# $InsP_3$ Signaling Induces Pulse-Modulated $Ca^{2+}$ Signals in the Nucleus of Airway Epithelial Ciliated Cells

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ABSTRACT The phenomenology of nuclear $Ca^{2+}$ dynamics has experienced important progress revealing the broad range of cellular processes that it regulates. Although several agonists can mobilize $Ca^{2+}$ from storage in the nuclear envelope (NE) to the intranuclear compartment (INC), the mechanisms of $Ca^{2+}$ signaling in the nucleus still remain uncertain. Here we report that the NE/INC complex can function as an inositol-1,4,5-trisphosphate ($InsP_3$)-controlled $Ca^{2+}$ oscillator. Thin optical sectioning combined with fluorescent labeling of $Ca^{2+}$ probes show in cultured airway epithelial ciliated cells that ATP can trigger periodic oscillations of $Ca^{2+}$ in the NE ($[Ca^{2+}]_{NE}$) and corresponding pulses of $Ca^{2+}$ release to the INC. Identical results were obtained in $InsP_3$-stimulated isolated nuclei of these cells. Our data show that $[Ca^{2+}]_{NE}$ oscillations and $Ca^{2+}$ release to the INC result from the interplay between the $Ca^{2+}/K^+$ ion-exchange properties of the intralumenal polyanionic matrix of the NE and two $Ca^{2+}$-sensitive ion channels—an $InsP_3$-receptor-$Ca^{2+}$ channel and an apamin-sensitive $K^+$ channel. A similar $Ca^{2+}$ signaling system operating under the same functional protocol and molecular hardware controls $Ca^{2+}$ oscillations and release in/to the endoplasmic reticulum/cytosol and in/to the granule/cytosol complexes in airway and mast cells. These observations suggest that these intracellular organelles share a remarkably conserved mechanism of $InsP_3$-controlled frequency-encoded $Ca^{2+}$ signaling.

## INTRODUCTION

$Ca^{2+}$ can critically influence the conformation of a large number of intracellular molecular effectors. Receptor proteins involved in a broad range of intracellular functions can be switched from resting to active functional conformation by small changes of $Ca^{2+}$ that act as efficient signaling relays. The specificity of $Ca^{2+}$ as second messenger is thought to result from encoding information via changes of amplitude, duration, and/or frequency of fluctuations of intracellular $Ca^{2+}$ (Berridge et al., 2003). A broad range of cytosolic and particularly nuclear processes respond to these encoding protocols (Dolmetsch et al., 1997, 1998; Li et al., 1998; Santella and Carafoli, 1997; Teruel et al., 2000). In addition, the spatial constrains resulting from specific site release, $Ca^{2+}$ diffusion, and intracellular buffering facilitates the confinement of signals to specific local addresses without spreading throughout the rest of the cell (Berridge et al., 2003). In this manner, cytosolic and nuclear processes may manage a level of independent control through regulation of specific local $Ca^{2+}$ signals in each compartment (Chawla et al., 1998; Hardingham et al., 1997, 2001; Leite et al., 2003; Pusl et al., 2002). It has been proposed that the nucleus may function as a signaling unit. This idea is supported by reports demonstrating that the nucleus possesses both the enzymatic machinery to synthesize second messengers, including inositol-1,4,5-trisphosphate ($InsP_3$) (Divecha et al., 1991) and cyclic ADP ribose (cADPr) (Adebanjo et al., 1999) and the corresponding ion-receptor channels to mobilize $Ca^{2+}$ between the nuclear envelope (NE) and the DNA-containing intranuclear compartment (INC) (Adebanjo et al., 1999; Echevarria et al., 2003; Gerasimenko et al., 1995, 2003; Leite et al., 2003; Quesada et al., 2002). Previous studies show that activation of these channels can produce single transient discharges of $Ca^{2+}$ into the INC (Adebanjo et al., 1999; Echevarria et al., 2003; Gerasimenko et al., 1995, 2003; Leite et al., 2003; Quesada et al., 2002). However, the mechanisms responsible for nuclear $Ca^{2+}$ fluctuations still remain uncertain (Santella and Carafoli, 1997). Here we report that in response to extracellular purinergic stimulation, the NE/INC complex of airway epithelial ciliated cells can function as a $Ca^{2+}$ oscillator. Exposure of isolated nuclei to the intracellular second messenger $InsP_3$ shows that activation of $InsP_3$-receptors located at the NE induces a standing wave of $[Ca^{2+}]_{NE}$ oscillations and a corresponding periodic $Ca^{2+}$ release from the NE to the INC.

We previously found that the endoplasmic reticulum (ER) of ciliated cells from the respiratory epithelium and the secretory granules of goblet cells can work as intracellular $Ca^{2+}$ oscillators by releasing trains of periodic quantal bursts of $Ca^{2+}$ to the neighboring cytosol (Nguyen et al., 1998; Quesada et al., 2001, 2003). This signaling process requires the interaction between two ion channels found in the ER and granular membranes that exhibit opposite $Ca^{2+}$ sensitivities—an $InsP_3$ receptor and an apamin-sensitive $Ca^{2+}$-sensitive $K^+$ channel ($ASK_{Ca}$)—with the polyanionic matrix of $Ca^{2+}$-sequestering glycoproteins found in the ER lumen and the intragranular matrix, which work as $Ca^{2+}/K^+$ exchangers (Nguyen et al., 1998; Quesada et al., 2001, 2003). These

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results show that both the molecular hardware and functional programming that implement $Ca^{2+}$ oscillations and $Ca^{2+}$ release in/from the ER and secretory granules are also present in the NE/INC complex of airway epithelial ciliated cells.

## MATERIALS AND METHODS

### Ciliated cells cultures, nuclei isolation, and labeling procedures

Epithelial ciliated cells were obtained from mice trachea according to protocols approved by the University of Washington Animal Care and Use Committee and cultured following procedures described elsewhere (Nguyen et al., 1998; Verdugo, 1980). To isolate nuclei, cultured cells were suspended in an intracellular buffer: 130 mM potassium glutamate, 10 mM KCl, 20 mM HEPES, 5 mM $MgSO_4$, and 100 nM $Ca^{2+}$ buffered with ethylene glycol bis($\beta$-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), pH = 7.2. Cell membrane was disrupted by brief sonication. Single isolated nuclei were then separated by centrifugation, resuspended in intracellular medium, and allowed to attach onto glass chambers following procedures described previously (Gerasimenko et al., 1995, 2003; Quesada et al., 2002). The identification and location of the NE in intact cells and in isolated nuclei were revealed by incubation for 5 min at 20°C in Hanks' solution or intracellular buffer, respectively, containing 1 $\mu$M of the ER marker rhodamine B hexyl ester (excitation wavelength $\lambda_{ex} = 556$ nms, emission wavelength $\lambda_{em} = 580$ nms; Molecular Probes, Eugene, OR; Quesada et al., 2002). The DNA-containing INC was labeled in intact cells and isolated nuclei by equilibrating for 5 min at 20°C in Hanks' and intracellular buffers, respectively, containing 1 $\mu$M of the DNA-labeling dye, ethidium homodimer-1 (5 min at 20°C; $\lambda_{ex} = 528$ nms, $\lambda_{em} = 617$ nms; Molecular Probes). These dyes provide an excellent guide to precisely identify in thin optical sections the areas from which $Ca^{2+}$ measurements were performed without photobleaching the $Ca^{2+}$ probes, as their excitation/emission wavelengths do not interfere with those of the $Ca^{2+}$ fluorophores (Quesada et al., 2002). An antibody to calnexin allowed us to readily detect ER contamination of the isolated nuclei preparation (Quesada et al., 2002; Radominska-Pandya et al., 2002). Fragments associated with the ER (Tabares et al., 1991) were present in <10% of nuclei and were easily recognized by both transmitted light microscopy and tomographyc serial thin sectioning of fluorescence images using rhodamine B hexyl ester labeling (Quesada et al., 2002). Only those nuclei free of ER debris were used for imaging experiments. In addition, the occurrence of the same $Ca^{2+}$ dynamics in the NE and INC of intact cells and isolated nuclei further discards the notion that the development of $InsP_3$-induced nuclear $Ca^{2+}$ oscillations can be explained by ER contamination in the nuclei preparation.

### $Ca^{2+}$ measurements

Intact cells were loaded for 30 min at 20°C in Hanks' solution containing either 2 $\mu$M Calcium Green-1/acetoxymethyl ester (AM; dissociation constant $K_d = 0.19$ $\mu$M; $\lambda_{ex} = 506$ nms, $\lambda_{em} = 531$ nms; Molecular Probes) or 2 $\mu$M Calcium Green-5N/AM ($K_d = 14$ $\mu$M; $\lambda_{ex} = 506$ nms, $\lambda_{em} = 532$ nms; Molecular Probes), in this latter case for 1 h at 37°C. These two protocols favor the incorporation of the probes either in the cytosol and INC or in the ER and NE, respectively (Echevarria et al., 2003; Gerasimenko et al., 1995, 2003; Quesada et al., 2002).

Recordings of $Ca^{2+}$ in isolated nuclei were performed with two $Ca^{2+}$-sensitive probes following protocols previously described (Adebanjo et al., 1999; Gerasimenko et al., 1995, 2003; Quesada et al., 2002). The NE was labeled by equilibrating the nuclei for 60 min in intracellular medium (pH = 7.2 at 4°C) containing 20 $\mu$M of the membrane permeant probe Calcium Green-5N/AM. The INC was labeled by equilibrating the nuclei for 30 min in intracellular medium (pH = 7.2 at 4°C) containing 10 $\mu$g ml$^{-1}$ of the nonpermeant, low-diffusivity dye Calcium Green-1/dextran ($K_d = 0.26$ $\mu$M; $\lambda_{ex} = 508$ nms, $\lambda_{em} = 533$ nms; Molecular Probes). The preferential localization of Calcium Green-1/dextran in the INC following this protocol has been previously verified (Gerasimenko et al., 1995, 2003; Petersen et al., 1998; Quesada et al., 2002). The $K_d$ of these two probes rendered excellent detection of both resting steady-state $Ca^{2+}$ and of $Ca^{2+}$ oscillations in the NE and INC. Nuclei were then washed twice with the intracellular medium and equilibrated in the same medium but supplemented with 1 mM ATP and 300 nM $Ca^{2+}$ (EGTA buffered) for 10 min to load nuclei with $Ca^{2+}$ as reported previously (Adebanjo et al., 1999; Gerasimenko et al., 1995, 2003; Quesada et al., 2002). Nuclei were then washed in ATP-free intracellular medium. The chambers containing the nuclei were mounted and kept at 37°C on the thermoregulated stage of a Nikon inverted fluorescence microscope, and experiments were immediately conducted in ATP-free intracellular buffer. $Ca^{2+}$ recordings lasted 20–30 s without a substantial fluorescence decay of the signal from the NE. However, after several minutes, fluorescence decayed probably due to photobleaching of the probe or to lack of $Ca^{2+}$ uptake by the sarcoplasmic/endoplasmic-reticulum $Ca^{2+}$-ATPases (SERCA) (Nicotera et al., 1989).

### Staining of $ASK_{Ca}$ channels

Labeling of $ASK_{Ca}$ channels in the nucleus was performed by incubation of isolated nuclei in intracellular buffer containing 100 nM of apamin-Alexa Fluor 488 conjugate ($\lambda_{ex} = 494$ nms, $\lambda_{em} = 520$ nms; Molecular Probes) for 30 min at 4°C. The NE was identified by incubating the isolated nuclei in intracellular buffer containing 1 $\mu$M rhodamine B hexyl ester.

### $Ca^{2+}/K^+$ exchange

To investigate $Ca^{2+}/K^+$ ion exchange in the NE matrix, isolated nuclei loaded with Calcium Green-5N/AM were equilibrated in intracellular buffer in the presence of heparin (100 $\mu$g ml$^{-1}$) and apamin (100 nM). Under these conditions, the $InsP_3$-receptor-$Ca^{2+}$ channel and apamin-sensitive $K^+$ channel are inactivated and the $Ca^{2+}$ in the NE ($[Ca^{2+}]_{NE}$) remained stable (Nguyen et al., 1998). To import $K^+$ across the membrane of the NE, nuclei were exposed to valinomycin (10 $\mu$M). Then, the $K^+$ in the intracellular buffer was varied from 1 mM to 140 mM while the $[Ca^{2+}]_{NE}$ was continuously monitored in single equatorial thin sections of nuclei. Ionic strength and osmolarity were kept constant by adjusting the concentration of the monovalent organic cation *N*-methyl-D-glucamine ($NMG^{+}$) (Nguyen et al., 1998; Quesada et al., 2001, 2003).

### Optical sectioning

Nuclei were imaged with a Nikon Diaphot inverted fluorescence microscope (Melville, NY) using a 100 W mercury vapor epifluorescence source and a 100×, 1.4 NA oil-immersion objective. Images were captured on a 336 × 243 charge-coupled device array of a thermoelectrically cooled, low dark noise (1.3 photoelectrons s$^{-1}$ at −36°C) frame transfer digital camera with 16-bit resolution and $10^5$ pixel s$^{-1}$ maximum readout rate (Spectra Source Model 400, Westlake Village, CA). The camera was attached to the photoport of the microscope using a 20× relay lens, yielding a final resolution of 10 pixels $\mu$m$^{-1}$. To increase the sampling rate, we acquired three line scans at a time, instead of the whole image. Data were sampled at a rate of 1–2 scans s$^{-1}$ with 300 ms exposure time. Scans sampled an area of 0.3 $\mu$m × 24 $\mu$m across the equatorial plane of single nuclei and were accumulated in a memory buffer forming 20–35 s long sequential scan stacks. A no-neighbors deconvolution algorithm was implemented to get optical sections of ~0.2–0.3 $\mu$m (Monck et al., 1992; Nguyen et al., 1998; Quesada et al., 2001, 2003). Validation of the optical sectioning method has been published elsewhere (Nguyen et al., 1998; Quesada et al., 2001, 2003). The time course of average fluorescence intensity in photoelectron counts per pixel s$^{-1}$ in the NE and in the INC was measured from the line scans.

## RESULTS

### $InsP_3$ induces $Ca^{2+}$ oscillations in nuclei of airway epithelial ciliated cells

As in previous studies (Monck et al., 1992; Nguyen et al., 1998; Quesada et al., 2001, 2003), optical sectioning proved to be an excellent method to investigate subcellular $Ca^{2+}$ dynamics. Thin sections of intact cells labeled with the ER marker rhodamine B hexyl ester revealed loops and segments of ER network contiguous to the NE (Fig. 1 *A*). An almost identical image was obtained in cells loaded with the $Ca^{2+}$-sensitive probe Calcium Green-5N/AM that, as shown earlier, exhibit preferential accumulation in the ER and NE (Echevarria et al., 2003; Fig. 1 *B*). Moreover, because of its low affinity ($K_d = 14\ \mu M$), Calcium Green-5N exhibits better quantum yield fluorescence in organelles containing higher $Ca^{2+}$—such as the ER or the NE—than in the cytosol (Echevarria et al., 2003). In isolated nuclei, Calcium Green-5N exhibits a similar preferential compartmentalization into the NE (Fig. 1, *C* and *D*), whereas the low diffusivity probe Calcium Green-1/dextran ($K_d = 0.19\ \mu M$) shows an almost identical distribution as the DNA marker ethidium homodimer-1 (Fig. 1, *E* and *F*). These observations are consistent with previous reports, confirming the confinement of Calcium Green-5N/AM and Calcium Green-1/dextran in the NE and INC, respectively (Adebanjo et al., 1999; Echevarria et al., 2003; Gerasimenko et al., 1995, 2003; Leite et al., 2003; Quesada et al., 2002). As shown by these authors, this combination of $Ca^{2+}$ probes allows their application to independently monitor changes of $Ca^{2+}$ in each of these two nuclear compartments (Adebanjo et al., 1999; Echevarria et al., 2003; Gerasimenko et al., 1995, 2003; Leite et al., 2003; Quesada et al., 2002).

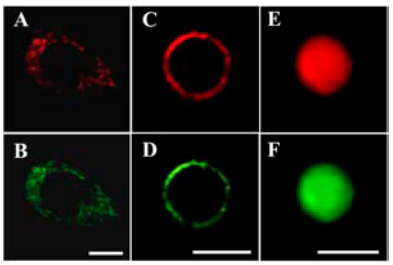


FIGURE 1 Loading of $Ca^{2+}$-sensitive dyes in the INC and NE of epithelial ciliated cells. Intact cells stained with the ER and NE probe rhodamine B hexyl ester (*A*) show a fluorescence distribution identical to that obtained with the $Ca^{2+}$-sensitive dye Calcium Green-5N/AM (*B*). The identification and location of the NE in isolated nuclei were also assessed by incubation with rhodamine B hexyl ester (*C*). This probe further confirmed the accumulation of the $Ca^{2+}$-sensitive dye Calcium Green-5N/AM in the NE (*D*). The INC of isolated nuclei identified by labeling with the DNA marker ethidium homodimer-1 (*E*) can accumulate z-1/dextran (*F*). Images are representative of more than 10 experiments either in intact cells or in isolated nuclei. These fluorescent markers for the NE and INC were also used to locate areas from which $Ca^{2+}$ records were made, avoiding unnecessary photobleaching of the $Ca^{2+}$ probes.

Activation of $InsP_3$ production relays the $Ca^{2+}$ signals that transduce stimulation of ciliary beating by ATP in respiratory ciliated cells (Nguyen et al., 1998). Application of 100 $\mu$M ATP to intact cells triggered $Ca^{2+}$ oscillations that exhibited the same spectral profile (~0.2 Hz) in the NE and in the INC (Fig. 2, *A* and *B*). To further verify that the NE undergoes $Ca^{2+}$ oscillations and its involvement as a source

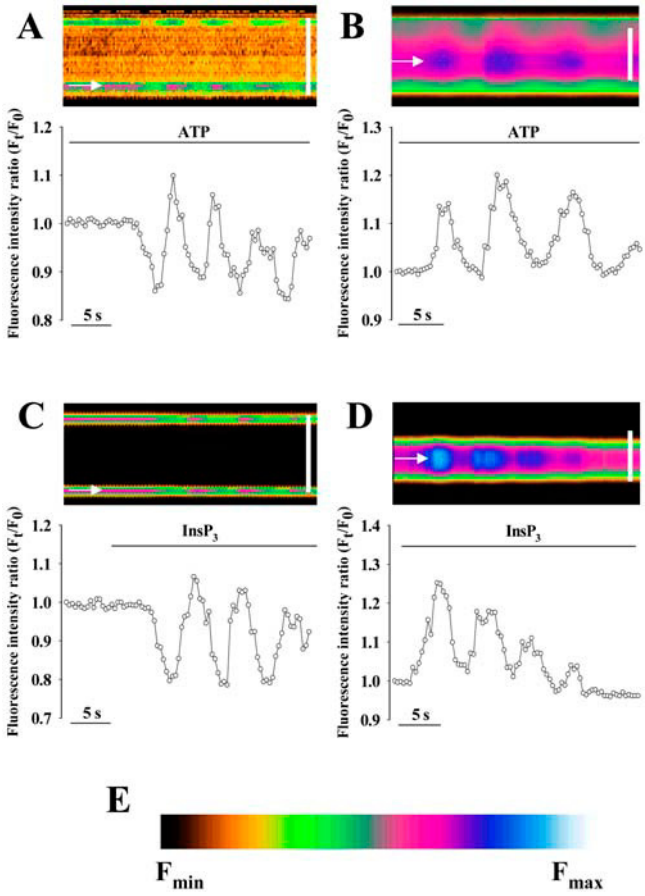


FIGURE 2 $InsP_3$-induced oscillatory $Ca^{2+}$ signals in the nucleus. (*A*) Exposure of ciliated cells loaded with Calcium Green-5N/AM to 100 $\mu$M ATP resulted in oscillations of $[Ca^{2+}]_{NE}$ with a frequency of 0.195 ± 0.013 Hz ($n = 4$). (*B*) A similar oscillatory pattern of $Ca^{2+}$ (~0.2 Hz), this time in the INC, follows application of ATP to ciliated cells loaded with Calcium Green-1/AM ($n = 4$). Experiments in isolated nuclei gave comparable results. (*C*) Nuclei loaded with Calcium Green-5N/AM exposed to 3 $\mu$M $InsP_3$ resulted in oscillations of $Ca^{2+}$ in the NE ($n = 7$) of 0.207 ± 0.018 Hz. (*D*) $InsP_3$ also induced a periodic release of $Ca^{2+}$ with a very similar frequency in the INC of isolated nuclei loaded with Calcium Green-1/dextran ($n = 9$). Exposure to stimuli produced very similar oscillatory $Ca^{2+}$ patterns in intact cells and isolated nuclei: it induced an initial $Ca^{2+}$ reduction in the NE followed by periodic $Ca^{2+}$ decreases, whereas an initial $Ca^{2+}$ rise in the INC with a subsequent train of $Ca^{2+}$ increases. Line scans were sampled across the equatorial plane of the nucleus in either intact cells or single nuclei and accumulated forming sequential scan stacks. Insets in figures show the line scan stacks corresponding to each graph in the same temporal scale. Arrows in the insets indicate the position where the measurement was made to plot each graph. Scale bar = 4 $\mu$m. (*E*) Pseudocolor scale corresponding to fluorescence changes in arbitrary units.

of periodic $Ca^{2+}$ release to the INC, we conducted experiments in isolated nuclei. In this preparation, the only source of $Ca^{2+}$ is the $Ca^{2+}$ stored in the matrix of the NE. Results show that application of $InsP_3$ (3 $\mu$M) generated a train of out of phase $Ca^{2+}$ oscillations in the NE and in the INC that mirror those found in intact cells (Fig. 2, *C* and *D*). It produced a periodic decrease of $[Ca^{2+}]_{NE}$ (Fig. 2 *C*), with a corresponding increase of $Ca^{2+}$ in the INC ($[Ca^{2+}]_{INC}$; Fig. 2 *D*). We have previously reported a similar pattern of $InsP_3$-induced out of phase $Ca^{2+}$ oscillations in ER/cytosol and granule/cytosol $Ca^{2+}$ signaling (Nguyen et al., 1998; Quesada et al., 2001, 2003). Our results agree with the idea that the NE is an extension of the ER and probably shares similar molecular components and properties (Echevarria et al., 2003), and the results are consistent with observations conducted in nuclei from liver and oocytes that suggest that $InsP_3$ receptors are found predominantly on the inner membrane of the NE (Gerasimenko et al., 1995, 2003; Humbert et al., 1996; Petersen et al., 1998). Although $InsP_3$ induced release of $Ca^{2+}$ from the NE to the INC, we did not observe significant changes of $Ca^{2+}$ in the neighboring space around isolated nuclei when the extranuclear space was labeled with Calcium Green-1/dextran (10 $\mu$grs/ml; not shown), suggesting that $InsP_3$-receptor-$Ca^{2+}$ channels might be predominantly facing the INC.

### Involvement of $InsP_3$ receptors and $ASK_{Ca}$ channels in nuclear $Ca^{2+}$ oscillations

The presence of functional $InsP_3$ receptors in the NE has been extensively documented. $InsP_3$ receptors together with ryanodine receptors channels have been found to mediate single discrete discharges of $Ca^{2+}$ to the INC (Nicotera et al., 1990; Gerasimenko et al., 1995; Adebanjo et al., 1999; Echevarria et al., 2003; Gerasimenko et al., 2003; Leite et al., 2003). We found that heparin (100 $\mu g \times ml^{-1}$), a blocker of the $InsP_3$ receptor, produced a complete inhibition of $Ca^{2+}$ oscillations in the NE and in the INC (Fig. 3, *A* and *B*). These results strongly suggest that $InsP_3$ receptors are involved in the generation of $[Ca^{2+}]_{NE}$ oscillations and corresponding periodic local release of $Ca^{2+}$ to the INC. Because experiments were conducted in ATP-free medium, it is unlikely that $Ca^{2+}$ oscillations resulted from the activity of SERCA. Moreover, application of 100 nM thapsigargin—an inhibitor of SERCA pumps—failed to block the $InsP_3$-induced oscillations of $[Ca^{2+}]_{NE}$ and $[Ca^{2+}]_{INC}$ (not shown; $n = 4$). Similar results have been previously found in the ER of ciliated cells (Nguyen et al., 1998) and further demonstrate that the SERCA pump is probably not involved in the generation of $InsP_3$-induced nuclear $Ca^{2+}$ oscillations. Nonetheless, SERCA pumps have been proven to control $Ca^{2+}$ uptake in the nucleus contributing to the maintenance of resting steady-state $[Ca^{2+}]_{NE}$ (Gerasimenko et al., 1995, 2003; Nicotera et al., 1989; Petersen et al., 1998; Quesada et al., 2002). In agreement with these reports, we observed

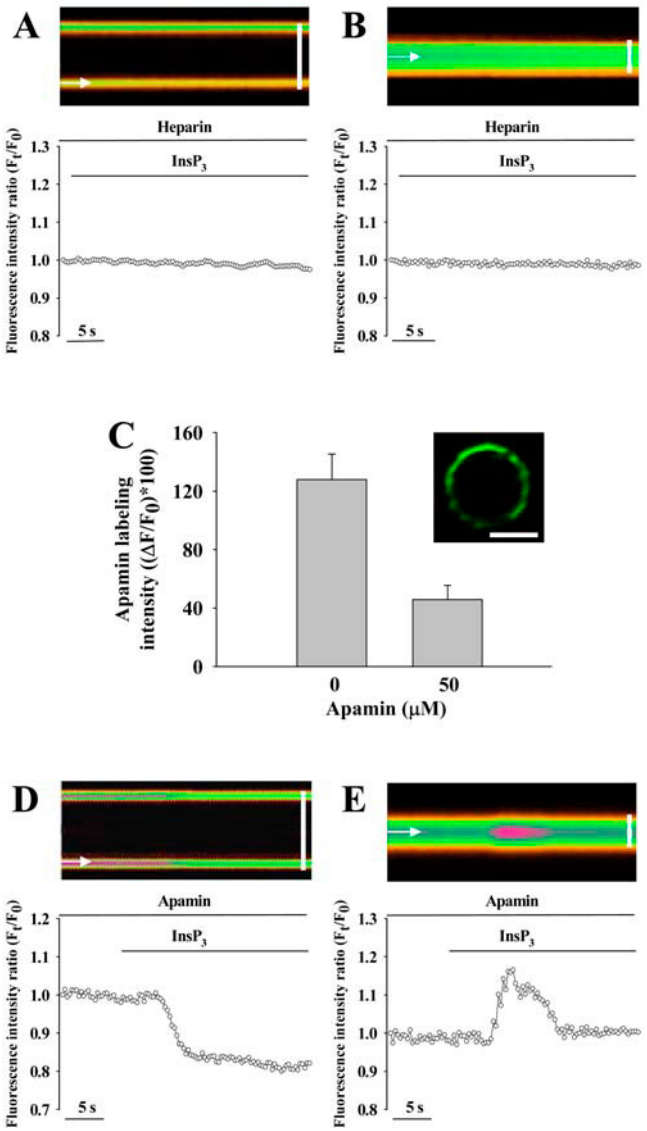


FIGURE 3 Nuclear $Ca^{2+}$ oscillations depend on the presence of functional $InsP_3$ receptors and $ASK_{Ca}$ channels in the NE. Heparin (100 $\mu g\ ml^{-1}$) blockade of $InsP_3$ receptors of isolated nuclei loaded with Calcium Green-5N/AM completely inhibited $InsP_3$-induced (3 $\mu$M) oscillations of $[Ca^{2+}]_{NE}$ ($n = 9$; *A*) or $[Ca^{2+}]_{INC}$ ($n = 16$; *B*). (*C*) Isolated nuclei incubated with 100 nM apamin conjugated with Alexa Fluor 488 conjugate (see Materials and Methods) exhibit a characteristic ringlike fluorescence corona (see *inset*; $n = 21$ nuclei from different random fields). This pattern was almost identical to that obtained by staining with the NE label rhodamine B hexyl ester (1 $\mu$M; not shown). Preincubation with 50 $\mu$M of nonconjugated nonfluorescent apamin resulted in a significant decrease of the fluorescence ($n = 19$; $p < 0.05$; mean ± SE), indicating the existence of binding competition and further confirming the presence of $ASK_{Ca}$ channels in the NE. In the presence of 100 nM apamin, $InsP_3$ failed to induce $Ca^{2+}$ oscillations in the NE or INC resulting instead in a slow decrease of $[Ca^{2+}]_{NE}$ (*D*) and a corresponding increase in the $[Ca^{2+}]_{INC}$ (*E*) in ~42% ($n = 12$) and ~39% ($n = 13$) of cases, respectively. These outcomes suggest that inflow of $K^+$ into the NE via $ASK_{Ca}$ channels is necessary for nuclear $Ca^{2+}$ oscillations. Insets in figures show the line scan stacks corresponding to each graph in the same temporal scale. Arrows in the insets indicate the position where the measurement was made to plot each graph. Scale bar = 4 $\mu$m.

that although thapsigargin failed to block $Ca^{2+}$ oscillations, it did produce a time-dependent decrease of the steady-state $[Ca^{2+}]_{NE}$ (not shown; $n = 4$).

The $ASK_{Ca}$ channel has been shown to play a critical role in the generation of $Ca^{2+}$ oscillations in the ER and in secretory granules (Nguyen et al., 1998; Quesada et al., 2001, 2003). The presence of $ASK_{Ca}$ in the nucleus was revealed by labeling isolated nuclei with the specific $ASK_{Ca}$ channel blocker apamin, conjugated in this case with a fluorescent reporter (see Materials and Methods). Preincubation of isolated nuclei with an excess of nonfluorescent apamin (50 $\mu$M) resulted in a decrease of the fluorescence associated with the labeling of the apamin-conjugated reporter, indicating that the observed binding was specific (Fig. 3 *C*). Complementary functional evidence indicated that $InsP_3$-induced $Ca^{2+}$ oscillations are suppressed in isolated nuclei exposed to 100 nM apamin. Instead, apamin produced a decrease of $[Ca^{2+}]_{NE}$ and a transient increase of $[Ca^{2+}]_{INC}$ (Fig. 3, *D* and *E*). These outcomes can be readily explained by the model we previously proposed for the ER (Nguyen et al., 1998); namely, a transient $Ca^{2+}$ release without oscillations takes place when inhibiting $ASK_{Ca}$ channels, as $InsP_3$ receptor activation would lead to the efflux of free $Ca^{2+}$ from the NE lumen until it reaches electrochemical equilibrium. A similar inhibition of $InsP_3$-induced $Ca^{2+}$ oscillations took place when we replaced $K^+$ by $NMG^+$ in the intracellular buffer (not shown). Several types of $K^+$ channels, including $Ca^{2+}$-sensitive $K^+$ channels, have been reported on the ER and NE (Burnham et al., 2002; Maruyama et al., 1995; Mazzanti et al., 2001). It has previously been proposed that these $Ca^{2+}$-dependent channels may play a role for controlling $Ca^{2+}$ release from the ER and NE (Maruyama et al., 1995; O'Rourke et al., 1994). $K^+$ influx in the lumen of the ER and nucleus has been thought to maintain electroneutrality during $Ca^{2+}$ movements (Maruyama et al., 1995; O'Rourke et al., 1994). Electroneutrality aside, these results and our previous reports (Nguyen et al., 1998; Quesada et al., 2001, 2003) give the $ASK_{Ca}$ channel a critical role as modulator of the $Ca^{2+}/K^+$ ion exchange process responsible for intralumenal unbinding of stored $Ca^{2+}$ (see next paragraph), placing the $ASK_{Ca}$ as a critical molecular component of the $Ca^{2+}$ oscillators present in the ER, the secretory granule, and now in the nucleus of airway epithelial ciliated cells.

## $Ca^{2+}/K^+$ ion exchange in the NE

Previous observations both in vivo (Nguyen et al., 1998) and in vitro (Mitchell et al., 1988) have shown that that the $Ca^{2+}$-buffering glycoprotein matrix of the ER can function as a $Ca^{2+}/K^+$ exchanger. This feature is crucial for the development of oscillatory $Ca^{2+}$ signals in the ER (Nguyen et al., 1998). Since the NE shares most of the $Ca^{2+}$-binding glycoproteins found in the ER (Villa et al., 1993), we evaluated whether a $Ca^{2+}/K^+$ exchange is also taking place in the NE. Fig. 4 shows results of an experiment in which isolated nuclei loaded with the $Ca^{2+}$ probe Calcium Green-5N/AM were exposed to an intracellular medium containing 10 $\mu$M of the $K^+$ ionophore valinomycin, heparin (100 $\mu$g $ml^{-1}$) to block the $InsP_3$ receptor, and apamin (100 nM) to block the $ASK_{Ca}$ channel and varying $K^+$ concentrations. Increasing $K^+$ in the bath resulted in an increase of the fluorescence of the $Ca^{2+}$ probe, indicating that in the NE, as in the ER (Nguyen et al., 1998), $K^+$ can indeed exchange for the $Ca^{2+}$ bound to the polyanionic sites of the lumenal matrix of these organelles.

## DISCUSSION

Our results show that the nucleus can function as an $InsP_3$-controlled $Ca^{2+}$ oscillator. Oscillations of $[Ca^{2+}]_{NE}$ and periodic pulsed release of $Ca^{2+}$ to the INC result from the interaction of two $Ca^{2+}$-modulated ion channels with opposite sensitivities to $Ca^{2+}$—an $InsP_3$-receptor $Ca^{2+}$ channel and an $ASK_{Ca}$ $K^+$ channel—and the $Ca^{2+}/K^+$ ion exchange properties of the $Ca^{2+}$-sequestering polyanionic matrix of the NE (Fig. 5). Binding of $InsP_3$ activates its receptor, turning on the efflux of $Ca^{2+}$, which leads to a decrease of $[Ca^{2+}]_{NE}$ and a corresponding increase of $[Ca^{2+}]_{INC}$. This transient increase of $[Ca^{2+}]_{INC}$ has a dual effect: it inhibits $InsP_3$ receptors (Finch et al., 1991), switching off the release of $Ca^{2+}$ from the NE, and activates $ASK_{Ca}$ channels, turning on the influx of $K^+$ to the NE and the $Ca^{2+}/K^+$ ion exchange. Since the $InsP_3$-receptor channels remain closed, the outcome is an increase of $[Ca^{2+}]_{NE}$. Meanwhile, $[Ca^{2+}]_{INC}$ diffuses away from the vicinity of the NE, turning on the $InsP_3$ receptors and $Ca^{2+}$ efflux to the INC and turning off the $ASK_{Ca}$ channels and the influx of $K^+$ to the NE, thereby starting a new cycle (Fig. 5; Nguyen et al., 1998).

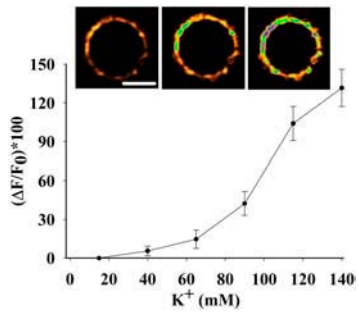


FIGURE 4 Ion-exchange properties of the matrix of the NE. $Ca^{2+}/K^+$ exchange was evaluated by equilibration of isolated nuclei loaded with Calcium Green-5N/AM in an intracellular medium containing 100 mg $ml^{-1}$ heparin, 100 nM apamin to block $InsP_3$ receptors and $ASK_{Ca}$ channels, respectively, and 10 $\mu$M valynomicin, a $K^+$ ionophore. Increasing the extralumenal $K^+$ led to $K^+$ inflow into the NE, resulting in a characteristic increase of $[Ca^{2+}]_{NE}$ ($n = 6$). Data are expressed as mean ± SE. The upper panel shows three images corresponding to an isolated nucleus loaded with the $Ca^{2+}$ probe in the presence of 40 mM $K^+$, 90 mM $K^+$, and 140 mM $K^+$, respectively (from *left* to *right*). Scale bar = 2 $\mu$m.

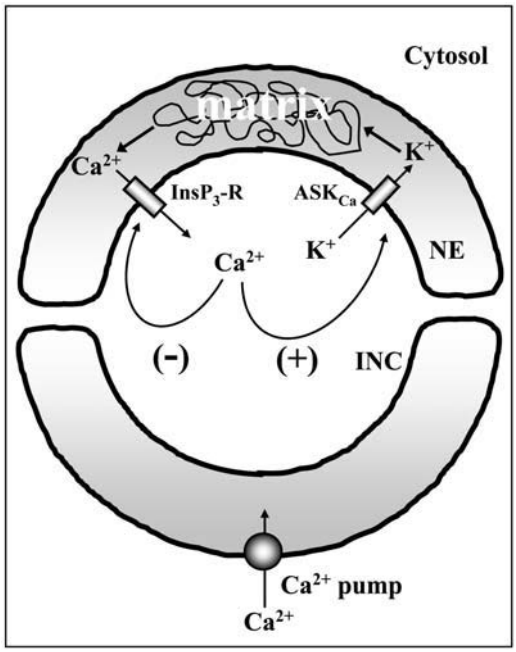


FIGURE 5 Model illustrating the mechanism involved in nuclear $Ca^{2+}$ oscillations. Free $Ca^{2+}$ in the NE is in equilibrium with $Ca^{2+}$ bound to the negative sites of the NE polyanionic matrix. Binding of $InsP_3$ molecules to $InsP_3$ receptors causes the release of free $Ca^{2+}$ from the NE to the INC, leading to a decrease in $[Ca^{2+}]_{NE}$ and an increase in $[Ca^{2+}]_{INC}$. The $Ca^{2+}$ rise in the INC induces a dual effect: it inactivates $InsP_3$ receptors (Finch et al., 1991) while opening $ASK_{Ca}$ channels, allowing for an influx of $K^+$. Influx of $K^+$ displaces $Ca^{2+}$ bound to the matrix by ion exchange, producing an increase in $[Ca^{2+}]_{NE}$ while $InsP_3$ receptors remain closed. Because of $Ca^{2+}$ diffusion—most probably through nuclear pores—and the buffering capacity of the nucleus, $Ca^{2+}$ decreases in the INC resulting in the reactivation of $InsP_3$ receptors $Ca^{2+}$ channel while closing $ASK_{Ca}$ channels starting a new cycle. Although reloading $Ca^{2+}$ in the NE is most likely mediated by SERCA pumps (Gerasimenko et al., 1995; Nicotera et al., 1989), results suggest that SERCA pumps are not directly involved in $Ca^{2+}$ oscillations. Our model suggests that the $InsP_3$ receptor $Ca^{2+}$ channel, the $ASK_{Ca}$ $K^+$ channel, and the ion exchange properties of the NE matrix are all necessary for nuclear $Ca^{2+}$ oscillations and that these oscillations must remain activated for as long as $InsP_3$ is bound to the $InsP_3$ receptor ($InsP_3$-R).

The mechanisms that control $Ca^{2+}$ dynamics in the nucleus have been the subject of multiple controversial studies. Although some reports support the idea that the nucleus offers no barrier to cytosolic $Ca^{2+}$ input through nuclear pores, other groups have shown that this intracellular compartment can indeed filter, attenuate, or even insulate cytosolic $Ca^{2+}$ signals (Al-Mohanna et al., 1994; Brini et al., 1993; Chamero et al., 2002; Gerasimenko et al., 1996). Notwithstanding the role of nuclear pores in $Ca^{2+}$ trafficking, the NE contains both $Ca^{2+}$ channels and the enzymatic machinery to synthesize second messengers—including $InsP_3$ and cADPr—that control these channels. Results obtained in isolated nuclei indicate that activation of $Ca^{2+}$-release channels on the NE leads to single transient rises in $[Ca^{2+}]_{INC}$ (Adebanjo et al., 1999; Echevarria et al., 2003; Gerasimenko et al., 1995, 2003; Leite et al., 2003; Quesada et al., 2002). Here we show that the nucleus can also generate periodic oscillations of $Ca^{2+}$. In airway epithelial ciliated cells, ATP stimulation, which triggers $InsP_3$ signaling in these cells, induces a train of $[Ca^{2+}]_{NE}$ oscillations and periodic pulses of $Ca^{2+}$ release to the INC resulting in a corresponding train of out of phase oscillations of $[Ca^{2+}]_{INC}$. Isolated nuclei stimulated with the intracellular second messenger $InsP_3$ exhibit an identical response.

Both single transient fluctuations and periodic oscillations of $Ca^{2+}$ can regulate cellular functions (Chawla et al., 1998; Dolmetsch et al., 1997, 1998; Goldbeter et al., 2000; Hardingham et al., 1997, 2001; Li et al., 1998; Pusl et al., 2002; Teruel et al., 2000). Although single transient changes of $[Ca^{2+}]_{INC}$ are thought to control transcriptional response and protein nuclear translocation (Chawla et al., 1998; Dolmetsch et al., 1997; Hardingham et al., 1997, 2001; Pusl et al., 2002), periodic oscillations of $Ca^{2+}$ have been implicated in enzymatic catalysis and the activity of several transcription factors in the nucleus (Dolmetsch et al., 1998; Hu et al., 1999; Li et al., 1998; Tomida et al., 2003). The activation of nuclear proteins such as calmodulin kinase II is known to be particularly sensitive to frequency-encoded $Ca^{2+}$ signaling (Bayer et al., 2002; De Koninck and Schulman, 1998; Kutcher et al., 2003). In addition to the potential intrinsic specificity of frequency-encoded $Ca^{2+}$ signaling, oscillations of $Ca^{2+}$ are thought to prevent desensitization of $Ca^{2+}$ receptors (Berridge et al., 2003). However, despite several observations that $Ca^{2+}$-receptor molecules and processes responding to oscillatory $Ca^{2+}$ signals are present in the nucleus (Bayer et al., 2002; De Koninck and Schulman, 1998; Dolmetsch et al., 1998; Kutcher et al., 2003; Li et al., 1998; Tomida et al., 2003), frequency-encoded nuclear $Ca^{2+}$ signaling had not been experimentally validated. The results presented here indicate that the $InsP_3$-controlled mechanism that implements periodic oscillations of $Ca^{2+}$ release from intracellular storage compartments—including ER, secretory granules, and the NE— shares the same $Ca^{2+}/K^+$ ion exchange scheme and a similar ion channel molecular hardware. This elegant and intriguing ATP-independent functional protocol produces trains of $Ca^{2+}$ oscillations and discrete pulses of $Ca^{2+}$ release in/to the NE/INC, or the ER/cytosol, or granule/cytosol, allowing precise time-resolved step-by-step local control of $Ca^{2+}$ inside the compartments where receptor/effector molecules are located (Nguyen et al., 1998; Quesada et al., 2001, 2003). $Ca^{2+}$ oscillations and release exhibit remarkably constant frequency and remain activated for as long as $InsP_3$ is bound to its receptor (Nguyen et al., 1998; Quesada et al., 2001, 2003). Although our observations show that $Ca^{2+}$ is delivered at a remarkably constant rate of $\sim$0.2 pulses $\times$ $s^{-1}$, the amount of $Ca^{2+}$ released per pulse to the INC can not be precisely estimated from our data.

An interesting implication is that the pulse-modulated quantal mode of $Ca^{2+}$ release we reported in the ER and the granule (Nguyen et al., 1998; Quesada et al., 2001, 2003) and

that we documented here in the nucleus might underlay previously reported single transient $Ca^{2+}$ fluctuations. Spatial or temporal integration of pulse trains of $Ca^{2+}$ fluctuations could indeed account for previously reported single longer transient fluctuations of intracellular $Ca^{2+}$; namely, in thick optical sections, fluctuations of $Ca^{2+}$ are integrated across the whole optical path of the imaging system, preventing the resolution of small $Ca^{2+}$ oscillations and yielding large $Ca^{2+}$ transients that could result from spatial integration of small $Ca^{2+}$ pulses. As shown earlier (Monck et al., 1992; Nguyen et al., 1998; Quesada et al., 2001, 2003), this problem is virtually avoided by the thin optical sectioning method used in these experiments. Alternatively, temporal integration could as well result in an apparent single $Ca^{2+}$ transient if the frequency of $Ca^{2+}$ release pulses saturates the buffering capacity of the local cytosol or the INC or if the sampling rate of the detector or the frequency response of the $Ca^{2+}$ probe aliases the $Ca^{2+}$ signal. Despite these uncertainties, intracellular single transients of $Ca^{2+}$ have been extensively reported in the literature and they probably represent a specific mode of amplitude modulated signal encoding. The membrane of intracellular organelles, and the NE in particular, contain a broad range of $Ca^{2+}$ ion channels as well as $Ca^{2+}$ pumps and ion exchangers (Adebanjo et al., 1999; Echevarria et al., 2003; Gerasimenko et al., 1995, 2003; Leite et al., 2003; Quesada et al., 2002; Santella and Carafoli, 1997) that could well implement a broad range of signaling modes including single fluctuations and the multiple oscillatory patterns reported by our group (Nguyen et al., 1998; Quesada et al., 2001, 2003).

These results indicate that the nucleus of airway epithelial ciliated cells possesses the molecular hardware to generate oscillatory $Ca^{2+}$ signals that are directly relayed to the INC, thereby enhancing the precise local delivery of nuclear $Ca^{2+}$ messages which could be important for the control of specific and local processes in the nucleus. Our observations introduce a novel paradigm by providing objective evidence that the cellular organelles, including the ER, secretory granule, and the NE, share similar molecular components and a similar working protocol, enabling them to function as intracellular $Ca^{2+}$ oscillators.

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