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# **AUTONOMOUS REGULATION OF FREE $Ca^{2+}$ CONCENTRATIONS IN ISOLATED PLANT CELL NUCLEI: A MATHEMATICAL ANALYSIS**

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Running Title: Modelling nuclear calcium homeostasis

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**Abbreviations:** ER, endoplasmic reticulum; NE, nuclear envelope; INM, inner nuclear membrane; NPC, nuclear pore complex;  $[Ca^{2+}]_{nuc}$ , nucleoplasmic free calcium concentration;  $[Ca^{2+}]_{nuc\_total}$ , nucleoplasmic total calcium concentration;  $[Ca^{2+}]_{store}$ , nuclear store free calcium concentration;  $[Ca^{2+}]_{store\_total}$ , nuclear store total calcium concentration.

## **Abstract**

Experiments performed on nuclei isolated from animal or plant cells have provided evidence that the nucleus generates directly specific nucleoplasmic calcium transients in response to external stimuli. Recent data suggest that isolated plant nuclei might be considered as a closed system where the nuclear concentration of free calcium would be regulated by reversible movements between the nucleoplasm and nuclear stores. We have addressed the relevance of this hypothesis by developing a mathematical approach to simulate nucleoplasmic calcium dynamics generated under various pH and temperature conditions. Here, we show that the experimental results could be explained provided that calcium-channels as well as systems transporting calcium are present on the inner nuclear membrane. The putative channels would allow the entry of calcium into the nucleoplasm whereas the elusive transporting system(s) would contribute to replenish the nuclear stores. The simple proposed model is versatile enough to explain and predict autonomous changes in free calcium in the nucleoplasm of isolated plant nuclei.

## 1. Introduction

In both plants and animals, the calcium ion is fully recognized as a second messenger that regulates a wide variety of biological processes triggered by biotic and abiotic external stimuli [1]. Changes in free calcium concentration do not proceed in a stereotypical manner. Rather, fluctuations of free calcium have time-, space-, intensity-, and frequency-characteristics which depend upon the nature and strength of the stimulus [2]. Concerning the spatial changes in free calcium, it is now established that calcium concentrations vary in cell organelles like chloroplasts [3], mitochondria [4] or nuclei [5-7]. Nuclei are separated from the other cell compartments by a double membrane system that is punctuated by nuclear pore complexes (NPC), which allow trafficking of molecules and ions between the nucleoplasm and the cytosol [8-10]. Indeed, the capacity of NPC to allow free diffusion of calcium from the cytosol to the nucleoplasm is largely accepted [11-13]. Thus, simple diffusion has been reported to explain fully the pattern of nuclear calcium upon stimulation in rat cardiac myocytes [14]. However, a series of experimental evidence suggests that the situation is more complex [13] and other mechanisms may be involved in the generation of nuclear calcium signals, depending on the cell type and/or the stimulus. Firstly, kinetic measurements of intranuclear calcium gradients have shown that calcium permeates through NPC in a strictly controlled manner [15]. Secondly, nuclei contain specific calcium stores in the nuclear envelope and in the recently identified nucleoplasmic reticulum which forms a continuum with the endoplasmic reticulum [16].

Recent experimental evidence, in both animals and plants, show that nuclear and cytosolic calcium may be regulated independently. In nuclei of HepG2 cells, nuclear calcium signals may be generated before any variation in the cytosol [17]. In tobacco cells, a hyper-osmotic shock induces an increase in the calcium concentration in the cytosol but not in the nucleus [6]. Cryptogein, an oomycete elicitor, induces calcium transients in both the cytosol and the nucleus, but the nuclear calcium peaks 15 min after the cytosolic peak [7]. According to Meyer et al. [18] if calcium signal kinetics in the cytosol and the nucleoplasm differ from each other by at least 1 sec, then the nuclear envelope is a substantial barrier to  $\text{Ca}^{2+}$  fluxes. Furthermore, isolated nuclei from either myocyte [19] or tobacco cells [5, 20] are able to respond to external stimuli by generating nucleoplasmic calcium signals directly and thus independently of the cytosolic concentration. Xiong et al. [20] showed further that high concentrations of calcium in the external medium or treatment with EGTA do not induce any particular calcium variation in isolated tobacco nuclei, thus showing that the nuclei are impermeable to external calcium.

Different calcium channels or transporters have been shown to localize to the nuclear envelope. The presence of  $\text{Ca}^{2+}$ -ATPases pumps on the outer membrane of the NE is well documented in animals [21-24] as well as in plants [25, 26]. InsP4- receptors that allow the InsP4-dependent entry of calcium into the NE calcium store have been located to the outer membrane of rat liver nuclei [24].

Systems mobilizing calcium from the NE and subsequently inducing calcium increases in the nucleoplasm have also been characterized. In animals, the inner membrane of the NE has been shown to contain InsP3-dependent calcium channels [12, 22, 24, 27] as well as ryanodin-receptors [28]. Mechano-sensitive  $\text{Ca}^{2+}$ -channels responsible for calcium elevation in the nucleus have been characterized by patch-clamping of nuclei isolated from MC3T3-E1 cells [19].

In plants, a  $\text{Ca}^{2+}$ - and voltage-dependent cation channel has been characterized by electrophysiology in nuclei isolated from beet [29], and a pharmacological approach suggests the existence of TRP-like channels in nuclei of tobacco cells [20].

Taken together, these data show that the nuclear envelope is able to store calcium and that the stored ion may be mobilized to increase the nucleoplasmic free calcium concentration. In order to understand and to explain calcium dynamics in the nucleoplasm, it is essential to consider calcium exchanges between the different sub-nuclear compartments (nucleoplasm, nuclear envelope stores, calcium buffers) taken as a whole. In this context, development of mathematical models may be instrumental for testing different hypotheses *in silico*, by verifying their consistency with experimental data. Here, we present a simple model of nuclear calcium dynamics that may explain most characteristics of the calcium patterns in isolated plant cell nuclei.

Because most of the molecular components which are likely to be implicated in the entry/mobilization of calcium remain to be identified in plants, the model is built on general assumptions based on known or well-accepted data (mostly in animal cells), and on more speculative hypotheses which emerged from the analysis of specific experimental data [20].

## **2. Material and methods**

### ***2.1. Calcium measurements***

Nuclei from BY2 tobacco cells expressing apoaequorin in the nucleus were isolated as described previously [20]. Coelentarazin (2.5  $\mu$ M final concentration) was added to the nuclei suspension and aequorin was allowed to reconstitute for at least 2 hours. Aliquots of 100 $\mu$ l of nuclei suspension containing about 30 000 nuclei per tube were prepared and stored on ice in the dark before measurements. Luminescence was measured using a Berthold Sirius luminometer and calcium concentrations calibrated as previously reported [30]. In a typical experiment, a 100 $\mu$ l nuclei sample was transferred into the luminometer at room temperature and luminescence recording was started immediately ( $t=0$ ). At  $t=20$ s a mechanical stimulus was applied by injecting (injection duration = approx. 1s) two volumes of buffer (0.4M sucrose, 5mM Bis-Tris, 10mM NaCl, 5mM MgCl<sub>2</sub>, pH adjusted at the required value) in the luminometer cuvette. At  $t=5$  min 300 $\mu$ l of lysis buffer containing 2% Nonidet were injected to discharge total aequorin in the sample.

### ***2.2. Model simulation***

Differential equations were solved by a 4<sup>th</sup> order Runge-Kutta method using the modeling software package Berkeley-Madonna (University of California, Berkeley, USA).

## **3. Model description**

### ***3.1. Experimental background***

The present mathematical model relies on experimental data from the work of Xiong et al. [20] who analyzed nucleoplasmic free calcium transients in isolated nuclei of tobacco BY2 cells, challenged with mechanical stimuli. These data have been completed by additional experiments reported in figure 1. The main conclusions are as follows:

a) Under acidic conditions, the resting value of the nucleoplasmic free calcium concentration  $[Ca^{2+}]_{nuc}$  is low (about 0.1 $\mu$ M) and a mechanical stimulus provokes a rapid (<1sec) increase in the free calcium level which is followed by a slow decrease (>1min) back to the background level. The intensity of the response is positively correlated to the acidity of the bathing medium and the nuclei responded to a series of stimuli without a refractory period or a loss in response intensity. At low pH the resting concentration is not dependent on temperature.

b) As shown in figure 1A, addition of 10 mM calcium in the incubation medium had no effect on the resting level of nucleoplasmic free calcium, confirming that isolated nuclei are not freely permeable to calcium. Upon mechanical stimulation, there was no difference in either the kinetic characteristics or the intensity of the calcium response between the control (no addition of calcium) and the assay (addition of calcium). Consequently, the presence of calcium in the external milieu did not increase the nuclear response, suggesting that no additional entry of calcium was elicited by the stimulus.

c) Under neutral or alkaline pH conditions, a mechanical stimulus has no effect on  $[Ca^{2+}]_{nuc}$  whose resting level becomes sensitive to temperature (Fig. 1B). After thermal equilibration at 20°C, the resting level is higher (about 0.4 μM) than under acidic conditions. A cold shock lowers rapidly  $[Ca^{2+}]_{nuc}$  to about 0.1 μM.  $[Ca^{2+}]_{nuc}$  increases gradually to 0.4 μM upon thermal equilibration.

From these data, we conclude that isolated nuclei from BY2 cells constitute a closed system (no calcium exchanges with the external medium). They respond to mechanical stimulation in a pH dependent manner and the resting levels of  $[Ca^{2+}]_{nuc}$  are regulated by temperature.

A rapid increase of the free calcium concentration in the nucleoplasm might be explained by the opening of  $Ca^{2+}$ -channels located on the INM, inducing a calcium influx from nuclear stores (nuclear envelope, nucleoplasmic reticulum). The slow decreasing phase of the process, which took up to 3 min to return to the initial calcium level, cannot be explained in such a simple way. A possible explanation would be the binding of free calcium to calcium chelators (proteins or negatively charged compounds). However, interactions between calcium and buffers proceed with time constants in the millisecond range [31], that are not thermodynamically consistent with the experimental data unless the existence of very slow calcium buffers is postulated. Another possibility would be to suppose a release of calcium from the nucleoplasm to the external medium by free diffusion through the nuclear pore. This would suit reports suggesting that ions can pass freely through NPC channels. However, this hypothesis is contradicted by the data reported in figure 1A. Indeed, increasing the calcium concentration in the external medium to reverse the calcium gradient between the nucleoplasm and the bathing medium has no effect on the nuclear responses. Thus, experiments with isolated nuclei show that (1) control of calcium diffusion through nuclear pores must exist to maintain the resting nucleoplasmic concentration at its observed level (about 0.1 μM), and (2) there must be a mechanism by which calcium ions are transported actively from the nucleoplasm to the nuclear store, in order to restore the resting level after stimulation.

In order to check the consistency of this hypothesis with experimental data, we developed a simple mathematical model to simulate the dynamics of calcium in isolated nuclei.

### ***3.2. Model hypotheses***

We will consider here the isolated nucleus as a closed system, where no calcium exchange with the environment takes place, as suggested by the experiments reported above. This system is thus composed of only two physical compartments, the nucleoplasm and the nuclear envelope. For the sake of simplicity, we will consider the NE and other putative nuclear stores as a single compartment. In each compartment, calcium is either in a free form or bound to calcium buffers. Following Neher [32] “calcium buffer” refers here to chemical species acting as calcium ligands with a rapid equilibrium between the free and bound forms of calcium. Free calcium can be mobilized from the NE to the nucleoplasm through calcium channels, some of them being activated by a mechanical stimulus. We will not consider here the mechanisms of channel activation, we just assume that a stimulus provokes a calcium influx. Therefore the model will be valid regardless of the (direct or indirect) activation

mechanism. Conversely, we suppose that calcium ions are actively transported from the nucleoplasm to the nuclear envelope by still hypothetical transporters.

### 3.3. Model formulation

Under these assumptions, the rates of change of the free  $\text{Ca}^{2+}$  concentration in the nucleoplasm  $[\text{Ca}^{2+}]_{\text{nuc}}$  and in the stores  $[\text{Ca}^{2+}]_{\text{store}}$  are represented by

$$\frac{d[\text{Ca}^{2+}]_{\text{nuc}}}{dt} = \beta(J_{\text{in}} - J_{\text{out}}) \quad (1a)$$

$$\frac{d[\text{Ca}^{2+}]_{\text{store}}}{dt} = -\alpha\rho(J_{\text{in}} - J_{\text{out}}) \quad (1b)$$

with

$J_{\text{in}}$  = inward flux from the stores to the nucleoplasm,

$J_{\text{out}}$  = outward flux from the nucleoplasm to the stores,

$\beta$  is in first approximation equal to the ratio of free versus total calcium in the nucleoplasm  $[\text{Ca}^{2+}]_{\text{nuc}} / [\text{Ca}]_{\text{nuc\_total}}$ ,

$\alpha$  is in first approximation equal to the ratio of free versus total calcium in the store  $[\text{Ca}^{2+}]_{\text{store}} / [\text{Ca}]_{\text{store\_total}}$ .

Because fluxes are expressed in term of nucleoplasmic concentration the rate of change of  $[\text{Ca}^{2+}]_{\text{store}}$  is weighted by the volume ratio  $\rho = \text{Vol}_{\text{nucleoplasm}} / \text{Vol}_{\text{store}}$

For  $\text{Ca}^{2+}$  buffering, we considered fast reactions between calcium and buffers and used the rapid equilibrium approximation [31] in order to simplify the model (see appendix). Calcium ratios depend on free calcium concentration, but as only small variations of  $[\text{Ca}^{2+}]$  are involved, in most simulations we assumed constant values for the free calcium ratios  $\alpha$  and  $\beta$ . An extended version of the model, accounting for variable buffering capacities, is described in the appendix.

From (1) we can derive the conservation equation expressing that the total quantity of calcium is constant:

$$\frac{[\text{Ca}^{2+}]_{\text{nuc}}}{\beta} + \frac{[\text{Ca}^{2+}]_{\text{store}}}{\gamma} = Q \quad (2)$$

where  $\gamma = \alpha\rho$ .

Formulations of the inward flux  $J_{\text{in}}$  and of the outward flux  $J_{\text{out}}$  used in the numerical simulations were derived from various models of calcium exchanges between the endoplasmic reticulum (ER) and the cytosol [33-36].  $J_{\text{in}}$  is the sum of the effects of (1) a calcium leak from nuclear stores to the nucleoplasm, and (2) of a mechanical stimulus which is supposed to open specific calcium channels. A calcium leak current has been observed between the ER and the cytosol in animal cells [37], and we assumed a similar leak between the nuclear store and the nucleoplasm. This leak is assumed to be proportional to the difference of concentrations between the two compartments, such that:

$$J_{\text{leak}} = k_s ([\text{Ca}^{2+}]_{\text{store}} - [\text{Ca}^{2+}]_{\text{nuc}}) \quad (3)$$

We assumed also that a mechanical stimulus induces a transient opening of  $\text{Ca}^{2+}$ -channels, resulting in a rapid and transient influx of calcium in the nucleoplasm from the nuclear stores:

$$J_{stimulus} = F(t)([\text{Ca}^{2+}]_{store} - [\text{Ca}^{2+}]_{nuc}) \quad (4)$$

The time-dependent function  $F(t)$  is related to the fractional activity of the channels [34, 36], and is modeled as :

$$F(t) = F_1 \left( 1 - e^{-\frac{t-t_s}{t_1}} \right) e^{-\frac{t-t_s}{t_2}} \quad (5)$$

where  $t_s$  is the time at which a stimulus is applied.

For calcium uptake from the nucleoplasm to the nuclear store we assumed a simple Hill function, such that:

$$J_{out} = V \frac{[\text{Ca}^{2+}]_{nuc}^p}{K^p + [\text{Ca}^{2+}]_{nuc}^p} \quad (6)$$

Such a function has been used often to model  $\text{Ca}^{2+}$  pumps [33, 34, 38] either in the plasma membrane or in the ER membrane.

Finally, the simplified model reduces to the conservation equation (2) and one differential equation

$$\frac{d[\text{Ca}^{2+}]_{nuc}}{dt} = \beta \left\{ (F(t) + ks)([\text{Ca}^{2+}]_{store} - [\text{Ca}^{2+}]_{nuc}) - V \frac{[\text{Ca}^{2+}]_{nuc}^p}{K^p + [\text{Ca}^{2+}]_{nuc}^p} \right\} \quad (7)$$

### 3.4. Steady-state analysis

When  $F = 0$ , the system equilibrates to a steady-state, whose value is the solution of the following equation:

$$\gamma Q - \left(1 + \frac{\gamma}{\beta}\right) C_{nuc} = \frac{V}{k_s} \frac{C_{nuc}^p}{K^p + C_{nuc}^p} \quad (8)$$

The equilibrium level is strongly dependent upon the ratio  $V/k_s$ , which determines the balance between inward flux and outward flux, and upon the free calcium ratio  $\beta$ . Its value reaches an upper limit  $\frac{\beta\gamma}{\beta + \gamma} Q$  when  $V/k_s \rightarrow 0$ , and for which  $C_{nuc} = C_{store}$ .

An increase of the equilibrium level may thus be obtained either by increasing the influx or by decreasing the uptake. But the kinetics of convergence towards the equilibrium are quite different according to whether  $ks$  is high or  $V$  is low. In the case of a weak uptake, the transient to the steady-state is slow. In contrast, in the case of a large basal influx, the system jumps immediately to the steady-state (Fig. 2A). If one simulates a stimulus, according to equation (5), in both cases the system evolves rapidly to the steady-state, and becomes essentially insensitive to a second stimulus (Fig. 2B).

### 3.5. Parameter values

The average nucleus size of BY2 cells was derived from microscopic measurements of isolated nuclei labeled with DAPI (average width =  $6.5 \pm 0.83 \mu\text{m}$ , average length =  $7.5 \pm 1 \mu\text{m}$ ,  $n=66$ ). Considering an ellipsoid shape for the nucleus, the mean volume of a nucleus was estimated to be  $160 \mu\text{m}^3$  and the surface area to  $150 \mu\text{m}^2$ . For a 50 nm thick nuclear envelope this gives a volume ratio  $\rho \approx 20$ .

The total amount of calcium in a nucleus of plant cells has been estimated to be about 1nmol/mg protein [26]. In BY2 cell nuclei we assessed the quantity of proteins at 150 pg / nucleus. Thus, for a mean volume of  $160 \mu\text{m}^3$  the total calcium concentration in the nucleus is approximately  $900 \mu\text{M}$ . This figure falls into the same order of magnitude as values reported for animal cell nuclei, namely,  $225 \mu\text{M}$  in nuclei of NIH 3T3 cells [39] and  $600 \mu\text{M}$  in breast epithelial cells [40]. Free calcium concentration in the nucleoplasm of plant nuclei at rest has been assessed at  $150 \text{nM}$  [26]; here, we found approximately  $100 \text{nM}$ . These values lead to an estimation of the nucleoplasmic free calcium ratio to be in the range 1000 – 5000. By comparison, the calcium binding ratio in the cytosol was found to vary from 1500 to 2000 in pancreatic cells [41], and was assessed at 50 in bovine adrenal chromaffin cells [42]. Because of the NE-ER continuity, expected calcium concentrations in the nuclear envelope should be in the same range as in the reticulum lumen, namely  $100\text{-}1000 \mu\text{M}$  [free calcium] and  $5\text{-}50 \text{mM}$  [total calcium] [28, 43, 44]. This leads to a calcium binding ratio between 10 and 50 in the ER lumen, which is lower than the ratio in the cytosol [42, 45].

From these published data, we assumed resting concentrations of total and free calcium in the nucleoplasm of about  $200 \mu\text{M}$  and  $100 \text{nM}$  respectively ( $\beta = 1/2000$ ). In the nuclear envelope the total calcium concentration was set to  $10 \text{mM}$  and the free calcium concentration to  $200 \mu\text{M}$  ( $\alpha = 1/50$ ), giving a total calcium concentration in the whole nucleus of approximately  $400 \mu\text{M}$ , a value close to the above mentioned estimate.

Kinetic parameter values used in mathematical models vary greatly according to the cell type and to the authors [33-36, 38, 41, 45, 46]. Table 1 summarizes the parameter values used in these various models. In our simulations, kinetic parameter values (table 2) were chosen such as to fit the model to experimental data.

## 4. Results

### 4.1. Simulation of changes in nucleoplasmic free calcium in isolated nuclei challenged with mechanical stimuli

In the following simulations, a low free calcium level ( $[\text{Ca}^{2+}]_{\text{nuc}} = 0.1 \mu\text{M}$ ) was set as an initial condition to mimic experiments in which nuclei were maintained at low temperature before being stimulated by addition of buffer at room temperature [20]. The effect of this mechanical stimulus was simulated by a rapid and transient influx of calcium from the store into the nucleoplasm (Fig3A, dotted line), according to equation (5). Figure 3A shows an example of the calcium response in isolated nuclei, after mechanical stimulation at acidic pH conditions (thin line), and the corresponding simulation (thick solid line). Nucleoplasmic concentration  $[\text{Ca}^{2+}]_{\text{nuc}}$  increased rapidly up to a maximum and then returned slowly to the initial equilibrium as expected. This variation of  $[\text{Ca}^{2+}]_{\text{nuc}}$  corresponds to a concomitant variation of  $[\text{Ca}^{2+}]_{\text{store}}$ , but in the opposite direction. After a rapid decrease, due to the outward flux from the store to the nucleoplasm, the calcium level in the store returns slowly to its initial level under the action of  $\text{Ca}^{2+}$ -transporter(s) (Fig. 3B). In this simulation, the free



calcium level in the store decreased dramatically, from  $160\mu\text{M}$  to about  $10\mu\text{M}$ . Because of the large volume ratio between the two compartments and of the high buffering capacity, a weak increase in the nucleoplasmic free calcium required a large influx of calcium from the store. The response of the model to a stimulus is qualitatively and quantitatively in good agreement with experiments.

The model predicts that repeated stimuli will induce a stable variation of  $[\text{Ca}^{2+}]_{\text{nuc}}$  as observed experimentally. After an increase due to the initial stimulation, subsequent stimulations do not modify the average value of  $[\text{Ca}^{2+}]_{\text{nuc}}$ . There is no accumulating or exhausting effect of repeated stimuli (Fig. 3C). This results from the large decrease of the  $\text{Ca}^{2+}$ -level in the store initiated by the first stimulus and by the effect of the re-uptake mechanism which restores partially the calcium level of the store (Fig. 3D). Such a behavior was observed for parameter values which fit experimental data shown in Figure 3A. When parameters values are set such that either the store level does not decrease enough or the outward flux is too low, the average level of  $[\text{Ca}^{2+}]_{\text{nuc}}$  increases progressively after each subsequent stimulus before reaching a plateau (result not shown).

At room temperature, the response of isolated nuclei to a mechanical stimulus depends upon the pH of the bathing buffer [20]. At acid pH, nuclei respond as described above, whereas at alkaline pH they are insensitive to stimulation. This is illustrated in Figure 4, showing the experimental responses of nuclei stored on ice before measurement, and subsequent effect of adding two volumes of buffer adjusted to acid or alkaline pH at room temperature. The stimulus induced a rapid increase of  $[\text{Ca}^{2+}]_{\text{nuc}}$  reaching a pH-dependent peak value, followed by a slow transient towards equilibrium values positively correlated to the pH of the medium. Experimental plots were simulated using the same procedure as above (Fig. 4) and coefficient values given in table 2. A good adjustment of the various curves was obtained by changing only three coefficients between different pH conditions. These include the rate constant  $k_s$  of the resting inward flux, and the two free calcium ratios  $\beta$  and  $\gamma$ . Changing  $k_s$  alone had an effect on the equilibrium value only, while changing the values of  $\beta$  and  $\gamma$  modified also the peak value. Thus, the pH value of the bathing buffer would influence both the kinetics of the calcium leak and the calcium binding capacity of the nuclei.

When repeated stimuli were simulated (Fig. 4B), different responses were obtained according to the parameter values. For values simulating a neutral or alkaline pH, the model was insensitive to stimulation, while for values corresponding to an acid pH, the amplitude of the response was pH dependent.

#### ***4.2. Simulation of temperature effects on the resting calcium level at alkaline pH***

Xiong et al (23) have shown that, the calcium resting level is independent upon temperature variations at acid pH, and positively correlated with temperature in alkaline conditions. When nuclei, stored at  $0^\circ\text{C}$  in an alkaline buffer (pH 7.5) before measurement, are transferred to room temperature, there is a progressive increase of the nucleoplasmic calcium concentration with temperature, reaching a plateau after about 5 min.

Temperature variations may affect the kinetics of calcium release and/or calcium uptake, or the kinetics of binding to calcium binding proteins. In the model (eq. 7) these kinetics are controlled essentially by the coefficients  $k_s$ ,  $V$ , and  $K$  and by the buffering capacity, represented by  $\beta$ . For example, according to eq. (8), an increase of  $k_s$  or a decrease of  $V$  will induce an increase of  $[\text{Ca}^{2+}]_{\text{nuc}}$ . As shown in Fig. 2A, a high value of  $k_s$  induces a rapid increase of  $[\text{Ca}^{2+}]_{\text{nuc}}$  to the steady-state, while a low value of  $V$  induces only a slow increase of  $[\text{Ca}^{2+}]_{\text{nuc}}$  towards the steady-state.  $[\text{Ca}^{2+}]_{\text{nuc}}$  may also increase if the affinity of calcium to buffers is decreased.

In order to simulate the effect of temperature variations on calcium dynamics we used the extended version of the model that is described in the appendix. The extended model accounts for time-dependent buffering coefficients.

Variations in temperature were simulated by time-variations of  $k_s$ ,  $V$ ,  $K$  and/or the calcium-buffer dissociation coefficient  $K_d$  (Fig. 5a-e) and the simulated response compared to experimental curves (Fig. 5f). To simulate a cold shock, following a slow increase in temperature, coefficients were reversed rapidly to their initial value. The responses of the model to variations of  $V$  or  $K$ , which control calcium re-uptake, are not in good qualitative agreement to the experimental data (Fig. 5a and 5c). The measured increase of  $[Ca^{2+}]_{nuc}$  with temperature is simulated adequately by a progressive increase of the influx rate constant  $k_s$  (Fig. 5a), but the effect of a cold shock is better predicted by a rapid decrease of  $K_d$  (Fig. 5d). The best fit to experimental measurements was obtained by simultaneously increasing  $k_s$  and the dissociation constant  $K_d$  (Fig. 5e).

Thus, according to these simulations, temperature might have a double effect: i) on the activity of  $Ca^{2+}$ -channels, by changing membrane dynamics, and ii) on the calcium-buffering capacity, by modifying the calcium-buffer dissociation constant.

## 5. Discussion

Active trafficking of information between the nucleus and the cytosol in intact cells has been largely established [15, 47, 48]. Moreover, it has become apparent that nuclei isolated either from plant cells [20] or from human cells [19] are able to convert mechanical stimuli into changes in the free calcium concentration, which then controls downstream calcium-dependent events. In nuclei isolated from tobacco cells, the calcium response depends highly on (i) the pH and (ii) the temperature of the incubation medium. Thus, at acidic pH, a train of mechanical stimuli induces a periodic stable variation of  $[Ca^{2+}]_{nuc}$  even at low temperature. In contrast, at neutral-alkaline pH, the system is apparently desensitized to mechanical stimulation. Rather, under these conditions, the concentration of nucleoplasmic free calcium depends on the temperature of the incubation medium which determines the steady state concentration level of calcium at equilibrium [20].

We have simulated these data by a simple mathematical model to describe calcium homeostasis and disturbance in isolated plant cell nuclei. This model is based upon the assumption that nucleoplasmic calcium is regulated by the balance between  $Ca^{2+}$ -channel and  $Ca^{2+}$ -transporter activities, both located on the inner nuclear membrane of the nuclear envelope.

Isolated nuclei from tobacco BY2 cells behave like a closed system, the nucleoplasmic calcium variations being independent from the external calcium concentration. Therefore, calcium diffusion through nuclear pores as a possible mechanism of regulation can be ruled out. A similar behavior has already been observed in isolated nuclei from pancreatic beta cells in which blockade of a nuclear  $K_{ATP}$  channel triggers nuclear  $Ca^{2+}$  transients [49]. Calcium binding to proteins or to negatively charged compounds could be a way of reducing free calcium level after stimulation. However, the kinetics of calcium buffering is very fast, with a time constant in the millisecond range [31] which is not compatible with observed kinetics, with a characteristic time higher than 1 min. Thus, to be consistent with kinetic data, the existence of a very slow calcium buffer in the nucleus should be assumed. Another argument against the "buffering" hypothesis comes from experiments with a train of mechanical stimuli. Essentially, we have observed that the maximal calcium concentration reached in the nucleoplasm after a stimulus remains constant whatever the frequency of the stimuli. A few minutes after stimulation, the system seems to be restored to its resting state and able to respond to a new stimulus. Such behavior cannot be explained only by calcium

buffering in the nucleoplasm, because in such a case the calcium stores would be rapidly depleted. For example, it has been shown that  $\text{Ca}^{2+}$ -concentration in the ER decreased by a factor of 10 after  $\text{Ca}^{2+}$  mobilization [50]. In the nucleus, because of the high ratio ( $> 1000$ ) between total and free calcium in the nucleoplasm and the low volume ratio ( $< 0.05$ ) between nuclear envelope and nucleoplasm, a small increase (e.g. 300nM) of  $[\text{Ca}^{2+}]$  in the nucleoplasm must correspond to a large decrease ( $> 6\text{mM}$ ) of  $[\text{Ca}^{2+}]$  in the nuclear envelope. Considering that the total calcium concentration in the nuclear envelope is in the 5-50mM range [28, 43, 44], such a calcium release after each stimulus should empty rapidly the calcium stores, leading to a decrease of the nucleoplasmic response to subsequent stimuli. This is not consistent with experimental data, which showed a sustained response after successive stimuli [20].

In the “calcium re-uptake” hypothesis, the nuclear calcium stores are continuously refilled, making it possible to respond to another stimulus. Thus, the present model, based on such a hypothesis, formally explains the response of isolated nuclei to repeated stimuli. Furthermore, calcium transporters like sarco/endoplasmic reticulum calcium ATPases have time constants in the minute range [51] that are in the time constant range of the calcium decrease measured in the nucleoplasm of isolated nuclei.

Variations of resting  $[\text{Ca}^{2+}]_{\text{nuc}}$  with temperature or pH result from modifications of the balance between inward and outward calcium fluxes, i.e. by a modulation of  $\text{Ca}^{2+}$ -channel or/and  $\text{Ca}^{2+}$ -transporter activities. Numerical simulations have shown that temperature effects on calcium kinetics are better described by an increase of the  $\text{Ca}^{2+}$ -channel kinetic constant with temperature rather than by a decrease in transport activity. An active transport activity that would be lower at room temperature than at low temperature seems very unlikely. Thus, ATPase activity increases with temperature [52] whereas  $\text{Ca}^{2+}$ -permeable channels are primary sensors of temperature in plants [53]. Plant cells respond to a cold shock by a rapid increase of  $[\text{Ca}^{2+}]_{\text{cyt}}$  but do not respond to a slow variation of temperature [54]. In mammalian cells, primary sensors of temperature are  $\text{Ca}^{2+}$ -channels of the TRP family [55] which can be activated either by cold (TRPM8) or by heat (TRPV1). Xiong et al [20] suggested the presence of TRP-like  $\text{Ca}^{2+}$ -channels on the inner membrane of the nuclear envelope. These channels would be activated by a slow increase rather than by a rapid variation of temperature. This hypothesis fits with the proposed model which simulates the effect of temperature increase by a progressive activation of  $\text{Ca}^{2+}$ -channels.

The numerical simulations suggest that the pH-dependence of the nuclear calcium dynamics may be due mainly to simultaneous changes of the  $\text{Ca}^{2+}$ -binding capacity and the modulation of  $\text{Ca}^{2+}$ -channel activity, rather than to  $\text{Ca}^{2+}$  re-uptake. Proton concentration is known to have a profound influence on a variety of biological processes and especially on ion channel, transporter activities, and the calcium-binding capacity of calcium-binding proteins.

Acidification of the extracellular medium reduces the conductivity of many voltage-gated and ligand-gated channels [56-59]. This is the case particularly for L-type calcium channels [56, 60] and sodium channels [61]. Conversely, it has been shown that acidification activates a particular class of channels referred to as Acid Sensitive Ion Channels (ASIC) [62] and stimulation of ASIC1a provides a pathway for calcium entry in neuronal cells [63].

The experimental data used to propose the model presented here show clearly that acidic pH values inhibit changes in nucleoplasmic calcium, this suggesting that ASICs are not key players in the process. Moreover, the pharmacological profile of the putative channels is more compatible with the channels being TRP-like rather than L-type channels. The putative channels become highly sensitive to activation by mechanical stimulations at acidic pH and not at alkaline pH. Changes in ionic charges of the channels may be the mechanism that controls their sensitivity to either mechanical or thermal stimulation. Clearly,

the molecular nature of the channels and the mechanism of their activation remain to be clarified.

In isolated nuclei, the resting calcium concentration  $[Ca^{2+}]_{nuc}$  is temperature independent at low pH (5.3). At low temperature (approx. 0°C),  $[Ca^{2+}]_{nuc}$  becomes pH independent.

Consequently, if temperature controls channel opening and pH controls uptake activity, the two processes must be coupled. The simple way to achieve such coupling is that both factors regulate the same process.

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## Tables and figure legends

Table 1: Kinetic parameter values taken from the literature

Ref.	Ca buffer ( $\mu\text{M}$ )	k1 ( $\mu\text{M}^{-1}\text{s}^{-1}$ )	k2 ( $\text{s}^{-1}$ )	Ca <sup>2+</sup> ratio	Vp ( $\mu\text{M}\cdot\text{s}^{-1}$ )	Kp ( $\mu\text{M}$ )
[46]	120	5	1			
[38]	300			0.02	45	0.1
[34]	300	601	97		500	0.1
[64]				0.053	11.2	0.4
[33]	195	10	10		350	0.219



Table 2. Kinetic parameter values used in the simulations.

<b>Figure</b>	$\gamma$	$\beta$	$k_s$ ( $s^{-1}$ )	$V_p$ ( $\mu M s^{-1}$ )	$K_p$ ( $\mu M$ )	$F_{max}$ ( $s^{-1}$ )
<b>2</b>	<b>0.2</b>	<b>5.0 10<sup>-4</sup></b>	<b>0.012 / 1</b>	<b>7.9 / 0.0</b>	<b>0.23</b>	<b>13</b>
<b>3</b>	<b>0.2</b>	<b>5.0 10<sup>-4</sup></b>	<b>0.012</b>	<b>7.9</b>	<b>0.23</b>	<b>13</b>
<b>4 (pH 4)</b>	<b>0.22</b>	<b>7.0 10<sup>-4</sup></b>	<b>0.04</b>	<b>13.0</b>	<b>0.2</b>	<b>6.0</b>
<b>4 (pH 6)</b>	<b>0.26</b>	<b>5.2 10<sup>-4</sup></b>	<b>0.06</b>	<b>13.0</b>	<b>0.2</b>	<b>6.0</b>
<b>4 (pH 7)</b>	<b>0.28</b>	<b>4.7 10<sup>-4</sup></b>	<b>1.0</b>	<b>13.0</b>	<b>0.2</b>	<b>6.0</b>
<b>4 (pH 9)</b>	<b>0.24</b>	<b>6.0 10<sup>-4</sup></b>	<b>1.0</b>	<b>13.0</b>	<b>0.2</b>	<b>6.0</b>
<b>5</b>	<b>0.25</b>	<b>6.0 10<sup>-4</sup> / 0.01</b>	<b>0.2 / 0.09</b>	<b>19</b>	<b>0.2</b>	<b>0</b>

In all simulations:  $[Ca^{2+}]_{nuc}(0) = 0.1$ ,  $[Ca^{2+}]_{store}(0) = 160$  (so that  $Q = 1 \text{ mM}$ ) and  $p = 1$ .

Fig. 1. (A) Nucleoplasmic free calcium ( $[Ca^{2+}]_{nuc}$ ) variations in isolated nuclei at rest ( $\Delta$  and %) or after mechanical stimulation ( $\circ$  and #) applied at  $t=20$  sec (arrow). Nuclei were incubated at room temperature in a buffered medium (pH 5.3) containing no (% and #) or 10 mM ( $\Delta$  and  $\circ$ ) calcium. (B) pH-dependence of nucleoplasmic free calcium concentration. Nuclei were stored on ice before measurement in a buffer adjusted to pH 5.3, 6.5 or 7.5, and thereafter transferred at room temperature in a luminometer for recording of light emission.

Fig. 2. (A) Simulations of calcium transients from the initial state to the steady-state value of model eq. (7), (B) Effect of repeated stimuli simulated by eq. (4) on the simulated calcium transient. Parameter values: (—)  $k_s=1$  and  $V=8$  (large basal influx), and (- - -)  $k_s=0.012$  and  $V=0$  (absence of calcium re-uptake).  $\gamma = 0.2$ ,  $\beta = 0.0005$ ,  $K_p = 0.228294$ ,  $p = 2$ ,  $F_{max} = 12.9521$ , initial conditions at  $t=0$ :  $[Ca^{2+}]_{nuc} = 0.1 \mu M$ ,  $[Ca^{2+}]_{store} = 160 \mu M$ .

Fig. 3. Simulations of nucleoplasmic free calcium ( $[Ca^{2+}]_{nuc}$ ) variation by eq. (7) after single (A and B) or repeated (C and D) mechanical stimulation (eq. 5). Thin line (A, C) = observed data for  $[Ca^{2+}]_{nuc}$ , thick line (A-D) =  $[Ca^{2+}]_{nuc}$  solution of eq. 7, dashed line (B, D) =  $[Ca^{2+}]_{store}$ , dotted line (A, C) = influx of  $Ca^{2+}$  after a stimulus simulated by eq. 4. Parameter values used in simulations (A,B) :  $\gamma = 0.2$ ,  $\beta = 0.0005$ ,  $k_s = 0.012$ ,  $V = 8$ ,  $K_p = 0.23$ ,  $p = 2$ ,  $F_{max} = 13$ , (C,D)  $\gamma = 0.2$ ,  $\beta = 0.00054$ ,  $k_s = 0.025$ ,  $V = 13.3$ ,  $K_p = 0.2$ ,  $p = 2$ , initial conditions at  $t=0$ :  $[Ca^{2+}]_{nuc} = 0.1 \mu M$ ,  $[Ca^{2+}]_{store} = 160 \mu M$ .

Fig. 4. Calcium variations induced by mechanical stimulation at different pH values. (A) Adjustment of the model (thick line) to experimental data (thin line). Stimulation was simulated according to eq. (7). Parameter values as in table 2. (B) Simulation of the response to repeated stimuli (same parameter values as in (A)).

Fig. 5. Effect of temperature variations on the nucleoplasmic calcium level. Temperature effect was simulated by time-dependent variations of single parameters:  $k_s$  (A),  $V$  (B),  $K$  (C),  $K_d$  (D), or both parameters  $k_s$  and  $K_d$  (E). Solid line = calcium level, dotted line = parameter value. (F) Experimental data showing the effect of temperature on the nucleoplasmic calcium level of nuclei in a basic buffer (pH 7.5).  $T$  was allowed to increase from 0 to 20°C, before applying a cold shock at  $t=200$  s (arrow).

## Appendix

A full model for nuclear calcium homeostasis reads:

$$\frac{d[Ca^{2+}]_{nuc}}{dt} = J_{in} - J_{out} - Jb_{nuc} \quad (A1)$$

$$\frac{d[CaB]_{nuc}}{dt} = Jb_{nuc} \quad (A2)$$

$$\frac{d[Ca^{2+}]_{store}}{dt} = -\rho(J_{in} - J_{out}) - Jb_{store} \quad (A3)$$

$$\frac{d[CaB]_{store}}{dt} = Jb_{store} \quad (A4)$$

with

$$Jb_{nuc} = k_{1nuc}[Ca^{2+}]_{nuc}([B]_{nuc} - [CaB]_{nuc}) - k_{2nuc}[CaB]_{nuc} \quad (A5)$$

$$Jb_{store} = k_{1store}[Ca^{2+}]_{store}([B]_{store} - [CaB]_{store}) - k_{2store}[CaB]_{store} \quad (A6)$$

where  $[Ca^{2+}]_{nuc}$  and  $[B]_{nuc}$  are free calcium and total buffer concentrations in the nucleosol,  $[Ca^{2+}]_{store}$  and  $[B]_{store}$  are free calcium and total buffer concentrations in the nuclear stores, and  $\rho$  is the volume ratio between the nucleosol and the nuclear stores.

Summing (A1)+(A2), and (A3)+(A4) implies :

$$\frac{d[Ca]_{nuc\_total}}{dt} = J_{in} - J_{out} \quad \text{and} \quad \frac{d[Ca]_{store\_total}}{dt} = -\rho(J_{in} - J_{out}) \quad (A7)$$

Assuming fast reaction kinetics for calcium buffering, the following equilibrium equation holds:

$$[CaB]_{nuc} = \frac{[Ca^{2+}]_{nuc} B_{nuc}}{[Ca^{2+}]_{nuc} + K_{nuc}} \quad (A8)$$

where  $K_{nuc}$  is the dissociation constant. It follows:

$$[Ca]_{nuc\_total} = [Ca^{2+}]_{nuc} + [CaB]_{nuc} = \left(1 + \frac{B_{nuc}}{[Ca^{2+}]_{nuc} + K_{nuc}}\right)[Ca^{2+}]_{nuc} \quad (A9)$$

By deriving (A9) and using (A7) we obtain

$$\frac{d[Ca^{2+}]_{nuc}}{dt} = \beta \left\{ J_{in} - J_{out} - \frac{[Ca^{2+}]_{nuc}}{[Ca^{2+}]_{nuc} + K_{nuc}} \frac{dB_{nuc}}{dt} + \frac{B_{nuc}[Ca^{2+}]_{nuc}}{([Ca^{2+}]_{nuc} + K_{nuc})^2} \frac{dK_{nuc}}{dt} \right\} \quad (A10)$$

$$\text{where } \beta = \left(1 + \frac{K_{nuc} \times B_{nuc}}{([Ca^{2+}]_{nuc} + K_{nuc})^2}\right)^{-1}$$

In the case where the buffering coefficients  $B_{nuc}$  and  $K_{nuc}$  are constant, (A10) reduces to:

$$\frac{d[Ca^{2+}]_{nuc}}{dt} = \beta(J_{in} - J_{out}) \quad (A11)$$

$$\text{where } \beta = \left(1 + \frac{K_{nuc} \times B_{nuc}}{([Ca^{2+}]_{nuc} + K_{nuc})^2}\right)^{-1}$$

A similar equation can be derived for the nuclear stores:

$$\frac{d[Ca^{2+}]_{store}}{dt} = -\alpha\rho(J_{in} - J_{out}) \quad (A12)$$

$$\text{where } \alpha = \left(1 + \frac{K_{store} \times B_{store}}{([Ca^{2+}]_{store} + K_{store})^2}\right)^{-1}$$

For low affinity buffers and small variations of  $[Ca^{2+}]$ , the two parameters  $\alpha$  and  $\beta$  can be considered as approximately constant, with:

$$\alpha \cong \frac{K_{store}}{K_{store} + B_{store}} \quad \text{and} \quad \beta \cong \frac{K_{nuc}}{K_{nuc} + B_{nuc}} \quad (A13)$$

Figure 1

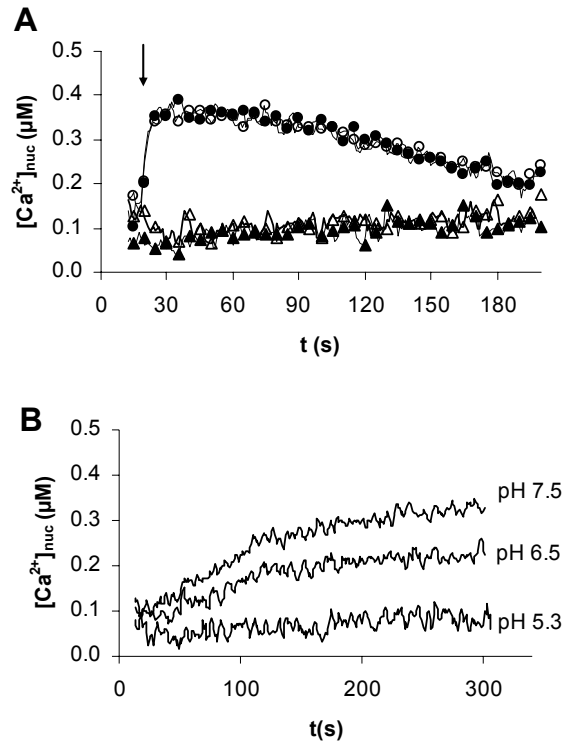


Figure 2

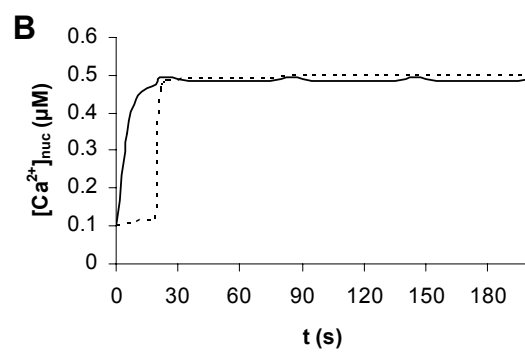
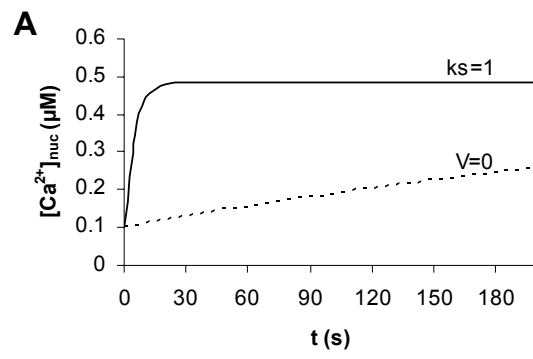


Figure 3

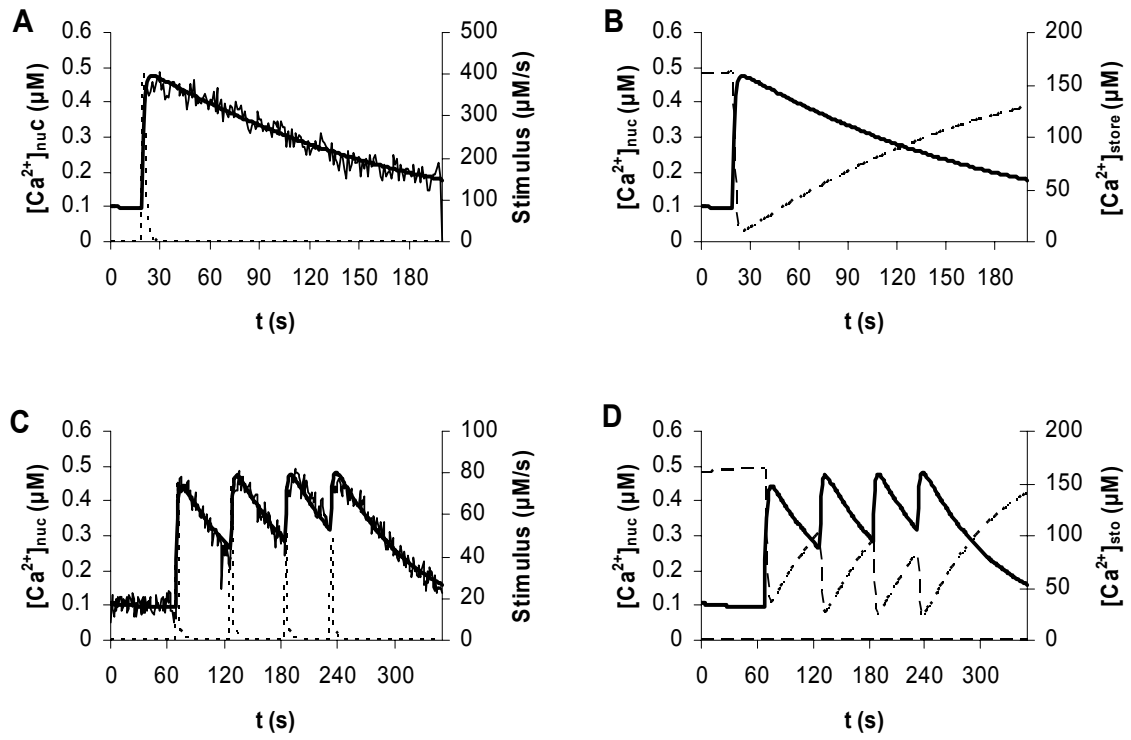


Figure 4

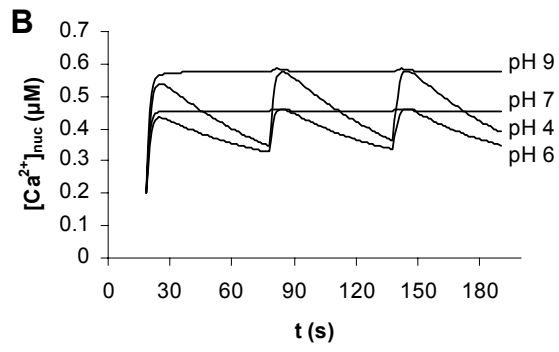
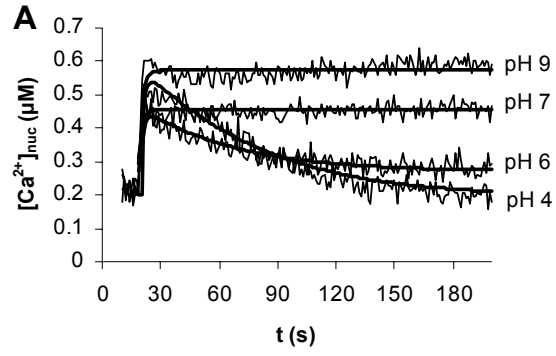




Figure 5

