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Revised version

**Role of the nuclear envelope in calcium signalling**

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## Summary

The endoplasmic reticulum is the major  $\text{Ca}^{2+}$  store inside the cell. Its organisation in specialised sub-domains allows the local delivery of  $\text{Ca}^{2+}$  to specific cell areas on stimulation. The nuclear envelope, which is continuous with the endoplasmic reticulum, has a double role: it insulates the nucleoplasm from the cytoplasm and it stores  $\text{Ca}^{2+}$  around the nucleus. Furthermore, all the constituents of the signalling cascade leading to  $\text{Ca}^{2+}$  mobilisation are found in the nuclear envelope; this allows the nuclear  $\text{Ca}^{2+}$  to be regulated autonomously. On the other hand, cytosolic  $\text{Ca}^{2+}$  transients can propagate within the nucleus via the nuclear pore complex. The variations in nuclear  $\text{Ca}^{2+}$  concentration are important for controlling gene transcription and for progression in the cell cycle. Recent data suggest that invaginations of the nuclear envelope modify the morphology of the nucleus and may affect  $\text{Ca}^{2+}$  dynamics in the nucleus and regulate transcriptional activity.

## Introduction

The endoplasmic reticulum (ER) is a three-dimensional network of membranous tubules and cisternae that spreads throughout the cell from the nucleus to the plasma membrane. The interphase ER can be divided into the nuclear and the peripheral ER and the ER network can have different morphologies in different cell areas, in conjunction with different functions (Baumann and Walz, 2001). It has many different cell functions, including translocation of proteins across the ER membrane, integration of proteins into the membrane, folding and modification of proteins in the lumen, synthesis of phospholipids and steroids (see Baumann and Walz, 2001, Voeltz, et al., 2002). The ER is also recognised as a major  $\text{Ca}^{2+}$  store inside the cells characterised by the presence of  $\text{Ca}^{2+}$  pumps that accumulate  $\text{Ca}^{2+}$ ,  $\text{Ca}^{2+}$ -binding proteins that retain the ion in the lumen of the store and  $\text{Ca}^{2+}$  channels to rapidly release  $\text{Ca}^{2+}$  on stimulation (for reviews, see Meldolesi and Pozzan, 1998, Papp, et al., 2003). The mobilisation of  $\text{Ca}^{2+}$  accumulated in this store plays a major role in agonist-induced  $\text{Ca}^{2+}$  signalling in stimulated cells and the  $\text{Ca}^{2+}$  ion is used as an intracellular signal that regulates a variety of cell functions (for reviews, see Berridge, et al., 2003, Rizzuto and Pozzan, 2006). The widespread distribution of the ER throughout the cell allows the delivery of  $\text{Ca}^{2+}$  in precise areas of the cell and the fine regulation of specific cell functions, like stimulation of ion channels in the plasma membrane, regulation of mitochondrial metabolism and regulation of gene expression in the nucleus (Rizzuto and Pozzan, 2006). Heterogeneity of the ER  $\text{Ca}^{2+}$  store depends on the non-random distribution of  $\text{Ca}^{2+}$ -handling proteins and the localisation of the  $\text{Ca}^{2+}$  release channels which can give rise to local  $\text{Ca}^{2+}$  signals and microdomains in the cytosol (Papp, et al., 2003). Regulation of the  $\text{Ca}^{2+}$  level in the nucleus is an example of a complex form of local  $\text{Ca}^{2+}$  signalling. Increases in  $\text{Ca}^{2+}$  concentration in the nucleus can have specific effects, different from those observed in the cytoplasm.  $\text{Ca}^{2+}$  is essential in signalling pathways that regulate gene expression by stimulating the translocation of transcription factors from the cytosol to the nucleus;  $\text{Ca}^{2+}$  also translocates or activates enzymes that regulate nuclear transcription factor activity and modifies the structure of chromatin (Flavell and Greenberg, 2008, Mellstrom, et al., 2008). The use of selective buffers for cytoplasmic or nucleoplasmic  $\text{Ca}^{2+}$  allowed the direct demonstration that cell proliferation is dependent on  $\text{Ca}^{2+}$  signals within the nucleus rather than in the cytoplasm (Rodrigues, et al., 2007). A similar approach in a liver cell line indicated that nucleoplasmic  $\text{Ca}^{2+}$  is required for mitogen-activated protein kinase-mediated gene transcription (Pusl, et al., 2002). The nucleus is bordered by the nuclear envelope (NE) which has a double function as it insulates the nucleoplasm from the cytoplasm and may be directly involved in  $\text{Ca}^{2+}$  signalling inside the nucleus. Recent reviews describe in detail various aspects of nuclear calcium signalling, (Alonso and Garcia-Sancho, 2011, Bootman, et al., 2009). In the present review I will focus on the role of the NE in nuclear  $\text{Ca}^{2+}$  homeostasis and in the mechanisms involved in nuclear  $\text{Ca}^{2+}$  signalling.

## Structure of the nuclear envelope

The use of fluorescent dyes which cannot exchange between discontinuous membranes provided evidence that the ER is a single membrane system (Terasaki and Jaffe, 1991) and the use of luminal GFP-tagged proteins demonstrates that large molecules can rapidly diffuse within the luminal space defined by the ER and the NE membranes (Subramanian and Meyer, 1997) (for review, see Voeltz, et al., 2002). This continuous membrane system is organised in different specialised sub-domains like sER, rER or NE, which are morphologically distinct and have different functions. So, most membrane proteins are shared between the sER and rER, but several proteins involved in translocation or processing of newly synthesised

proteins are enriched in the rER (for reviews, see Levine and Rabouille, 2005, Voeltz, et al., 2002). The NE separates the nuclear and the cytoplasmic compartments of interphase cells. When observed by light microscopy, the nucleus appears delimited by a membrane in continuity with the ER, but electron microscopy reveals a double membrane layer around the nucleus. Therefore, the NE can be further subdivided into three morphologically and biochemically different structures: the outer nuclear membrane (ONM), the inner nuclear membrane (INM) and the nuclear pore complex (NPC) (for reviews, see Baumann and Walz, 2001, Gerace and Burke, 1988). The ONM is continuous with the ER, is decorated with ribosomes and contains a lot of proteins in common with the ER. It also displays a set of unique proteins, some of which may tether the nucleus to the actin cytoskeleton and the centrosome; others interact through the lumen with proteins of the INM. The INM contains many distinct proteins that contact the underlying lamina and chromatin (Batrakou, et al., 2009, Hetzer, 2010, Prunuske and Ullman, 2006). Proteomic analysis of the NE reveals cell type-specific differences in the composition of protein sub-complexes and suggests a functional complexity of the NE (Schirmer and Gerace, 2005). The ONM and the INM are continuous at each NPC that span the NE and regulates transport between the nucleus and the cytoplasm. The NPC is a 120 MDa protein complex which shows eight-fold rotational symmetry with an outer diameter of 100 nm and a central transport channel measuring 40 nm in diameter. It is composed of about 30 distinct protein components, some of high molecular weight and others duplicated many times per NPC (Batrakou, et al., 2009). The NPC mediates communication and selective exchange between the nucleoplasm and the cytoplasm.

The NE can be observed either by electron microscopy or by conventional microscopy after the immunostaining of proteins present in, or associated with, either of the membranes of the nuclear envelope. These studies illustrate the variety of the nuclear shape depending on cell type or cell physiology. Such approaches allow the identification of dynamic tubular channels which appear as deep, narrow invaginations of the nuclear envelope. These invaginations, or infoldings, first described in 1979 (Bourgeois, et al., 1979), have been observed in a lot of cell types (Clubb and Locke, 1998, Collado-Hilly, et al., 2010, Fricker, et al., 1997, Johnson, et al., 2003, Langevin, et al., 2010, Lui, et al., 2003, Wittmann, et al., 2009). The invaginations of the NE have been recently classified into two main classes depending on whether the ONM is involved (Malhas, et al., 2011). Type I invaginations are those where the only the INM invaginates into the nucleoplasm, they can be branched and ramified. The type II invaginations involve both the INM and the ONM. Studies by electron microscopy and recently by three-dimensional structured illumination microscopy have resolved the bilamellar structure of these type II invaginations and revealed the presence of NPC (Schermelleh, et al., 2008). The tubules delimited by these type II invaginations contain cytoplasmic components like actin (Clubb and Locke, 1998, Johnson, et al., 2003), and mitochondria (Clubb and Locke, 1998, Lui, et al., 2003). These invaginations, which have been called the nucleoplasmic reticulum increase the area of exchange between the cytoplasm and the nucleoplasm and make contact deep inside the nucleus with chromatin and sometimes extend to near the nucleolus. Fig. 1 illustrates the presence of invaginations of the NE in polarised MDCK epithelial cells.

It has been suggested that cells that are highly de-differentiated or cancerous have an increased incidence of invaginations (Johnson, et al., 2003). Nuclear infoldings are dynamic structures which can also be formed following various physiological stimuli. Stimulation of synaptic NMDA receptors increases the percentage of infolded nuclei in hippocampal neurons via a process that requires the ERK-MAP kinase pathway and new protein synthesis. In contrast, death signalling pathways triggered by extrasynaptic NMDA receptors cause a rapid

loss of infoldings (Wittmann, et al., 2009). The presence of NE invaginations could also depend on the physiological status of the cell, we have found that they are much more prominent in polarised MDCK epithelial cells than in non-polarised proliferating cells (Collado-Hilly, et al., 2010). The mechanical environment of the cells could also influence the cell nucleus and stretching of fibroblasts induces loss of nuclear invaginations (Langevin, et al., 2010). The formation of invaginations of the nuclear envelope increases the number of NPCs, which may provide more anchoring sites for active genes and enhance gene expression (Akhtar and Gasser, 2007). Furthermore, the infoldings dynamically modify the geometry of the nucleus and form unequally sized nuclear compartments (Wittmann, et al., 2009). These modifications of nuclear geometry and the generation of signalling microdomains may be particularly relevant for diffusion in and out of the nucleus.

### **Nuclear envelope as a Ca<sup>2+</sup> store**

The lumen of the nuclear envelope is continuous with the lumen of the ER (Subramanian and Meyer, 1997), so it can be assumed that Ca<sup>2+</sup> accumulated anywhere in the ER can diffuse freely to the nuclear envelope. However, this does not imply that Ca<sup>2+</sup> is uniformly transported within the ER throughout the cell and we can ask whether the nucleus has its own Ca<sup>2+</sup> transporting system. An active ATP-dependent Ca<sup>2+</sup> accumulation has been measured in isolated liver nuclei and this was associated with an increase in the intranuclear free Ca<sup>2+</sup> concentration (Nicotera, et al., 1989). The use of Ca<sup>2+</sup>-sensitive fluorescent probes and confocal analysis indicates that Ca<sup>2+</sup> is accumulated within the NE of isolated liver nuclei (Gerasimenko, et al., 1995), and measurements of the Ca<sup>2+</sup> concentration in the NE with low-affinity fluorescent Ca<sup>2+</sup> probes in intact cells gave a resting value of 250-350 μM (Petersen, et al., 1998). The SERCAs are responsible for the active Ca<sup>2+</sup> transport in the ER and it has been demonstrated that the Ca<sup>2+</sup> pumps of the NE are identical to that of the ER (Lanini, et al., 1992). The SERCA family includes the products of three genes, named SERCA1, SERCA2 and SERCA3, each giving rise to alternatively spliced mRNA and protein isoforms (for reviews, see Wuytack, et al., 2002). Some studies have reported the presence of SERCA on the NE by using BODIPY-thapsigargin, but the hydrophobic nature of this molecule casts some doubt on the specificity of the labelling (Collado-Hilly, et al., 2010).

Immunohistochemical experiments directly demonstrated the presence of the SERCA2 isoform on the NE of epithelial cells (Collado-Hilly, et al., 2010, Lee, et al., 1997a) or muscle cells (Abrenica and Gilchrist, 2000). The Ca<sup>2+</sup> pump has also been found in the invaginations of the NE (Avedanian, et al., 2011, Collado-Hilly, et al., 2010). Since the ONM is continuous with the ER membrane, the SERCA is more likely present on the ONM, but to our knowledge there is no EM study indicating the presence of SERCA on the INM. In some cell types a sodium-calcium exchanger located in the INM may function to transfer Ca<sup>2+</sup> between the nucleoplasm and the lumen of the envelope (Xie, et al., 2002). Functional studies indicated that Ca<sup>2+</sup> concentration in the nucleoplasm decreases after stimulation. This could be due either to direct pumping through the INM or diffusion through the NPC.

The Ca<sup>2+</sup> pumps can accumulate Ca<sup>2+</sup> in specific areas of the cell and Ca<sup>2+</sup>-binding proteins in the lumen of the ER provide a releasable Ca<sup>2+</sup> pool near the Ca<sup>2+</sup> release channels (Papp, et al., 2003). The Ca<sup>2+</sup>-storage protein calreticulin has been localised by immunocytochemistry in the NE and in invaginations of the NE of various tissues, including human oocytes and embryos and epithelial cells (Balakier, et al., 2002, Bedard, et al., 2005, Collado-Hilly, et al., 2010, Echevarria, et al., 2003). Therefore, the presence of the Ca<sup>2+</sup>-binding protein in the nuclear envelope confirms its role as a Ca<sup>2+</sup> store which may provide Ca<sup>2+</sup> to the nucleus.

The localisation of  $\text{Ca}^{2+}$ -releasing channels is critical for generating spatially complex  $\text{Ca}^{2+}$  signals and in this case to induce nuclear  $\text{Ca}^{2+}$  signals. Inositol trisphosphate ( $\text{IP}_3$ ), produced from membrane phosphoinositides on hormonal stimulation, binds to the  $\text{IP}_3$  receptor ( $\text{IP}_3\text{R}$ ) and releases  $\text{Ca}^{2+}$  from intracellular stores. The  $\text{IP}_3\text{R}$  family includes the products of three genes, named  $\text{IP}_3\text{R1}$ ,  $\text{IP}_3\text{R2}$ ,  $\text{IP}_3\text{R3}$  which display specific tissue distribution and sometimes different sub-cellular localisations (Mikoshiha, 2007, Vermassen, et al., 2004). The presence of  $\text{IP}_3\text{R}$  in the nucleus is suspected from direct measurements of  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  release from liver nuclei and from radiolabelled  $\text{IP}_3$  binding to isolated nuclei (Malviya, et al., 1990, Nicotera, et al., 1990). However, there are few reports directly demonstrating the localisation of  $\text{IP}_3\text{R}$  in the nucleus or the NE, possibly because of the low density of receptors found in this organelle. Immunohistochemical experiments performed *in situ* confirmed the presence of  $\text{IP}_3\text{R}$  on the perinuclear ER and on the nuclear envelope in epithelial cells (Collado-Hilly, et al., 2010, Lee, et al., 1997b, Leite, et al., 2003, Nakanishi, et al., 1996, Siefjediers, et al., 2007, Yule, et al., 1997), cardiomyocytes (Bare, et al., 2005) skeletal muscle cells (Cardenas, et al., 2005, Kusnier, et al., 2006), smooth muscle cells (Vermassen, et al., 2003), and *Xenopus* oocytes (Kume, et al., 1993).  $\text{IP}_3\text{R}$  has also been detected on the nucleoplasmic reticulum of HeLa cells (Lui, et al., 2003), liver cells (Echevarria, et al., 2003), MDCK cells (Collado-Hilly, et al., 2010), cardiomyocytes (Guatimosim, et al., 2008) and smooth muscle cells (Avedanian, et al., 2011). Some studies suggest that  $\text{IP}_3\text{R2}$  is more often associated with the nucleus (Bare, et al., 2005, Laflamme, et al., 2002, Leite, et al., 2003), but according to the tissue, any receptor subtype could be localised at the nucleus (for review, see Vermassen, et al., 2004). However, immunohistochemical experiments performed by conventional microscopy failed to determine whether the  $\text{IP}_3\text{Rs}$  are localised on the ONM or the INM. The ONM shares many features with the ER membranes and, indeed,  $\text{IP}_3\text{Rs}$  have been found on the ONM of isolated nuclei by electrophysiological measurements of  $\text{IP}_3$ -induced currents (Mak and Foskett, 1994, Stehnbittel, et al., 1995a). However, the contamination of the preparation by ER membranes cannot be completely excluded. Recently, the cytoplasmic domain of  $\text{IP}_3\text{R}$  has been directly visualized on isolated nuclei of Sf9 cells by freeze-dry rotary shadowing for electron microscopy, and was located on the cytoplasmic side of the NE (Cardenas, et al., 2010). While there is direct evidence for the localisation of  $\text{IP}_3$  on the ONM, its presence on the INM is debated. Functional experiments suggest that  $\text{IP}_3$  can induce the release of  $\text{Ca}^{2+}$  directly through the INM (see below), but to our knowledge there is no immunohistochemical evidence at the EM level for the localisation of  $\text{IP}_3\text{R}$  on the INM. It should be noted that some studies describe the presence of  $\text{IP}_3\text{R}$  on small vesicles inside the nucleus that could be responsible for nuclear  $\text{Ca}^{2+}$  transients (Huh, et al., 2006, Yoo, et al., 2005). Whether these vesicles are different from invaginations of the NE is not clear. Finally, ryanodine receptors have directly been visualised with BODIPY-Ry on the NE of nuclei isolated from pancreatic cells (Gerasimenko, et al., 2003). They have also been located by immunohistochemical experiments in the NE of cardiac cells (Abrenica and Gilchrist, 2000) and in the NE and the nucleoplasmic reticulum of myoblastic cells (Marius, et al., 2006). These data clearly indicate that the NE of many cell types has all the characteristics of a  $\text{Ca}^{2+}$  store which can be mobilised on stimulation to deliver a  $\text{Ca}^{2+}$  signal within or in the close proximity of the nucleus.

### **Production of $\text{IP}_3$ in the NE**

The canonical pathway that leads to the mobilisation of intracellular  $\text{Ca}^{2+}$  involves the activation of either seven-transmembrane domains receptors or receptor tyrosine kinases, which induces the hydrolysis of phosphatidylinositol 4,5-bisphosphate and the production of  $\text{IP}_3$  (Berridge, et al., 2003). The mobility of  $\text{IP}_3$  released from the plasma membrane is

sufficient to mobilise  $\text{Ca}^{2+}$  from the ER anywhere in the cytoplasm, including the nuclear envelope (Allbritton, et al., 1992). However, the various components of the signalling cascade leading to  $\text{Ca}^{2+}$  mobilisation have also been found in the nucleus. Phosphoinositides (as well as the enzymes responsible for their synthesis, namely phosphatidylinositol and phosphatidylinositol 4-phosphate kinases) have long been known to be present in the nucleus (for review, see Irvine, 2003). The fact that these nuclear inositol lipids resist detergents suggests that they are localised inside the nucleus, possibly on structures called speckles, and virtually not in the nuclear envelope. But it is possible that invaginations of the NE could be enriched in PIP2 such that it appears to be intranuclear (Irvine, 2006). Different phospholipase C isoforms have been identified in the nucleus and may provide in situ production of  $\text{IP}_3$  and DAG. PLC- $\beta$  contains at its C-terminal domain a region responsible for nuclear localisation (Cocco, et al., 2006). PLC- $\beta$  is present both in whole nuclei and in nuclei treated with detergent, suggesting that it is probably not present on NE (for review, see (Visnjic and Banfic, 2007).

If we assume that the local production of  $\text{IP}_3$  in the nucleus mobilises  $\text{Ca}^{2+}$  locally, it is still unclear how the PLC is stimulated. Several studies have described the translocation to the nucleus of growth factor receptors after stimulation (for review, see Wells and Marti, 2002), but their role in the change in nuclear  $\text{Ca}^{2+}$  concentration and growth factor-mediated responses have only recently been investigated. For example, experiments performed on a hepatic cell line indicated that the HGF receptor, c-Met, rapidly translocated to the nucleus upon stimulation with HGF and appeared both at the NE and inside the nucleus. The c-Met-induced nuclear  $\text{Ca}^{2+}$  signal depended on the hydrolysis of PIP2 and formation of  $\text{IP}_3$  in the nucleus. This is different from the  $\text{Ca}^{2+}$  signal induced by vasopressin which depended on the formation of  $\text{IP}_3$  in the cytoplasm (Gomes, et al., 2008). Insulin also induced  $\text{Ca}^{2+}$  oscillations in rat hepatocytes and data suggest that the insulin receptor translocated to the nucleus to initiate an  $\text{IP}_3$ -dependent nuclear  $\text{Ca}^{2+}$  signal (Rodrigues, et al., 2008). Various G protein-coupled receptors have also been found in the nucleus in various cell types (for review, see Gobeil, et al., 2006). For example, the metabotropic glutamate receptor, mGlu5R, has been found in the nucleus of neurons, and immunohistochemical experiments using confocal or electron microscopy suggest that it is localised on the membrane of the NE (O'Malley, et al., 2003). Experiments performed on HEK293 cells expressing mutant mGlu5 receptor leading to the loss of G-protein coupling, indicated that the nuclear mGlu5 receptor couples to  $\text{G}_{q/11}$  and PLC to generate  $\text{IP}_3$  and mobilise nuclear  $\text{Ca}^{2+}$  (Kumar, et al., 2008). In intact cells, extracellular ligands like glutamate or quisqualate reach nuclear receptors via both sodium-dependent transporters and cystine glutamate exchangers (Jong, et al., 2005). Another study in cardiomyocytes indicated that the type 1 and 2 angiotensin receptors (AT1R and AT2R, respectively) are localised on nuclear membranes. Application of angiotensin II to these receptors increased NF $\kappa$ B mRNA expression, and stimulation of AT1R induced a  $\text{Ca}^{2+}$  signal in isolated nuclei via an  $\text{IP}_3$ R-mediated mechanism. Angiotensin II can be produced in situ in intact cardiomyocytes and activates this intranuclear pathway (Tadevosyan, et al., 2010).

### **Role of nuclear envelope in nuclear $\text{Ca}^{2+}$ signalling**

The nucleus is separated from the surrounding cytoplasm by the NE, but the two compartments can communicate through the NPC. It is admitted that molecules larger than 70 kDa do not redistribute passively between nucleus and cytoplasm and so need a targeting sequence to be transported through the NPC by facilitated diffusion or active transport. Conversely, metal ions, small metabolites and molecules up to 10 kDa in mass freely exchange between the cytoplasm and the nucleus by passive diffusion through the NPC



(Torok, 2007). The permeability of the pore to proteins of intermediate size may be regulated. Among the factors that modulate the permeability of the nuclear pore, the  $\text{Ca}^{2+}$  ion is of special interest as both the cytosolic  $\text{Ca}^{2+}$  concentration and the amount of  $\text{Ca}^{2+}$  within the NE may be involved. Greber and Gerace (Greber and Gerace, 1995) first demonstrated in mammalian cells that depletion of  $\text{Ca}^{2+}$  from the NE inhibits both the signal mediated transport of proteins into the nucleus and the passive diffusion of 10 kD fluorescent dextran through the NPC. Similarly, experiments performed in intact nuclei or nuclear membrane from *Xenopus laevis* oocytes demonstrated that depletion of the nuclear  $\text{Ca}^{2+}$  store regulates movements of 10 kD molecules through the NE (Stehnobittel, et al., 1995b). However the measurement of the diffusion of GFP through the NE of intact cell by real time imaging indicated that it was not affected by depletion of perinuclear  $\text{Ca}^{2+}$  store (Wei, et al., 2003). A recent study in liver cells, using localised two-photon activation of photoactivatable GFP, demonstrates that hormones that increase cytosolic  $\text{Ca}^{2+}$  concentration increased the permeability of the nuclear membrane (O'Brien, et al., 2007). These studies and others illustrate the discrepancies on the role of  $\text{Ca}^{2+}$  on the permeability of the NPC, they also indicate that both luminal and cytoplasmic  $\text{Ca}^{2+}$  may have an important role in regulating trafficking of macromolecules between the cytoplasm and the nucleoplasm (for review and discussion see Bootman, et al., 2009, Sarma and Yang, 2011). This may provide a mechanism to mediate the entry of transcription factors or other regulatory molecules and to regulate gene transcription in target cells. It is admitted that  $\text{Ca}^{2+}$  can diffuse freely through the NPC however, whether it can be regulated by  $\text{Ca}^{2+}$  within the NE is also controversial (Gerasimenko and Gerasimenko, 2004). The regulatory role of  $\text{Ca}^{2+}$  stored in the lumen of the NE is associated with a switch in the conformation of the nuclear pore complex which may control the diffusion of intermediate sized molecules. Atomic force microscopy (AFM) showed the displacement of a central granule to the cytoplasmic face of the NE under  $\text{Ca}^{2+}$  depleting conditions (Moore-Nichols, et al., 2002, PerezTerzic, et al., 1996, Stoffler, et al., 2006).

The  $\text{Ca}^{2+}$  ion could also act directly in the nucleoplasm and regulate gene transcription. It is a small molecule which is assumed to diffuse freely through the NPC, so it can be expected that the  $\text{Ca}^{2+}$  concentration in the nucleus rapidly equilibrates with the cytosolic  $\text{Ca}^{2+}$  concentration. Indeed, studies on intact RBL cells (Allbritton, et al., 1994), or neurons (Eder and Bading, 2007, Omalley, 1994), and isolated liver nuclei (Gerasimenko, et al., 1995), demonstrate that the  $\text{Ca}^{2+}$  signal from the cytosol to the nucleus is not substantially delayed by the NE. Therefore,  $\text{Ca}^{2+}$  waves that occur in the cytosol of stimulated cells could propagate to the nucleoplasm. However, a major question concerns the  $\text{Ca}^{2+}$  transients observed in the nucleoplasm and whether they could be generated autonomously and distinguished from cytosolic  $\text{Ca}^{2+}$  variations. Several groups using fluorescent  $\text{Ca}^{2+}$  imaging in living cells reported that the  $\text{Ca}^{2+}$  concentration can be different in the cytosol and in the nucleus. However, it has been recognised that the fluorescent  $\text{Ca}^{2+}$  indicators can behave differently in the nucleus as they are much brighter in the nucleus than in the cytosol. Both  $\text{Ca}^{2+}$  binding to the probe and dynamic range of the indicator may be affected. This results in technical artefacts that led to misinterpretation of the data and controversy on the regulation of nuclear  $\text{Ca}^{2+}$  concentration (for discussion see Bootman, et al., 2009)).

Several experimental approaches have been used to study nuclear  $\text{Ca}^{2+}$  signals, such as kinetic analysis of  $\text{Ca}^{2+}$  transients in the cytoplasm and nucleoplasm, and the direct manipulation of the cytoplasmic and nucleoplasmic  $\text{Ca}^{2+}$  contents in intact cells. Several studies indicate that the  $\text{Ca}^{2+}$  signal can originate in the nucleus (Fig. 2). Stimulation of HepG2 liver cells with submaximal concentrations of ATP revealed that the  $\text{Ca}^{2+}$  signal started earlier and was

greater in the nucleus than in the cytosol. This was confirmed by the photorelease of IP<sub>3</sub> inside the cells, which showed that the nuclear IP<sub>3</sub>R channels were more sensitive to IP<sub>3</sub> than the cytoplasmic IP<sub>3</sub>R, and this was correlated with a higher sensitivity of the type 2 IP<sub>3</sub>R present on the nuclear envelope (Leite, et al., 2003). The selective measurements of changes of Ca<sup>2+</sup> in the cytosol or in the nucleus by targeted aequorin in GH3 pituitary cells indicated that the IP<sub>3</sub>-induced nuclear response is not inhibited by the addition of cytosolic heparin, which does not permeate the nuclear pore. Furthermore, the nuclear Ca<sup>2+</sup> response to IP<sub>3</sub> showed a higher sensitivity and again this was correlated with the presence of IP<sub>3</sub>R-2 in the nucleus (Chamero, et al., 2008). In functional experiments on isolated liver nuclei, IP<sub>3</sub> or cyclic ADP ribose evoked intranuclear Ca<sup>2+</sup> elevations (Gerasimenko, et al., 1995). Similarly, in isolated acinar pancreatic nuclei, NAADP, cyclic ADP ribose or IP<sub>3</sub> reduced the Ca<sup>2+</sup> concentration inside the nuclear envelope, and this was associated with a transient Ca<sup>2+</sup> rise in the nucleoplasm (Gerasimenko, et al., 2003).

Cardiomyocytes are of special interest because Ca<sup>2+</sup>-dependent signalling pathways are regulated under conditions of continual Ca<sup>2+</sup> transients that mediate cardiac contraction during each heart beat (Molkentin, 2006). Stimulation of ventricular myocytes with endothelin 1, which produces intracellular IP<sub>3</sub>, induced local Ca<sup>2+</sup> release from the NE via IP<sub>3</sub>R, and again these data are correlated with the presence of type 2 IP<sub>3</sub>R on the nuclear envelope. This local Ca<sup>2+</sup> release triggers histone deacetylase 5 phosphorylation and nuclear export, which do not occur during the global Ca<sup>2+</sup> transients at each heart beat (Wu, et al., 2006). The involvement of IP<sub>3</sub>-induced Ca<sup>2+</sup> release in the nuclear Ca<sup>2+</sup> transients was confirmed by the direct addition of IP<sub>3</sub> to permeabilised cardiomyocytes, which increases nuclear Ca<sup>2+</sup> (Zima, et al., 2007). Direct labelling of Ca<sup>2+</sup> in the lumen of the nuclear envelope indicates that it is mobilised to increase the Ca<sup>2+</sup> concentration in the nucleoplasm. Experiments performed on isolated nuclei also demonstrate that InsP<sub>3</sub> induces the release of Ca<sup>2+</sup> from the nuclear envelope and allows the genesis of Ca<sup>2+</sup> transients in the nucleoplasm (Zima, et al., 2007). Similar results have been obtained in electrically stimulated atrial myocytes, where addition of endothelin 1 caused an increase of nuclear Ca<sup>2+</sup>, an effect mediated by the production of IP<sub>3</sub> and the activation of IP<sub>3</sub>R (Kockskamper, et al., 2008). The elevation of nuclear Ca<sup>2+</sup> concentration induced by endothelin 1 in cardiomyocytes is involved in the pathway leading to cardiac hypertrophy (Higazi, et al., 2009).

It is now acknowledged that Ca<sup>2+</sup> release from the NE participates in the genesis and regulation of Ca<sup>2+</sup> transients in the nucleoplasm. However, a major controversy concerns the orientation of the Ca<sup>2+</sup> flux from the NE. Ca<sup>2+</sup> can be either directly released to the nucleoplasm through the INM or released to the cytoplasm and secondly enter the nucleus through the NPC. One approach to determine whether Ca<sup>2+</sup> is released directly to the nucleoplasm is to apply IP<sub>3</sub> or IP<sub>3</sub> antagonists to the cytoplasm or to the nucleoplasm and to consider the effects on the cytoplasmic or nucleoplasmic Ca<sup>2+</sup> transients. These approaches have given conflicting results, possibly due to the cell type used in the experiments or to the geometry of the nucleus (see below). In an experiment performed in rat basophilic leukaemia cells stimulated by IP<sub>3</sub>-generating agonist, the injection of heparin-dextran, an inhibitor of the IP<sub>3</sub>R which is excluded from the nucleus, suppressed Ca<sup>2+</sup> transients in the cytosol and in the nucleus, suggesting that nuclear Ca<sup>2+</sup> increases followed cytosolic Ca<sup>2+</sup> increases (Allbritton, et al., 1994). But other experiments performed on intact cells led to a different conclusion. So, the injection of heparin in the nucleus of HeLa cells abolished Ca<sup>2+</sup> increase induced by histamine, while an increase in cytosolic Ca<sup>2+</sup> was observed (Lui, et al., 1998a). Injection of IP<sub>3</sub> in the nucleus of *Xenopus* oocytes induced Ca<sup>2+</sup> increases in the nucleus even if heparin was present in the cytosol (Hennager, et al., 1995). Moreover, the observation of Ca<sup>2+</sup>

transients in isolated nuclei suggests that  $\text{Ca}^{2+}$  was directly delivered from the lumen of the NE to the nucleoplasm, because it is assumed that  $\text{Ca}^{2+}$  released to the incubation medium will immediately diffuse and be diluted far from the nucleus (Gerasimenko, et al., 2003, Gerasimenko, et al., 1995, Zima, et al., 2007). Furthermore, patch-clamp recording from the inner nuclear membrane of Purkinje neurons revealed the presence of  $\text{InsP}_3\text{R}$ -activated channels (Marchenko, et al., 2005). Today, there is some consensus that  $\text{Ca}^{2+}$  can be delivered directly from the lumen of the NE to the nucleoplasm through the INM, more likely through  $\text{IP}_3\text{R}$  or in some cases through  $\text{RyR}$ . It should be noted that small vesicles have been described inside the nucleoplasm that could accumulate  $\text{Ca}^{2+}$  and release it in the presence of  $\text{IP}_3$  (Yoo, et al., 2005). These putative  $\text{Ca}^{2+}$  stores in the nucleoplasm can also be involved in some cell types in the genesis of nuclear  $\text{Ca}^{2+}$  signals. It is not clear whether autonomous nuclear  $\text{Ca}^{2+}$  signals play a role in physiological responses in intact cells. The whole machinery needed to produce  $\text{IP}_3$  locally within the nucleoplasm is present in the nucleus. This could induce  $\text{Ca}^{2+}$  release through the INM or nucleoplasmic vesicles if we assume that stimuli (hormone or agonist) can access the nucleus as a complex with a membrane receptor or by endocytosis or by local intracellular production.

The presence of  $\text{IP}_3\text{R}$  on the ONM is better documented (see above), but its involvement in the regulation of nuclear  $\text{Ca}^{2+}$  concentration is difficult to differentiate from the role of  $\text{IP}_3\text{R}$  on the ER in close proximity to the nuclear membrane. Careful analysis of the subcellular origin of nuclear  $\text{Ca}^{2+}$  signals in Fluo-3-loaded HeLa cells indicates that all signals that increase nuclear  $\text{Ca}^{2+}$  concentration are of cytoplasmic origin.  $\text{Ca}^{2+}$  puffs that originate within a 2–3  $\mu\text{m}$  perinuclear zone propagate anisotropically across the entire nucleus. Furthermore, the low buffering capacity of the nucleus allow the substantial prolongation of the  $\text{Ca}^{2+}$  signal in the nucleus as compared to the small cytosolic signal (Lipp, et al., 1997). In this case, it can be assumed that  $\text{Ca}^{2+}$  released through the  $\text{IP}_3\text{R}$  toward the cytosol will diffuse to the nucleoplasm through neighbouring NPC and, the absence of  $\text{Ca}^{2+}$  uptake mechanism in the nucleus allows the  $\text{Ca}^{2+}$  to rapidly diffuse over the entire nucleoplasm (Lipp, et al., 1997). Several studies agree with this model. So, the direct injection of  $\text{IP}_3$  in the nucleus of hamster oocyte in close proximity to the INM did not induce  $\text{Ca}^{2+}$  release, but when  $\text{IP}_3$  was uniformly applied to the cytoplasm it initiated a  $\text{Ca}^{2+}$  rise in the perinuclear cytoplasm and then in the nucleoplasm. It was concluded that the rise in  $\text{Ca}^{2+}$  concentration in the nucleoplasm was a consequence of  $\text{Ca}^{2+}$  release in the perinuclear cytoplasm and diffusion through the NPC. Accordingly, clusters of ER are localised in the perinuclear cytoplasm and serve as a trigger zone for  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  release and propagation to the nucleoplasm (Shirakawa and Miyazaki, 1996). In neonatal myocytes, phenylephrine- or  $\text{IP}_3$ -evoked nuclear  $\text{Ca}^{2+}$  waves engulf the entire nucleus without spreading into the bulk cytosol (Luo, et al., 2008). The  $\text{IP}_3\text{R}$  located at the ONM can be activated by  $\text{IP}_3$  produced at the plasma membrane in the presence of agonists. This pathway can be facilitated by the structure of the cell as, for example, in the cardiac myocytes, the  $\text{IP}_3\text{R}$  present on the ONM is in close proximity to the T tubules (a likely source of  $\text{IP}_3$ ) and dyads containing  $\text{RyR2}$  which can establish local perinuclear  $\text{Ca}^{2+}$  microdomains and activate  $\text{IP}_3\text{R}$  (Escobar, et al., 2011). On another hand, it is interesting to notice that in some cell types, NPC are also found in an ER subdomain known as annulate lamellae. It was recently found that  $\text{IP}_3\text{R}$  activity is decreased within annulate lamellae and the relief of this inhibition is correlated with the dismantling of the NPC from this ER subdomain (Boulware and Marchant, 2008). It would be interesting to study whether NPC could also regulate  $\text{Ca}^{2+}$  fluxes and  $\text{IP}_3\text{R}$  activity within the NE.

## Influence of the geometry of the nucleus

The geometry of the nucleus may participate in the genesis and pattern of the nuclear  $\text{Ca}^{2+}$  signal. We have seen above that invaginations of the double membrane of the NE, form a  $\text{Ca}^{2+}$  store deep inside the nucleus in close proximity to the chromatin. They increase the nuclear surface and the number of NPC and may then facilitate the exchanges between the cytoplasm and nucleus. Wittmann et al (Wittmann, et al., 2009) used a mathematical modelling approach that revealed that the division of the nucleus into unequally sized compartments by infoldings can function as microdomains which generate distinct  $\text{Ca}^{2+}$  signals. It is assumed that nuclear infoldings decrease the diffusion distances,  $\text{Ca}^{2+}$  reaches central sites faster and is cleared more quickly. This means that “nuclear inertia” is reduced by membrane infoldings (Queisser, et al., 2011). Both modelling and experimental approaches in hippocampal neurons show that nuclear  $\text{Ca}^{2+}$  transients evoked by cytosolic  $\text{Ca}^{2+}$  transients are larger in small nuclear compartments than in large compartments of the same nucleus. Moreover, frequency information in the  $\text{Ca}^{2+}$  signal is preserved and resolved better in small compartments of infolded nuclei. The authors demonstrated that the presence of nuclear infoldings correlates with transcription-relevant events since synaptic activity-induced phosphorylation of histone H3 on serine 10 was more robust in neurons with infolded nuclei than in neurons with near spherical nuclei (Wittmann, et al., 2009). This work highlights the role of the geometry of neuronal nuclei in  $\text{Ca}^{2+}$  signals and the activation of transcription-regulating events. The formation of nuclear infoldings in synaptically activated neurons constitutes a structural plasticity that may allow an adaptation to metabolic states related to the regulation of gene expression.

In the preceding example, the invaginations of the NE passively regulate the  $\text{Ca}^{2+}$  signal generated in the cytosol, they can also be directly involved in the genesis of  $\text{Ca}^{2+}$  transients in the nucleus. In HeLa cells, the NE invaginations define an extension of the cytosol inside the nucleus where  $\text{Ca}^{2+}$  can be released or transported after ionomycin stimulation (Lui, et al., 1998b). This demonstrates that the nucleoplasmic reticulum is a  $\text{Ca}^{2+}$  store. Recently, the  $\text{IP}_3$ -3-kinase-B has been found, together with  $\text{IP}_3\text{R}$ , in nuclear invaginations of a lung carcinoma cell line, and may modulate the  $\text{IP}_3$  concentration locally within the nucleus (Nalaskowski, et al., 2011).

The role of the nucleoplasmic reticulum in  $\text{Ca}^{2+}$  signalling was characterised in experiments performed in a liver cell line by Echevarria et al (Echevarria, et al., 2003), who demonstrated that a branching intranuclear network which is continuous with the ER and the NE contains calreticulin,  $\text{IP}_3\text{R}$  and  $\text{Ca}^{2+}$  storing activity. Stimulation of the cells with HGF preferentially induced nuclear  $\text{Ca}^{2+}$  signals. Furthermore, local photorelease of a small amount of  $\text{IP}_3$  resulted in a small increase in  $\text{Ca}^{2+}$  which began near this nucleoplasmic reticulum. These authors also show that release of  $\text{Ca}^{2+}$  in the nucleus causes nuclear PKC to translocate to the nuclear envelope, demonstrating a specific effect of localized nuclear  $\text{Ca}^{2+}$  signals. We emphasized above the question concerning the direction of the  $\text{Ca}^{2+}$  release from the nuclear envelope, either directly to the nucleoplasm or to the cytoplasm and diffusion through the NPC. The presence of NE invaginations can facilitate the diffusion of  $\text{Ca}^{2+}$  from the cytoplasm to the nucleoplasm. The membrane bordering the lumen of the tubule is identical to the ONM and therefore delineates a confined cytosolic space in the deeper part of the nucleus. Brasen et al (Brasen, et al., 2010) calculated that the wrinkled surfaces of cells provide a mechanism for generating microdomains where the  $\text{Ca}^{2+}$  concentration may be much higher than in the bulk cytosol, and long narrow membrane wrinkles will be most effective in

generating such a high  $\text{Ca}^{2+}$  microdomain. The ONM lining the tubules more likely contains  $\text{IP}_3\text{R}$  which can release the  $\text{Ca}^{2+}$  ions toward the cytosol within the narrow wrinkles formed by the NE invagination. This should give rise locally to high concentrations of free  $\text{Ca}^{2+}$ , whose diffusion into the bulk cytosol is restricted. The  $\text{Ca}^{2+}$  retained in this confined space of the cytoplasm may diffuse into the nucleoplasm more easily, through the nuclear pores identified in invaginations by electron microscopy (Fig. 2).

### **Differences between the NE and the peripheral ER**

The NE is often presented as a sub-domain of the ER and the peripheral ER is described as an extensive network that branches out of the NE. One can ask what are the respective roles of the NE and the peripheral ER in  $\text{Ca}^{2+}$  signalling? The protein composition of the ONM is mainly similar to that of the peripheral ER independently of differences which may characterise specialised sub-domains of the ER. The main differences in the protein composition of the peripheral ER and the NE concern the INM which has its own protein components and is underlined by lamina rich in intermediate filaments. Particularly, the NE is riddled with NPC that regulate transport of molecules between the cytoplasm and the nucleoplasm. We have seen above that the  $\text{Ca}^{2+}$  handling proteins are equally distributed in the peripheral ER and in the NE, this allows the accumulation of  $\text{Ca}^{2+}$  in a single luminal space and the  $\text{Ca}^{2+}$  release in the cytoplasm or the nucleoplasm. One important difference between the two structures concerns their spatial organisation. The peripheral ER consists of a dynamic network of membrane sheets and interconnected tubules and the NE forms membrane sheets around the chromatin and nuclear lamins. This has direct involvements in the mechanisms of cell signalling.

The peripheral ER is a network that expands within the cytosol and covers every part of the cell. This ER network is stabilised partly through interactions with cellular organelles such as mitochondria, Golgi, endosomes or plasma membrane. These close juxtapositions of ER membranes with other cell membranes are important for interorganelle exchanges of lipids. These interactions also play an important role in  $\text{Ca}^{2+}$  signalling. Protein complexes including  $\text{IP}_3$  receptor have been extensively studied these last few years and allow the mitochondrial-ER attachment and the transfer of  $\text{Ca}^{2+}$  to the mitochondria (de Brito and Scorrano, 2010). In the same way, interactions of ER with the plasma membrane have been described in epithelial cells where the  $\text{IP}_3\text{R}$  is concentrated near the apical domain of the plasma membrane (Vermassen, et al., 2004). The ER proteins STIM sense a decrease in the ER  $\text{Ca}^{2+}$  concentration and activate the ORAI  $\text{Ca}^{2+}$  channel of the plasma membrane across the junction between the two membranes (Shen, et al., 2011). These local interactions of the peripheral ER associated with the fact that it is constantly rearranged and moves along microtubules, allow the comparison to an octopus which expands its tentacles within the cytosol and regulates locally the  $\text{Ca}^{2+}$  fluxes.

The NE, although being in continuity with the peripheral ER has an additional role as it separates two cell compartments, the cytoplasm and the nucleus, and consequently, it has a static role. It regulates the transfer of molecules, including  $\text{Ca}^{2+}$ , between the cytoplasm and the nucleus and it is a  $\text{Ca}^{2+}$  store that allows the delivery of  $\text{Ca}^{2+}$  to the nucleoplasm. The dynamics of the NE is limited to the formation of infoldings that increases the surface of exchange between the cytoplasm and the nucleoplasm. The invaginations of the NE modulate the transfer of the  $\text{Ca}^{2+}$  signal from the cytoplasm to the nucleus and allow the release of  $\text{Ca}^{2+}$  deeply near the chromatin.

## Concluding remarks

Over recent years, it has become more and more evident that nuclear  $\text{Ca}^{2+}$  signals can specifically influence gene transcription and cell-cycle progression. At the molecular level, all the components constituting the  $\text{Ca}^{2+}$  signalling cascade (from the hormone receptor to the  $\text{Ca}^{2+}$  release channels) have been found in the nucleus. The nuclear envelope plays a major role by integrating all the molecules that constitute a  $\text{Ca}^{2+}$  store and allows the local release of  $\text{Ca}^{2+}$  when stimulated by  $\text{Ca}^{2+}$ -mobilising agents like  $\text{IP}_3$ , also produced locally. All this machinery suggests that  $\text{Ca}^{2+}$  signalling in the nucleus can be independent of cytosolic variations even if the mechanisms involved are not yet clearly established. On the other hand, the global cytosolic  $\text{Ca}^{2+}$  signals can be easily transmitted to the nucleoplasm through the NPCs, which are freely permeable to small molecules like calcium ions. The exchanges between the cytoplasm and the nucleoplasm depend on the area of exchange and the number of NPCs and these two features are increased in some cell types and under some physiological conditions by the formation of invaginations of the NE. These structures, recently called nucleoplasmic reticulum, have long been observed, but very interesting recent data suggest that they could play important roles in nuclear functions. The nucleoplasmic reticulum allows the delivery of  $\text{Ca}^{2+}$  locally deep inside the nucleus. It also forms separate compartments of varying sizes within the nucleoplasm and could therefore determine whether the nucleus functions as an integrator or a detector of oscillating  $\text{Ca}^{2+}$  signals (Queisser, et al., 2011). Until now we have described the generation of  $\text{Ca}^{2+}$  microdomains by considering their different subcellular location and how each organelle has its own distinct  $\text{Ca}^{2+}$ -handling properties. A future challenge will be to characterise the signalling pathways and/or the physiological conditions that regulate the formation of the nucleoplasmic reticulum and modify the geometry of the nucleus. These regulatory mechanisms more likely participate in the pattern of the  $\text{Ca}^{2+}$  signal and possibly in other nuclear processes. The morphology regulation of the  $\text{Ca}^{2+}$  signal described in the nucleus could be extended to the  $\text{Ca}^{2+}$  dynamics in the entire cells (Queisser, et al., 2011). In this way, the manipulation of the cell shape and microenvironnement by the micropatterning techniques could be of considerable help (They, 2010). Modification of the morphology of the organelles like the nucleus can modulate  $\text{Ca}^{2+}$  signals and this opens up new ways to describe the generation and propagation of  $\text{Ca}^{2+}$  signals at the nuclear level and, possibly, at the level of the entire cell.

**Abbreviations used:** EM, electron microscopy; ER, endoplasmic reticulum; GFP, green fluorescent protein; INM, inner nuclear membrane; IP<sub>3</sub>, inositol-1,4,5-trisphosphate; IP<sub>3</sub>R, inositol-1,4,5-trisphosphate receptor; MDCK, Madin Darby canine kidney; NE, nuclear envelope; NPC, nuclear pore complex; ONM, outer nuclear membrane; PIP<sub>2</sub>, phosphatidylinositol-4,5-bisphosphate; PLC, phospholipase C; RyR, ryanodine receptor, SERCA, sarco/endoplasmic reticulum Ca<sup>2+</sup>-ATPase.

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## Figure legends

### Figure 1

#### **Polarised MDCK cells develop invaginations in the nuclear envelope.**

MDCK cells were plated at  $10^4$  cells/ cm<sup>2</sup> and cultured for 12 to 15 days. (Left):

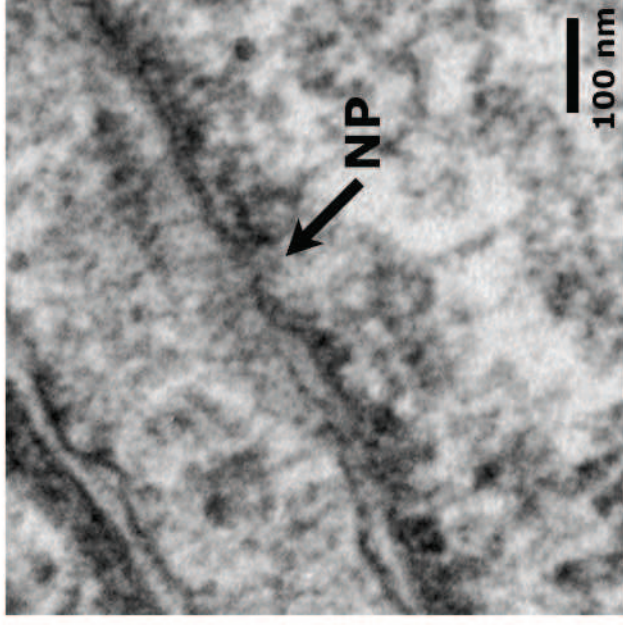
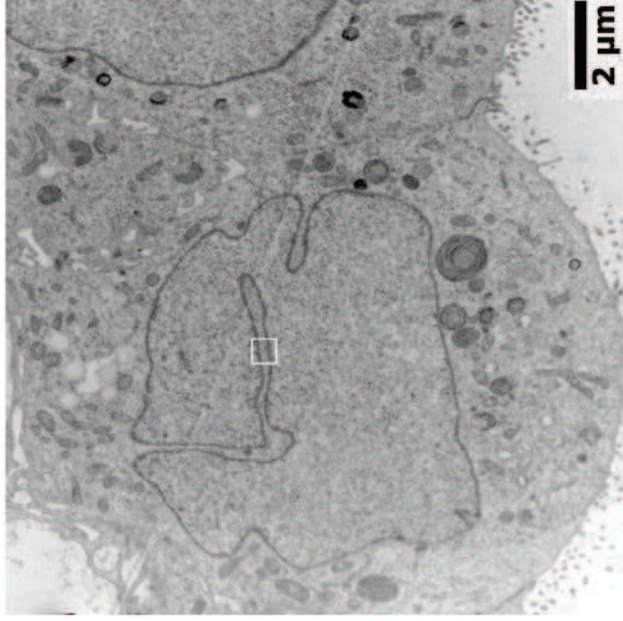
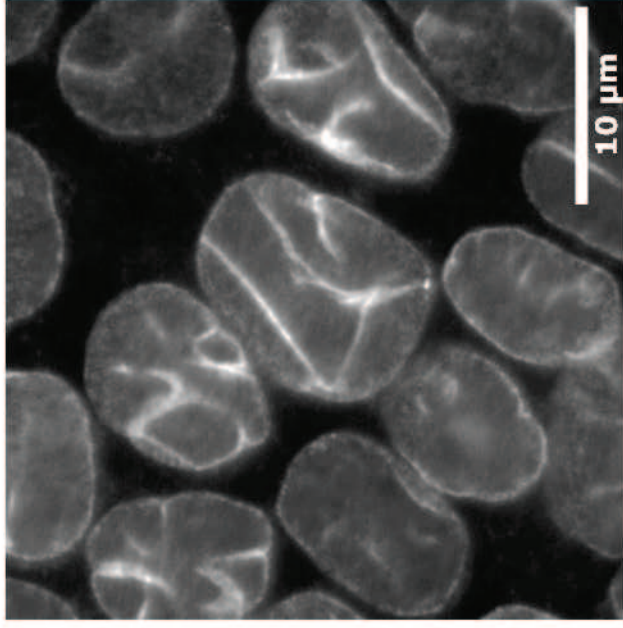
Epifluorescence image of polarised MDCK cells stained with antibodies against emerlin, a protein associated with the inner nuclear membrane. The antibodies reveal the presence of invaginations of the nuclear envelope. (Middle): Electron micrograph of polarised MDCK cells shows the narrow invaginations of the nuclear envelope which penetrate deeply the nucleus. (Right): Observations at high magnification of the inset in the middle panel revealed that both the inner and the outer membrane of the nuclear envelope form the invaginations. The presence of a nuclear pore (NP) in invaginations is indicated by the arrow.

### Figure 2

#### **Summary of the different mechanisms that regulate nuclear Ca<sup>2+</sup> concentration.**

Ca<sup>2+</sup> is accumulated from the cytoplasm into the NE by the Ca<sup>2+</sup> pump (SERCA) localised in the outer nuclear membrane (ONM). Ca<sup>2+</sup> can be released by IP<sub>3</sub> either through the inner nuclear membrane (INM) directly to the nucleoplasm or through the ONM to the cytoplasm and secondly through the nuclear pore complex (NPC) to the nucleoplasm. The presence of invaginations of the nuclear envelope generate cytosolic microdomains where the Ca<sup>2+</sup> concentration may be much higher than in the bulk cytosol, this can favour the transfer of Ca<sup>2+</sup> from the cytoplasm to the nucleoplasm. The local production of IP<sub>3</sub> in the nucleus is indicated.





**Fig. 1**

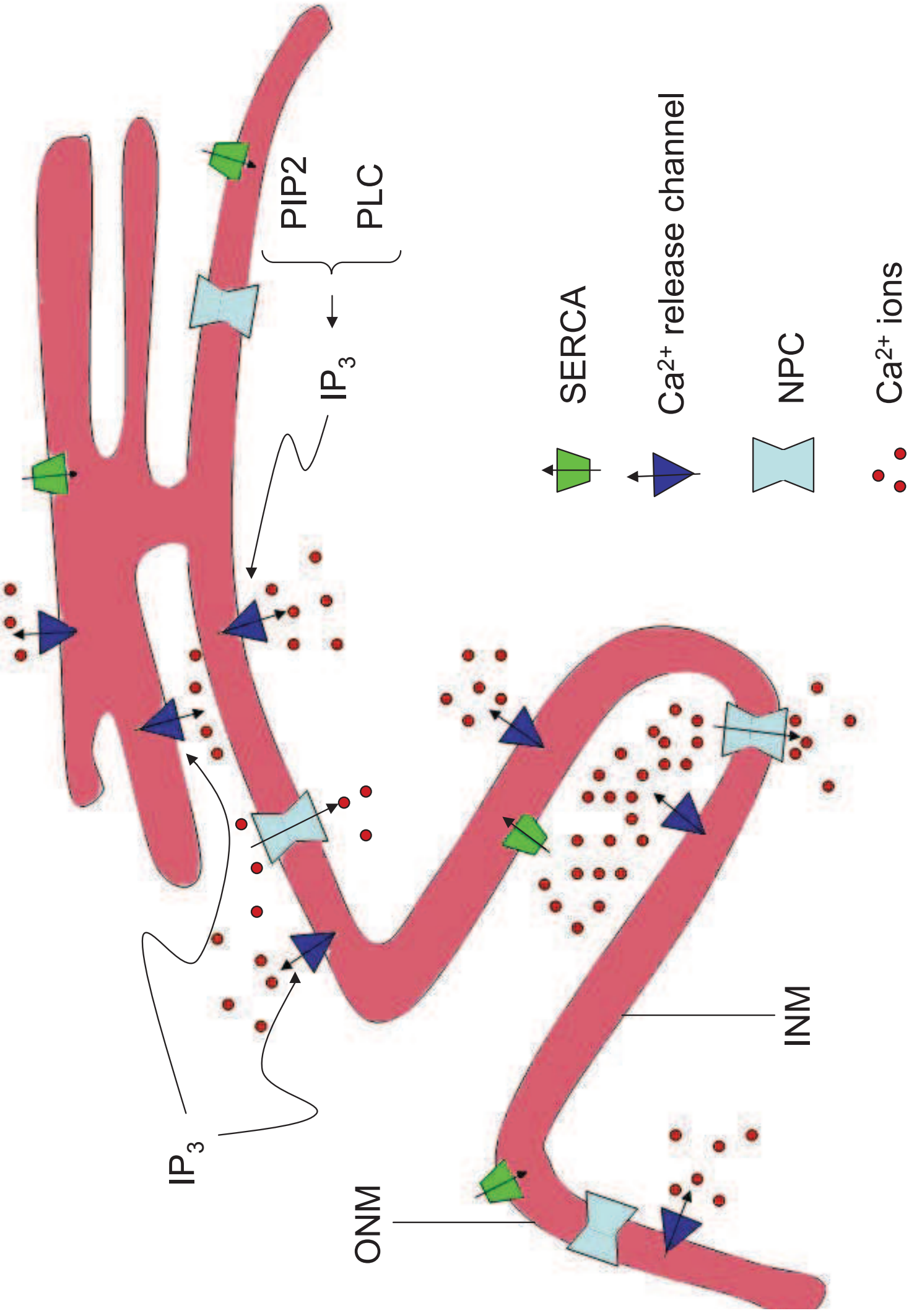


Fig. 2