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High concentration of calcium ions in Golgi apparatus

XUE Shaobai[1], M. Robert NICOUD, Jie CUI, D.J. Arndt JOVIN
Department of Biology, Beijing Normal University, Beijing 100875, China
Max-Planck-Institute fSr Biophysikalische Chemie, GSttingen, Germany

ABSTRACT

The interphase NIH3T3 cells were vitally fluorescent-stained with calcium indicator fluo-3 and Glogi probe C_6-NBD-ceramide, and then the single cells were examined by laser scanning confocal microscopy (LSCFM) for subcellular distributions of Ca^{2+} and the location of Golgi apparatus. In these cells, the intracellular Ca^{2+} were found to be highly concentrated in the Golgi apparatus. The changes of distribution of cytosolic high Ca^{2+} region and the Golgi apparatus coincided with the cell cycle phase. In calcium free medium, when the plasma membrane of the cells which had been loaded with fluo-3/AM were permeated by digitonin, the fluorescence of the Golgi region decreased far less than that of the cytosol. Our results indicated that the Glogi lumen retained significantly high concentration of free calcium.

Key words: *intracellular free calcium, fluo-3/AM, Golgi apparatus, C_6-NBD-ceramide, laser scanning confocal microscopy, intracellular calcium store.*

INTRODUCTION

A lot of studies demonstrated that many aspects of cell functions are regulated by the concentration of cytosolic free calcium[1]. Most cells produce changes in intracel-

1. Corresponding author

lular free calcium for signaling via Ca^{2+} channels in the surface membrane, and/or discharge calcium from intracellular stores[2,3]. But high [Ca^{2+}]i may do harm to the cell if it maintains a long time. Cells return [Ca^{2+}]i toward its low physiological level through its Ca^{2+} -ATPase on the plasma membrane or membrane of internal calcium pools. Thus, special structure for storing intracellular Ca^{2+} plays an essential role in calcium homeostasis. Although there is no question that sarcoplasmic reticulum (SR) is the intracellular calcium pools for muscle cells[4], the localization of non-mitochondrial calcium pool is much less clear in non-muscle system. Some authors considered that endoplasmic reticulum(ER) served as pools[5], while others though of a separate organelle termed as calciosome, playing the role[6,7]. For technical reason, it is impossible to measure the [Ca^{2+}]i precisely in ER or calciosomes. That is to say the anatomical localization of the[Ca^{2+}]i store in non-muscle system remains unclear. Although Chandra et al observed a prominent perinuclear regional concentration of total calcium (both free and binding calcium) associated with the Golgi apparatus by laser scanning confocal microscopy(LSCFM) and ion microscopy[8], the storage of cytosolic free calcium has not been clearly defined yet. In the present study, calcium indicator fluo-3 /AM and Golgi probe C_6-NBD-ceramide were used to localize the cytosolic free calcium and the Golgi apparatus by imaging their fluorescence individually. The results showed that the fluorescence of C6-NBD-ceramide and the highest fluorescence of fluo-3 were distributed identically in each phase of the cell cycle. The Golgi apparatus is especially retentive of Ca^{2+} when the plasma membrane was permeated by 10 μM digitonin. It seems, therefore, that high concentration of free calcium is localized mainly in the Golgi apparatus.

MATERIALS AND METHODS

Cells cultures

NIH3T3 cells were seeded onto the glass coverslips and incubated in DMEM supplemented with 20% fetal bovine serum at 37 ℃ in 5% CO_2 atmosphere. Cells were used for experiments when they reached logarithmic phase.

Vital staining of cytosolic free calcium and Golgi apparatus

The cells grown on the coverslips were stained with 10 μM fluo-3/ AM (molecular probe) for 30 min at 37 ℃, and washed with a standard medium (135 m*M* NaCl, 10 m*M* HEPES, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% D-glucose, 0.1 % bovine serum albumin, pH 7.3) at least 3 times. Then the cells were mounted on a small chamber containing the standard medium to maintain them in living state. For vital staining of Golgi apparatus, the cells were loaded with C_6-NBD-ceramid (molecular probe) as previously described[9].

Microscopy and image analysis systems[10]

Comparable microscopic observations were done by using LSCFM (Carl Zeiss, Oberkochen, Germany) equipped with laser 488 nm excitation. An oil objective × 40/1.30 was used. Observations were done at an image resolution of 512 × 512 pixels (8 bit). For digital processing the images were transferred to a DEC micro VAX II computer through an IEEE connector. TIPS (Delft University of Technology, the Netherlands) was used for digital processing. The images were taken by a timing

videoprint. The stereo pairs of 3-D reconstructional images were obtained by processing a set of optical sections with the improved stereovisualization method described by Schormann et al[10].

Opening the plasma membrane with digitonin[11]

Exposing the cells loaded with fluo-3/AM in Ca^{2+}-free standard medium containing 10 μM digitonin, the cytosol calcium flowed out through the millipores on the plasma membrane.

RESULTS

The 3-D reconstructional image revealeda high intracellular calcium region in a perinuclear area

In non-confocal image (Fig 1a), the fluorescence of fluo-3 distributed homogeneously throughout the NIH3T3 cell and did not show any high Ca^{2+} region. Using LSCFM serial optical sections were obtained (Fig 1b-1f), and some of them showed that there was a high Ca^{2+} region in the perinuclear area. Reconstruction of these optical sections also showed clearly that the highest Ca^{2+} concentration was in the perinuclear region (Fig 2).

The regions of high perinuclear [Ca^{2+}]i are associated with the Golgi apparatus in interphase cell

When different NIH3T3 cells were separately stained with fluo-3/AM and C_6-NBD-ceramide, one could see that the highest Ca^{2+} concentration was detected in an area adjacent to the nucleus (Fig 3a) and the C_6-NBD-ceramide fluorescence was also found to be located asymmetrically around the nucleus (Fig 3b). When the same cell was first stained with fluo-3/AM and imaged by LSCFM, the cell was then exposed to liposomes containing C_6-NBD-ceramide for 10 min and then post-incubated at room temperature for 45 min to traffic the lipid probe to the Golgi apparatus before capturing another image. Such colocalization stainning was made possible since the fluorescence of C_6-NBD-cermamide was far brighter than that of fluo-3, so that the interference of fluo-3 fluorescence to the former was almost negligible. In the same NIH3T3 cell. the hightest concentrations of [Ca^{2+}]i in perinuclear area (Fig 4b) closely coincided with the location of the Golgi apparatus (Fig 4a). This study provided direct evidences that the Golgi apparatus might concentrate a high amount of free calcium.

The changes of distribution of intracellular free calcium and the Golgi apparatus in different phases of cell cycle

The distribution of cytosolic Ca^{2+} and Golgi apparatus in different phases of cell cycle was shown in Fig 5 and Fig 6. In metaphase (Fig 5a and 5b),the fluorescence of fluo-3 was uniformly distributed throughout the cell and there was no special high Ca^{2+} region. Since the Golgi complex disassembled during mitosis, the fluorescence of C_6-NBD-ceramide was also diffusely dispersed throughout the cell. However, in telophase (Fig 6a and 6b), cytosolic free calcium became concentrated at the region

of spindle remnants, and so did the fluorescence of C $_{6}$-NBD-ceramide.

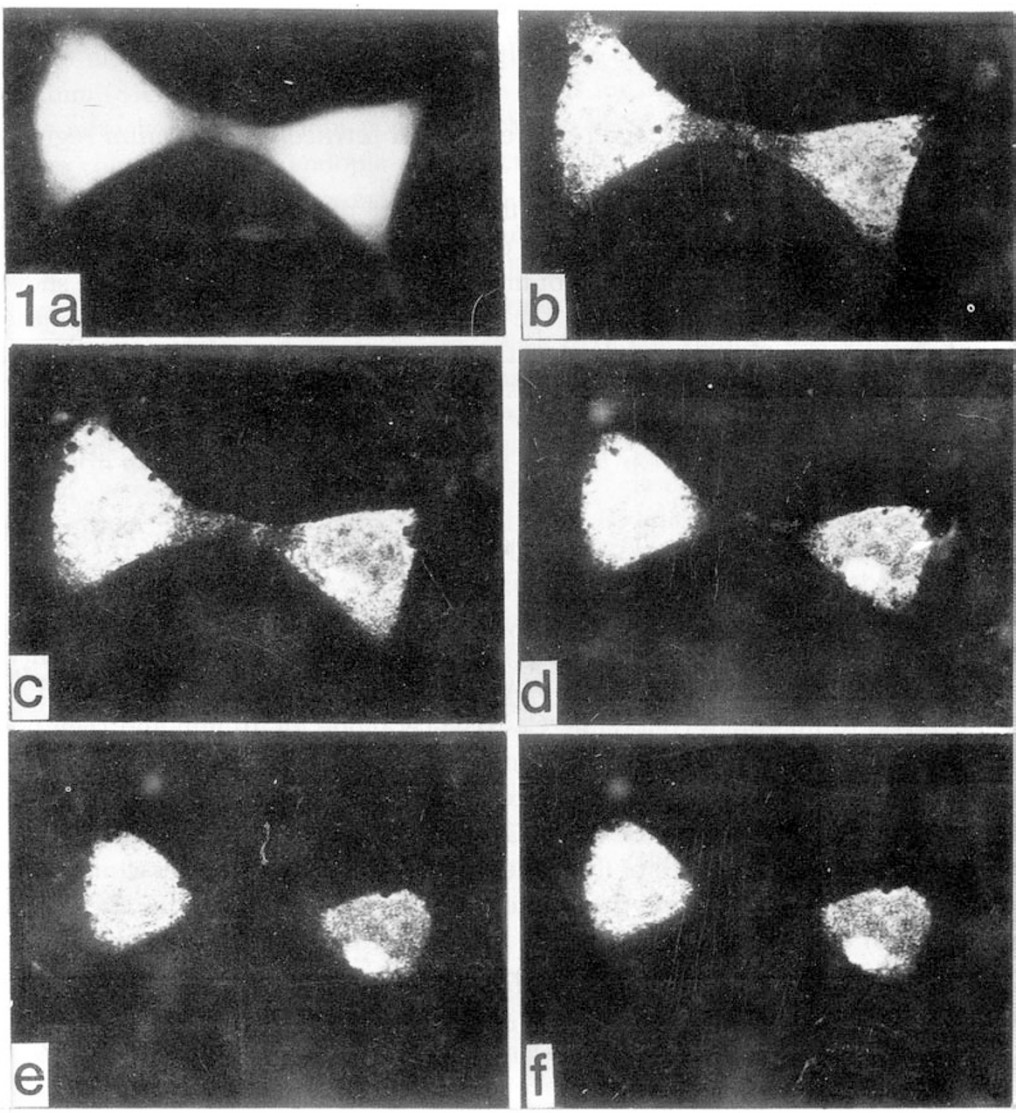

Fig 1. Fluo-3 fluorescence images of NIH3T3 cells. (a) The image was obtained without confocal micrscopy. (b-f) The LSCFM images of a serial optical sections from the matrix surface upwards. Some of the sections indicate a high-calcium perinuclear region.

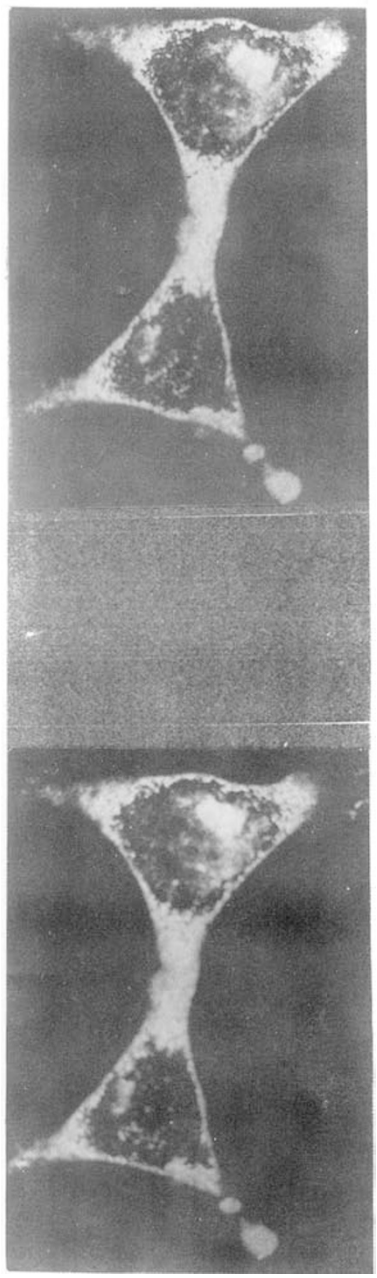

Fig 2. A stereo pair of photographs (should be observed under reflective steroscope), showing distribution of intracellular free calcium. NIH3T3 cells stained with fluo-3/AM.

High retention of cytosolic free calcium in Golgi lumen

The decrease of cytosol calcium was investigated by permeating the cells with 10 μM digitonin. The NIH3T3 cells were first loaded with 10 μM fluo-3/ AM, then exposed to a calcium-free medium containing 10 μM digitonin. Tab 1 showed the

changes of fluorescence in the Golgi and non-Golgi regions. It can be seen that the fluorescence in non-Golgi regions decreased 40 % more than that in the Golgi area, suggesting a high retention of free calcium concentrated in the lumen of the Golgi apparatus.

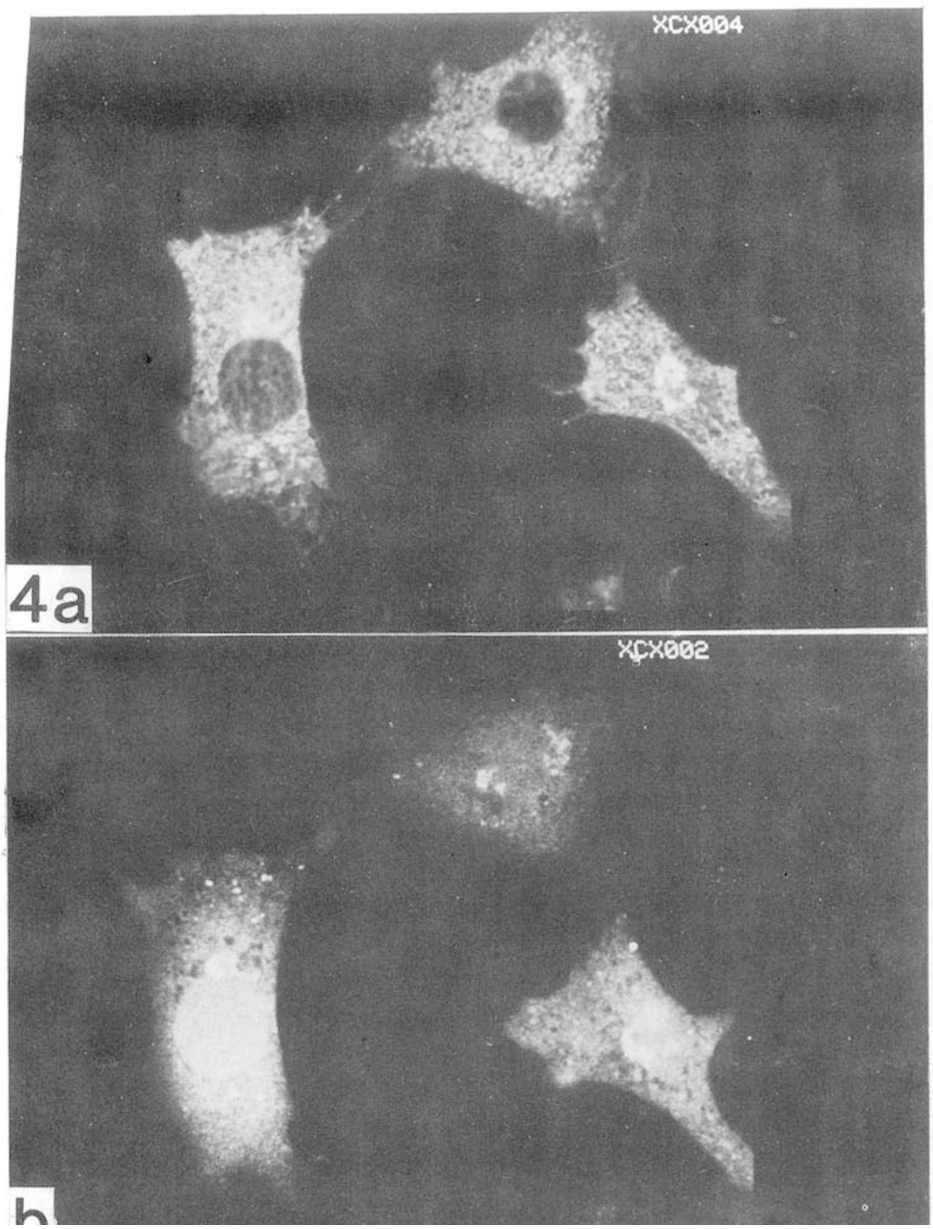

Fig 3. The LSFCM images of (a) fluo-3 fluorescence and (b) C_6-NBD-ceramide fluorescence of 2 different NIH3T3 cells.

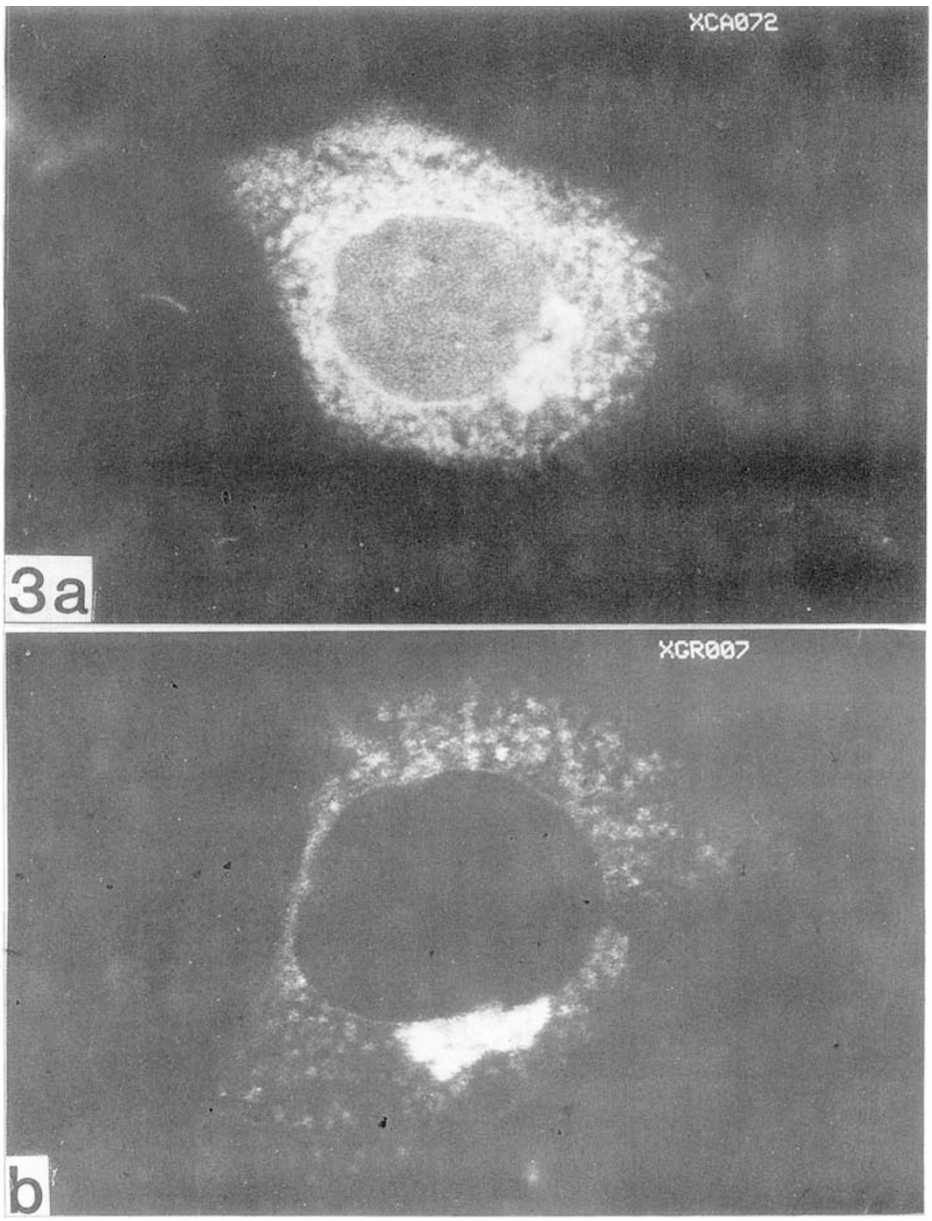

Fig 4. Co-localization of intracellular free calcium and Golgi apparatus in the same interphase NIH3T3 cell. (a) The fluorescence of C_6-NBD-ceramide for Golgi apparatus. (b) The fluorescence of fluo-3 for Ca^{2+}. The cell was first loaded with fluo-3/AM and imaged by LSCFM and then stained with C_6-NBD-ceramide. The image of C_6-NBD-ceramide was taken under the condition that the magnification of the signal was reduced to an absence of detectable fluo-3 fluorescence by decreasing the sensitivity of image detector.

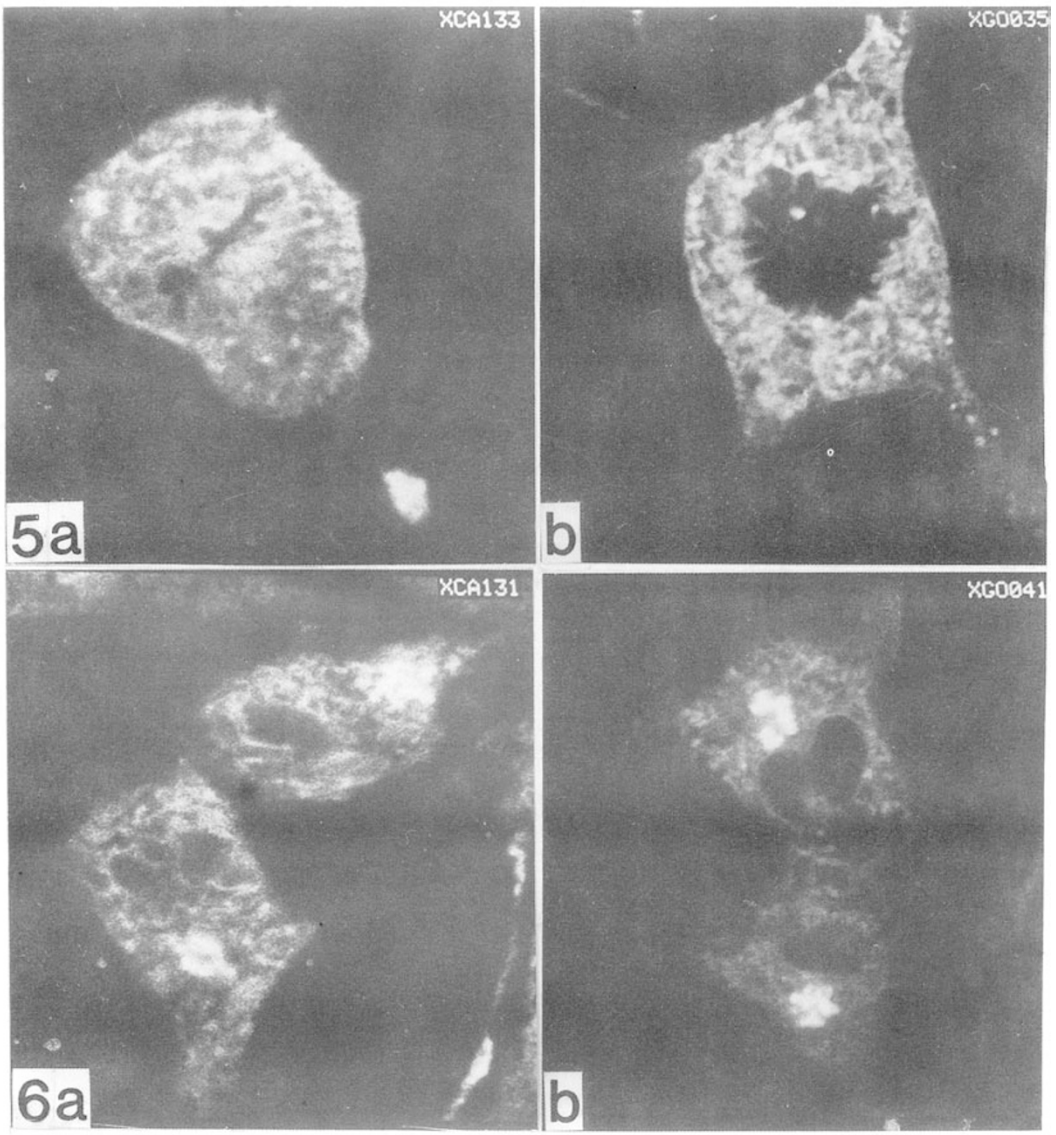

Fig 5. and Fig 6. The LSCFM images of fluo-3 fluorescence for intracellular free calcium (Fig 5a and Fig 6a) and corresponding C_6 - NBD-ceramide fluorescence revealing Golgi apparatus (Fig 5b and Fig 6b) of metaphase (Fig 5) and telophase (Fig 6) cells.

Tab 1. The fluo-3 fluorescence of the cells with or without 10 μM digitonin treatment

	Fluo-3 Fluorescence[a]	
	Golgi regions[b]	Non -Golgi region of cytosol[c]
Control cells	143.46	64.68
1min, 10 μM digitonin	92. 75	16.07
Fluorescence retention%	64.7%	24.8%

(a) Relative intensity of fluo-3 fluorescence.
(b) Total relative fluorescent intensity in Golgi regions of more than 30 cells in 1 image.
(c) Total relative fluorescent intensity in non-Golgi cytosol of more than 30 cells in 1 image.

DISCUSSION

It has been noticed that there is a high Ca^{2+} region at the perinuclear area of a cell through the use of different calcium fluorescent indicator and other new techniques[12-15]. However, as the objective of conventional microscopy was limited by its axial resolution, the image of a point source produced by a diffraction-limited optical system is not only periodic around the point of focus in the focal plane, but also periodic above and below the focal plane along the axis of the microscope (out-of-focus) [16]. It is difficult to determine whether the perinuclear high Ca^{2+} region is only a mask image since this area of the cell is thicker than that of any other regions. Since the Golgi apparatus is usually located at the perinuclear region and can be visualized with the fluorescent probe C_6-NBD-ceramide, it is possible to colocalize the high Ca^{2+} region and the Golgi apparatus in a single cell. Recently Chandra et al demonstrated that Golgi apparatus sequestrated a high concentration of total calcium with LSCFM and ion microscopy[8]. Wahl et al have compared the spatial distribution of free Ca^{2+} gradients with the subcellular distribution of cytoplasmic organelles using calcium probe fura-2 and organelle-specific fluorescent dyes and found the Golgi apparatus to be closely coincident with the highest concentration of $[Ca^{2+}]i$[21]. The present work confirmed these observations with LSCFM. The precise optical sections and the 3-D reconstruction images showed clearly the spatial distribution of the highest concentration of $[Ca^{2+}]i$ at perinuclear region in the living cell. Futher experiment for co-localization of the subcellular distribution of free calcium and Golgi apparatus with fluo-3/AM and C_6-NBD-ceramide showed that the highest concentration of $[Ca^{2+}]i$ was associated with the Golgi apparatus in the same interphase NIH3T3 cell.In every cell cycle phase the distributions of highest concentration of $[Ca^{2+}]i$ and the Golgi apparatus were identical (Fig 4 – 6). The results of digitonin experiment also revealed a prominent Ca^{2+} concentration in the Golgi lumen. Thus, it is the Golgi apparatus that concentrates a high amount of calcium ions.

Golgi apparatus concentrates high [Ca^{2+}]i

In the present study, we also reported a technique for co-localizing the intracellular free calcium and the Golgi apparatus using the calcium probe fluo-3/AM and a fluorescent lipid C_6-NBD-ceramide stains the Golgi apparatus prominently. C_6-NBD-ceramide can also label mitochondria and ER, but the fluorescence emanating from these structures was indistinguishable from the background after 45 min post-staining incubation[9]. Although both fluo-3 and C_6-NBD-ceramide can be excited at 488 nm and their fluorescence emission can not be separated by available barrier filter, the fluorescence of C_6-NBD-ceramide was much brighter than that of fluo-3. When the image of the cells preloaded with fluo-3/AM was taken, the magnification of the fluorescence signal was reduced by decreasing the sensitivity of the image detector so that the fluorescence of fluo-3 can not be detected. And then the cell was stained with C_6-NBD-ceramide. Under such a condition, there is no contribution of the fluo-3 fluorescence to the C_6-NBD-ceramide fluorescent images. Therefore, it is possible to co-localize both the [Ca^{2+}]i and the Golgi apparatus.

As for the subcellular storage of calcium in non-muscle system, besides mitochodria, it commonly attributed to the ER or calciosome. Besides containing high concentrations of Ca^{2+} , the [Ca^{2+}]i stores should contain at least 3 kinds of proteins: Ca^{2+}-ATPase, the low-affinity high-capacity calcium binding protein (such as calreticulin), and the calcium release channel (IP3 receptor et al). Although a number of experiments revealed that the ER or calciosome contained the IP3 receptor and the low-affinity high-capacity calcium binding protein[22], neither of them showing accurate localization with calcium. Several lines of indirect evidences also implicated the calcium store in the Golgi apparatus. It was demonstated that the Golgi apparatus presented a Ca^{2+} pump in human neutrophils[17] and lactating mammary epithelial cells[18 – 20]. Since the membrane traffic between ER and Golgi apparatus is a bidirectional one: in both forward and backword pathways [23], it is possible that the ER membrane protein, including calreticulin and IP3 receptor, can also be transferred from the ER to the Golgi membrane through transport vesicles. It is known that calcium plays an essential role in the function of Golgi apparatus, such as aggregation of proteins in sectetory vesicles[24] and exocytosis of calcium through secretory vesicles. Although whether Golgi apparatus is an adjustable [Ca^{2+}]i pool remains to be clarified, it is out of question that high calcium ions are concentrated in the Golgi apparatus.

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