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Dampening of Cytosolic Ca^{2+} Oscillations on Propagation to Nucleus*

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Ca^{2+} signals may regulate gene expression. The increase of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) promotes activation and/or nuclear import of some transcription factors, but others require the increase of the nuclear Ca^{2+} concentration ($[Ca^{2+}]_N$) for activation. Whether the nuclear envelope may act as a diffusion barrier for propagation of $[Ca^{2+}]_c$ signals remains controversial. We have studied the spreading of Ca^{2+} from the cytosol to the nucleus by comparing the cytosolic and the nuclear Ca^{2+} signals reported by targeted aequorins in adrenal chromaffin, PC12, and GH_3 pituitary cells. Strong stimulation of either Ca^{2+} entry (by depolarization with high K^+ or acethylcholine) or Ca^{2+} release from the intracellular Ca^{2+} stores (by stimulation with caffeine, UTP, bradykinin, or thyrotropin-releasing hormone (TRH)) produced similar Ca^{2+} signals in cytosol and nucleus. In contrast, both spontaneous and TRH-stimulated oscillations of cytosolic Ca^{2+} in single GH_3 cells were considerably dampened during propagation to the nucleus. These results are consistent with the existence of a kinetic barrier that filters high frequency physiological $[Ca^{2+}]_c$ oscillations without disturbing sustained $[Ca^{2+}]_c$ increases. Thus, encoding of the Ca^{2+} signal may allow differential control of Ca^{2+}-dependent mechanisms located at either the cytosol or the nucleus.

Ca^{2+} signals regulate many cell functions including motility, secretion, proliferation, differentiation, programmed cell death, and gene expression (1). The increase of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$)[1] promotes activation and/or nuclear import of some transcription factors, such as NFAT or NFκB (2, 3), but changes of the nuclear Ca^{2+} concentration ($[Ca^{2+}]_N$) are required for regulation of other transcriptional mechanisms, such as the ones mediated by CREB (1, 4) or DREAM-DRE (5).

The mechanisms involved in controlling $[Ca^{2+}]_N$ are poorly known and controversial (6). The nucleus is surrounded by a double membrane, the nuclear envelope (NE), which can accumulate Ca^{2+} inside its lumen. It has been proposed that Ca^{2+} can be released directly from NE cisterna into the nucleoplasm through ion channels associated to either inositol trisphosphate or ryanodine receptors of the inner NE membrane (7–9). On the other hand, cytosol and nucleoplasm communicate through 10-nm diameter nuclear pores with large ion conductance (10). However, conductance can be drastically reduced by either ATP-dependent accumulation of Ca^{2+} inside NE (gating) or macromolecular transport through nuclear pore complexes (plugging) (11). The permeability of the NE to cytosolic Ca^{2+} is a matter of controversy. While several authors report that $[Ca^{2+}]_N$ follows passively changes of $[Ca^{2+}]_c$ (12–14) others identify a significant diffusion barrier at the NE (8, 15–17). This question is not trivial, as it may determine whether or not cytosolic Ca^{2+} signals shall be transduced into changes of gene expression.

In the present work we have compared the cytosolic and the nuclear Ca^{2+} signals reported by targeted aequorins in several resting and stimulated cell systems (adrenal chromaffin cells, PC12 cells, GH_3 pituitary cells). We find that strong stimulation of either Ca^{2+} entry or Ca^{2+} release from the intracellular Ca^{2+} stores produced similar Ca^{2+} signals in cytosol and nucleus. However, both spontaneous and thyrotropin-releasing hormone (TRH)-stimulated $[Ca^{2+}]$ oscillations in GH_3 cells were considerably dampened in nucleus as compared with cytosol, suggesting that nucleus can filter high frequency physiological $[Ca^{2+}]_c$ signals.

EXPERIMENTAL PROCEDURES

Cell Culture and Expression of Aequorins—Bovine adrenal chromaffin cells, kindly provided by Professor Antonio García, Instituto Teófilo Hernando, Madrid, Spain, were handled as described previously (18, 19). HEK-293 cells were maintained in DMEM medium (Invitrogen) supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics. Culture and handling of GH_3 pituitary cells (20, 21) and PC12 cells (22) were as described previously. Cytosolic and nuclear (nucleoplasmin chimera) aequorin cDNAs were obtained from Molecular Probes and cloned in the pHSVpUC plasmid (23). Packaging and titration of the pHSVnucAEQ (nuclear) and pHSVcytAEQ (cytosolic) viruses were performed as reported (22). The multiplicity of infection ranged between 0.01 and 0.1 for batch luminescence measurements and between 0.3 and 1 for bioluminescence imaging (19, 23, 24). Cells were cultured for 12–24 h before measurements.

Measurements of Aequorin Bioluminescence—Cells expressing apoaequorins were incubated for 1–2 h at room temperature with 1 μM coelenterazine. Coelenterazine h (from Molecular Probes), which increases the affinity of reconstituted aequorin for Ca^{2+} (25), was used for imaging experiments. The standard incubation medium had the following composition (in mM): NaCl, 145; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; glucose, 10; sodium-HEPES, 10, pH 7.4. Batch cell aequorin photoluminescence measurements were performed as described previously (19, 24), and calibrations in $[Ca^{2+}]$ were done using the values published for the constant (26). For bioluminescence imaging measurements (23, 24), cells were placed into a perfusion chamber thermostatted to 37 °C under a Zeiss Axiovert 100 TV microscope and perfused at 5–10 ml/min with the test solutions, prewarmed at 37 °C. At the end of each experiment cells were permeabilized with 0.1 mM digitonin in 10 mM $CaCl_2$ to release all the residual aequorin counts. Images were taken with a Hamamatsu VIM photon counting camera handled with an Argus-20

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[1] The abbreviations used are: $[Ca^{2+}]_c$, cytosolic $[Ca^{2+}]$; $[Ca^{2+}]_N$, nuclear $[Ca^{2+}]$; NE, nuclear envelope; TRH, thyrotropin-releasing hormone; cps, counts per second; HEDTA, *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid.

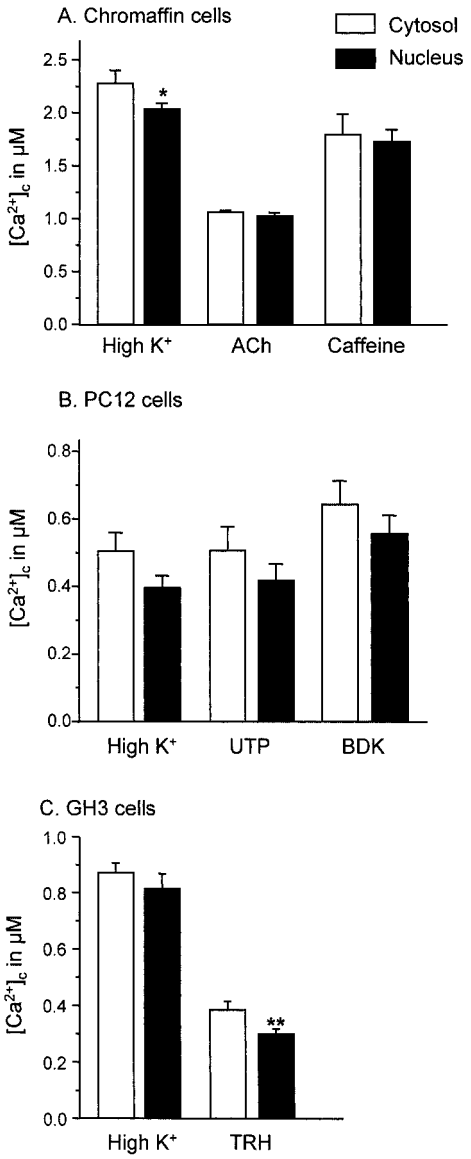

FIG. 1. **Comparison of the changes of $[Ca^{2+}]_c$ and $[Ca^{2+}]_N$ produced by different stimuli.** Cells were infected with either cytosolic or nuclear aequorin, and $[Ca^{2+}]$ was estimated from photoluminescence emission (see "Experimental Procedures"). Mean ± S.E. values of 7–32 experiments are shown. *A*, bovine adrenal chromaffin cells. *B*, PC12 cells. *C*, GH_3 pituitary cells. Stimuli, applied for 30 s, were: high K^+ (70 mM), acethylcholine (*ACh*, 100 μM), caffeine (50 mM), UTP (100 μM), bradykinin (*BDK*, 1 μM), TRH (0.1 μM). Significance (Student's *t* test): *, $p < 0.05$; **, $p < 0.01$.

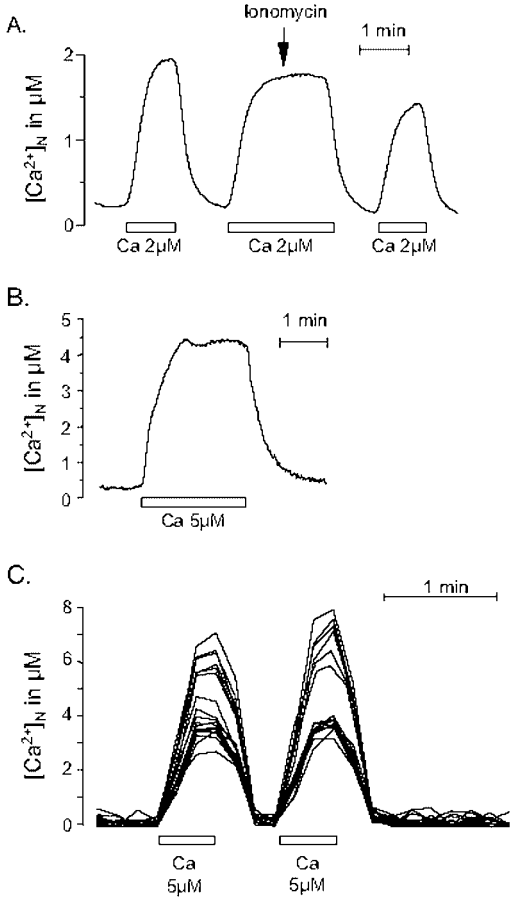

FIG. 2. **Changes of $[Ca^{2+}]_N$ by perfusion of plasma membrane-permeabilized cells with Ca^{2+}-containing solutions.** HEK-293 cells (*A* and *C*) or GH_3 cells (*B*) infected with nuclear aequorin were permeabilized by perfusion with 0.1 mM digitonin for 1 min in "intracellular" solution (composition, in mM: NaCl, 10; KCl, 140; $MgCl_2$, 1; KH_2PO_4, 1; Mg-ATP, 2; EGTA, 2; K-HEPES, 20, pH 7). Then the cells were perfused with intracellular solution containing 0.1 μM Ca^{2+} (1 mM $CaCl_2$ and 2 mM EGTA) for 5 min before starting luminescence measurements. At the times shown perfusion was switched to intracellular solution containing 2 or 5 μM Ca^{2+} (buffered with HEDTA). *A*, a representative batch experiment with HEK-293 cells. The concentration of ionomycin was 1 μM. *B*, a representative experiment with GH_3 cells. *C*, a representative imaging experiment with HEK-293 cells. The traces from 19 single cells present in the same microscope field have been superimposed.

image processor and integrated for 10-s periods. Photons/cell in each image were quantified using the Hamamatsu Aquacosmos software. Total counts per cell ranged between 2×10^3 and 2×10^5, and noise was (mean ± S.D.) 1 ± 1 cps typical cell area (about 2000 pixels). Data were quantified as rates of photoluminescence emission/total cps remaining at each time and divided by the integration period (L/L_{TOTAL} in s^{-1}). Emission values of less than 4 cps were not used for calculations. Calibrations for $[Ca^{2+}]$ are shown in Fig. 3. For calculation of oscillation indexes the differences (in absolute value) between each L/L_{TOTAL} value and the following one were added and divided by the total number of measurements during the integration period. This parameter is sensitive to both the amplitude and the frequency of oscillations (20).

RESULTS AND DISCUSSION

To assess whether the NE affects the progression of the Ca^{2+} wave we elicited a $[Ca^{2+}]_c$ increase, either by activation of Ca^{2+} entry or of Ca^{2+} release from the endoplasmic reticulum, and compared the size of the Ca^{2+} peaks measured in cytosol and nucleus. Fig. 1*A* summarizes results in bovine adrenal chromaffin cells. Ca^{2+} entry was elicited either by depolarization with high K^+ (70 mM) solution or by stimulation with acetylcholine. The $[Ca^{2+}]$ peaks were very similar in the cytosol (*open bars*) and in the nucleus (*filled bars*). Ca^{2+} release from the endoplasmic reticulum was induced by stimulation with caffeine (18). Again the $[Ca^{2+}]$ peaks elicited by caffeine in the cytosol and the nucleus were very similar (Fig. 1*A*). In PC12 cells the Ca^{2+} peaks elicited in cytosol and nucleus by either Ca^{2+} entry (High K^+) or Ca^{2+} release from the intracellular Ca^{2+} stores (UTP or bradykinin) were similar. In GH_3 pituitary cells depolarization with high K^+ or release of Ca^{2+} from the stores by stimulation with 0.1 μM TRH (22) also produced similar Ca^{2+} peaks in cytosol and nucleus (Fig. 1*C*). In all the cases the increase of $[Ca^{2+}]_c$ tended to be somewhat smaller in the nucleus but the differences were small, at most 25%, even though some of them were statistically significant (Fig. 1).

The apparent permeability of the NE was directly assessed by monitoring changes of $[Ca^{2+}]_N$ on exposure of permeabilized HEK-293 cells to bathing solutions with controlled Ca^{2+} con-

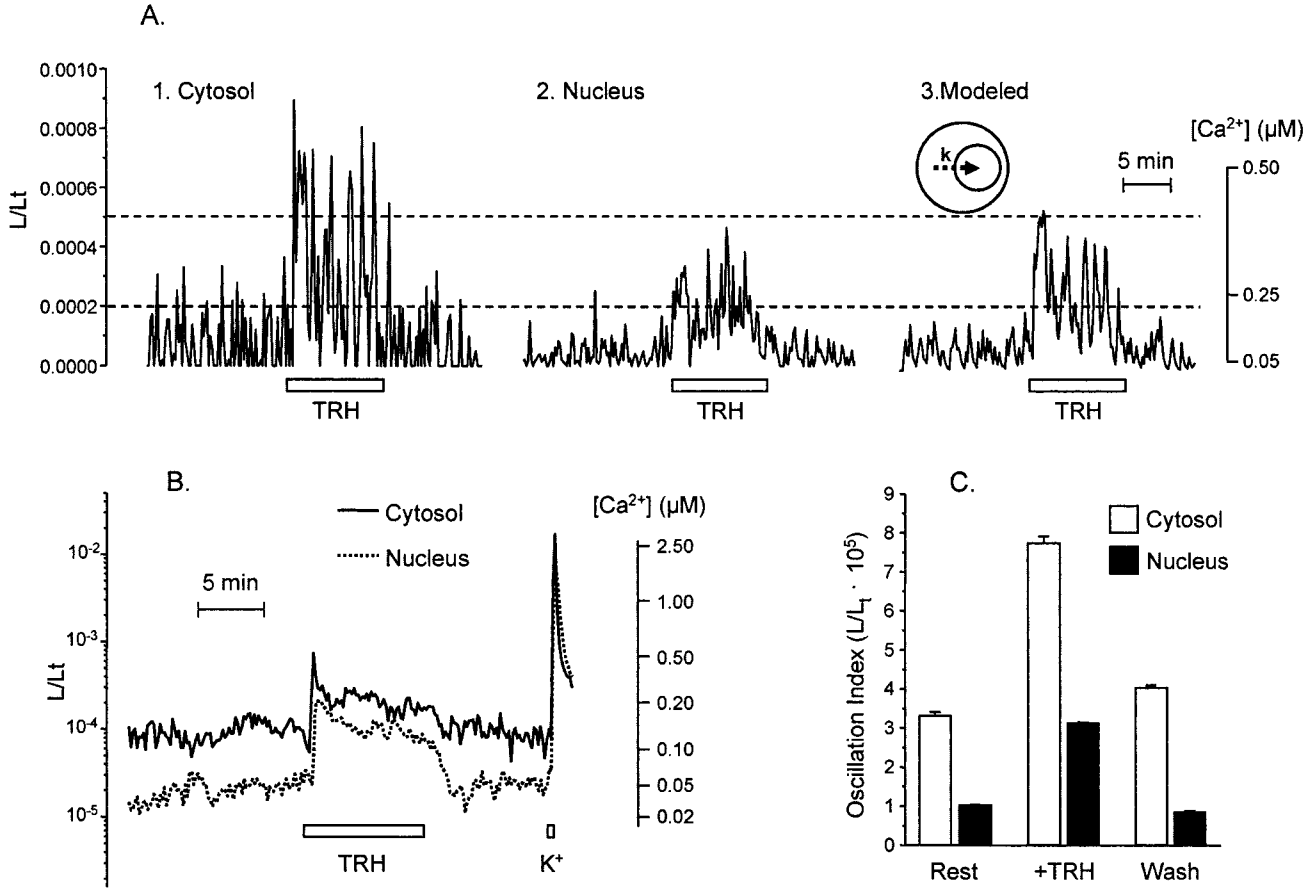

FIG. 3. **Comparison of cytosolic and nuclear Ca^{2+} oscillations.** GH_3 cells expressing either the cytosolic or the nuclear aequorin were imaged for photoluminescence emission. *A*, typical single-cell traces from cytosol (*trace 1*) and nucleus (*trace 2*). *Trace 3* was computed from *trace 1*, assuming diffusion with $T_{½} = 10$ s. *B*, average values of 55 (cytosol) or 72 (nucleus) single cells present in the same microscope field. *C*, oscillation indexes of cytosolic and nuclear Ca^{2+} oscillations integrated during 5-min periods with or without TRH. Values are mean ± S.E. of 131–199 cells from four different experiments. The concentration of TRH was 2 nM.

centrations. When external $[Ca^{2+}]$ was increased from 0.1 to 2 μM, $[Ca^{2+}]_N$ increased quickly to near thermodynamic equilibrium (Fig. 2*A*). Addition of the Ca^{2+} ionophore ionomycin at this stage did not modify $[Ca^{2+}]_N$. On switching perfusion to the low $[Ca^{2+}]$ solution, $[Ca^{2+}]_N$ returned quickly to the resting level. Half-equilibration times were about 10 s for both the increase and the decrease of $[Ca^{2+}]_N$. Similar results were obtained with permeabilized GH_3 pituitary cells (Fig. 2*B*). Single-cell measurements produced similar results (Fig. 2*C*). Several putative regulatory mechanisms of nuclear permeability to Ca^{2+} were explored pharmacologically. Pretreatment of the cells with activators of either protein-kinase A (forskolin, 5 μM, 5 min) or protein kinase C (phorbol myristate acetate, 0.1 μM, 5 min) did not modify results in experiments similar to those in Fig. 2*A*. Replacement of K^+ by Na^+, addition of protonophores (carbonyl cyanide *m*-chlorophenylhydrazone, 10 μM) or inhibitors of the endoplasmic reticulum Ca^{2+} pump (*tert*-butylhydroquinone, 40 μM) did not have any effect either.

The above results suggest that the NE does not act as a significant permeability barrier for Ca^{2+} diffusion, and hence $[Ca^{2+}]_N$ mimics $[Ca^{2+}]_c$, at least for events that last for several seconds, such as the ones tested here. However, we were surprised to find that the measured resting $[Ca^{2+}]_N$ in GH_3 cells was consistently much smaller than $[Ca^{2+}]_c$. The values found were (mean ± S.E.) 56 ± 14 (n = 11) and 177 ± 39 nM (n = 12), respectively. In the other cell types we found no such differences between the resting values of $[Ca^{2+}]_c$ and $[Ca^{2+}]_N$. A relevant difference among these cell types is that GH_3 cells display spontaneous electric activity, Ca^{2+} action potentials, and $[Ca^{2+}]_c$ oscillations (27, 28). We reasoned that a kinetic barrier could filter propagation of high frequency $[Ca^{2+}]_c$ oscillations to the nucleus. This would result in a lower value of the average resting $[Ca^{2+}]_c$ in GH_3 cells, but not in the other cell types tested, which do not display spontaneous Ca^{2+} oscillations. On the other hand, this kinetic barrier would not disturb significantly the propagation to nucleus of sustained $[Ca^{2+}]_c$ increases, such as those shown in Fig. 1. To test this working hypothesis we compared single-cell spontaneous $[Ca^{2+}]_c$ and $[Ca^{2+}]_N$ oscillations in GH_3 cells. Hypothalamic releasing factors regulate hormone secretion in several anterior pituitary cell types by increasing (TRH, growth hormone-releasing hormone) or decreasing (dopamine, somatostatin) the spontaneous activity (29). In GH_3 cells, TRH increases the rate of action potential firing, $[Ca^{2+}]_c$ oscillations, and prolactin secretion (27–30).

Fig. 3*A* compares typical traces of spontaneous and TRH-induced $[Ca^{2+}]_c$ and $[Ca^{2+}]_N$ activity in single GH_3 cells. TRH reversibly increased activity in both compartments (*traces 1* and *2*). Overall, the size of the $[Ca^{2+}]_N$ peaks tended to be smaller than the $[Ca^{2+}]_c$ ones, both during spontaneous and during TRH-induced activity. Fig. 3*B* shows the averaged $[Ca^{2+}]_c$ and $[Ca^{2+}]_N$ traces of all the cells present in the microscope field. Since Ca^{2+} oscillations are not synchronic among cells, they disappeared on averaging. The average $[Ca^{2+}]_N$ values were smaller than the $[Ca^{2+}]_c$ ones, especially during the resting periods. During stimulation with high K^+, $[Ca^{2+}]_N$ and $[Ca^{2+}]_c$ values tended to converge. This is consistent with the results obtained in the batch experiments described above

(Fig. 1*C*). Fig 3*C* compares the $[Ca^{2+}]_c$ and the $[Ca^{2+}]_N$ oscillations, quantified as oscillation indexes. These indexes are computed by adding the differences (in absolute value) between every $[Ca^{2+}]$ value and the subsequent one along the whole integration period (20). The average oscillation index was three to four times smaller in the nucleus than in the cytosol during the resting periods (*Rest* or *Wash*). During stimulation with TRH the oscillation index increased both in cytosol and in nucleus, but still it was two to three times smaller in the nucleus (Fig. 3*C*). In *trace 3* of Fig. 3*A* we have modeled the effects of a nuclear permeability barrier with $T_{½} = 10$ s on the transmission of the $[Ca^{2+}]_c$ oscillation (*trace 1*) to the nucleus (*trace 3*). The outcome was very similar to the experimental observation (compare *traces 2* and *3*). The oscillation index computed from *trace 1* (cytosol) was 3.9 times larger than the one computed from *trace 3* (nucleus), a difference very similar to the one measured experimentally (Fig. 3*C*). The kinetic barrier proposed here could fit equally well for either slowed diffusion at the NE or increased buffering of Ca^{2+} inside nucleus (31). In both cases the appearance of free Ca^{2+} inside the nucleus would be slower than expected.

In conclusion, our results indicate that nucleus dampens Ca^{2+} oscillations of cytosolic origin in GH_3 cells, both the spontaneous and the TRH-induced. This nuclear barrier is, however, much less efficient to filter sustained increases of $[Ca^{2+}]_c$ such as those elicited here by depolarization with high K^+ solutions for several seconds or maximal stimulation with inositol 1,4,5-trisphosphate-generating agonists or caffeine. In practical terms, this means that propagation of the $[Ca^{2+}]_c$ signal to the nucleus is very much dependent on signal encoding, the higher frequency signals being much less efficient than the more sustained ones. This may be a clue for understanding the effects of different stimulation patterns on nuclear events. For example, $[Ca^{2+}]_c$ oscillations increase the efficiency of gene expression via NFAT or NFκB (3). Another example is the differential activation of serum response element-regulated genes and cyclic AMP response element-regulated genes by either cytosolic or nuclear calcium rises (32). According to our results, expression of genes controlled directly by $[Ca^{2+}]_N$ should preferentially respond to sustained $[Ca^{2+}]_c$ changes.

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