration in the nucleus tracked that in the cytosol up to ~300 nM, after which they were insulated against further rises (al-Mohanna et al., 1994) – is compromised by the lack of in situ calibration (O’Malley et al., 1999). We (Thomas et al., 2000b) and others (O’Malley et al., 1999; Perez-Terzic et al., 1997) have demonstrated that commonly used fluorescent Ca\(^{2+}\) indicators are much brighter in the nucleus than in the cytosol; they differ with respect to both their affinity for Ca\(^{2+}\) and their dynamic range. Therefore, to obtain absolute values for Ca\(^{2+}\) concentration in different cellular compartments, it is crucial to perform independent calibrations with the indicator in exactly the same environment in which it will be used (Thomas et al., 2000b). Typically, when quiescent cells are loaded with a Ca\(^{2+}\) indicator such as fura2 or fluo3, it is evident that the nucleus has a greater fluorescence than the cytosol. The difference in the intensity of the indicator fluorescence between the nucleus and cytosol is maintained or even exaggerated as the Ca\(^{2+}\) concentration globally increases. This exaggerated nuclear fluorescence has led some studies to erroneously conclude that nuclei amplify cellular Ca\(^{2+}\) signals, and even that there are intranuclear Ca\(^{2+}\) gradients (Birch et al., 1992). It is not known why fluorescent Ca\(^{2+}\) indicators behave differently in the nucleus, although it has been established that factors such as ionic strength, viscosity and pH can alter Ca\(^{2+}\) binding to the indicator. In addition, it appears that Ca\(^{2+}\) indicators interact with nuclear contents; for example, in Xenopus oocyte nuclei that had been loaded with fluo3, it was found that the indicator did not diffuse freely because it became somehow ‘trapped’ in chromatin and/or DNA (Perez-Terzic et al., 1997). These technical artefacts are one of the main reasons why nuclear Ca\(^{2+}\) signalling has such a controversial history.

### Table 1. Selected examples of observed differences in cytosolic and nucleoplasmic Ca\(^{2+}\) levels

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<tr>
<td>Nucleoplasmic Ca(^{2+}) signal lagged ~4 seconds behind elevation in cytosolic Ca(^{2+})</td>
<td>Cardiac myocytes</td>
<td>Fluorescence imaging</td>
<td>(Bootman et al., 2007; Kockskamper et al., 2008a)</td>
</tr>
<tr>
<td>Nucleoplasmic Ca(^{2+}) signal lagged ~100 milliseconds behind elevation in cytosolic Ca(^{2+})</td>
<td>Liver-cell line</td>
<td>Fluorescence imaging</td>
<td>(Leite et al., 2003; Echevarria et al., 2003)</td>
</tr>
<tr>
<td>Lower threshold for InsP(_3)-evoked Ca(^{2+}) release in the nucleus</td>
<td>HeLa cells</td>
<td>Bioluminescence (aequorin)</td>
<td>(Brini et al., 1993)</td>
</tr>
<tr>
<td>Larger increase in Ca(^{2+}) in the nucleoplasm than in the cytosol, but with the same kinetics</td>
<td>HeLa cells</td>
<td>Bioluminescence (aequorin)</td>
<td>(Badminton et al., 1996; Badminton et al., 1995)</td>
</tr>
<tr>
<td>Smaller increase in Ca(^{2+}) in nucleoplasm than in the cytosol, but with the same kinetics</td>
<td>Cardiac myocytes</td>
<td>Fluorescence imaging</td>
<td>(Higazi et al., 2009)</td>
</tr>
<tr>
<td>Nucleoplasmic Ca(^{2+}) signals occurred in the absence of Ca(^{2+}) changes in the surrounding cytosol</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The interplay between voltage-gated Ca\textsuperscript{2+} channels on the plasma membrane and RyRs on the sarcoplasmic reticulum regulates excitation-contraction (EC) coupling in the heart, allowing the cells to contract and pump blood. Studies have shown that independent nuclear Ca\textsuperscript{2+} signals exist in non-excitable cell types (Lipp et al., 1997). Many of these signals have specific effects on nucleoplasmic Ca\textsuperscript{2+} levels. When Ca\textsuperscript{2+} puffs are activated, they release Ca\textsuperscript{2+} into the nucleoplasm, engulfs the entire nucleus, and can even diffuse through the nucleus from sites distant to the NE. Perinuclear Ca\textsuperscript{2+} signals have been shown to have a long-lasting effect on gene transcription in cardiac myocytes (Luo et al., 2006), whereas distal signals are limited in duration.

Perinuclear Ca\textsuperscript{2+} release and the consequent elevation in nuclear Ca\textsuperscript{2+} have a specific spatial and temporal effect on gene transcription. Studies have shown that perinuclear Ca\textsuperscript{2+} release promotes the phosphorylation of histone deacetylase 5 via Ca\textsuperscript{2+}-calmodulin-dependent kinase II, leading to changes in gene expression. Hormones such as phenylephrine induce hypertrophy by activating perinuclear Ca\textsuperscript{2+} release, and the activation of Ca\textsuperscript{2+} release in close proximity to the NE triggers nucleus-specific Ca\textsuperscript{2+} signals.

Few micrometres. If such events occur in the cytosol at sites that are distant from the nucleus, they probably have only a modest impact on cell behaviour, but if they occur next to the nucleus, they can have a long-lasting and integrating effect on nucleoplasmic Ca\textsuperscript{2+}. Such observations have been made in both excitable and non-excitable cell types (Lipp et al., 1997). Indeed, many of the studies that proposed the existence of independent nuclear Ca\textsuperscript{2+} signals could be explained by such perinuclear cytosolic Ca\textsuperscript{2+} release, followed by anisotropic diffusion of Ca\textsuperscript{2+} through NPCs into the nucleoplasm. In particular, this probably applies to studies in which the nuclear Ca\textsuperscript{2+} signal lags behind the cytosolic Ca\textsuperscript{2+} response (Huh et al., 2007).

Perinuclear Ca\textsuperscript{2+} release, and the consequent elevation in nuclear Ca\textsuperscript{2+} that it induces, has recently become a particular focus in studies of cardiomyocytes (Higazi et al., 2009; Kockskamper et al., 2008b; Wu et al., 2006). Ca\textsuperscript{2+} signals occur rhythmically in cardiac myocytes and promote the interaction of protein filaments that make the cells contract and thereby permit the influx of a small amount of Ca\textsuperscript{2+}. This Ca\textsuperscript{2+} signal is greatly amplified by RyRs that are close to the voltage-gated Ca\textsuperscript{2+} channels. Ca\textsuperscript{2+} diffuses from the RyRs to the actin/myosin filaments and initiates cell contraction. RyRs are therefore considered to be the main Ca\textsuperscript{2+}-release channel in the heart. However, cardiac myocytes also express InsP\textsubscript{3}Rs (Lipp et al., 2000; Perez et al., 1997), and recent studies of neonatal cardiomyocytes have shown that these Ca\textsuperscript{2+} channels are often densely concentrated around the nucleus (Bootman et al., 2001; Shirakawa and Miyazaki, 1996; Thomas et al., 2000a; Vermassen et al., 2003). It therefore seems probable that modulation of nucleoplasmic Ca\textsuperscript{2+} concentration by perinuclear Ca\textsuperscript{2+} release is a generic signalling paradigm.
Independent nuclear Ca\textsuperscript{2+} signalling

The studies described above substantiate the idea that perinuclear Ca\textsuperscript{2+} release significantly affects nucleoplasmic Ca\textsuperscript{2+} concentration. However, the observations also raise questions about the function of Ca\textsuperscript{2+} channels located on the inner NE and in the nucleoplasmic reticulum. In particular, why don’t the Ca\textsuperscript{2+} channels on the inner NE, or other putative nuclear Ca\textsuperscript{2+} stores, generate a Ca\textsuperscript{2+} signal that is independent from the cytosol? Or do Ca\textsuperscript{2+} signals that arise in the cytosol always dominate what happens in the nucleus? As mentioned earlier, it has been shown that isolated nuclei have the machinery to release Ca\textsuperscript{2+}, but the majority of intact cell studies indicate that nucleoplasmic Ca\textsuperscript{2+} signals originate outside the nucleus (Lipp et al., 1997; Luo et al., 2008). Indeed, in one notable study, InsP\textsubscript{3} was introduced specifically to the nucleus or cytosol to examine the relative ability of this messenger to trigger Ca\textsuperscript{2+} signals in each compartment. As expected, when released in the cytosol, InsP\textsubscript{3} evoked a cytosolic Ca\textsuperscript{2+} signal that invaded the nucleus via diffusion. However, when InsP\textsubscript{3} was released in the nucleus, the resulting Ca\textsuperscript{2+} signal also initiated in the cytosol, and then invaded the nucleus (Shirakawa and Miyazaki, 1996). This indicates that even when InsP\textsubscript{3} is given immediate access to the InsP\textsubscript{3}Rs on the NE, the messenger diffuses out of the nucleus and preferentially triggers cytosolic Ca\textsuperscript{2+} release. A similar conclusion was reached when an InsP\textsubscript{3} antagonist was introduced in the cytosol of cells during stimulation with an InsP\textsubscript{3}-generating agonist. The antagonist, which was too large to enter the nucleus, blocked both cytosolic and nucleoplasmic Ca\textsuperscript{2+} transients (Allbritton et al., 1994). The obvious conclusion from such studies is that, although nuclei have the capacity to generate Ca\textsuperscript{2+} transients, their contribution is ‘swamped’ by the larger cytosolic Ca\textsuperscript{2+} release [but see Lui et al. (Lui et al., 1998) for a contradictory observation].

Perhaps it is only under specific stimulation conditions that substantial Ca\textsuperscript{2+} responses are evoked from nuclear InsP\textsubscript{3}Rs. For example, a comparison between the Ca\textsuperscript{2+} signals that are evoked by vasopressin and hepatocyte growth factor (HGF) in hepatic carcinoma cells revealed that vasopressin triggered Ca\textsuperscript{2+} release in the cytosol, whereas HGF caused nuclear Ca\textsuperscript{2+} mobilisation (Gomes et al., 2008). In the latter case, it was suggested that the HGF receptor was internalised and translocated to the nucleus, where it triggered local InsP\textsubscript{3} production. Vasopressin, on the other hand, stimulated InsP\textsubscript{3} production in the cytosol. It is therefore plausible that different stimuli activate InsP\textsubscript{3}Rs in alternative cellular compartments, depending on where in the cell the InsP\textsubscript{3} is generated.

An example of nuclear-specific Ca\textsuperscript{2+} signalling is presented in Fig. 4A, which shows the spatial profile of spontaneous Ca\textsuperscript{2+} transients that occur in a neonatal rat cardiac myocyte. Such Ca\textsuperscript{2+} signals are largely generated from nuclear InsP\textsubscript{3}Rs and RyRs (Luo et al., 2008). The black trace shows substantial nuclear Ca\textsuperscript{2+} elevations, but a corresponding increase in cytosolic Ca\textsuperscript{2+} on either side of the nucleus is not observed. It was not determined whether the nuclear Ca\textsuperscript{2+} signals were due to activation of Ca\textsuperscript{2+} channels on the inner NE, or to perinuclear Ca\textsuperscript{2+} release. However, the figure presents an example in which substantial Ca\textsuperscript{2+} changes can be clearly observed solely in the nucleus.

**Autonomous generation of Ca\textsuperscript{2+}-mobilising messengers in the nucleus**

If nuclei are truly independent of cytosolic signalling, they must have the ability to generate Ca\textsuperscript{2+}-mobilising messengers that then act locally on nucleoplasmic InsP\textsubscript{3}Rs, RyRs and NAADPs.

Numerous studies have demonstrated that the biochemical machinery that is required for the generation of Ca\textsuperscript{2+}-mobilising messengers is present in the nucleus. For example, the ADP-ribosyl cyclase enzyme that is responsible for the production of cyclic ADP ribose (cADPR) and/or NAADP is localised in the nucleus (Adebanjo et al., 1999; Bezin et al., 2008a; Trubiani et al., 2008). In addition, it has been well established that nuclei possess phosphoinositide signalling mechanisms that are similar to, but distinct from, those that occur at the plasma membrane, and which can lead to InsP\textsubscript{3} production (Visnjic and Banfic, 2007; Ye and Ahn, 2008). Indeed, nuclei have substantial amounts of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P\textsubscript{2}]...
and phosphoinositide-specific phospholipase C (PtdIns-PLC) (Downes et al., 2005). Interestingly, it has been reported that PtdIns(4,5)P₂ and PtdIns-PLC localise in particles known as nuclear speckles (see Fig. 1). These small nuclear substructures are believed to be the sites of storage and/or assembly of pre-mRNA splicing factors. The exact function of PtdIns(4,5)P₂ and PtdIns-PLC in nuclear speckles is currently under investigation, but they are involved in linking the production of diacylglycerol (DAG) and InsP₃ (and their metabolites) to the control of RNA processing (Alcazar-Roman and Wente, 2008).

Mammalian cells express at least 13 different PtdIns-PLC isoforms that can be grouped into six subfamilies (β, γ, δ, ε, η and ζ) (Cockcroft, 2006; Suh et al., 2008), of which PtdIns-PLCβ₁ is considered to be the predominant nuclear form (Faenza et al., 2008; Ye and Ahn, 2008). In some cell types, PtdIns-PLCβ₁ is constitutively present in nucleus, whereas in others, growth-factor stimulation causes the enzyme to translocate from the cytosol to the nucleus. This is facilitated by C-terminal sequences in PtdIns-PLCβ₁ that act either as a NLS or cause the retention of the protein in the nucleus (Kim et al., 1996). It is known that PtdIns-PLCβ isoforms are activated at the plasma membrane by G-protein-coupled receptors and are responsible for rapid InsP₃ production and acute Ca²⁺ signals (see Box 2). In the nucleus, however, PtdIns-PLCβ₁ plays an important role in signalling events that are evoked by growth-factor-receptor stimulation (Visnjic and Banfi, 2007). For example, PtdIns-PLCβ₁ was found to be essential during the differentiation of skeletal muscle myoblasts (Ramazzotti et al., 2008). The expression of the enzyme increases during myogenesis, and is required for the induction of cyclin D3 expression and the formation of the retinoblastoma-cyclin D3 complex that underlies differentiation (De Santa et al., 2007). The nuclear localisation of PtdIns-PLCβ₁ is essential in this context, as a cytosolically targeted form does not support myoblast differentiation (Faenza et al., 2007).

These studies highlight the importance of nuclear PtdIns-PLCs in growth factor responses. However, they mainly describe the roles of nuclear DAG, protein kinase C translocation or inositol polyphosphate formation that results from PtdIns-PLC activation (Divecha et al., 1991). Considerably less attention has been focussed on whether changes in the levels of nuclear Ca²⁺ participate in growth-factor-mediated responses. However, several recent reports have suggested a crucial role for Ca²⁺ downstream of nuclear PtdIns-PLC activation. For example, insulin receptors are found in the nucleus following insulin stimulation of cells, where they promote PtdIns(4,5)P₂ turnover and nuclear Ca²⁺ signalling independently of cytosolic Ca²⁺ signalling (Rodrigues et al., 2008). The specific action of insulin on nuclear InsP₃ production was confirmed by expressing the InsP₃-binding portion of the InsP₃R (known as InsP₃ ‘sponge’) to buffer changes in InsP₃ concentration. To affect its subcellular location, the Ins₃P₃ sponge was fused to either a NLS or a NES. The expression of the nucleus-targeted InsP₃ sponge significantly attenuated responses to insulin, whereas the cytosol-targeted Ins₃P₃ sponge did not (Rodrigues et al., 2008). The opposite effect was observed if the cells were stimulated with vasopressin, which acts on the plasma membrane pool of PtdIns(4,5)P₂ via cytosolic PtdIns-PLC isoforms (Gomes et al., 2008).

In addition to growth factor receptors being present in the nucleus, a number of studies have demonstrated that functional hormone receptors are also internalised and move across the NE. For example, a C-terminal NLS directs the nuclear import of the bradykinin B₂ receptor (Savard et al., 2008). Specific bradykinin-binding sites are present in hepatocyte nuclear extracts, and the addition of bradykinin to isolated nuclei was found to evoke a rapid, transient nucleoplasmic Ca²⁺ signal. Furthermore, it has been demonstrated that endogenous and ectopically expressed mGluR₅ metabotropic glutamate receptors are present on the nuclear membranes of primary neurons and of cultured cells, respectively (Kumar et al., 2008). Studies of isolated nuclei indicate that these receptors activate PtdIns-PLCs in the nucleus, leading to nucleoplasmic InsP₃ generation and Ca²⁺ signals.

Exactly how G-protein-coupled receptors in the nucleus become activated is unclear. The lumen of the NE is topologically the same as the extracellular space, so the ligand-binding domain of nuclear hormone receptors would be exposed within the NE lumen. It has been proposed that specific transporters allow the uptake of neurotransmitters such as glutamate into the NE, where they can bind to their cognate receptors and activate signalling (Jong et al., 2005). Similarly, receptors for endothelin, angiotensin and prostaglandin, the activation of which can also stimulate Ca²⁺-mobilising pathways, are reportedly expressed on nuclear membranes (Bkaily et al., 2006; Coffey et al., 1997).

Although many studies have focussed on investigating the nuclear functions of PtdIns-PLCβ₁, other PtdIns-PLC isoforms are also found in the nucleus, in particular PLCδ₁ (Okada et al., 2005) and PtdIns-PLCζ₁ (Gomes et al., 2008). In quiescent cells, PtdIns-PLCδ₁ is localised at the plasma membrane and in the cytosol, but following stimulation, it translocates to the nucleus in a Ca²⁺-dependent manner (Okada et al., 2005), where it plays a role in regulating PtdIns(4,5)P₂ levels, DNA synthesis and cell proliferation (Stallings et al., 2008). Growth-factor-induced Ca²⁺ signals, as well as non-specific Ca²⁺ transients caused by ionophores, promote nuclear PtdIns-PLCδ₁ translocation, thereby acting as positive feedback for further nuclear Ca²⁺ signalling (Yagisawa, 2006). Although data regarding the functions of different PtdIns-PLC isoforms in the nucleus are still emerging, current evidence suggests that they all play roles in cell survival, division and differentiation.

PtdIns-PLCζ₁ is also known to accumulate in the nucleus owing to an NLS, but in this case, the translocation of the enzyme prevents Ca²⁺ signalling (Ito et al., 2008). Repetitive Ca²⁺ oscillations occur immediately following fertilisation in mammalian oocytes, and are believed to be driven by InsP₃ production that is caused by the introduction of PtdIns-PLCζ₁ into the oocyte from the fused sperm. The Ca²⁺ oscillations cease around the time when the pronucleus is formed, owing to the sequestration of PtdIns-PLCζ₁ in the nucleus (Larman et al., 2004). Unlike the other PtdIns-PLC isoforms described above, when PtdIns-PLCζ₁ is in the nucleus, it does not evoke InsP₃ production and Ca²⁺ signalling.

The Ca²⁺-mobilising pathways described above have well-characterised effects on nuclear Ca²⁺ signalling. However, there are numerous additional messengers and signalling pathways by which cellular Ca²⁺ signals can be evoked (Boothman et al., 2002), and some of these have been shown to function in the nucleus. Another example of a putative nuclear messenger is arachidonic acid: this lipid is known to increase cytosolic Ca²⁺ concentration. Although little is known about the effect of arachidonic acid on nucleoplasmic Ca²⁺, it is plausibly an additional regulator of nuclear Ca²⁺ concentration. Indeed, phospholipase A2, an enzyme involved in the production of arachidonic acid, translocates into the nucleus in a Ca²⁺-dependent manner (Schievella et al., 1995). Furthermore, arachidonic acid can be metabolised into further Ca²⁺ signalling moieties by the action of cyclooxygenase-2, which translocates into the nucleus in response to growth-factor-stimulation (Coffey et al., 1997). Although the bulk of studies have focussed on InsP₃-mediated Ca²⁺ release in
the nucleus, it is probable that nuclear Ca\textsuperscript{2+} signalling utilises the same diverse range of messengers and channels as the cytosol.

Recovery of nucleoplasmic Ca\textsuperscript{2+} signals

It has been noted in several studies that nuclear Ca\textsuperscript{2+} signals persist considerably longer than equivalent cytosolic Ca\textsuperscript{2+} transients (Bootman et al., 2007; Kockskamper et al., 2008a; Lipp et al., 1997). The reason for this is the lack of Ca\textsuperscript{2+} ATPases on the inner NE (Humbert et al., 1996). Because of the slow dissipation of nuclear Ca\textsuperscript{2+}, it is plausible that rapid cellular stimulation could cause a progressive accumulation of Ca\textsuperscript{2+} in the nucleoplasm. Fig. 4B,C shows an example of the kinetic delay in both the rise and dissipation of nuclear Ca\textsuperscript{2+}. The recordings were obtained by imaging fluo4 fluorescence from a single atrial cardiac myocyte that was activated by regular electrical depolarisation. In addition to the kinetic delay in recovery of the nuclear Ca\textsuperscript{2+} transient, it is evident that the nuclear fluo4 fluorescence was consistently higher than that in the cytosol, even when both signals had recovered. This is because fluo4 is brighter in the nucleus than in the cytosol (as described above), not because there was a persistent gradient of Ca\textsuperscript{2+} between the cytosol and nucleus. The calibrated diastolic Ca\textsuperscript{2+} levels are actually very similar in the cytosol and nucleus. The differences in the kinetics of the nuclear and cytosolic Ca\textsuperscript{2+} signals are illustrated in the line-scan image shown in Fig. 4C; it is evident that the nucleus takes longer to fill with Ca\textsuperscript{2+}, and considerably longer for the Ca\textsuperscript{2+} signal to dissipate.

A major mechanism for the dissipation of nuclear Ca\textsuperscript{2+} signals is via simple diffusion through NPCs. To leave the nucleus, a Ca\textsuperscript{2+} ion has to pass through the nucleoplasm and then encounter and traverse a NPC. Once outside the nucleus, Ca\textsuperscript{2+} can be quickly sequestered by Ca\textsuperscript{2+} ATPases on the outer NE, ER and plasma membrane, or by other buffers such as mitochondria. The NE can be regarded as a diffusion barrier for both Ca\textsuperscript{2+} entry and exit. Although Ca\textsuperscript{2+} ATPases are not expressed on the inner NE, there is growing evidence that a splice variant of type 1 sodium-calcium exchanger (NCX) is expressed on the inner NE (Ledeen and Wu, 2007). It is suggested that the nuclear NCX uniquely associates with a ganglioside known as GM1, which potentiates Ca\textsuperscript{2+} transport and prevents Ca\textsuperscript{2+} overloading within the nucleus (Ledeen and Wu, 2008). NCX is a mechanism of Ca\textsuperscript{2+} transport that is usually associated with electrically excitable cells in which larger and rapid fluxes of Ca\textsuperscript{2+} occur. However, many non-excitable cells also express NCX and, in some cells, expression of the transporters appears to be restricted to the nucleus (Xie et al., 2004). The configuration of the NCX on the inner NE is the same as on the plasma membrane, but the interaction with GM1 is atypical. In the presence of a Na\textsuperscript{+} gradient, nuclei sequester Ca\textsuperscript{2+}, verifying the functionality of NCX (Xie et al., 2002). The Na\textsuperscript{+} gradient might be driven by a Na\textsuperscript{+}/K\textsuperscript{+} ATPase or Na\textsuperscript{+}H\textsuperscript{+} exchanger, all of which are also present on the NE (Bkaily et al., 2006; Garner, 2002). Therefore, in addition to passive diffusion out of the nucleus, nucleoplasmic Ca\textsuperscript{2+} signals can be reversed by active uptake processes.

Replenishment of Ca\textsuperscript{2+} in the NE lumen is probably mediated by ER-localised Ca\textsuperscript{2+} ATPases. Although these enzymes sequester cytosolic Ca\textsuperscript{2+} into the ER, the fact that the lumen of the NE and ER are connected means that Ca\textsuperscript{2+} can diffuse from one membrane system to the other (Wu and Bers, 2006). In addition, it has been reported that Ca\textsuperscript{2+} accumulates in the NE via a curious mechanism that is driven solely by InsP\textsubscript{3} (Hsu et al., 1998; Malviya and Klein, 2006). It has been reported that specific InsP\textsubscript{3} receptors are present on the outer NE (Malviya, 1994), but exactly how these binding sites activate Ca\textsuperscript{2+} uptake is unknown. A major problem with this proposed uptake pathway is that the ER and NE possess a substantially higher Ca\textsuperscript{2+} concentration than the cytosol, and, unless the Ca\textsuperscript{2+} content of these stores was completely emptied, the flux of Ca\textsuperscript{2+} would always be towards the cytosol. Complete depletion of ER Ca\textsuperscript{2+} is a pathological state that culminates in cell death.

In the process of signal transduction, the reversal of a signalling event is as important as its induction. Exactly how nucleoplasmic Ca\textsuperscript{2+} signals decline back to pre-stimulation levels is not entirely clear, as the majority of studies have focussed solely on the generation of nucleoplasmic Ca\textsuperscript{2+} transients. Currently, passive diffusion through NPCs and uptake into the NE by NCX appear to be the main mechanisms by which nuclear Ca\textsuperscript{2+} transients are limited and reversed.

Conclusions

Over the past decade there has been a tremendous increase in the knowledge of Ca\textsuperscript{2+} signalling systems in the nucleus, and progress has been made in unravelling the impact of changes in nuclear Ca\textsuperscript{2+} on cellular activity. Recent studies have clearly highlighted the idea that gene transcription and cell-cycle progression can be specifically influenced by nuclear Ca\textsuperscript{2+} signals. It is probable that many more cellular processes will be shown to be dependent on nucleoplasmic Ca\textsuperscript{2+} changes. Despite this understanding of the functions of nuclear Ca\textsuperscript{2+} signals, there is still some uncertainty concerning how nuclear Ca\textsuperscript{2+} signals are generated and whether Ca\textsuperscript{2+} signalling in the nucleus is really autonomous. This is a surprisingly contentious issue that has been debated for many years. It has been shown that NPCs do not prevent Ca\textsuperscript{2+} fluxes from crossing the NE, which would imply that Ca\textsuperscript{2+} signalling in the nucleus must follow cytosolic Ca\textsuperscript{2+} changes (providing that cytosolic Ca\textsuperscript{2+} changes persist for sufficient time). Yet, several studies have shown that Ca\textsuperscript{2+} signals initiate within, and are restricted to, the nucleus. Exactly how Ca\textsuperscript{2+} signals within the nucleus or cytosol are prevented from equilibrating with the other compartment remains unclear.

In future studies, it will be important to work towards defining unified views on several topics, including nuclear Ca\textsuperscript{2+}-release mechanisms and the regulation of NPC structure and function by Ca\textsuperscript{2+}. As some of the controversy in nuclear Ca\textsuperscript{2+} signalling might be owing to the use of different cell types in different laboratories, it will also be necessary to consider cell-type-specific versus generic Ca\textsuperscript{2+} signalling paradigms. Finally, another goal of future research will be to link putative nucleoplasmic Ca\textsuperscript{2+} signals with cellular processes.

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