ATP-dependent regulation of nuclear Ca^{2+} levels in plant cells

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Abstract Localised alterations in cytoplasmic Ca^{2+} levels are an integral part of the response of eukaryotic cells to a plethora of external stimuli. Due to the large size of nuclear pores, it has generally been assumed that intranuclear Ca^{2+} levels reflect the prevailing cytoplasmic Ca^{2+} levels. Using nuclei prepared from carrot (*Daucus carota* L.) cells, we now show that Ca^{2+} can be transported across nuclear membranes in an ATP-dependent manner and that over 95% of Ca^{2+} is accumulated into a pool releasable by the Ca^{2+} ionophore A.23187. ATP-dependent nuclear Ca^{2+} uptake did not occur in the presence of ADP or ADPγS and was abolished by orthovanadate. Confocal microscopy of nuclei loaded with dextran-linked Indo-1 showed that the initial ATP-induced rise in $[Ca^{2+}]$ occurs in the nuclear periphery. The occurrence of ATP-dependent Ca^{2+} uptake in plant nuclei suggests that alterations of intranuclear Ca^{2+} levels may occur independently of cytoplasmic $[Ca^{2+}]$ changes.

Key words: Nucleus; Plant; Ca^{2+} uptake; Signal transduction; Imaging; Nuclear pore complex

1. Introduction

Ca^{2+} is an ubiquitous second messenger in eukaryotic cells and the role of Ca^{2+} in plant cell signalling is now firmly established. Alterations in intracellular Ca^{2+} levels and the generation of Ca^{2+} gradients and Ca^{2+} waves are integral features of the cellular responses to a wide range of stimuli [1–7]. An important current question is how signal-specific information is conveyed by spatial and temporal Ca^{2+} changes and how these changes lead to selective upregulation of signal-responsive genes.

In mammalian cells, it has been hypothesised that cytoplasmic Ca^{2+} transients may act as a trigger for nuclear events and it has been assumed that the intranuclear Ca^{2+} concentration is determined largely by the cytoplasmic Ca^{2+} levels and equilibration across nuclear pores. However, it has been shown that ATP-dependent Ca^{2+} uptake systems exist in mammalian nuclei [8,9] and several recent pieces of evidence suggest that the flux of molecules and ions across nuclear pore complexes is far more rigidly controlled than previously envisaged [10]. Thus, changes in intranuclear Ca^{2+} levels are unlikely to be controlled simply by the rate of diffusion from the cytoplasm [11] and it has been demonstrated that gene expression in pituitary cells can be differentially controlled by specific changes in the concentration of either nuclear or cytoplasmic Ca^{2+} [12].

There are several pieces of evidence which point to a specific physiological role for nuclear Ca^{2+} in plants. Ca^{2+}-dependent protein kinases and Ca^{2+}-binding proteins have been shown to be present in nuclei and the Ca^{2+}-dependent phosphorylation of a range of nuclear proteins has been demonstrated [13,14]. There is also evidence which suggests that the nuclear region can act as a focal point for the generation of signal-induced Ca^{2+}-spiking [1,15]. It also deserves mentioning that antibodies raised to a plant homologue of the mammalian SERCA pump recognise specific antigens in plant nuclei [16].

In this study, we show that ATP-dependent Ca^{2+} transport systems are associated with the plant nucleus. These findings may have a number of implications for the understanding of how Ca^{2+}-dependent nuclear processes are regulated and how cytoplasmic Ca^{2+} signals are transduced to, and within, the nucleus.

2. Materials and methods

2.1. Chemicals

10 kDa dextran-linked Indo-1 and $DiOC_6$ were purchased from Molecular Probes Inc. (Eugene, OR, USA), $^{45}CaCl_2$ (specific activity 1.85 GBq/mg) was purchased from NEN-Dupont (UK) and other chemicals from Sigma (Poole, UK).

2.2. Cell culture and nuclear isolation

Non-embryonic carrot (*Daucus carota* L.) cell cultures were maintained as described previously [17] and protoplasts were prepared from 4–5 day old cells by incubation overnight in growth medium supplemented with 0.4 M mannitol, 0.01% (w/v) pectinase (Sigma, UK) and 0.1% (w/v) cellulase-RS (Yakult Pharmaceutical Co. Ltd., Japan). Nuclei were prepared essentially as described by Beven et al. [18] except that Triton X-100 was omitted throughout, and protoplasts were disrupted by passing the protoplast suspension through a 25–26 G needle fitted to a syringe. The homogenate was layered over a sucrose gradient (1.6–2.3 M sucrose in isolation buffer: 0.4 M sucrose, 10 mM MES (pH 5.3), 10 mM NaCl, 0.1 mM dithiothreitol, 5 mM EDTA, Complete™ EDTA-free protease inhibitors (Boehringer Mannheim, Germany)) overlying a 0.5 ml Maxidens (Nycomed, Norway) cushion. The nuclear preparation was centrifuged at $100\,000\times g$ for 2 h and the nuclei were collected at the sucrose/Maxidens interface and resuspended in isolation buffer. When large quantities of nuclei were required for multiple uptake experiments, the sucrose gradient step was omitted and debris instead removed by filtration through a 10 μm nylon mesh filter and nuclei harvested by centrifugation for 15 min at $100\times g$ onto a Maxidens cushion. Nuclei were used immediately after isolation for $^{45}Ca^{2+}$ uptake experiments.

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Abbreviations: $[Ca^{2+}]_{cyt}$, cytoplasmic Ca^{2+} concentration; $[Ca^{2+}]_{nuc}$, intranuclear Ca^{2+} concentration; $[Ca^{2+}]_{ext}$, extranuclear Ca^{2+} concentration

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2.3. $^{45}Ca^{2+}$ uptake

Nuclei were resuspended in Ca^{2+} uptake buffer: 0.4 M sucrose, 25 mM BTP-MES (pH 7.25), 5 mM $MgCl_2$, 100 mM KCl, 0.1 mM spermine, protease inhibitors. The free Ca^{2+} concentration was adjusted to between 100 nM and 1 μM using EGTA/Ca^{2+} buffers and $^{45}CaCl_2$ (final specific activity 30 kBq/ml) was added. Experiments were carried out at room temperature and uptake reactions started by the addition of ATP. Nuclear samples were collected by filtration under vacuum onto prewetted 0.45 μm pore size cellulose nitrate filters (Whatman, UK). Filters were rapidly washed with 2.5 ml of ice-cold buffer and transferred to scintillation vials. Radioactivity was determined by liquid scintillation spectrophotometry (Wallac 1410, Turku, Finland) using Picofluor scintillation cocktail (Canberra-Packard, UK).

2.4. Light and confocal microscopy

Light microscopy was carried out using a Zeiss universal microscope. For confocal Ca^{2+} imaging, nuclei were loaded with Indo-1 10 kDa dextran in dye-loading buffer (250 mM sucrose, 25 mM BTP-MES (pH 7.25), 5 mM $MgCl_2$, 100 mM KCl and protease inhibitors) for 10 min at 4°C. Dye-loaded nuclei were washed twice in buffer and placed in a sealed perfusion chamber on an APTES coated coverslip [19]. Nuclei were perfused with dye-loading buffer with free Ca^{2+} concentrations adjusted using Ca^{2+}/EGTA buffers. Nuclei were imaged using a Bio-Rad MRC-1000 UV confocal microscope with a Nikon 40×-fluor oil immersion objective (NA 1.3) and Bio-Rad Comos software. Indo-1 was excited by the 351 nm line of the argon ion laser (Coherent Innova Enterprise 662) and the emission was recorded using 405/30 and 460LP emission filters into two detector channels. Ratio images for the Indo-1 labeling were calculated by dividing the 405 nm image by the 460 nm image. Data were analysed using NIH-image software and images were produced using PhotoShop 5.0.

2.5. Miscellaneous methods

The method of Fohr et al. [20] was used for the preparation of stable solutions of vanadate. Standard protocols were used for the fixation of nuclei for transmission electron microscopy. Agarose blocks embedded in LR-white resin were used for the preparation of ultrathin sections. Sections were post-stained with freshly prepared potassium permanganate (1%, w/v), uranyl acetate and lead citrate in 0.1 M phosphate buffer or with silicotungstic acid as described previously [21].

3. Results

3.1. Characterisation of nuclei isolated in the absence of detergent

As intact nuclear membranes are a prerequisite for carrying out ion-flux studies, we developed a nuclear isolation procedure which did not rely on the use of detergents. Fig. 1 shows images of nuclei isolated using this protocol. Fig. 1A and B show nuclei incubated in nuclear isolation buffer containing 70 kDa dextran-linked FITC, a fluorochrome which does not pass through intact membranes or nuclear pore complexes. The transmission DIC image (Fig. 1A) shows a well preserved nuclear ultrastructure, with clearly defined nuclear membranes and nucleoli. The confocal fluorescence image (Fig. 1B) further shows that the dextran-linked fluorochrome is excluded from the nuclei indicating that the nuclear membranes are intact. If nuclei were incubated in the presence of even low concentrations of detergents (i.e. >0.0025% w/v Triton X-100), they lost the ability to exclude the fluorochrome. Isolated nuclei were also examined by transmission electron microscopy and can be seen to be intact (Fig. 1C). The nuclear membranes appear as double-membranes (Fig. 1D) and nuclear pore complexes are visible on the surface (arrow, Fig. 1E).

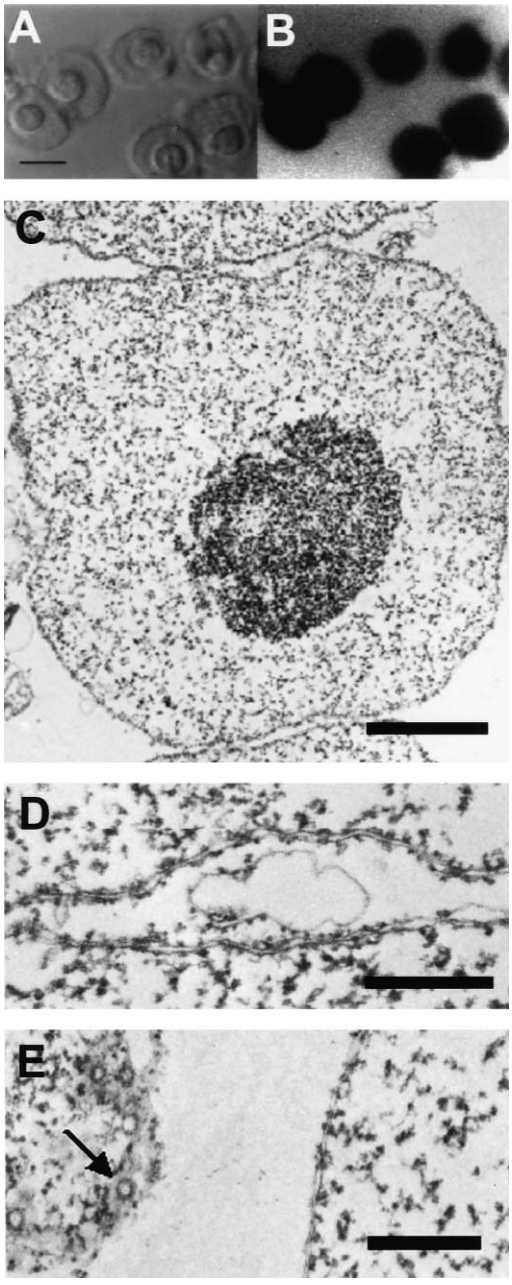

Fig. 1. DIC image (A) and confocal fluorescence image (B) of carrot nuclei isolated in the absence of detergent and incubated for 5 min in the presence of 1 mg/ml 70 kDa dextran-linked FITC. Transmission electron micrographs showing (C) intact nuclei, (D) intact nuclear membranes and nuclear envelope and (E) nuclear pore complexes (arrow). Scale bars: (A) 6 μm; (C) 2 μm; (D) 500 nm; (E) 500 nm.

3.2. ATP-dependent Ca^{2+} uptake by isolated nuclei

To investigate the possibility of Ca^{2+} transport across nuclear membranes, experiments were carried out using $^{45}Ca^{2+}$ as tracer. The results of these experiments are shown in Fig. 2. In the absence of ATP, there is negligible nuclear Ca^{2+} uptake. However, the addition of 1 mM ATP triggers the immediate onset of Ca^{2+} uptake. Ca^{2+} uptake continues at a near linear rate for up to 10 min at which time a nuclear Ca^{2+} concentration of approximately 1 nmol/mg nuclear protein is reached. Following this peak level, we consistently observed a decrease in Ca^{2+} levels until a plateau of around

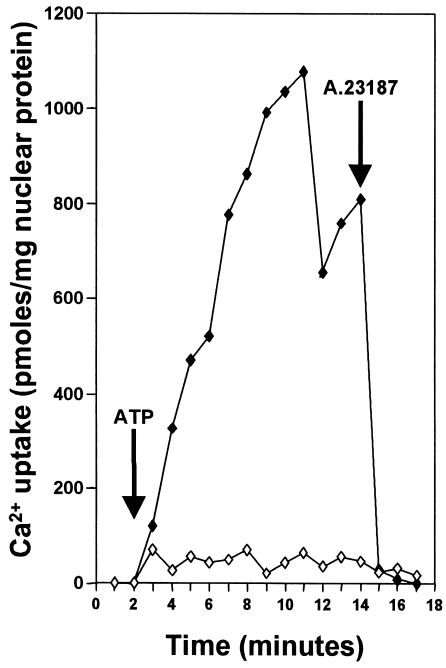

Fig. 2. ATP-dependent Ca^{2+} uptake by intact plant nuclei. The timing of ATP and A.23187 additions is indicated by arrows, $[Ca^{2+}]_{ext}$ was clamped at 100 nM using Ca^{2+}/EGTA buffers. Solid diamonds: +1 mM ATP; open diamonds: −ATP. Data are typical of seven independent experiments.

600–800 pmol/mg nuclear protein was reached. We ascribe the decrease in $[Ca^{2+}]_{nuc}$ seen after prolonged incubations to a gradual increase in leakiness of the nuclear membranes. Addition of the Ca^{2+} ionophore A.23187 results in virtually

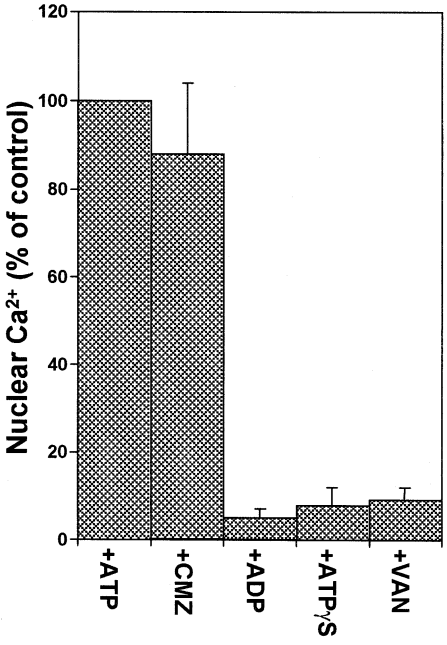

Fig. 3. Effect of various pharmacological agents on nuclear Ca^{2+} uptake. ATP, control experiment with the addition of ATP (1 mM) has been set to 100%. CMZ, calmidazolium (10 mM); ADP, adenosine diphosphate (1 mM); ATPγS, adenosine 5′-diphospho-γ-S (1 mM); VAN, orthovanadate (1 mM). Nuclear $[Ca^{2+}]$ was determined after 9 min incubation. Data are the mean of three independent experiments and vertical bars indicate S.D.

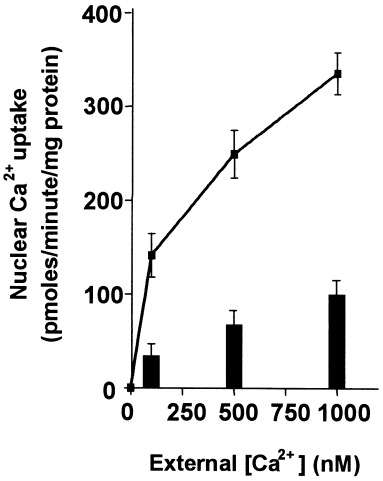

Fig. 4. Effect of varying $[Ca^{2+}]_{ext}$ on the rate of nuclear Ca^{2+} uptake. The solid squares show the rate of Ca^{2+} uptake by nuclei incubated in the presence of ATP. The solid bars illustrate the rate of nuclear Ca^{2+} uptake in the absence of ATP. Data are the mean of five independent experiments and vertical bars indicate S.D.

100% release of Ca^{2+}. Fig. 3 shows that the addition of the calmodulin antagonist calmidazolium (10 mM) had no significant effect on nuclear Ca^{2+} uptake. In contrast, the addition of orthovanadate (1 mM) resulted in a near complete inhibi-

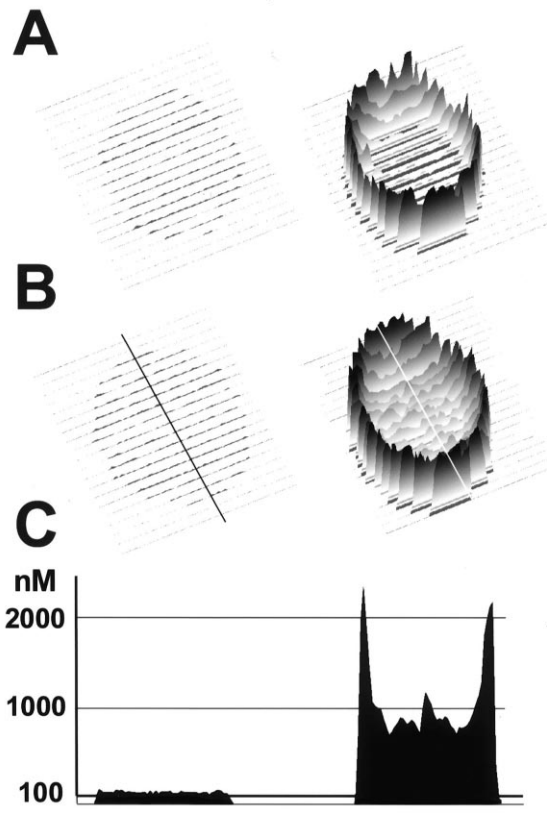

Fig. 5. Ca^{2+} profiles of two nuclei loaded with the ratiometric Ca^{2+} dye, 10 kDa dextran-linked Indo-1 and imaged by confocal microscopy. The images shown in the left lanes of A and B were obtained from nuclei which had been perfused for 10 min in the absence of ATP. The right lanes in A and B show the nuclear Ca^{2+} profiles obtained 60 s after the addition of ATP to the perfusion buffer. C shows the quantification of nuclear Ca^{2+} across the two images shown in B.

tion of ATP-dependent uptake. Neither ADP (1 mM) nor the non-hydrolyzable ATP analogue, ATPγS (1 mM), supported nuclear Ca^{2+} uptake.

3.3. Kinetic properties of nuclear Ca^{2+} uptake

The effect of varying the extranuclear free Ca^{2+} concentration, $[Ca^{2+}]_{ext}$, on the rate of nuclear Ca^{2+} uptake is shown in Fig. 4. The rate of nuclear Ca^{2+} uptake in the presence of ATP (1 mM) was determined during the linear phase of the uptake curve and was found to be approximately 140 pmol/min/mg nuclear protein at $[Ca^{2+}]_{ext}$ of 100 nM. A significant increase in the Ca^{2+} uptake rate can be seen at increased $[Ca^{2+}]_{ext}$. In the absence of ATP, a near linear relationship exists between $[Ca^{2+}]_{ext}$ and the intranuclear Ca^{2+} concentration, $[Ca^{2+}]_{nuc}$. It is likely that a component of $[Ca^{2+}]_{nuc}$ determined both in the presence and absence of ATP can be accounted for by Ca^{2+} association to multi-affinity nuclear Ca^{2+}-binding sites. The Ca^{2+} K_m for the ATP-dependent component of the nuclear Ca^{2+} uptake was calculated to be approximately 200 nM.

3.4. Laser scanning confocal microscopic studies

To pinpoint the initial site of Ca^{2+} uptake, we carried out a number of confocal imaging experiments. Fig. 5 shows the effect of ATP addition (1 mM) on the Ca^{2+} profile in nuclei loaded with the ratiometric Ca^{2+} reporter dye, 10 kDa dextran-linked Indo-1. The images shown in Fig. 5A–C (left lanes) indicate a homogenous intranuclear resting $[Ca^{2+}]_{nuc}$ of approximately 150–200 nM. Shortly after the addition of ATP (1 mM), a significant increase in the fluorescence signal can be seen with $[Ca^{2+}]_{nuc}$ in the nuclear periphery reaching levels of above 2 μM (Fig. 5A–C, right lanes).

4. Discussion

4.1. ATP-dependent Ca^{2+} uptake in plant nuclei

The data obtained in this study show that plant nuclei possess an ATP-dependent Ca^{2+} uptake system and that significant Ca^{2+} accumulation occurs even when extranuclear Ca^{2+} concentrations are as low as 100 nM. Confocal data confirm that the observed Ca^{2+} uptake is not due to contaminating organelles such as e.g. nuclear-associated endoplasmic reticulum. The fact that as little as 100 nM extranuclear Ca^{2+} supports uptake is interesting as these Ca^{2+} levels are close to the lowest levels of free $[Ca^{2+}]_{cyt}$ reported to exist in plant cells [22]. Nicotera et al. [8] made similar observations in rat liver nuclei which were found to accumulate Ca^{2+} in an ATP-dependent manner when incubated in a medium containing submicromolar concentrations of Ca^{2+}. These findings have been corroborated by Stehno-Bittel and Clapham [23].

The ability of A.23187 to release nearly 100% of $[Ca^{2+}]_{nuc}$ shows that nuclear-associated Ca^{2+} accumulated in the presence of ATP is contained within a membrane bound pool and not irreversibly sequestered. Pharmacological data indicate that ATP hydrolysis is necessary for the observed changes in $[Ca^{2+}]_{nuc}$ as neither ATPγS nor ADP lead to increases in $[Ca^{2+}]_{nuc}$. The abolition of ATP-dependent nuclear Ca^{2+} uptake by vanadate further suggests that a Ca^{2+}-ATPase may be responsible for the observed Ca^{2+} uptake, but this needs further confirmation. The image data shown in Fig. 5 indicate a spatially homogenous $[Ca^{2+}]_{nuc}$ of approximately 150–200 nM in the absence of ATP. Addition of ATP (1 mM) leads to a rapid increase in $[Ca^{2+}]_{nuc}$ with levels of 0.8–2.2 μM being reached. One minute after ATP addition, the nucleoplasmic Ca^{2+} levels were found to increase significantly but the highest levels of $[Ca^{2+}]_{nuc}$ were consistently found to appear as a broad 'ring' at the periphery of the nucleus. Although 10 kDa dextran-linked molecules are unlikely to cross the nuclear membranes, we cannot entirely rule out that a small, but significant, proportion of the dextran-linked dye might have become unlinked and entered the nuclear envelope. However, we find this an unlikely explanation for the results obtained in these experiments. First, the size of the peripheral 'ring' argues against the changes occurring within the very narrow confines of the inner nuclear membrane space and the dextran-unlinked dyes would also be expected to leach rapidly from the inner nuclear membrane compartment should they have entered.

An interesting point concerning the use of dextran-linked dyes in the study of nuclear Ca^{2+} fluxes emerged in a recent study by Rogue et al. [24] who showed that phosphorylation of the Ca^{2+}-ATPase of rat liver nuclei by cyclic AMP-dependent protein kinase (protein kinase A) markedly enhances the transport of 10 kDa fluorescent-labeled dextrans across the nuclear envelope. As the dye-loading in our experiments was carried out in the absence of ATP, it is unlikely that this effect, if indeed occurring in plant cells, would have had any significant influence on the experiments.

That there is a correlation between $[Ca^{2+}]_{ext}$ and $[Ca^{2+}]_{nuc}$ both in the presence and absence of ATP can be seen from the data in Fig. 4 but it is clear that the $[Ca^{2+}]_{nuc}$ is not determined simply by equilibration with $[Ca^{2+}]_{ext}$.

There are two likely scenarios that can explain the observed data: either, (I) Ca^{2+} is taken up initially at the cytosolic face of the outer nuclear membrane and transported into the nuclear envelope (possibly courtesy of an ATPase) and from there Ca^{2+} may be transported further into the nucleoplasm, or, (II) Ca^{2+} uptake occurs at the nucleoplasmic face of the inner nuclear membrane, and Ca^{2+} is taken up directly from the nucleoplasm. The confocal microscopy data which show that the initial rise in $[Ca^{2+}]_{nuc}$ occurs at the nuclear periphery has lead us currently to favour scenario I described above. The possibility that $[Ca^{2+}]_{nuc}$ can supersede $[Ca^{2+}]_{cyt}$ in vivo in eukaryotic cells is still controversial [25] and an added complication in the interpretation of this type of data is that Ca^{2+} also has been shown to affect the transport properties of the nuclear pore complex [10].

4.2. Physiological significance of changes in intranuclear free Ca^{2+} concentrations

Although the physiological consequences of changes in the Ca^{2+} concentrations within the nuclear membranes and the nucleoplasm in plant cells remain obscure, there are several pieces of evidence which indicate that such events could have implications for a range of nuclear processes. Datta et al. [13] have shown how several proteins in pea nuclei are phosphorylated by Ca^{2+}-dependent kinases and Roux [14] demonstrated that nuclear enzymes in pea can be stimulated by phytochrome in a Ca^{2+}-dependent manner. Ca^{2+}-binding proteins are also known to be present in plant nuclei [26]. In a recent study employing a nucleoplasmin–aequorin fusion protein expressed in tobacco, van der Luit et al. [27] further showed that the kinetics of Ca^{2+} changes in the nucleus and

cytoplasm differ in response to stresses induced by wind and cold, respectively.

A pertinent question in Ca^{2+} signalling research at the moment is whether nuclear Ca^{2+} changes are capable of directly influencing gene expression. A study by Hardingham et al. [12] using nuclear microinjection of non-diffusible Ca^{2+} chelators suggests that the answer to this question is affirmative. Further support for a role of Ca^{2+} in gene transcription comes from a recent study by Carrion et al. [28] who identified a new human transcriptional repressor, DREAM (prodynorphin downstream regulatory element antagonist modulator). DREAM is capable of binding specifically to DNA and represents the first Ca^{2+}-binding protein to function as a direct repressor of transcription. The finding that Ca^{2+} can reduce the DREAM DNA affinity is the first evidence for a direct mechanism of differential regulation of gene expression not depending on changes in the activity of other transcriptional effectors or direct protein–protein interactions. That similar scenarios might exist in plant cells is not unlikely, but as yet the necessary experiments to test this hypothesis remain to be carried out.

Although we primarily have focused on the plant nucleus as a Ca^{2+} accumulating organelle, the possibility should not be overlooked that the nucleus also could act as a locus for the generation of cytoplasmic Ca^{2+} signals. Ehrhardt et al. [15] studying nod-factor signalling in root hairs thus observed that Ca^{2+}-spiking could originate within the nuclear region and our own studies of Ca^{2+} waves in pollen tubes suggest that a nuclear origin of Ca^{2+} waves is a distinct possibility [1]. Nuclear membranes are also known to act as inositol(1,4,5)-trisphosphate and inositol(1,3,4,5)-tetrakisphosphate releasable Ca^{2+} stores in mammalian cells [29,30].

We think that our findings may provide a new starting point for research into how extracellular stimuli, transduced via intracellular Ca^{2+}-dependent pathways, regulate signal-induced gene expression in plants.

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