Imaging of Calcium Release in Inositol 1,4,5-trisphosphate-sensitive Internal Stores in Permeabilized HSY Cells Using Fluorescent Indicators

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ABSTRACT. We described methods for imaging the IP3-induced Ca2+ release in Ca2+ storage organelles. IP3-induced changes in Ca2+ concentrations within Ca2+ stores ([Ca2+]L) in permeabilized HSY cells were monitored using the low affinity Ca2+ indicators, mag-fura-2 and mag-fura-red. The ratio images of mag-fura-2 were used to estimate the [Ca2+]L in the store. The apparent [Ca2+]L was 300–1000 μM at the cell periphery, whereas the [Ca2+]L in the cytoplasm around the nucleus was 70–150 μM. The [Ca2+]L throughout the cytoplasm was reduced by the application of 10 μM IP3 to 30–70 μM, and could be largely recovered after removal of IP3. The structure of IP3-sensitive Ca2+ stores was investigated by confocal microscopy using mag-fura-red. An IP3-induced increase in fluorescence was observed in the ER-like network and reticulum structures of the cytoplasm, and also in the nuclear envelope, suggesting that these organelles serve as IP3-sensitive Ca2+ stores. An analogous localization of the network and tubular elements of the ER was also demonstrated by electron microscopy. These observations suggest that these fluorescence techniques are useful to study the correlation between the distribution and function of Ca2+ stores.

Intracellular Ca2+ is an essential mediator of many cellular functions when cells are stimulated with hormones, growth factors, or neurotransmitters (1). In most non-excitable cells, agonist-induced Ca2+ signaling is initiated by a Ca2+ release from Ca2+ storage organelles through the inositol trisphosphate (IP3)-sensitive Ca2+ channel (2, 18). The endoplasmic reticulum (ER) is considered to be a primary source of IP3-releasable Ca2+ (22), although the nuclear envelope (21), the lysosome (9), and the secretory granules (4) have also been proposed to be Ca2+ stores in certain cell types.

Information about the distribution of Ca2+ stores is especially important, as the distribution has a direct relation to the spacial regulation of cytosolic Ca2+ concentration ([Ca2+]i). The structure and subcellular distributions of Ca2+ stores have been studied by determining the Ca2+ channels (IP3 receptors), Ca2+ pumps, or Ca2+ binding proteins using immunocytochemical approaches (18). These studies are based on the assumption that the distribution of the marker proteins is ultimately correlated to the distribution of Ca2+ stores. However, this assumption may not always be applicable.

Recently, fluorescent Ca2+ indicators, which accumulate in Ca2+ stores, have been used to monitor IP3-induced changes in Ca2+ concentration within the Ca2+ stores ([Ca2+]L) (6, 11, 25). The remarkable advantage of these methods is that the function of Ca2+ stores can be investigated directly with IP3-stimulation. These techniques have been used to investigate the mechanism of the quantal Ca2+ release and Ca2+ oscillation in permeabilized HSY cell, a continuous salivary ductal cell line from human parotid, and other cell types (3, 10, 23, 24, 26).

In the present study, these fluorescent techniques were extended to visualize functional Ca2+ stores. We estimated the [Ca2+]L and the distribution of IP3-sensitive Ca2+ stores using ratio images of mag-fura-red fluorescence in permeabilized HSY cells. Structures of the Ca2+ releasable organelles were visualized with confocal images of mag-fura-red. The latter methods were particularly useful to determine the function of individual IP3-sensitive Ca2+ stores in permeabilized cells. The advantage and applicability of these methods will also be discussed.
MATERIALS AND METHOD

Media
We used Hanks’ balanced salt solution with HEPES (HBSS-HB) containing 137 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl₂, 0.41 mM MgSO₄, 0.49 mM MgCl₂, 0.34 mM Na₂HPO₄, 0.44 mM NaH₂PO₄, 5.5 mM glucose, 20 mM HEPES (pH 7.4) and 1% bovine serum albumin (Sigma, St. Louis, USA). Intracellular-like medium (ICM) contained 125 mM KCl, 19 mM NaCl, 10 mM HEPES (pH 7.3 with KOH), 3 mM ATP, 1.4 mM MgCl₂, 330 μM CaCl₂ and 1 mM EGTA (free Ca²⁺ and Mg²⁺ concentrations were 50 nM and 0.1 mM, respectively).

Dye loading and permeabilization
HSY cells (28), a generous gift from Dr. Mitsunobu Sato (Tokushima University Japan), were cultured in a 50:50 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 medium supplemented with 10% newborn calf serum, 2 mM glutamine, and 100 μg/ml each of penicillin and streptomycin (all from Gibco, Gaithersburg, VA, USA) as previously described (16). Experiments were carried out on cells cultured for at least 36 h in a sample chamber precoated with fibronectin. Cells were loaded with mag-fura-2 or mag-fura-red (Molecular Probes, Eugene, OR USA) and permeabilized essentially as previously described (23). Briefly, cells were incubated with 8 μM mag-fura-2/AM for 25 min or with 8 μM mag-fura-red/AM for 45–60 min at 37°C in HBSS-HB in the dark. The dye-loaded HSY cells were washed with BSA-free HBSS-HB and Mg²⁺/ATP-free ICM, followed by exposure to 70 μg/ml (w/v) saponin (ICN, Cleveland, OH USA) in Mg²⁺/ATP-free ICM for 90 sec at room temperature. The permeabilized cells were washed with Mg²⁺/ATP-free ICM and then incubated in the complete ICM for at least 5 min to allow complete filling of their intracellular stores. All experiments were carried out at room temperature.

Mag-fura-2 fluorescence imaging
Mag-fura-2 fluorescence images were acquired using an ARGUS 5.0 imaging system (Hamamatsu Photonics, Shizuoka, Japan) coupled to a Nikon Diaphot microscope (TMD-300), equipped with a Nikon CFFluor 100 × objective. Saponin-permeabilized HSY cells were excited alternately at 344 nm and 360 nm at 1 sec interval. Images (8 frame summations, 8 bits/pixel) were acquired using emission centered at 510 nm (20 nm bandwidth) with a silicon intensifier target camera attached to the side port of the microscope. We used an excitation light reduced by a neutro-density filter, to minimize photobleaching. Free Ca²⁺ concentrations were calculated from mag-fura-2 fluorescence ratios according to the procedure of Grynkiewicz et al. (8). The fluorescence ratios (344/360) at saturating and zero free [Ca²⁺] (R_max and R_min, respectively) required for these calculations were determined from 5 μM mag-fura-2 solutions in Mg²⁺/ATP-free ICM containing 9 mM CaCl₂ or no added CaCl₂, respectively. The Kd value for Ca²⁺ used in this calculation is 53 μM (19). In our experiments, R_max and R_min of mag-fura-2 fluorescence ranged from 2.0 to 2.4 and from 0.3 to 0.4, respectively.

Confocal microscopy
Confocal microscopy was carried out using a Leica TCS 4D system (Leica, Heidelberg, Germany) equipped with a 40 × PL FLUOTAR objective. Confocal images of mag-fura-red fluorescence were obtained at an excitation wavelength of 488 nm and at an emission wavelength of above 665 nm, using a 57.5 μm pinhole (axial resolution is approximately 2 μm) or a 170 μm pinhole (axial resolution is approximately 5 μm). In order to minimize the photobleaching of mag-fura-red, the excitation beam was attenuated (approximately 90%) by passage through a 500 nm cut-off filter in some experiments (shown in Fig. 2E).

For the staining of mitochondria, intact HSY cells were incubated in culture medium containing 100 nM of MitoTracker green FM for 1 hr at 37°C. After the permeabilization, confocal images for MitoTracker-green (Molecular Probes) were obtained using excitation and emission wavelengths of 488 nm and above 530 nm, respectively.

Electron microscopy
Intact or saponin-permeabilized HSY cells were fixed with 2% paraformaldehyde-2% glutaraldehyde in 0.1 M cacodylate buffer, and post fixed with 1% OsO₄ in the same buffer. Preparations were dehydrated and embedded in Epon 812. Sections were cut with an ultramicrotome and stained using uranyl acetate and lead citrate, and observed with an electron microscope.

Data analysis
The image analysis was performed on a Macintosh Performa 6310 computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, Virginia, part number PB95-500195GEI). Unless otherwise stated, results shown are representative of at least 8 experiments carried out under identical experimental conditions and yielding the same results. Quantitative results are given as means ± S.E.

RESULTS
Imaging of IP₃-sensitive changes in [Ca²⁺]₅
A low affinity Ca²⁺-sensitive fluorescent probe, mag-fura-2 (Kd for Ca²⁺ approximately 53 μM) (19), accumulates in IP₃-sensitive Ca²⁺ stores and other organelles of HSY cell (23). The mag-fura-2 loaded HSY cells were permeabilized with saponin to monitor IP₃-dependent changes in [Ca²⁺]₅. The fluorescent image of the permeabilized HSY cells at 344 nm excitation (the isosbestic wavelength of mag-fura-2) is shown in Fig. 1A. This image indicates distributions of the compart-
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mentalized mag-fura-2. The darkened central area of cells corresponded to the nucleus. Changes in \([\text{Ca}^{2+}]_\text{L}\) within the IP\(_3\)-sensitive Ca\(^{2+}\) stores was analyzed by the sequential ratio imaging of the fluorescence. The apparent \([\text{Ca}^{2+}]_\text{L}\) in the non-stimulated cells was estimated to be 300 \(\mu\text{M}\) to over 1 mM at the cell periphery, whereas the \([\text{Ca}^{2+}]_\text{L}\) around the nucleus was 70-150 \(\mu\text{M}\) (n=135). The stimulation with IP\(_3\) (10 \(\mu\text{M}\)) decreased the ratio throughout the cells (Fig. 1 C), resulting in the apparent \([\text{Ca}^{2+}]_\text{L}\) of 30-70 \(\mu\text{M}\).

The effect of IP\(_3\) on fluorescence and the 344/360 ratio was illustrated in Fig. 1D. The ratio was decreased maximally within 2 min after application of IP\(_3\), and then recovered close to the unstimulated level after the removal of IP\(_3\). This recovery was due to the Ca\(^{2+}\) uptake via sarco/endoplasmic Ca\(^{2+}\)-ATPase (SERCA pump), since it was attenuated in the absence of Mg\(^{2+}\) (Fig. 1D) or the presence of thapsigargin, a microsomal Ca\(^{2+}\)-ATPase inhibitor (data not shown). This method provided information about the distribution of Ca\(^{2+}\).

![Fig. 1. The effect of IP\(_3\) on mag-fura-2 fluorescence in a permeabilized HSY cell. Mag-fura-2 loaded HSY cells were permeabilized by saponin and incubated in ICM at least for 5 min. The permeabilized cell was exposed to Mg\(^{2+}\)-free ICM and stimulated with 10 \(\mu\text{M}\) IP\(_3\), and then incubated in the complete ICM (without IP\(_3\)) as indicated in panel D. A pair of fluorescent images was acquired every 1 min with alternative excitation at 344 nm and 360 nm. A: Fluorescent image obtained with excitation at 344 nm just before the application of IP\(_3\). B: Ratio Image (344/360) just before the application of IP\(_3\). C: Ratio image at 6 min after application of 10 \(\mu\text{M}\) IP\(_3\). D: Changes in fluorescence with excitation at 344 nm (closed square) and 360 nm (open square) and the ratio (open circle). Scale bar: 10 \(\mu\text{m}\).](image1)

![Fig. 2. Distribution of IP\(_3\)-responsive organelles in a permeabilized HSY cell. Mag-fura-red loaded HSY cells were permeabilized with saponin, and confocal images were acquired using a 57.5 \(\mu\text{m}\) pinhole. The permeabilized cell was incubated in ICM at least for 5 min (A and C), and then exposed to 10 \(\mu\text{M}\) IP\(_3\) for 2 min (B and D). Images A and B are represented with different brightness in images C and D, respectively. The gray scale represents pixel values of mag-fura-red fluorescence images. Scale bar: 10 \(\mu\text{m}\). E: A single cell in ICM was stimulated with 10 \(\mu\text{M}\) IP\(_3\), and then washed with ICM. The fluorescent image of a permeabilized HSY cells was acquired every 17 sec with a 500 nm cut-off filter as described in Materials and Method. Changes in fluorescence intensity were determined from the sequential images and represented as arbitrary units.](image2)
stores, though the structure of individual organelles could not be resolved.

**Monitoring of IP$_3$-induced changes in [Ca$^{2+}$]$_L$ by confocal microscopy**

In order to visualize functions of individual Ca$^{2+}$ stores, we employed confocal laser scanning microscopy. In this study we used mag-fura-red (Kd for Ca$^{2+}$ approximately 55 μM) (29), because the excitation beam available in our system was 488 nm. As shown in Fig. 2A and B, the IP$_3$-induced changes in fluorescence were observed throughout the cytoplasm (small arrows), but not in the vesicles around the nucleus (Fig. 2C and D). The fluorescence in the cytoplasm was increased 1.35 ± 0.04 fold (mean ± S.E., n = 29) by the application of 10 μM IP$_3$. In the absence of IP$_3$, there was a small decrease of fluorescence (approximately 5%) probably due to photobleaching. Fig. 2E illustrates the change in fluorescence intensity obtained from sequential confocal images of a permeabilized HSY cell. This experiment was performed with a 500 nm cut-off filter to minimize photobleaching. The increase in mag-fura-red fluorescence correlated with the IP$_3$-induced Ca$^{2+}$ release, and then returned to the unstimulated level after removal of IP$_3$.

**Imaging of IP$_3$-sensitive Ca$^{2+}$ stores**

The fluorescence of mag-fura-red increases as a function of the decrease in Ca$^{2+}$ concentration. Thus, the fall in [Ca$^{2+}$]$_L$ in the stores can be observed as an increase in fluorescence. In order to reveal the structure of the IP$_3$-sensitive Ca$^{2+}$ store, confocal images with high-

![Fig. 3. Structure of IP$_3$-sensitive organelles in a permeabilized HSY cell. Permeabilized HSY cells were incubated in ICM for at least 5 min (A, D and G). Cells were then stimulated with 10 μM IP$_3$ for 2 min (B, E and H). The relative increase in fluorescence (C, F, and I) was calculated by dividing the image after the application of IP$_3$ (B, E and H) by the image before the application (A, D and G). The gray scale represents the ratio values of images C, F, and I. Images A and B were obtained using a 170 μm pinhole. A 57.5 μm pinhole was used in images D, E, G, and H. N; nucleus, arrows; IP$_3$-sensitive organelles, dashed line; cell border, scale bar; 5 μm.]
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Images were obtained before (A, D, and G) and after (B, E, and H) stimulation with 10 \(\mu\)M IP\(_3\). The relative increase in fluorescence is also demonstrated in images C, F, and I. Comparison of the two images of Fig. 3A and B shows that the IP\(_3\)-dependent increase in fluorescence occurred at the border between the nucleus and the cytoplasm (arrows in Fig. 3B), suggesting that \([\text{Ca}^{2+}]_L\) in the nuclear envelope is depleted by IP\(_3\). Also, a fine network (Fig. 3E and F) and reticulum structures (Fig. 3H and I), whose fluorescence was increased by applications of IP\(_3\), were observed in the cytoplasm. These findings strongly suggest that the nuclear envelope and the ER-like reticulum structure function as the IP\(_3\)-sensitive Ca\(^{2+}\) store. There were many IP\(_3\)-insensitive vesicles around the nucleus (Fig. 2C and D, and the large arrow in Fig. 2B). We found that similar vesicular structures were stained with a specific mitochondrial marker (17), MitoTracker green (data not shown), suggesting that they are probably mitochondria.

Ultrastructural observations of intact and permeabilized HSY cells

In order to determine the structure and the distribution of intracellular organelles, we observed the ultrastructures of the intact and saponin-permeabilized HSY cells (Fig. 4). The electron micrograph showed that free-ribosomes were almost completely eliminated after the permeabilization (Fig. 4B), suggesting that the soluble proteins in the cytosol had been removed in the permeabilized HSY cell. Nevertheless, the structure and distribution of the ER and the nuclear envelope were well preserved. The network and tubulo-vesicular elements of the ER were located predominantly at the cell periphery. ER fragments, the Golgi apparatus, and mitochondria were often found around the nucleus.

DISCUSSION

The main aim of the present study was to develop methods for studying the IP\(_3\)-induced Ca\(^{2+}\) release in a single Ca\(^{2+}\) storage organelle. We visualized the Ca\(^{2+}\) store by imaging compartmentalized mag-fura-red using a confocal microscope. The confocal images demonstrated that IP\(_3\) decreased \([\text{Ca}^{2+}]_L\) in the network and reticulum structures of the cytoplasm, as the mag-fura-red fluorescence in these structures was increased by IP\(_3\). These structures correlated with the network of rough ER and tubular elements of the smooth ER observed by electron microscopy. These observations are consistent with the generally accepted knowledge that the ER is the main Ca\(^{2+}\) storage organelle (18). It also appeared that Ca\(^{2+}\) was released from the nuclear envelope after IP\(_3\)-stimulation. The nuclear envelope has now been accepted as the IP\(_3\)-sensitive Ca\(^{2+}\) store (5, 14, 21). On the other hand, IP\(_3\) evoked little or no change in \([\text{Ca}^{2+}]_L\) in vesicular structures around the nucleus. These vesicles were most likely the mitochondria. Therefore, we conclude that the entire ER and the nuclear envelope, but not the mitochondria, serve as IP\(_3\)-sensitive Ca\(^{2+}\) stores in HSY cells. This result is quite consistent with the current knowledge with respect to the IP\(_3\)-sensitive Ca\(^{2+}\) storage organelles. Therefore, it is considered that the
methods described here are suitable to characterize functional Ca$^{2+}$ storage organelles in permeabilized cells.

The digital image analysis of compartmentalized fluorescent indicators has been performed in several cell types. Terasaki and Sardet (25) demonstrated the presence of Ca$^{2+}$ stores in the form of ER-like network structures in a flou-3 loaded sea urchin egg, using a conventional microscope. In a similar way, the distribution of IP$_3$-sensitive Ca$^{2+}$ stores has been investigated in AR4-2J cells (6), DDT$_3$MF-2 cells (20), BHK-21 cells (12) and rat pancreatic acinar cells (27). However, this method has not been as successful in showing the structure of the Ca$^{2+}$ stores in these mammalian cells. As we have shown here, the confocal image analysis, with a low affinity Ca$^{2+}$ indicator, is adequate to identify the intracellular organelles. Very recently, a similar confocal technique has been reported to demonstrate functional Ca$^{2+}$ stores in intact cells (7).

Although the conventional microscopy was not sufficient to demonstrate the Ca$^{2+}$ storage organelle, it was able to estimate [Ca$^{2+}$]$_L$ within the stores. The ratio image of mag-fura-2 loaded cells indicated that the apparent [Ca$^{2+}$]$_L$ in the cell periphery (300–1,000 nM) was considerably higher than that around the nucleus (70–150 nM). The confocal images and electron microscopy revealed that the cell periphery was predominantly composed of the ER including the network structure and tubular elements, whereas the area around the nucleus contained not only the ER but also the mitochondria and Golgi complex. Therefore, we assumed the difference in the apparent [Ca$^{2+}$]$_L$ was due to, at least in part, the distribution of the low [Ca$^{2+}$]$_L$ compartments, i.e., the mitochondria. On the other hand, the apparent [Ca$^{2+}$]$_L$ in the cell periphery corresponds to the [Ca$^{2+}$]$_L$ in the IP$_3$-sensitive Ca$^{2+}$ stores, since the ER is predominantly located in this area. In fact, the [Ca$^{2+}$]$_L$ in the cell periphery (300–1,000 nM) is reasonably close to the [Ca$^{2+}$]$_L$ in the IP$_3$-sensitive Ca$^{2+}$ store (approximately 700 nM) reported in our previous paper (23). The similar values have been estimated in BHK 21 cells (approximately 540 nM) with mag-fura-2 (13) and in HeLa cells (2 mM) with erAEQ, aequorin-chimera targeted to the ER (15). Although we attempted to estimate [Ca$^{2+}$]$_L$ in individual Ca$^{2+}$ stores with confocal images of mag-fura-red, it was not achieved in this study.

Immunocytochemical studies have provided significant information concerning the structure and subcellular distributions of Ca$^{2+}$ stores (18). The fluorescent techniques with Ca$^{2+}$ sensitive probes are particularly useful to investigate the functional characteristics of these Ca$^{2+}$ stores. The methods described here may provide additional information concerning the distributed nature and functional heterogeneity of Ca$^{2+}$ stores.

In conclusion, we have described methods to visualize Ca$^{2+}$ stores by monitoring dynamic changes in [Ca$^{2+}$]$_L$, using ratio images of mag-fura-2 and confocal images of mag-fura-red. The results indicate that the ratio imaging technique has an advantage for the quantitative analysis of [Ca$^{2+}$]$_L$, whereas the confocal images preferably characterize the function of a single Ca$^{2+}$ store.

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