Imaging of Quantal Calcium Release in the Inositol 1,4,5-trisphosphate-sensitive Organelles of Permeabilized HSY Cells

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ABSTRACT. The spatial characteristics of inositol 1,4,5-trisphosphate (IP_3)-induced quantal Ca^{2+} release were examined by imaging Ca^{2+} concentrations within Ca^{2+} stores ($[\text{Ca}^{2+}]_L$) in permeabilized HSY cells. The image of mag-fura-2 fluorescent ratio with dual excitation (344 nm/360 nm) demonstrated that a sequential application of different concentrations of IP_3 (0.1, 0.3, 10 μM) resulted in a stepwise decrease in the ratio at all regions of the cytoplasm. This change in the ratio suggests that the stepwise decrease in $[\text{Ca}^{2+}]_L$ is associated with the quantal Ca^{2+} release. To monitor the change in $[\text{Ca}^{2+}]_L$ within a single organelle, IP_3 -dependent changes in the mag-fura-red fluorescence of permeabilized cells were studied by confocal microscopy. Applications of increasing concentrations of IP_3 caused a stepwise increase in fluorescence within ER-like reticulum structures of the cytoplasm. This finding suggests that the $[\text{Ca}^{2+}]_L$ in a single Ca^{2+} store was not depleted by submaximal concentrations of IP_3 , and supports the steady-state model of quantal Ca^{2+} release.

Many physiological stimulations that activate phospholipase C cause increases in cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) through the production of inositol 1,4,5-trisphosphate (IP_3) (1). IP_3 is an intracellular messenger that mobilizes Ca^{2+} from the internal Ca^{2+} pool by opening a specific channel (IP_3 receptor) located on Ca^{2+} storage organelles such as the endoplasmic reticulum (ER) (4, 5, 11, 12) and the nuclear envelope (6, 23). Muallem *et al.* (17) reported an interesting observation concerning the nature of IP_3 -induced Ca^{2+} release. They found that application of submaximal concentrations of IP_3 to permeabilized cells resulted in the release of only part of the Ca^{2+} in the store. This phenomenon has now been observed in many cell types and referred to as “quantal” or “incremental” Ca^{2+} release (2, 15).

The mechanisms underlying quantal responses to IP_3 are unknown, although two major hypotheses known as “all-or-none model” (17) and “steady-state model” (10) have been proposed to explain this phenomenon. The all-or-none model is based on the view that only a fraction of the stores discharge their Ca^{2+} content at a submaximal IP_3 level. This model hypothesizes the pre-

sence of multiple intracellular Ca^{2+} stores with different sensitivities to IP_3 . On the other hand, the steady-state model proposes that all Ca^{2+} stores are depleted to some extent by submaximal concentrations of IP_3 , and that a decrease in $[\text{Ca}^{2+}]_L$ results in the closure of the IP_3 -sensitive Ca^{2+} channel. The two models have been examined in various laboratories (2, 3, 7, 13, 15, 22, 24), and continue to be the subject of considerable debate.

These two possible mechanisms for quantal Ca^{2+} release predict quite distinctive behavior in a single Ca^{2+} store, on the one hand all-or-none depletion of Ca^{2+} , and on the other hand, partial depletion by submaximal concentrations of IP_3 . To address this issue we have attempted to monitor the IP_3 -induced quantal Ca^{2+} release in individual Ca^{2+} stores. Recently, we have demonstrated techniques to visualize changes in Ca^{2+} concentrations within Ca^{2+} stores ($[\text{Ca}^{2+}]_L$) by low-affinity Ca^{2+} indicators, mag-fura-2 and mag-fura-red (23). Using these fluorescent techniques, we here demonstrate that a sequential application of increasing IP_3 concentrations causes a stepwise decrease in $[\text{Ca}^{2+}]_L$ in the endoplasmic reticulum in a saponin-permeabilized HSY cell. This result suggests that Ca^{2+} stores are not completely depleted by submaximal concentrations of IP_3 , as proposed by the steady-state model.

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Abbreviations: $[\text{Ca}^{2+}]_i$, cytosolic Ca^{2+} concentration; $[\text{Ca}^{2+}]_L$, Ca^{2+} concentrations within Ca^{2+} stores; HBSS-HB, Hanks's balanced salt solution with Hepes; ICM, intracellular-like medium; IP_3 , inositol 1,4,5-trisphosphate.

MATERIALS AND METHODS

Media

Hanks's balanced salt solution with Hepes (HBSS-HB) contained 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 Chemical Co., St. Louis, MO, 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 μ M CaCl₂ and 1 mM EGTA (free Ca²⁺ and Mg²⁺ concentrations 50 nM and 0.1 mM, respectively).

Cell culture

HSY cells were cultured in DMEM/F12 medium supplemented with 10% newborn calf serum, 2 mM glutamine, and 100 μ g/ml each of penicillin and streptomycin (all from Gibco, Gaithersburg, MD, USA) as previously described (16).

Dye loading and permeabilization

Experiments were carried out on cells cultured for at least 3 days in a sample chamber precoated with fibronectin. Cells were loaded with mag-fura-2 or mag-fura-red and permeabilized essentially as previously described (24). Briefly, cells were incubated with 8 μ M mag-fura-2/AM or mag-fura-red/AM (Molecular Probes, Eugene, OR, USA) in HBSS-HB for 30–60 min at 37°C 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 μ g/ml (w/v) saponin (ICN, Cleveland, OH, USA) in Mg²⁺/ATP-free ICM for 90 sec. The permeabilized cells were washed with Mg²⁺/ATP-free ICM and then incubated in 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 with dual excitation (344 nm and 360 nm) were acquired at an emission wavelength of 500–530 nm using an ARGUS 50 imaging system (Hamamatsu Photonics, Shizuoka, Japan) coupled to an inverted microscope (Diaphot: Nikon Inc., Tokyo, Japan), equipped with a Nikon CF Fluor 40 $\times$ or 100 $\times$ objective.

Confocal microscopy

Confocal microscopy was carried out using a Leica TCS 4D system (Leica, Heidelberg, Germany) equipped with a 40 $\times$ PL FLUOTAR objective. Confocal images of mag-fura-red fluorescence were obtained by excitation with the lowest laser energy of a 488 nm excitation beam and at an emission wavelength of above 515 nm. Experiments were carried out with two different confocal set-ups. To visualize organelles, five serial images with a 57.5 μ m pinhole (axial resolution, $\sim$ 2 μ m) were acquired at the time of interest. The noise level of each image was reduced by averaging of the values of 8 consecutive

line scanings. To study the time course, images (4 line scanning average) were acquired every 17 sec with a 170 μ m pinhole (axial resolution, $\sim$ 5 μ m). In this experiment, the excitation beam was further reduced to $\sim$ 10% by passage through a 500 nm cut-off filter to minimize for photobleaching.

Data analysis

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).

RESULTS AND DISCUSSIONS

Imaging of IP₃-induced quantal Ca²⁺ release with mag-fura-2

It has been shown that the low affinity Ca²⁺-sensitive fluorescent dye, mag-fura-2 (K_d for Ca²⁺ $\sim$ 53 μ M) (20), accumulates in IP₃-sensitive stores under appropriate loading conditions and can be used to monitor [Ca²⁺]_L of permeabilized cells (7, 9, 24, 25). We studied IP₃-induced quantal Ca²⁺ release by monitoring the change in [Ca²⁺]_L on sequential ratio images (344 nm/360 nm) of mag-fura-2 fluorescence in saponin-permeabilized HSY cells. Fig. 1A demonstrates that sequential application of increasing IP₃ concentrations resulted in a stepwise decrease of the mag-fura-2 ratio. This stepwise pattern in [Ca²⁺]_L decrease is characteristic of quantal Ca²⁺ release, as previously indicated using the same cell type (24).

The question is whether the quantal Ca²⁺ release reflects an all-or-none depletion of Ca²⁺ at specific subcellular compartments by different concentrations of IP₃ or a stepwise decrease in [Ca²⁺]_L within the same subcellular region. Fig. 1B–E shows pseudocolor images of the ratio at the time indicated in Fig. 1A. In non-stimulated cells (Fig. 1B), the apparent [Ca²⁺]_L at the cell periphery was higher than that around the nucleus. Our previous study indicates that the apparent [Ca²⁺]_L around the nucleus is underestimated by the fluorescence in the mitochondria (23).

As shown in the Fig. 1A (trace I and II) the stepwise pattern was observed at all subcellular regions of the permeabilized cell. Stimulation with 0.1 μ M IP₃ decreased the ratio throughout the cell (Fig. 1C), and the decreased level remained unchanged until the next increase in IP₃ concentration. Subsequent applications of 0.3 μ M (Fig. 1D) and 10 μ M IP₃ (Fig. 1E) elicited further decreases in the ratio at all subcellular regions. This experiment suggests that the increasing concentrations of IP₃ elicited a stepwise decrease in [Ca²⁺]_L in all subcellular regions. These observations are consistent with

Fig. 1. The effect of different concentrations of IP₃ on mag-fura-2 fluorescence in a permeabilized HSY cell. Mag-fura-2-loaded HSY cells were permeabilized with saponin and incubated in ICM at least for 5 min. The permeabilized cell was exposed to Mg²⁺-free ICM and stimulated sequentially with 0.1 μM, 0.3 μM, and then 10 μM IP₃ as indicated in panel A. A pair of fluorescent images was acquired every min with alternative excitation at 344 nm and 360 nm, and the ratio (344/360) was calculated. A: Change in the fluorescence ratio in the area depicted in panel B. The labels b, c, d, and e indicate the times the images B, C, D, and E, respectively, were obtained. B: Ratio image obtained in Mg²⁺-free ICM. C: Ratio image obtained 8 min after the application of 0.1 μM IP₃. D: Ratio image obtained 7 min after application of 0.3 μM IP₃. E: Ratio image obtained 2 min after application of 10 μM IP₃. A representative of 8 independent experiments. The color scale represents pixel values of the mag-fura-2 fluorescence ratio. Scale bar: 10 μm.

the view that quantal Ca^{2+} release is associated with the partial depletion of Ca^{2+} stores by submaximal concentrations of IP₃, as proposed in the steady-state model. Short *et al.* (21) and van de Put and Elliott (25) have reported a similar IP₃-induced stepwise decrease in $[\text{Ca}^{2+}]_L$ in permeabilized DDT₁MF-2 cells and permeabilized pancreatic acinar cells. The imaging technique, using conventional microscopy, identifies the regions of the cell responding to IP₃, but does not allow one to distinguish the specific organelles involved. Thus, we next employed confocal microscopy to visualize the subcellular structures of the IP₃-sensitive Ca^{2+} stores

Monitoring of $[\text{Ca}^{2+}]_L$ with confocal microscopy

In confocal microscopy we used another Ca^{2+} indicator, mag-fura-red (Kd for Ca^{2+} ~55 μM) (27), because the excitation beam available in our system was 488 nm. As indicated in Fig. 2, the sequential application of IP₃ (0.1, 0.3, 10 μM) elicited a stepwise increase in fluores-

cence, confirming that quantal Ca^{2+} release is observed in the mag-fura-red loaded HSY cell. This experiment indicated that Ca^{2+} release with 0.1, 0.3, and 10 μM IP₃ reached the steady-state phase within 5 min, 2 min, and 1 min, respectively.

Quantal Ca^{2+} release in the endoplasmic reticulum-like organelles

In order to study IP₃-induced Ca^{2+} release in a single Ca^{2+} store, the mag-fura-red images were acquired at 30 sec before application of IP₃ (Fig. 3A and E), 7 min after exposure to 0.1 μM IP₃ (Fig. 3B and F), 4 min after exposure to 0.3 μM IP₃ (Fig. 3C and G), and 2 min after exposure to 10 μM IP₃ (Fig. 3D and H). The IP₃-dependent increase in fluorescence was seen in the ER-like reticulum structures in the cytoplasm (small arrows). The stimulation with 0.1 μM IP₃ slightly increased the fluorescence in the ER-like organelles (Fig. 3B and F), and subsequent applications of 0.3 μM and

Fig. 2. Effects of a sequential application of IP_3 on mag-fura-red fluorescence in a permeabilized HSY cell. Mag-fura-red loaded HSY cells were permeabilized with saponin and incubated in ICM and Mg^{2+} -free ICM as described in Fig. 1. The permeabilized cell was stimulated sequentially with 0.1 μM , 0.3 μM , and then 10 μM IP_3 as indicated at the top of the panel. The change in fluorescence intensity in a permeabilized cell was determined from the sequential image and is represented as the average pixel value for the whole cell. Images were acquired every 17 sec with a 500 nm cut-off filter to minimize photobleaching (Materials and Methods). The result is representative of 10 independent experiments.

10 μM IP_3 resulted in further increases in fluorescence within the same organelles. This stepwise change in fluorescence was observed in most of the IP_3 -sensitive ER-like organelles, though this pattern was not clear in some organelles.

Fig. 3I shows the change in the fluorescence intensity in the IP_3 -sensitive ER-like organelle depicted in Fig. 3E (dashed line). The increases in fluorescence evoked by submaximal concentrations of IP_3 (0.1 and 0.3 μM) were normalized by dividing them by the maximal increase achieved with 10 μM IP_3 (Fig. 3I). This normalization was carried out at 63 randomly selected organelles in 25 cells. The histogram represents the number of Ca^{2+} stores (Y axis) plotted as a function of the normalized increase (X axis) in the fluorescence of the store (Fig. 4A and B). With 0.1 μM IP_3 , 46 out of 63 organelles increased their fluorescence to 10–60% of the maximal response, indicating that these organelles were not completely depleted of Ca^{2+} even after exposure to 0.1 μM IP_3 for as long as 7 min. As shown in Figs. 1E and 2, a 7 min incubation is sufficient to decrease the $[\text{Ca}^{2+}]_{\text{L}}$ to a steady-state level. Application of 0.3 μM IP_3 elicited a further decrease in $[\text{Ca}^{2+}]_{\text{L}}$ of these organelles, but did not completely deplete the Ca^{2+} of these stores. The average value of the normalized increase with 0.1 μM and 0.3 μM IP_3 was $26.3 \pm 3.1\%$ (Mean $\pm$ S.E., $n=63$) and $57.0 \pm 3.2\%$, respectively.

There was little or no change in fluorescence ($<10\%$) when the cells were incubated without IP_3 (Fig. 4A, insert).

Mechanism for quantal Ca^{2+} release

Using confocal microscopy it was demonstrated that the ER-like organelle released Ca^{2+} after application of IP_3 at concentrations of 0.1 or 0.3 μM , and that a following application of the maximum amount of IP_3 (10 μM) resulted in a further decrease in $[\text{Ca}^{2+}]_{\text{L}}$ in the same organelle. Consistent with this observation, sequential ratio images of mag-fura-2 revealed a stepwise decrease in $[\text{Ca}^{2+}]_{\text{L}}$ with increasing IP_3 concentrations in all subcellular regions of the permeabilized HSY cells. These results strongly suggest that the $[\text{Ca}^{2+}]_{\text{L}}$ in these organelles is only partially depleted by submaximal IP_3 concentrations.

The steady-state model explains that the quantal Ca^{2+} release is caused by an attenuation of Ca^{2+} release before complete depletion of the stored Ca^{2+} (10). The present results are consistent with the steady-state model, since this model predicts the partial depletion of a single Ca^{2+} store with submaximal concentrations of IP_3 . Several reports have suggested that $[\text{Ca}^{2+}]_{\text{L}}$ modulates the sensitivity of the Ca^{2+} store (14, 18, 19). It has also been shown that the permeability of the IP_3 -sensitive Ca^{2+} channel decreases with decreasing $[\text{Ca}^{2+}]_{\text{L}}$ in single HSY cells (24). The present imaging experiment provides additional support for the steady-state mechanism in HSY cells.

On the other hand, the all-or-none model anticipates that submaximal concentrations of IP_3 discharge Ca^{2+} from a fraction of the Ca^{2+} stores (17). However, submaximal levels of IP_3 do not deplete Ca^{2+} in most of the IP_3 -sensitive ER-like organelles, and are so at variance with one of the tenets of the all-or-none model. All-or-none type depletion may still occur in a few organelles, but it is unlikely that this type of mechanism plays a major role in the quantal Ca^{2+} release in HSY cells.

Heterogeneity of Ca^{2+} stores

Our observations have shown that most of the IP_3 -sensitive Ca^{2+} stores released Ca^{2+} with 0.1 μM IP_3 , while some stores did not respond to this IP_3 concentration. This apparent heterogeneity of the Ca^{2+} stores suggests that there are differences in the sensitivity to IP_3 in the Ca^{2+} stores. The partial depletion of Ca^{2+} stores explains the quantal release in the permeabilized HSY cells. It is also possible, however, that heterogeneous Ca^{2+} stores contribute to the quantal Ca^{2+} release at low IP_3 concentrations. It was not possible to determine the mechanism giving rise to the heterogeneity, but it is possible that a compartmentalization of ER-structures occurs after the permeabilization and that this causes the heterogeneity of the Ca^{2+} stores. Differ-

Fig. 3. IP_3 -induced quantal release of Ca^{2+} from ER-like organelles in a permeabilized HSY cell. Mag-fura-red loaded HSY cells were permeabilized, incubated in ICM, and then Mg^{2+} -free ICM as described in Fig. 1. High-resolution confocal images were acquired using a 57.5 μm pinhole. (A) Image obtained at 30 sec before application of IP_3 and (E) higher magnification of the area indicated in panel A. (B) Image obtained 7 min after the application of 0.1 μM IP_3 and (F) its higher magnification. (C) Image obtained 4 min after the application of 0.3 μM IP_3 and (G) its higher magnification. (D) Image obtained 2 min after the application of 10 μM IP_3 and (H) its higher magnification. The gray scale represents pixel values of mag-fura-red fluorescence. Scale bar: 5 μm . ER-like IP_3 -sensitive organelles (small arrows) and IP_3 -insensitive organelles (large arrows) are shown. A representative image of 25 permeabilized cells from 18 independent experiments. I: Changes in fluorescence intensity in an ER-like organelle. The average of the fluorescence intensity within the selected area, which is indicated with a dashed line in panel E and the same areas in Fig. F, G, and H, is plotted against the IP_3 concentration. The changes in fluorescence intensities with 0.1 μM and 0.3 μM IP_3 stimulations were normalized against the fluorescence level before stimulation (0%) and after 10 μM IP_3 stimulation (100%). A representative of 63 ER-like organelles from 18 independent experiments.

Fig. 4. Effect of submaximal concentrations of IP₃ on the mag-fura-red fluorescence in ER-like organelles. IP₃-induced changes in fluorescence were quantified, and normalized in 63 organelles as described in Fig. 3I. The histogram indicates the number of ER-like organelles plotted as a function of the normalized increase in fluorescence with 0.1 μM IP₃ (A) and 0.3 μM IP₃ (B). In ER-like organelles, the fluorescence intensity increased 1.45 ± 0.03 ($n=63$) fold after the application of 10 μM IP₃. In the control experiment (A, insert), changes in fluorescence in the ER-like organelle were examined at the beginning, 3–7 min after incubation without IP₃, and 2 min after stimulation with 10 μM IP₃. The relative fluorescence after incubation without IP₃ was 0.99 ± 0.01 ($n=18$).

ent subtypes and alternative splicing of the IP₃ receptors (1, 8, 26) or different densities of IP₃ receptors (7) may also be reasons for the heterogeneity. Alternatively, if there is variability in Ca²⁺ loading of these stores, the sensitivity of the IP₃ receptors would be modulated by the [Ca²⁺]_L and cause heterogeneity in the Ca²⁺ stores (14, 18, 19, 24). Heterogeneity of Ca²⁺ stores is necessary for the all-or-none model to hold, but this would not be contrary to the steady-state model.

In conclusion, IP₃-induced quantal release of Ca²⁺ was observed by monitoring [Ca²⁺]_L using imaging techniques. The result suggests that submaximal concentrations of IP₃ partially deplete Ca²⁺ within an ER-like organelle of the permeabilized HSY cells. This findings is consistent with the steady-state model for quantal Ca²⁺ release.

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