Thapsigargin-induced nuclear calcium signals in rat basophilic leukaemia cells

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By a confocal fluorescence microscope with an argon-ion laser (488 nm) and a He–Cd laser (325 nm) we have studied thapsigargin-induced calcium signals in individual rat basophilic leukaemia (RBL-2H3) cells. In the presence or absence of external calcium ions, thapsigargin-induced calcium signals were transferred to the nucleus as well as to the cytoplasm of RBL-2H3 cells. The calcium signals were generally much stronger in the nucleus than in the cytoplasm. However, some of the RBL-2H3 cells had apparently reduced nuclear calcium signals. They had a basophil-like bilobed (multilobed) nucleus, although most RBL-2H3 cells had a mast-cell-like monolobed nucleus. In the cells with a bilobed nucleus, IgE-receptor-mediated calcium signals were neither transferred to the nucleus nor to the cytoplasm. The results gave a new insight into the understanding of the mechanism of the nuclear calcium signals in RBL-2H3 cells.

INTRODUCTION

Rat basophilic leukaemia 2H3 cloned cell line (RBL-2H3) contains, like mast cells and blood basophils, receptors for IgE and can be stimulated to secrete histamine by aggregation of the receptors with antigen [1]. Before histamine secretion, the aggregation of the surface receptors for IgE is known to induce a sustained increase in intracellular free calcium concentration ($[Ca^{2+}]_i$) [2]. In addition to measuring $[Ca^{2+}]_i$ in cell suspensions, single cell observations for $[Ca^{2+}]_i$ in RBL-2H3 cells have been performed using digital-imaging fluorescence microscopes [3–8]. In these experiments, we and Millard et al. have shown that single RBL-2H3 cells respond much more rapidly to antigen than previously suggested by studies on populations of the cells. The experiments indicated that the lag time between addition of antigen and initiation of the increase in $[Ca^{2+}]_i$ varied considerably between individual cells. In addition to these results, we have recently found by confocal fluorescence microscopy that IgE-receptor-mediated calcium signals were transferred not only to the cytoplasm but also to the nucleus in RBL-2H3 cells [9]. This finding suggests the possibility that the increased intranuclear calcium ions may act as a nuclear third-messenger in RBL-2H3 cells [9]. In addition to this finding, we have revealed that the receptor-mediated calcium signals were transferred not only to the cytoplasm but also to the nucleus in lymphocytes [10,11]. However, it has not been determined yet how the signals are transferred from the plasma membrane to the nucleus to increase the calcium ion concentration in the nucleus [9–12].

To understand the mechanisms of the nuclear calcium signals in more detail, we have studied the effects of thapsigargin, an inhibitor of the intracellular Ca^{2+} pump, on the nuclear calcium signals in RBL-2H3 cells using a confocal fluorescence microscope with an argon-ion laser (488 nm) and a He–Cd laser (325 nm).

MATERIALS AND METHODS

Fluo-3 AM, Fura-2 AM and Hepes were obtained from Dojindo (Kumamoto, Japan). Hoechst 33342 (bisbenzimide H 33342) was obtained from Calbiochem (La Jolla, CA, U.S.A.). 1-(4-Trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) was obtained from Molecular Probes (Eugene, OR, U.S.A.). MEM (Eagle's minimum essential medium) was obtained from Nissui (Tokyo). Thapsigargin was obtained from Sigma (St. Louis, MO, U.S.A.). Mouse anti-dinitrophenyl monoclonal IgE (IgE-53-569) [13] was provided by Professor T. Kishimoto (Osaka University, Osaka, Japan). Mouse monoclonal antibody (BC4) [14] raised against the α-subunit of IgE receptor was given by Dr. R. P. Siraganian. Goat fluorescein isothiocyanate (FITC)-labelled anti-(mouse IgG) was obtained from MBL (Nagoya, Japan). Preparation of 2,4-dinitrophenol(DNP)-conjugated BSA was described previously [7,9]. In our present experiments, an average of six DNP groups were conjugated with BSA (DNP_6–BSA). All experiments were performed on a secreting sub-line of rat basophilic leukaemia cells, RBL-2H3 [1]. Cell lines were cloned by the limiting dilution technique and a single sub-clone (1-6) was obtained [7,9]. Cells from the single sub-clone (1-6) were maintained in a culture medium containing MEM supplemented with 10% (v/v) fetal-calf serum. In these culture conditions, most RBL-2H3 cells had mast-cell-like monolobed nucleus. However, a part of the cells (5–10%) had a basophil-like bilobed (multilobed) nucleus. These cells arose spontaneously in culture and have not been subcloned yet, because the cell growth was low in our present experimental conditions.

Single-cell observation using a confocal fluorescence microscope with an argon-ion laser (488 nm) and a He–Cd laser (325 nm) was performed by the following procedure [9]. RBL-2H3 cells were harvested from culture dishes and transferred to an observation chamber from Elekon (Chiba, Japan). The chamber was then settled in a CO_2 incubator overnight. After incubation, cells were treated with a medium containing 10 μM Fluo-3-AM (or Fura-2-AM) and 0.5 μg/ml anti-DNP IgE for 30 min at 37 °C. Then they were washed with 10 mM Hepes, 140 mM NaCl, 5 mM KCl and 1 mM $CaCl_2$ at pH 7.2 to remove free Fluo-3-AM (or Fura-2-AM) and unbound IgE in solution. Confocal fluorescence microscopic images of the Fluo-3-loaded

Abbreviations used: $[Ca^{2+}]_i$, intracellular free calcium concentration; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene; MEM, Eagle's minimum essential medium; FITC, fluorescein isothiocyanate.

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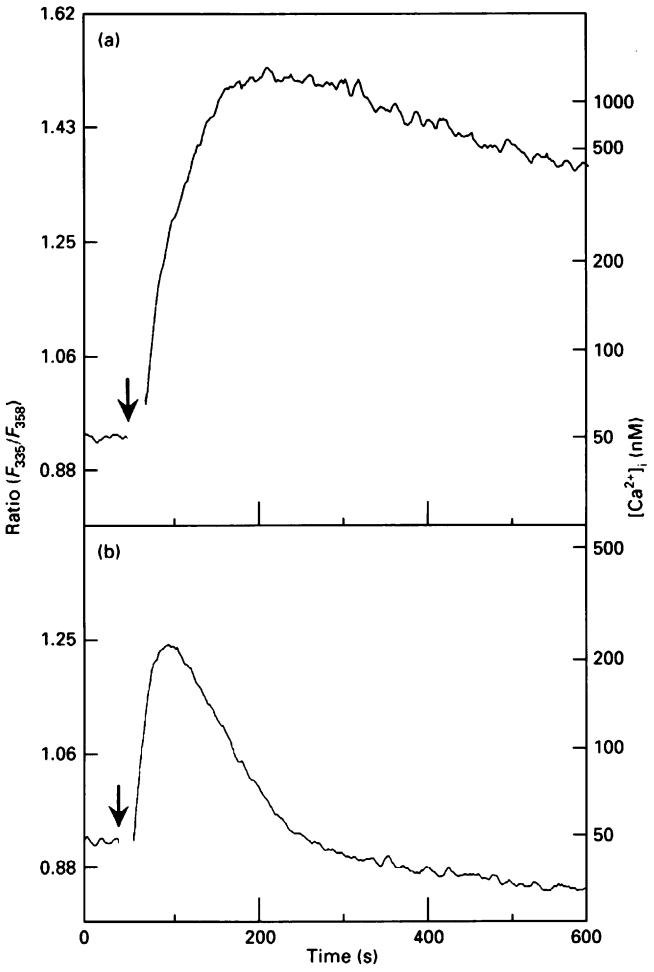

Figure 1 Time-courses of $[Ca^{2+}]_i$ in Fura-2-loaded RBL-2H3 cell suspensions after addition of thapsigargin (20 nM) observed using a fluorescence spectrophotometer at 37 °C

(**a**) In the presence of external free calcium ions, and (**b**) in the absence of external free calcium ions (with 1 mM EGTA).

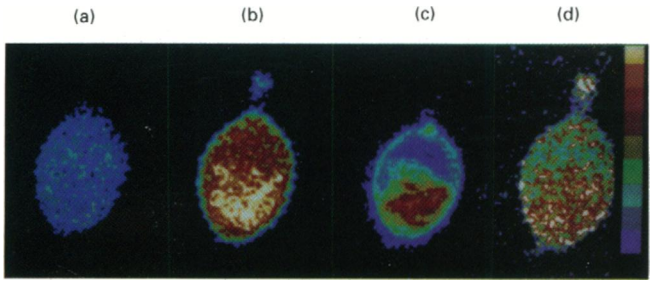

Figure 2 Confocal fluorescence pseudo-images of a Fluo-3 and Hoechst 33342 co-loaded RBL-2H3 cell after addition of thapsigargin (20 nM) in the presence of external free calcium ions at 37 °C

Fluo-3 fluorescence intensities are shown in a colour bar. Confocal fluorescence calcium images before (**a**) and after (**b**) stimulation of thapsigargin, which were excited by an argon-ion laser (488 nm). (**c**) A confocal fluorescence DNA image of a Hoechst 33342-loaded RBL-2H3 cell which was excited by a He–Cd laser (325 nm). (**d**) A ratio of the Fluo-3 fluorescence images after thapsigargin stimulation. Here, ratio determination was done by using the fluorescence images of (**a**) and (**b**).

RBL-2H3 cells were taken under a confocal-fluorescence-microscopic system (MRC-600, Bio-Rad), with an inverted epifluorescence microscope (Nikon TMD-EFQ). Fluo-3-loaded RBL-2H3 cells were excited at 488 nm using an argon-ion laser and the fluorescence emission was observed above 515 nm. The temperature of the observation chamber was maintained at 37 °C. Confocal fluorescence images of nuclei in RBL-2H3 cells were measured by a He–Cd laser (325 nm) from Omnichrome together with a DNA-specific fluorescence probe, Hoechst 33342 [15]. To determine the location of the nucleus exactly, we added the membrane-specific fluorescent probe, TMA-DPH, into the observation chamber. TMA-DPH indicated outlines of RBL-2H3 cells.

Fluorescence measurements of Fura-2-loaded RBL-2H3 cell suspensions were performed in a 1 cm quartz cuvette using a Shimadzu RF-5000 spectrofluorometer at 37 °C [9].

RESULTS

To study what kinds of mechanisms regulate intranuclear calcium signals in RBL-2H3 cells, we examined the effects of thapsigargin, an inhibitor of the intracellular Ca^{2+} pump [16–18], on the nuclear calcium signals in RBL-2H3 cells. First, we measured the $[Ca^{2+}]_i$ in Fura-2-loaded RBL-2H3 cell suspensions after addition of thapsigargin (1–100 nM) using a fluorescence spectrophotometer. A concentration of 20 nM thapsigargin was enough to induce the maximum level of the $[Ca^{2+}]_i$, as shown in Figure 1 [18]. From the Fura-2 ratio intensities [19], it was estimated that $[Ca^{2+}]_i$ in RBL-2H3 cells increased from 50 nM at the resting levels to 1000 nM at the activated levels in the presence of external calcium ions. In the absence of external calcium ions (with 1 mM EGTA), the $[Ca^{2+}]_i$ increased to 250 nM at the activated levels.

We then measured the fluorescence images of Fluo-3-loaded RBL-2H3 cells using a confocal fluorescence microscope [9]. Figure 2 shows a typical example of the confocal fluorescence images of Fluo-3-loaded RBL-2H3 cells before and after addition of thapsigargin (20 nM). In the presence of external calcium ions, Fluo-3 fluorescence intensities in RBL-2H3 cells increased gradually, responding to thapsigargin in a similar way to the cell suspensions shown in Figure 3(a). Before thapsigargin stimulation the confocal fluorescence images of Fluo-3-loaded RBL-2H3 cells were dim and non-homogeneous (Figure 2a). However, they increased in intensity and became more non-homogeneous after thapsigargin stimulation (Figure 2b). From the confocal fluorescence images of Hoechst 33342 (a DNA-specific fluorescence probe) (Figure 2c), the regions of bright fluorescence in the Fluo-3-loaded cells were shown to belong to the nucleus in the RBL-2H3 cells [9]. This indicated that the thapsigargin-induced calcium signals were transferred not only to the cytoplasm but also to the nucleus of RBL-2H3 cells.

To correct for inhomogeneities in dye intensity and distribution inside RBL-2H3 cells, we measured the ratio of a Fluo-3-fluorescence image after thapsigargin stimulation to a Fluo-3-fluorescence image before stimulation, as shown in Figure 2(d). While true ratio calculation was not possible with Fluo-3, the ratio using a fluorescence image before stimulation showed the existence of a heterogeneous fluorescence image in Figure 2(d). Although it is difficult to determine directly $[Ca^{2+}]_i$ from the Fluo-3-fluorescence intensities by confocal fluorescence microscopy, percentages of the increments in the ratio of Fluo-3 fluorescence intensities were much higher in the nucleus than in the cytoplasm.

Typical examples of the time-dependent thapsigargin-induced calcium signals are shown in Figure 3. The average time course of individual cells provides a trace which can be compared with

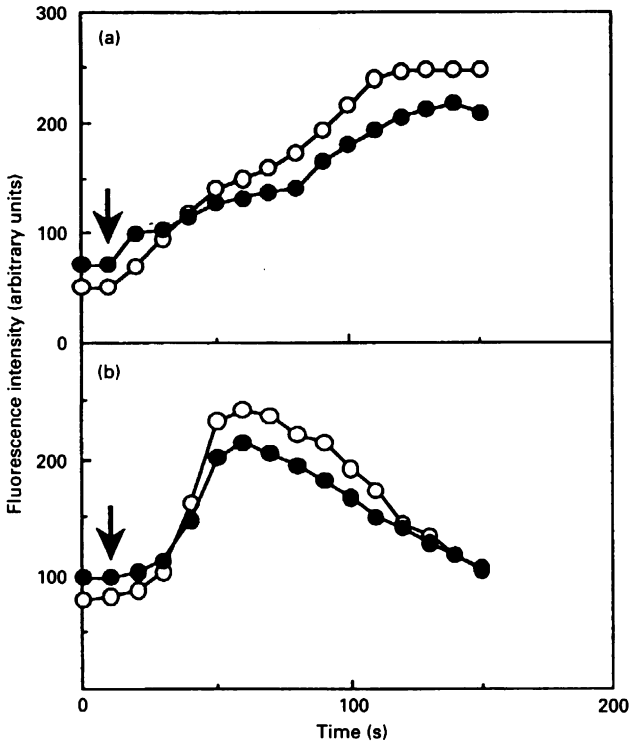

Figure 3 Time courses of the fluorescence intensity changes of the nucleus and cytosol in RBL-2H3 cells with a mast-cell-like monolobed nucleus after addition of thapsigargin (20 nM) at 37 °C

The vertical axes are relative fluorescence intensities which were obtained by subtraction of the background fluorescence intensities. Open circles indicate the Fluo-3 fluorescence intensities in the nucleus while closed circles indicate those in the cytoplasm. These fluorescence intensities are calculated from the appropriate areas of the nucleus and cytoplasm in Fluo-3 and Hoechst 33342 co-loaded RBL-2H3 cells. We checked that Hoechst 33342 did not affect the Fluo-3 fluorescence images in RBL-2H3 cells and that Fluo-3 did not affect the Hoechst 33342 fluorescence images. (**a**) In the presence of external calcium ions, and (**b**) in the absence of external calcium ions (1 mM EGTA).

fluorimetric measurements of the mean time course of a suspension of RBL-2H3 cells shown in Figure 1(a). Time-dependent increases of $[Ca^{2+}]_i$ by thapsigargin occurred more slowly than those induced by antigen (DNP_6–BSA) in the presence or absence of external calcium ions [9].

Subsequent examination of the effects of Ca^{2+} influx from the external medium on the nuclear calcium signals in RBL-2H3 cells revealed that intranuclear calcium signals as well as cytosolic calcium signals were still observable even when 1 mM EGTA was present in the external medium. A typical example of the time courses of the fluorescence intensity changes of Fluo-3-loaded invididual RBL-2H3 cells after addition of thapsigargin is shown in Figure 3(b). Here, the $[Ca^{2+}]_i$ both in the cytosol and in the nucleus increased more rapidly than those in the presence of external calcium ions. At the present time it is difficult to determine directly $[Ca^{2+}]_i$ from the Fluo-3 fluorescence images using confocal fluorescence microscopy. However, the time-dependent profiles shown in Figure 3 closely resembled the profiles of the Fura-2 fluorescence ratio intensities of the cell suspension (Figure 1). These results showed the idea that the Fluo-3 fluorescence images in RBL-2H3 cells reflected the pattern of $[Ca^{2+}]_i$ in RBL-2H3 cells [9].

In the present study we performed these kinds of experiments a large number of times and measured confocal fluorescence images in several hundred RBL-2H3 cells after addition of thapsigargin. Most of RBL-2H3 cells responded to thapsigargin

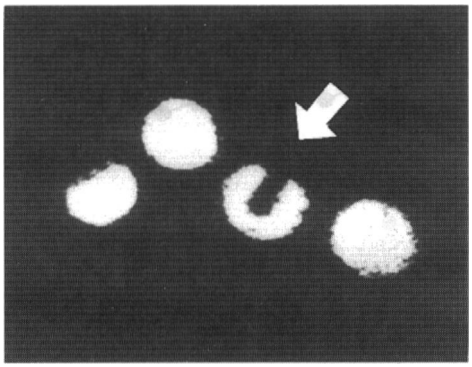

Figure 4 Confocal fluorescence nuclear images of Hoechst 33342-loaded RBL-2H3 cells with a basophil-like bilobed (multilobed) nucleus and a mast-cell-like monolobed nucleus

A cell with a bilobed (multilobed) nucleus is indicated by an arrow. The DNA images of Hoechst 33342-loaded RBL-2H3 cells were obtained by excitation with a He–Cd laser (325 nm).

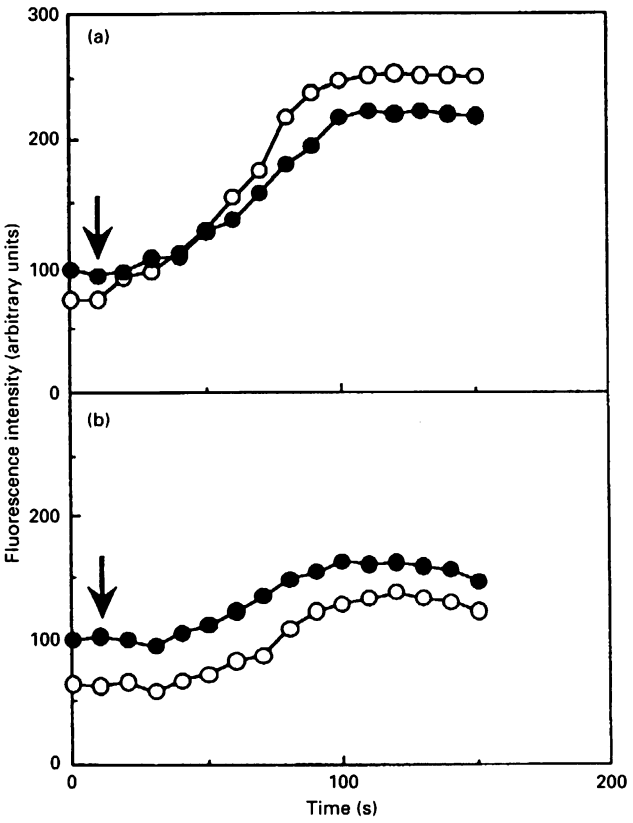

Figure 5 Typical examples of the time-dependent fluorescence intensity changes in Fluo-3-loaded RBL-2H3 cells with a basophil-like bilobed (multilobed) nucleus after addition of thapsigargin (20 nM)

Open circles indicate the Fluo-3 fluorescence intensities in the nucleus and closed circles indicate those in the cytoplasm. (**a**) In the presence of external free calcium ions. (**b**) In the absence of external free calcium ions (with 1 mM EGTA).

stimulation and the Fluo-3 fluorescence intensities increased more in the nucleus than in the cytoplasm. However, some RBL-2H3 cells responded somewhat differently to thapsigargin. Such cells had a basophil-like bilobed (multilobed) nucleus and the others had a mast-cell-like monolobed nucleus as shown in Figure 4. In RBL-2H3 cells with a basophil-like bilobed (multilobed) nucleus the calcium signals were apparently weaker in the nucleus

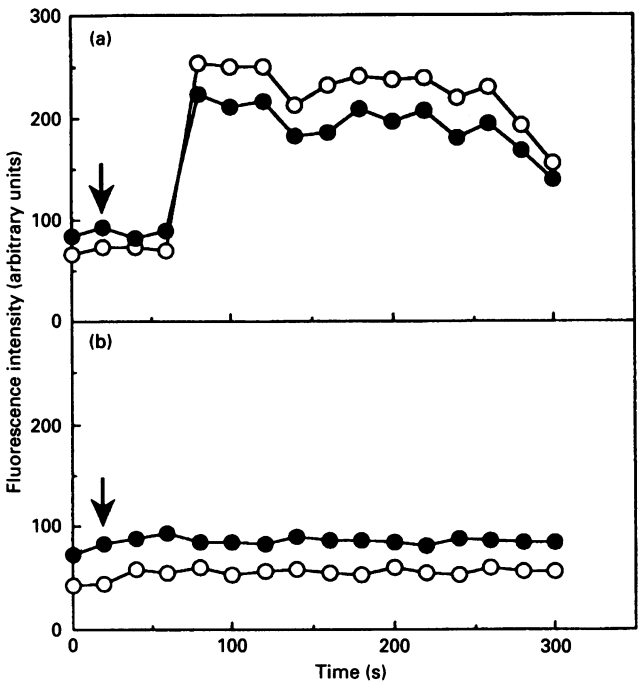

Figure 6 Time courses of the fluorescence intensity changes of Fluo-3-loaded RBL-2H3 cells after addition of antigen (DNP_6–BSA) in the presence of external calcium ions

Open circles indicate the Fluo-3 fluorescence intensities in the nucleus while closed circles indicate those in the cytoplasm. (**a**) A RBL-2H3 cell with a mast-cell-like monolobed nucleus, and (**b**) a RBL-2H3 cell with a basophil-like bilobed (multilobed) nucleus.

than in the cytoplasm in the absence of external calcium ions, though they were still higher in the nucleus than in the cytoplasm in the presence of external calcium ions (Figure 5). We found that under our present experimental conditions the proportion of such cells was 5–10% of the whole RBL-2H3 cell population.

Furthermore, we found that in RBL-2H3 cells with a bilobed nucleus, IgE-receptor-mediated calcium signals were neither transferred to the nucleus nor to the cytoplasm, as shown in Figure 6(b). We then added antigen (DNP_6–BSA) to Fluo-3-loaded RBL-2H3 cells with a basophil-like bilobed (multilobed) nucleus. However, in RBL-2H3 cells with a mast-cell-like monolobed nucleus, DNP_6–BSA was able to induce calcium signals not only in the cytoplasm but also in the nucleus, as shown in Figure 6(a) [9].

DISCUSSION

In this paper we have shown that the thapsigargin-induced calcium signals were transferred not only to the cytoplasm but also to the nucleus of RBL-2H3 cells. The calcium signals were generally much stronger in the nucleus than in the cytoplasm. However, some RBL-2H3 cells had apparently reduced nuclear calcium signals. The proportion of these kinds of cells was 5–10% of the whole RBL-2H3 cell population and they had a basophil-like bilobed (multilobed) nucleus. These cells did not respond to antigen (DNP_6–BSA) stimulation, although they had as many IgE receptors as normal RBL-2H3 cells did. This was confirmed by the confocal fluorescence images of RBL-2H3 cells with mouse anti-(IgE-receptor) IgG (BC4) and goat FITC-labelled anti-(mouse IgG). This suggested the possibility that the transmembrane signalling pathways may have some modification in RBL-2H3 cells with a bilobed (multilobed) nucleus. At the present time we have not determined what kinds of molecules in the signalling pathways may inhibit the IgE-receptor-mediated calcium signals and reduce the thapsigargin-induced nuclear calcium signals in RBL-2H3 cells with a bilobed (multilobed) nucleus. However, the present results were very interesting for understanding how the calcium signals were transferred to the nucleus after stimulation.

We showed, in the present experiment, that the thapsigargin-induced nuclear calcium signals in RBL-2H3 cells with a mast-cell-like monolobed nucleus were independent of the concentrations of external calcium ions. However, in RBL-2H3 cells with a basophil-like bilobed (multilobed) nucleus the thapsigargin-induced nuclear calcium signals were dependent on the concentration of external calcium ions. In addition, in the absence of external free calcium ions, the calcium signals in RBL-2H3 cells with a bilobed (multilobed) nucleus were apparently weaker in the nucleus than in the cytoplasm. This suggested that the amplitudes of the nuclear calcium signals are not merely dependent on the concentration of calcium ions in the cytosol of the cells but that there exist some active transporting mechanisms to regulate $[Ca^{2+}]_i$ in the nucleus of RBL-2H3 cells.

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