Nuclear Ca^{2+} signaling

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SUMMARY

Ca^{2+} signalling is important for controlling gene transcription. Changes of the cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_C$) may promote migration of transcription factors or transcriptional regulators to the nucleus. Changes of the nucleoplasmic Ca^{2+} ($[\text{Ca}^{2+}]_N$) can also regulate directly gene expression. $[\text{Ca}^{2+}]_N$ may change by propagation of $[\text{Ca}^{2+}]_C$ changes through the nuclear envelope or by direct release of Ca^{2+} inside the nucleus. In the last case nuclear and cytosolic signaling can be dissociated. Phosphatidylinositol biphosphate, phospholipase C and cyclic ADP-ribosyl cyclase are present inside the nucleus. Inositol trisphosphate receptors (IP_3R) and ryanodine receptors (RyR) have also been found in the nucleus and can be activated by agonists. Furthermore, nuclear location of the synthesizing enzymes and receptors may be atypical, not associated to the nuclear envelope or other membranes. The possible role of nuclear subdomains such as speckles, nucleoplasmic reticulum, multi-macromolecular complexes and nuclear nanovesicles is discussed.

Ca^{2+} is a pleiotropic messenger that regulates many cellular functions, including some specifically performed in the nucleus. Modifications of gene expression are triggered through changes in both the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_C$) and/or the nucleoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_N$) [1]. Changes of $[\text{Ca}^{2+}]_C$ promote messages that travel to the nucleus to reach the adequate effectors and to modify gene expression. In addition, changes of $[\text{Ca}^{2+}]_N$ modify the activity of several transcription factors. Reliable and selective monitoring of $[\text{Ca}^{2+}]_N$ is critical for investigation of Ca^{2+} signaling and methodology will also be briefly reviewed. The idea of an independent Ca^{2+} signaling system in the nucleus is most intriguing, favoured and the existence of intranuclear ryanodine receptors (RyR), inositol trisphosphate receptors (IP_3R) and the enzymatic machinery necessary for the synthesis of the messengers involved in these transduction systems will be addressed. Another fascinating facet that will be discussed here is the existence of structures such as invaginations of the NE (nucleoplasmic reticulum), nanovesicles, speckles or phospholipid-protein complexes. Previous reviews have addressed other aspects of nuclear calcium not treated so extensively in this review [2, 3].

1. Ca^{2+} and nuclear function

It has been proposed that Ca^{2+} signaling is important for cell proliferation, development and differentiation [4-8] and for plasticity of neurons and muscles [9-11]. Regulation of these processes is performed, ultimately, through changes in gene expression which can be attained by several pathways, as summarized in Fig. 1.

In first place (Fig. 1A), changes of $[\text{Ca}^{2+}]_C$ can promote translocation of transcription factors from the cytosol to the nucleus. The best known examples are those of NFkB (*nuclear factor kappa-light-chain-enhancer of activated B cells*) and NFAT (*Nuclear factor of activated T-cells*).

In the resting cell NFkB is anchored in the cytoplasm by another protein called IkB (Inhibitor of kB) through multiple ankyrin repeat motifs that mask the nuclear localization signals (NLS) of NFkB proteins and keep them inactive. Activation of NFkB is triggered by an increase of $[\text{Ca}^{2+}]_C$ which induces a phosphorylation cascade. IkB is phosphorylated by IkB kinase (not shown in the figure), which is, in turn, activated either by Ca^{2+} /calmodulin (CaM) dependent protein kinase (CaMK, type I, II or III) [12] or by PKC [13, 14] (Fig. 1A). Phosphorylated IkB dissociates from NFkB, which now exposes its NLS, translocates to the nucleus and turns on transcription of specific genes, generally related to inflammatory or immune responses, cell survival responses or cell proliferation.

The NFAT is a family of transcription factors important for immune response and for development of heart, skeletal muscle and nervous system. NFAT has a cytoplasmic location in the resting cell. On cell activation, calcineurin (CaN), a Ca^{2+} -CaM-dependent enzyme, dephosphorylates NFAT with the result of NLS exposure. Dephosphorylated NFAT migrates to the nucleus where it binds to the cis-regulatory elements of its target genes [15] (Fig. 1A). CaN action is opposed by “maintenance” kinases in the cytosol and by export kinases in the nucleus (not shown in the figure).

CaN has a higher affinity for Ca^{2+} than CaMK II. As a result, small and maintained $[\text{Ca}^{2+}]_c$ increases are enough for activation of NFAT, but not for NFkB, which is typically activated by high amplitude $[\text{Ca}^{2+}]_c$ oscillations [14, 16]. Amplification of the signal generated by Ca^{2+} release from the stores by *store operated Ca^{2+} entry* (SOCE) is essential for NFAT activation in lymphocytes [17]. In general, Ca^{2+} oscillations are a more efficient stimulus than sustained changes for NFAT activation [18, 19].

L-type voltage-gated calcium channels (LTCCs) may also control directly gene expression by another signaling mechanism in which a C-terminal fragment of the channel (*Calcium channel activated transcriptional regulator*, CCAT) translocates to the nucleus in a calcium-dependent manner and regulates transcription [20, 21] (Fig. 1A). CCAT acts on transcription indirectly, via other nucleoproteins. This mechanism has been identified in specific brain neurons [21] and in atrial cardiomyocytes [22]. Cleavage of CCAT from LTCC is performed by a constitutive proteolytic activity which is proportional to the number of channels. On the other hand, the increase of $[\text{Ca}^{2+}]_n$ promotes dissociation of CCAT from the DNA and migration to cytoplasm [20, 21]. Therefore, CCAT performs a quick and direct activation of target genes bypassing adaptor proteins and kinase cascades.

The increase of $[\text{Ca}^{2+}]_c$ also induces shuttling of phospholipase C- δ 1 (PLC- δ 1) to the nucleus, where it can induce the production of IP_3 and diacylglycerol, this affecting several nuclear functions [23, 24]. The increase of $[\text{Ca}^{2+}]_c$ also triggers translocation of ADP-ribosyl cyclase (CD38), an enzyme involved in the biosynthesis of two Ca^{2+} -regulating second messengers, cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate (NAADP) [25]. Moreover, it has been shown that CD38 is present on the inner side of NE of a variety of tissues including brain, liver, eye and spleen [26, 27].

Cyclic AMP response element-binding (CREB) protein binds to CRE sequences of DNA and increases or decreases transcription of downstream genes. CREB has a well-documented role in neuronal plasticity and long-term memory formation in the brain [10, 11, 28-30]. Some of the genes regulated by CREB are c-fos, the neurotrophin brain-derived neurotrophic factor (BDNF) and many peptidic neurotransmitters. Coactivators of the CREB pathway include *CREB binding protein* (CBP) and *transducers of regulated CREB proteins* (TORCs). CREB is activated by phosphorylation via protein kinase A, C, the mitogen-activated kinases (MAPK; ERK and p38) or CaMK. One of the pathways of activation by Ca^{2+} is shown in the Fig. 1A. The high $[\text{Ca}^{2+}]_c$ microdomain formed at the mouth of the L type plasma membrane Ca^{2+} channels activates CaMKII, which translocates to the nucleus and activates CREB [31, 32]. Apart from increases in the $[\text{Ca}^{2+}]_c$, CREB activity is also enhanced by $[\text{Ca}^{2+}]_n$. CREB binds to CRE together with CBP and TORC and switches the downstream gene on or off. Binding of CBP and TORC is also stimulated by $[\text{Ca}^{2+}]_n$ [14, 29, 30, 33]. Protein phosphatase 1, which is activated by CaN, dephosphorylates CREB and terminates CRE-dependent transcription. Excitation-transcription coupling is extraordinarily tight in sympathetic neurons [31] and allows encoding the frequency of L-type Ca^{2+} channel openings in the dendritic tree into gene expression in the nucleus. In other neurons a similar mechanism

couples plasma membrane glutamate channels and kinases other than CaMKII (for example MAPK) to gene expression [28, 32, 34, 35]. On the other hand, extrasynaptic glutamate receptors may shut down CREB activity [36].

Myocyte enhancer factor 2 (MEF2) family includes several stress response-related transcription factors that play an important role during embryonic development in several organs. MEF2 is normally repressed by a nucleoprotein complex that contains MEF2 binding domains. This repressor competes with Ca^{2+} -CaM for binding to MEF2, so that the increase of $[\text{Ca}^{2+}]_{\text{N}}$ activates MEF2 [14, 28] (Fig. 1A). In addition, both CaN and NFAT activate MEF-2 through a poorly known mechanism.

Downstream Regulatory Element (DRE) Antagonist Modulator (DREAM, also called *K channel interacting protein 3* and *calsenilin*) is encoded by KCNIP3, a gene that codifies a family of K channels that interacts with Ca^{2+} . The protein is a Ca^{2+} sensor containing four EF hands. It works as a transcriptional repressor that binds to DRE sites of DREAM-target genes and blocks the action of the *transcription initiation complex* (TIC) [14, 37]. When $[\text{Ca}^{2+}]_{\text{N}}$ increases, Ca^{2+} binds to DREAM and decreases its affinity for DRE. This results in DREAM dissociation from DNA and derepression of the transcription of the target gene (Fig. 1B). DREAM is widely expressed in brain, particularly in sensory neurons where it represses the expression of prodynorphin, a gene related to pain modulation [14, 37].

Apart from the above mechanisms, changes of $[\text{Ca}^{2+}]_{\text{N}}$ can affect gene expression through its interaction with histones and high mobility group (HMG) proteins, which can be phosphorylated by CaMK and give rise to changes in chromatin structure that may expose or mask gene regulatory sites [14]. In addition, Ca^{2+} -dependent proteins such as calmodulin, S-100 or calreticulin are present in the nucleus where they may be able to, directly or indirectly, interact with the chromatin.

2. Regulation of $[\text{Ca}^{2+}]_{\text{N}}$

As mentioned above, some Ca^{2+} -dependent changes of gene expression are really regulated by changes of $[\text{Ca}^{2+}]_{\text{C}}$, which in turn trigger translation of a Ca^{2+} -regulated proteins to the nucleus. In other occasions, transduction includes changes of $[\text{Ca}^{2+}]_{\text{N}}$. In this section we will first deal with the current methods to study nuclear Ca^{2+} and then we will discuss different mechanisms that may regulate $[\text{Ca}^{2+}]_{\text{N}}$

2.1 Probes to measure $[\text{Ca}^{2+}]_{\text{N}}$

Reliable monitoring of $[\text{Ca}^{2+}]_{\text{N}}$ poses some problems. The common fluorescent low molecular weight probes such as fluo-3 concentrate and are brighter in the nucleoplasm than in the cytosol. In addition, it has been suggested that their affinity for Ca^{2+} could be different depending on the environment [38]. This makes difficult to compare the Ca^{2+} concentrations in the nucleus and the surrounding cytosol during measurements performed either by conventional epifluorescence, confocal or two-

photon microscopy. High molecular weight dextran-bound dyes injected into the nucleus [7, 39] should have the same limitations. In addition, when separation of the nuclear and the cytoplasmic areas is only based on imaging, other biases may arise. It has been demonstrated, for example, that what seemed high Ca^{2+} hotspots inside the nucleus did really correspond to cytosolic changes taking place in invaginations of the cytosol inside the nucleus [40, 41]. Such cytoplasmic invaginations are very common in many cell types [40-44] (see below).

Protein-based Ca^{2+} probes such as cameleons [45], pericams [46] or aequorins [47] have been successfully targeted to the nucleus. Differences in behaviour between $[\text{Ca}^{2+}]_{\text{N}}$ and $[\text{Ca}^{2+}]_{\text{C}}$ have been reported in neurons using improved cameleon [48], and ratiometric pericam has been successfully used to monitor $[\text{Ca}^{2+}]_{\text{N}}$ in beating cardiomyocytes [49]. One of the difficulties with these fluorescent probes is its sensitivity to changes in pH within the physiological pH range [45, 46], a problem that has been addressed to in the improved cameleons [50]. Aequorins, on the contrary, are little sensitive to pH above pH 6.5 [51]. In Fig. 2 we compare the properties of the most frequently used nucleus-targeted probes: a cameleon (YC2.1), a pericam (R-pericam) and aequorin. Fluo-3 is also shown for comparison [52]. The dynamic range of aequorin, near three orders of magnitude, is larger than that of the other three fluorescence indicators, so that a wider range of Ca^{2+} concentration changes can be monitored with this probe. To make the range even wider, the affinity of aequorin can be further modified by reconstituting it with different coelenterazines; for example, coelenterazine n to decrease the Ca^{2+} affinity or coelenterazine h to increase it [53-55]. The gain of aequorin is also much larger than the one of the other probes. The Hill number for aequorin is about 2.1 compared to 0.85 for fluo-3, 0.36 for R-pericam and 0.18 for yellow cameleon 2.1 (Fig. 2). As a consequence, the change in light emission with changes of $[\text{Ca}^{2+}]_{\text{C}}$ is larger with aequorin and this produces a better contrast (see simulation in the rightmost part Fig. 2B) and a more sensitive detection and delimitation of high Ca^{2+} microdomains [56]. On the other hand, the nucleoplasmic environment does not seem to modify the behaviour of aequorin [57]. The main limitation of aequorins is low light output, which can be partly circumvented using a photon-counting camera [58], although the spatial resolution is still quite poor. The new Electron Multiplying Charged Coupled Device (EM-CCD) cameras are, in our hands, inferior to the photon-counting ones for aequorin imaging. This is so because the last ones are more sensitive and, more importantly, have a much wider dynamic range, which is required to avoid saturation of the camera during calibrations. Other potential advantages of aequorin include very low background and no competition with endogenous calmodulin.

By combining high-sensitivity detection with high expression, attained using a herpes virus-based system [54], we have been able to image individual cell $[\text{Ca}^{2+}]_{\text{N}}$ changes in chromaffin cells [59], HEK293 cells [60], GH₃ pituitary cells [60, 61] and pancreatic beta-cells [62]. Fig. 3A and 3B illustrate the response to high glucose of 5 single cells within a pancreatic islet (see also supplemental movie1). Fig. 3C illustrates spontaneous and thyrotropin-releasing hormone- (TRH) induced $[\text{Ca}^{2+}]_{\text{N}}$ oscillations in a single GH₃ cell. Chimeric proteins containing GFP or RFP fused to aequorin have been recently constructed and successfully targeted to the nucleus [61, 63]. These chimeric proteins seem brighter than the native aequorins [64], most probably because the fusion makes

them more stable. These new probes are very useful for imaging, as they allow identifying the aequorin-expressing cells by their fluorescence prior to performing the photoluminescence measurements. This is very useful to choose an adequate microscopic field containing aequorin-expressing cells for the ensuing experiment. **Fig. 3 D-F** shows the distribution of the nuclear GFP-aequorin in the GH₃ cell where the measurements shown in **Fig. 3C** were performed. Finally, aequorins of different colours can be co-expressed in the same cells to perform simultaneous measurements of the Ca²⁺ signals coming from two different subcellular compartments [63].

2.2. Propagation of [Ca²⁺]_C changes through nuclear envelope.

The nucleus is surrounded by the nuclear envelope (NE), a double membrane that separates the cytoplasm from the nucleoplasm (**Fig. 4**). The space between the internal and the external membranes of the NE, the perinuclear cisterna, is communicated with the lumen of the ER and contains high concentrations of Ca²⁺, which is accumulated by the sarcoendoplasmic reticulum Ca²⁺-activated ATPase (SERCA) units present in the external membrane of the NE [39, 65]. Cytoplasm and nucleoplasm communicate through enormous, 9 nm inner diameter, nuclear pore complexes (NPC), which are involved in transport of macromolecules from the cytoplasm to nucleoplasm and *vice versa* [66]. The ion conductance of NPC is very large and this should allow free solute and ion diffusion. Then, [Ca²⁺]_C signals should generate identical [Ca²⁺]_N signals (**Fig. 4.1**). It has been reported, however, that the NPC conductance can be dramatically reduced by accumulation of Ca²⁺ inside nuclear cisterna (gating) or by transport of macromolecules through the NPC (plugging) [67], although this view is controversial [3]. Opinions on the Ca²⁺ permeability of NPC are also far from univocal. Both, reports of free [47, 68] and restricted [39, 69, 70] permeability of Ca²⁺ through NPC have been published. The two-dimensional propagation of Ca²⁺ waves shows inhomogeneities at the nuclear envelope, which can be attributed to restricted diffusion [71, 72] or differences in buffering power between nucleus and cytosol [73]. In our hands NE acts as a kinetic barrier that dampens high-frequency [Ca²⁺]_C oscillations [60].

It has been proposed that, in some instances, Ca²⁺ release takes place from stores especially close to the nucleus such as nuclear cisterna (**Fig. 4.2**) [74, 75] or the perinuclear stores [68, 76-79]. It has been suggested that these signals have specific effects on [Ca²⁺]_N and that they are important for developmental and plastic changes of cardiomyocytes [74, 76-78, 80-82]. Stimulation of perinuclear Ca²⁺ stores could involve specific IP₃-transducing pathways [2] as recently shown in cardiomyocytes, where endothelin-1 stimulates IP₃-induced Ca²⁺ release from perinuclear IP₃Rs, causing an elevation in nuclear Ca²⁺ [76].

2.3. Calcium release from nuclear Ca²⁺ stores.

The NE works as a Ca²⁺ storage organelle because of the presence of a thapsigargin-sensitive SERCA in the outer NE membrane [39, 83]. In order to return to the resting [Ca²⁺]_N concentrations after a [Ca²⁺]_N peak, the Ca²⁺ released to the

nucleoplasm can diffuse to the cytosol through the NPCs and then be re-sequestered by the SERCA of the outer nuclear membrane. The inner nuclear membrane seems to lack SERCA [65, 83]. A most intriguing alternative Ca^{2+} uptake mechanism activated by inositol-1,3,4,5-tetrakisphosphate has been found in the outer NE membrane [84]. In the inner membrane, only an alternative spliced version of sodium-calcium exchanger (NCX) has been recently described [85, 86]. The exchanger needs to be bound to GM1 ganglioside to transfer Ca^{2+} from the nucleoplasm to the NE lumen.

It is accepted that the nucleus possesses both the substrates and the enzymes necessary for the production of IP_3 . Although the presence of phosphatidylinositol 4,5-bisphosphate (PIP_2) in the nucleus is beyond dispute, it is not clear whether PIP_2 is in the lipid bilayer or it is outside, associated to “soluble” proteins. Interestingly, a significant proportion of the nuclear inositol lipid is resistant to detergent extraction. On the other hand, this pool seems to act as a ‘privileged’ substrate for the phosphatidylinositol kinases present in the NE. For example, residual phosphatidylinositol 4 phosphate (PI4P) is only 5% of the total, but is able to sustain more than 50% of the phosphatidylinositol kinase activity [87]. Moreover, PIP_2 and the enzyme that produce it, PI4P 5 kinase, colocalize at the nuclear speckles, a dynamic undefined structure with unknown function [87]. These findings have led to propose that some nuclear proteins could be complexed in the speckles, where they could act as a sink for PIP_2 [88, 89]. Indeed, using anti- PIP_2 antibodies, or a PIP_2 -specific PH domain probe, it has been shown extensive accumulation of PIP_2 in the nucleus, but it locates at the nucleoplasm rather than at the nuclear envelope [90, 91]. Alternatively, PIP_2 could be contained within a lipid bilayer forming intranuclear membrane invaginations with a privileged access to the enzymes [87].

Regarding to the enzymes, a phosphoinositide-specific phospholipase C (PI-PLC) associated to the nuclear membranes has been detected [92, 93]. The most abundant isoform is the PI-PLC- $\beta 1$, although other isoforms such as PLC- $\gamma 1$ [4] or PLC- $\delta 1$ [23, 87, 94] have also been found in the nucleus. Activation of nuclear PLC triggers the production of nuclear diacylglycerol [87, 92] and IP_3 [95, 96]. In some cell types the PLC is found constitutively inside the nucleus, but in other cell types it is translocated from the cytosol, its usual location, upon stimulation with growth factors [24]. For example, nuclear PI-PLC- $\beta 1$ activity is up-regulated in response to insulin-like growth factor-1 (IGF-1) or insulin stimulation [97]. One unsolved issue is how the nuclear phospholipases would be activated. Conversely to their plasma membrane partners, who are activated by protein $\text{G}\alpha$, there are no evidences for the presence of nuclear $\text{G}\alpha$. A reported pathway is through MAPK phosphorylation and shuttling to the nucleus after IGF-1 stimulation. In other cases, influx of Ca would directly trigger the nuclear import of PLC, via Ca^{2+} -dependent interaction with importin- $\beta 1$ [23]. In summary, nuclear PLCs could have specific functions, distinct from their cytosolic counterparts, in growth factor signalling, leading to activation programs for cell division and differentiation [98]. For example, the nuclear localization of PLC- β is required for myoblast differentiation, which is impaired when the enzyme is shuttled to a cytosolic location [99]. A special case is the PLC- ζ , which is injected together with the sperm into the fertilized oocyte. This PLC- ζ seems responsible for the generation of repetitive $[\text{Ca}^{2+}]_c$ oscillations, which cease when the PLC is translocated to the nucleus [100]. Conversely to the other PLC isoenzymes,

the PLC ζ accumulation in the nucleus is not associated with IP₃ production and blocks Ca²⁺ signalling.

Several recent reports have documented the intriguing presence of growth factor receptors in the nucleus. For example, after insulin stimulation of hepatocytes insulin receptor translocates to the nucleus where it promotes PIP₂ hydrolysis and nuclear Ca²⁺ signalling independent of the cytosol [101]. Translocation of other receptors from cytosol to nucleus have also been reported for c-Met, which migrates to the nucleus on stimulation with hepatocyte growth factor (HGF) [4], , bradykinin B₂ receptor [102] and metabotropic glutamate receptor 5 (mGluR5) [103]. At the moment it is not fully understood how a G-protein-coupled receptor would be activated in the nucleus upon cell stimulation. It has been speculated that glutamate would reach its intracellular receptor via both sodium-dependent transporters and cystine/glutamate exchangers [103]. In any case, the receptor occupancy would activate nuclear PLCs, giving rise to IP₃ and generating an autonomous nuclear Ca²⁺ signalling pathway dissociated from the cytosolic inositol lipid cycle [97].

The presence of IP₃Rs has been well documented by immunostaining with specific antibodies anti-IP₃Rs both in the inner and in the outer NE membrane and in different cell types. The activation of the IP₃R enables the release of Ca²⁺ directly into the nucleoplasm (Fig. 4.3 and 4.4). Of the three IP₃R isoforms described, IP₃R type 2 is the one that has been found most often in the nucleus of a number of cell types like liver [43, 104, 105] or smooth muscle cells [105]. Using specific antibodies against the different isoforms, we found that pituitary GH₃ cells express type 2 IP₃R in the nucleus, in contrast to type 1 IP₃R, which is exclusively expressed in the cytosol (Fig. 5. Compare A and B) [61]. This expression pattern seems cell-type specific, as in HeLa cells immunostaining of IP₃R type 2 with the same antibody is exclusively cytosolic (Fig. 5C). During anaphase, when the NE is dissolved, IP₃R₂ can also be seen in the nuclear area (Fig. 5D).

IP₃R localization data are consistent with functional evidence. For example, addition of IP₃ to isolated nuclei from pancreatic acinar cells or Aplysia neurons [65, 106] has been shown to induce a [Ca²⁺]_N transient. In Purkinje neurons patch clamp data recording demonstrated the presence of functional IP₃Rs in the inner NE membrane [107]. Using targeted aequorins to selectively measure and compare nuclear and cytosolic Ca²⁺ signals in GH₃ pituitary cells, we find an independent activation of nuclear Ca²⁺ signalling with IP₃ [61]. An additional evidence for independent nuclear Ca²⁺ release is the differential sensitivity to inhibitors, as shown by the fact that high molecular weight heparin, which cannot enter the nucleus through NE, was unable to block most of the [Ca²⁺]_N response. ATP removal evidenced additional differences between the cytosolic and the nuclear peaks [61, 70, 108, 109].

The presence of RyRs in the NE has been demonstrated by immunostaining with anti-RyR antibodies [44] and by the binding of BODIPY FL ryanodine [3]. Moreover, addition of NAADP or cADPR was shown to induce nuclear Ca²⁺ mobilization in isolated pancreatic nuclei [65, 110] or intact neurones [25, 106]. In skeletal muscle cells, photorelease of caged Ca²⁺ in the nuclear region evoked nuclear Ca²⁺-induced Ca²⁺

release (CICR) [44]. In addition, it has been shown that ADP-ribosyl cyclase (CD38), the enzyme that cleaves NAD^+ and NADP^+ and generates cADPR, NAADP, and ADPR, translocates, upon cell depolarization, from the inner membrane of NE, where it locates at rest [111], to the nucleoplasm [25, 27]. Apart from RyRs, two other potential NAADP receptors have been proposed: the transient receptor potential mucolipin 1 channel (TRP-ML1) and the recently discovered family of two pore channels (TPC) [112]. At the moment, this issue is a matter of debate and more work would be needed to demonstrate the presence of these receptors in the nucleus, and its direct activation by NAADP.

2.4. Unconventional nuclear discharge docks: nucleoplasmic reticulum and nuclear nanovesicles.

The existence of NE invaginations into the nucleoplasm would increase the efficiency of Ca^{2+} release from the nuclear cisterna both by decreasing the diffusion distances and by increasing the membrane surface. The outer NE membrane is physically and functionally continuous with the ER. The presence of invaginations of the NE in the interior of the nucleus (Fig. 4a and 4b) has been demonstrated in a number of cell types by electron microscopy [42], immunofluorescence [41-43] and, more recently, by three-dimensional structured illumination microscopy [113]. This so-called *nucleoplasmic reticulum* [43] would be lined by both, the inner and the outer nuclear membranes and filled by cytosol, ER and even mitochondria (Fig. 4b; note difference with 4a) [40, 61]. The nucleoplasmic reticulum expresses functional IP_3Rs , both in the inner and the outer nuclear membrane. Fig. 6A shows invaginations of the NE in a tridimensional reconstruction of a GH₃ cell containing cytosolic (cytGFP), mitochondrial (mitoTracker red) and nuclear (DAPI) markers. Volume rendering of serial 0.35 μm optical sections permitted to follow the three-dimensional projection inside the nucleoplasm. The spaces occupied by these invaginations corresponded to hollow spaces in the chromatin distribution. The invagination itself contained green (left) and red (middle) fluorescence corresponding to the cytGFP-AEQ and the mitoTracker (mitochondria) present inside the invaginations. The invaginations were also visible with the inner NE marker lamin B and, moreover, contained endoplasmic reticulum inside, as evidenced by the green fluorescence of the erGFP-AEQ reporter (Fig. 6B). Nucleoplasmic reticulum may be cell-specific, as in nuclei of intact primary neurones it was observed by confocal or electron microscopy [106]

Even though the existence of a nucleoplasmic reticulum helps to explain the existence of selective $[\text{Ca}^{2+}]_{\text{N}}$ peaks by direct release of the Ca^{2+} it contains to the nucleoplasm, it is doubtful whether the quantitative relations would be satisfactory. The volume of the nucleoplasmic reticulum is only a small fraction of the total nuclear volume, so that released Ca^{2+} would be very much diluted into the nucleoplasm and the resulting $[\text{Ca}^{2+}]_{\text{N}}$ peaks would be small. It has been proposed that the Ca^{2+} would be accumulated inside intranuclear stores at very large concentrations because it would be chelated by macromolecules. The total calcium content of the nucleus is very high, above 10 mmole/l, the second highest after the secretory granules [82]. The high capacity/low affinity Ca^{2+} binding proteins chromogranin and secretogranin are present in large

amounts in the nucleus [114, 115], but, surprisingly, they apparently do not overlap with NE nor nucleoplasmic reticulum membranes. Apparently chromogranin B, phospholipid and IP₃R colocalize in complexes of 2-3·10⁷ Da [116] not associated to morphologic differentiations. This is reminiscent of speckles, where PIP2 and PIP kinases accumulate (see above). Recent studies with high voltage electron microscopy have suggested that the phospholipid-chromogranin-IP₃R complexes are associated to very small (<50 nm) vesicular structures that are located all along the nucleoplasm interspersed between the heterochromatin. Added IP₃ rapidly released Ca²⁺ from these structures, but other inositol phosphates did not [82]. Should these facts be confirmed, a fascinating view emerges where this intricate Ca²⁺ signalling machinery could induce the release of Ca²⁺ restricted to very discrete subdomains, perhaps close to the target gene or protein in the interior nucleus. At the moment more work is needed to firmly establish the existence and significance of such nuclear nanovesicles in different cell types.

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Figure Legends.

Fig. 1. Calcium effects on gene expression. A. Cytosolic Ca^{2+} -calmodulin can activate several pathways as shown. CaMKII, calmodulin-dependent protein kinase II. NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells. Ikb, inhibitor of κB . PKC, protein kinase C. CaN, calcineurin. NFAT, Nuclear factor of activated T-cells. CaMK, calmodulin-dependent protein kinase. MAPK, mitogen-activated protein kinase. CREB, cyclic AMP response element-binding protein. MEF2, myocyte enhancer factor 2; CCAT, calcium channel activated transcriptional regulator. P.M., plasma membrane. **B.** DREAM system. DREAM, downstream regulatory element antagonist modulator. DRE, downstream regulatory element, TIC, transcription initiation complex.

Fig. 2. Comparison of the light emission by different Ca^{2+} probes as a function of the Ca^{2+} concentration. Light emission is expressed as F/F_{max} for fluo-3 [52], R/R_{max} for yellow cameleon 2.1 (YC2.1) [50] and $R/\text{pericam}$ [46] and L/L_{TOTAL} for aequorin (AEQ) [117]. Note logarithmic scales in both axes. The Hill coefficients were: AEQ, 2.1; Fluo-3, .85; R-pericam, 0.36; YC2.1, 0.18. The image at right compares the two-dimensional profile of a $[\text{Ca}^{2+}]$ hotspot as it would be reported by either fluo-3 or aequorin. For simulation of hotspot, the center was assigned maximum brightness with exponential decay with distance ($S_x/S_0 = e^{-k \cdot x}$). The light output was estimated from the Michaelis equation for fluo-3 or as described by Montero et al. [117] for aequorin.

Fig. 3. Imaging of $[\text{Ca}^{2+}]_{\text{N}}$ with nucleus-targeted aequorin. A, B. Effects of stimulation with high glucose in a pancreatic islet expressing nucleus-targeted aequorin [62]. **A.** The average trace of the whole islet is shown in white, superimposed to the bright field image of the islet. **B.** The light emitted during the whole stimulation period was integrated and is represented in pseudocolor (scale at right) superimposed to the bright field image. Five high-emitting regions were identified, as shown by numerals 1-5. 4 and 5 were defined as two different regions on the basis of asynchrony in light-emitting. See supplemental movie 1. **C.** Oscillations of $[\text{Ca}^{2+}]_{\text{N}}$ in a single GH₃ pituitary cell. Spontaneous and TRH-induced oscillations are compared. Cells had been infected with an amplicon herpes virus vector carrying the nuclear GFP-aequorin [63]. Other details as in reference [60]. Panels **D**, **E** and **F** show the bright field image, the green fluorescence and the merged image corresponding to the cell shown in **C**, respectively.

Fig. 4. Schematic view of different mechanisms that may produce an increase of $[\text{Ca}^{2+}]_{\text{N}}$. PM, plasma membrane; CYT, cytoplasm; NE, nuclear envelope; NPL, nucleoplasm. Ca^{2+} may enter the nucleus from the cytosol during a $[\text{Ca}^{2+}]_{\text{C}}$ peak (1). Alternatively, it can be released from the nuclear cisterna either to the cytosol, and then enter through nuclear pores (2), or directly to the nucleoplasm (3). An invagination of the NE ("nucleoplasmic reticulum") could release Ca^{2+} to the nuclear core. The invagination may (4b) or may not (4a) contain cytoplasm. Finally, Ca^{2+} could be released to the nucleoplasm from intranuclear pools bound to macromolecules or from microvesicles too small to be seen by the usual procedures [82, 87, 118]. Redrawn with permission from [56].

Fig.5. Expression of IP₃ receptors in the nucleus of GH₃ pituitary cells. Cells were stained with isoform-specific anti-IP₃R antibodies (green) and counterstained with DAPI (blue). The merging of both images is shown at right. Results for IP₃R1 (A.) and IP₃R2 (B.) are shown. Note that HeLa cells did not show nuclear immunoreactivity for αIP₃R2 antibody (C.). During cell division of HeLa cells IP₃R2 was able to enter the nuclear space and appeared between the two sets of chromatids at anaphase (D.). Calibration bar, 10 μm. For other details see [61].

Fig. 6. Invaginations of the cytoplasm in the nucleus of GH₃ pituitary cells. A. Volume rendering of 15 consecutive sections taken every 0.35 μm. Sections have been tilted 30°. From left to right, green fluorescence of cytosolic GFP, red fluorescence of mitoTracker red and blue fluorescence of DAPI. Note that the cytosolic invagination fits to a hole in the chromatin and has mitochondria inside (arrows). Taken with permission from [61]. For further details see Movie 2 in Supplementary data. **B.** Section of a GH₃ pituitary cell stained with lamin B (red), endoplasmic reticulum targeted GFP (erGFP, green) and DAPI (blue). Calibration mark, 10 μm.

Fig. 1

Fig.2

Fig.3

Fig. 4

Fig.5

Fig. 6

Supplementary data

Movies (2)

Movie 1. Effects of high glucose (11 mM) on the nuclear Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{N}}$) in pancreatic B cells. High glucose increases $[\text{Ca}^{2+}]_{\text{N}}$. The movie shows the bright field image on an islet infected with nucleus-targeted aequorin to which pseudocolor-coded luminescence emission has been superimposed. Perfusion was switched to high-glucose (11 mM) when shown. The duration of the experiment shown is about 15 min. Images were taken every 10 seconds. Pseudocolor code: blue, , yellow and red represent 1, 2, and 3/10 s, respectively.

Movie 2. Rotating three-dimensional reconstruction of two GH₃ cells. Images of cytoplasm (cytoplasmic GFP, in green), mitochondria (mitotracker red, in red) and chromatin (DAPI, in blue) are shown. The cytoplasmic GFP was expressed in only one of the cells. Rendering was done with 15 0.35 μm -sections taken with the Zeiss ApoTome^R system using Volume J software (M.D).

Abràmoff, and M.A. Viergever. Computation and Visualization of Three Dimensional Motion in the Orbit. IEEE Trans Med Imag., 21 (4), 2002)(<http://bij.isi.uu.nl/vr.htm>).