Visualization of Cytosolic Free Calcium Distribution and Mobilization in Guinea Pig Gastric Chief Cells

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ABSTRACT. Distribution and temporal change of free calcium concentration ([Ca\(^{2+}\)]\(_{i}\)) in single guinea pig gastric chief cells were visualized by a digital imaging microscope equipped with a microspectrofluorometer. The distribution was not homogeneous; a higher [Ca\(^{2+}\)] area was often localized in some restricted regions of the endoplasm and also at the peripheral cytoplasm just beneath the plasma membrane. When stimulated with cholecystokinin, [Ca\(^{2+}\)], increased transiently in the apical peripheral cytoplasm and in the endoplasmic regions. This Ca\(^{2+}\) mobilization which precedes the biphasic pepsinogen secretion was composed of a rapid Ca\(^{2+}\) release from the intracellular store(s) as well as a rapid and a more sustained Ca\(^{2+}\) entry from the extracellular space.

Ca\(^{2+}\) plays key roles in stimulus-secretion coupling of various exocrine and endocrine cells (22, 23). Pepsinogen secretion from gastric chief cells is also regulated by Ca\(^{2+}\) following the stimulation of either a peptidergic (cholecystokinin) or a cholinergic pathway (2, 7, 15, 18), and it has been suggested that the Ca\(^{2+}\) mobilization is composed of three components; a rapid mobilization of internal Ca\(^{2+}\), a rapid Ca\(^{2+}\) entry from the extracellular space and a more prolonged Ca\(^{2+}\) entry from the extracellular space (2, 7, 15, 18). In all these studies gastric glands or isolated chief cell suspension previously loaded with either fura-2, quin2 or aequorin have been analysed by conventional fluorescence or luminescence spectrophotometers and the results obtained have been interpreted assuming that the cells respond homogeneously. Gastric glands, however, consist of several types of cells, such as parietal cells, mucous neck cells, chief cells and some unidentified or unidentified mucosal cells (10). In order to clarify the Ca\(^{2+}\) mobilization in the chief cells, it is necessary to examine more directly the spatial distribution and temporal change of free calcium concentration, [Ca\(^{2+}\)]\(_i\), in individual chief cells in intact gastric glands.

Recently [Ca\(^{2+}\)]\(_i\) in single cells has been analysed by a microspectrofluorometer and visualized using a digital imaging microscope (6, 8, 11, 21). We have applied these techniques to the chief cells in guinea pig gastric glands. Data of the single cells obtained by digital imaging microscopy and microspectrofluorometry were compared with those on the cell suspension obtained by conventional fluorescence and luminescence spectrophotometry.

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MATERIALS AND METHODS

Preparation of gastric glands and chief cells. Gastric glands were prepared from guinea pig (Hartley, male, 250 g) as described previously (12, 17). Gastric mucosal cells were dissociated from the glands and fractionated by Percoll density gradient ultracentrifugation (30,000 × g for 15 min at 4°C) (15, 18). A fraction enriched in chief cells was recovered at a density region from 1.062 to 1.077 g/ml.

Fura-2 loading. Chief cells (10^7) were suspended in 10 ml of a tissue culture medium, Medium-199 (Nissui, Japan), containing 10 mM Mops and 25 mM NaHCO3 (pH 7.4), and were loaded with fura-2 by incubating the cell suspension with 20 µl of fura-2 acetoxymethyl ester (1 mM stock solution, Dojindo, Japan) and 10 µl of 25% W/W Pluronic F-127 (BASF Wyandotte, USA). After constant gassing with 95% O2/5% CO2 for 15 min at 37°C, the cells were rinsed, resuspended and kept at 4°C in the fresh Medium-199 till just before use.

Digital imaging microscopy and microspectrofluorometry. Digital imaging microscopy and microfluorometry were carried out as described by several authors (6, 8, 11, 21) with some modifications (20). For digital imaging microscopy, fura-2-loaded glandular chief cells (population, 40%; viability, 97.5%; 10^6 cells/2.5 ml) in the Medium-199 were layered on a thin cover-glass slip (thickness < 0.12 mm) previously coated with adhesive protein, Cell Tak (Cosmo-Bio, Japan), and mounted in a Rose chamber (Ikemoto, Japan). The chamber was put on a microscopic stage with temperature control unit kept at 37°C.

Fluorescence spectrophotometry. For fluorescence spectrophotometry, fura-2-loaded isolated chief cells (population, 85%; viability, 90%; 5 × 10^5 cells/0.5 ml) in Mops-Tyrode's solution were transferred to a cuvette without adding CaCl2. CaCl2 or EGTA was added at a final concentration of 1 mM, 5 min and 1 min just before assay, respectively. The fluorescence intensity was read on a CAF 100 dual-spectrophotometer (Japan Spectroscopic Co., Japan) equipped with automatic cuvette stirrer and temperature control unit kept at 37°C. Emission wavelength was 500 nm, while the excitation wavelength was 340 nm/380 nm for the ratio method or was kept at 380 nm or 340 nm for the continuous measurement. [Ca2+]i was calibrated by the method of Grynkiewicz et al. (3).

Luminescence spectrophotometry. For luminescence spectrophotometry, aequorin-loaded isolated chief cells (10^6 cells/ml) were transferred to a cuvette. The luminescence read on a Platelet Ionized Calcium Aggregometer (PICA, Chrono Log, U.S.A) equipped to allow for continuous stirring of the sample kept at 37°C. Aequorin loading using dimethylsulfoxide was described in (5, 24) as modified by us (16, 19). Aequorin bioluminescence was expressed by an arbitrary unit and was not calibrated in terms of [Ca2+]i.

Pepsinogen measurement. Pepsinogen released to the medium and stocked in the cells was measured by the method of Anson and Mirsky (1) as modified by us (15, 18). Optical density at 640 nm was read using tyrosine as a standard. Pepsinogen secretion was expressed as a percentage of total pepsinogen activity present in the cells plus in the medium.

Electron microscopy. Gastric glands were fixed in 2.5% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h and postfixed in 1% (wt/vol) osmium tetroxide for 1 h at 24°C. The sample was dehydrated in graded alcohol solutions and the ultrathin sections were observed under a JEM 100 C electron microscope (JEOL, Japan).

Materials. All chemicals and instruments used have been described in our previous papers (12–20) unless otherwise noted.

RESULTS

Fig. 1 shows electron micrographs of typical gastric glands (A) and chief cells (B).
Fig. 1. Electron micrographs of guinea pig gastric glands (A) and chief cells (B). In Fig. 1A, C and P indicate chief cells and parietal cells, respectively. In Fig. 1B, LS and BS indicate luminal (apical) and basolateral surfaces of the chief cells, and G and N indicate secretory granules and nucleus, respectively. Fig. 1A; ×3,000, Fig. 1B; ×6,400.

Fig. 2. Three dimensional computer graphics of $\left[Ca^{2+}\right]_i$ distribution in a chief cell of the gastric glands (A) and pixel intensity of the cell after stimulation with cholecystokinin (CCK) (10^{-8} M) (B). The diameter of the chief cell was 10–12 μm. The luminal and basolateral surfaces of the chief cell were indicated by LS and BS, respectively. Pixel intensities of 30, 40, 50, 60, 70, and 80 correspond to $\left[Ca^{2+}\right]_i$ of 35, 62.5, 90, 110, 130, and 150 nM, respectively.
The chief cells present in the terminal portion of the gastric gland such as indicated by an arrow with star in Fig. 1A were analysed by digital imaging microscopy. The chief cells contain a number of pepsinogen granules and clearly showed polarized structure with luminal (apical) and basolateral cells surfaces (Fig. 1B).

Fig. 2A shows the distribution of [Ca^{2+}] in a resting single chief cell. Comparing Fig. 2A with the electron micrographs of chief cells (Fig. 1B), the plasma membrane domains, presumably corresponding to the luminal (apical) and basolateral cell surface were estimated and indicated by LS and BS as shown in Fig. 2A, respectively. In the resting chief cells, [Ca^{2+}] distribution was non-unimodal and a higher [Ca^{2+}] area was localized at the peripheral cytoplasm increasing towards the plasma membrane and often in some restricted regions of the endoplasm.

Fig. 2B (cholecystokinin 0 sec) shows pixel for pixel mapping of a single chief cell

![Fig. 2B](image-url)

**Fig. 2B.** Pixel for pixel mapping of a single chief cell.

**Fig. 3.** Three dimensional computer graphics (A) and histogram (B) of [Ca^{2+}] distribution in an isolated chief cell and their changes after stimulation with cholecystokinin (10^{-8} M). N: frequency. In Fig. 3A, domain "a" is presumable an apical surface and its [Ca^{2+}], was about 75 nM. Domain "b" is an endoplasm and its [Ca^{2+}], was about 50 nM. After stimulation with CCK, [Ca^{2+}], increased up to 200 nM in both areas.
shown in Fig. 2A. When the cells were stimulated with cholecystokinin (10$^{-8}$ M), the pixel intensity increased both at the endoplasmic regions (enclosed by a circle) and at the apical cytoplasm just beneath the luminal plasma membrane (30 sec), and even after 60 sec the pixel intensity at the apical cytoplasm was higher than that of the rest of the cytoplasm.

Fig. 3A shows three dimensional computer graphics of [Ca$^{2+}$]$_i$ distribution in an isolated single chief cell before and after cholecystokinin (10$^{-8}$ M) stimulation.

Fig. 4. Ca$^{2+}$ mobilization (A, B, C) and pepsinogen secretion (D) in the chief cells. Temporal change of [Ca$^{2+}$]$_i$ in a chief cell after stimulation with CCK was measured by microspectrofluorometry of individual chief cells previously loaded with fura-2 (A) or by fluorescence spectrophotometry of the chief cell suspension previously loaded with fura-2 (B) or by luminescence spectrophotometry with aequorin (C), in the presence and absence of Ca$^{2+}$. Pepsinogen secretion from the chief cells was stimulated by cholecystokinin in the presence and absence of Ca$^{2+}$. All data including Figs. 2 and 3 are typical representatives of at least three to thirteen experiments. When the experiments were to be carried out in a Ca$^{2+}$-free medium, CaCl$_2$ was omitted from the MOPS-Tyrodes solution and 1 mM EDTA was added just before measurements. The resting [Ca$^{2+}$]$_i$ in cell populations measured by fura-2 (260.2±27.8 nM, mean±SEM, n=6) was somewhat higher than that in single cells. Peak [Ca$^{2+}$]$_i$ induced by CCK was about 510 nM in the presence of Ca$^{2+}$.
Before stimulation (CCK 0 sec), average level of \([\text{Ca}^{2+}]_i\) over the cytoplasm in the individual chief cells was 50.1 nM (fluorescence ratio = 0.35 ± 0.037, mean ± SEM, n = 11). There were again two high \([\text{Ca}^{2+}]_i\) areas similar to Fig. 2A. After stimulation (30 sec), an increase in \([\text{Ca}^{2+}]_i\) was observed in the peripheral cytoplasm just beneath the apical plasma membrane and in endoplasmic regions, as indicated by arrows "a" (75 nM to 200 nM) and "b" (50 nM to 200 nM) in Fig. 3A, respectively. In the former domain "a" the \([\text{Ca}^{2+}]_i\) level was kept at a high level even after 60 sec.

Histogram of \([\text{Ca}^{2+}]_i\) distribution in the same chief cell is shown in Fig. 3B. Each pixel intensity was multiplied 1.25-fold compared to that of Fig. 2B (enhanced data). When treated with cholecystokinin, distribution of pixel intensity clearly shifted rightwards up to 200, reflecting a marked increase in \([\text{Ca}^{2+}]_i\).

Time course of a cholecystokinin-induced \(\text{Ca}^{2+}\) mobilization in a single chief cell was chased by the microspectrofluorometer attached to the digital imaging microscope, which is composed of a photomultiplier tube, a turret pin-hole and a photometric controller unit. A pin-hole was placed in the image plane in front of the photomultiplier tube to limit the field of view to an area of 10 μm in diameter. As shown in Fig. 4A, \(\text{Ca}^{2+}\) mobilization is composed of two components showing a high, fast and more global rise of \([\text{Ca}^{2+}]_i\) and a prolonged small elevation of \([\text{Ca}^{2+}]_i\) showing some oscillatory modes. Addition of \(\text{Ca}^{2+}\) ionophore ionomycin (2.5 μM) after cholecystokinin stimulation led to a further substantial increase in \([\text{Ca}^{2+}]_i\).

Similar results were obtained in the cell suspension measured by fura-2 fluorescence spectrophotometry and by photoprotein aequorin luminescence spectrophotometry. An initial peak of each signal was consisted of \(\text{Ca}^{2+}\) mobilization both from the internal store(s) and external \(\text{Ca}^{2+}\) influx to the cytoplasm followed by a sustained but small influx of extracellular \(\text{Ca}^{2+}\) (Fig. 4B, C). The ratio of \(\text{Ca}^{2+}\) released from the store(s) to that of \(\text{Ca}^{2+}\) entered from the extracellular space in the initial phase was about 1:2.5. The sustained small increase in \([\text{Ca}^{2+}]_i\) disappeared when lanthanum, a \(\text{Ca}^{2+}\) entry blocker, was added after \([\text{Ca}^{2+}]_i\) had reached near the steady-state level (18).

As shown in Fig. 4D, when cholecystokinin (10⁻⁸ M) was added, there was a biphasic response of pepsinogen secretion. The initial response reaching a peak at 5 min was independent of medium \(\text{Ca}^{2+}\) and was followed by a sustained response reaching a peak at 30 min that was dependent on medium \(\text{Ca}^{2+}\). The lack of sustained response in the absence of medium \(\text{Ca}^{2+}\) by cholecystokinin stimulation was due not to the depletion of \(\text{Ca}^{2+}\) in the store(s) but to the absence of extracellular \(\text{Ca}^{2+}\) (2, 7, 15, 18).

**DISCUSSION**

Analyses of single chief cells in the gastric glands by a digital imaging microscope equipped with a microspectrofluorometer and of the isolated chief cell suspension by fluorescence and luminescence spectrophotometers provided several new findings concerning the distribution and mobilization of \([\text{Ca}^{2+}]_i\):

1. Distribution of \([\text{Ca}^{2+}]_i\) in a single resting chief cell was non-unimodal and a high \([\text{Ca}^{2+}]_i\) area was localized at the peripheral cytoplasm increasing towards the plasma membrane and often in some restricted regions in the endoplasm.
2. When chief cells were stimulated with cholecystokinin, \([\text{Ca}^{2+}]_i\) increased transiently in both the apical regions of peripheral cytoplasm and the endoplasm.
3. Mobilization of \(\text{Ca}^{2+}\) induced by cholecystokinin was composed of three
components; a rapid Ca\(^{2+}\) release from the intracellular store(s), a rapid Ca\(^{2+}\) entry from the extracellular space and a more sustained but localized Ca\(^{2+}\) entry from the extracellular space.

The exact role and contribution of these Ca\(^{2+}\) mobilizations for exerting biphasic pepsinogen secretion have not yet been determined. The first initial response may be caused by intracellular Ca\(^{2+}\) release from the store(s), possibly through myo-inositol 1, 4, 5-trisphosphate (IP\(_3\)) mediation (2, 15). The second sustained response requires Ca\(^{2+}\) entry from the extracellular space (15, 18) and is mediated by diacylglycerol synergistically combined with the first response, possibly through protein kinase C activation (7, 18).

We could not identify the exact location of the high Ca\(^{2+}\) region just beneath the plasma membrane. However, the cytoskeletal system appears to provide a compartmentation which may restrict the movement of intracellular molecules and ions (4). In gastric chief cells, pretreatment with microtubule and microfilament poisons such as colchicine and cytochalasin D decreased the average resting level of [Ca\(^{2+}\)]\(_i\) and inhibited Ca\(^{2+}\) mobilization and pepsinogen secretion evoked by cholecystokinin (15). It is, therefore, suggested that the microtubular-microfilamentous system which forms the meshwork just beneath the plasma membrane may be related to this high [Ca\(^{2+}\)]\(_i\). High [Ca\(^{2+}\)]\(_i\) just beneath the plasma membrane in the chief cell may be reasonably assumed since the Ca\(^{2+}\) pump or Ca\(^{2+}\) binding protein, calmodulin, of the plasma membrane generally requires a micromolar of Ca\(^{2+}\) for its full activation (9, 18).

It has not yet been clarified why and how high [Ca\(^{2+}\)]\(_i\) areas in the endoplasm are created and maintained at the resting state and enlarged by stimulation. Our previous study suggested that the [Ca\(^{2+}\)]\(_i\) plateaus are probably related with an internal Ca\(^{2+}\) mobilization (20).

It is well known that the mobilization of Ca\(^{2+}\) in the chief cell is composed of three types of flux (2, 7, 15, 18). Initial Ca\(^{2+}\) release from the intracellular store(s), presumably the endoplasmic reticulum, is mediated by IP\(_3\) (2, 15). The mechanism for entering Ca\(^{2+}\) from the extracellular space at the initial stage has not yet been elucidated. However, a more prolonged but localized Ca\(^{2+}\) entry from the extracellular space may be mediated by diacylglycerols (18). This prolonged Ca\(^{2+}\) mobilization, as reflected by the fura-2 or aequorin signal shoulder, is presumably inhibited by hormone dissociation from the receptor, resulting in a replenishment of Ca\(^{2+}\) into the store(s) and leading to the termination of the cell response (2).

In this study, we tried to visualize these Ca\(^{2+}\) fluxes using a digital imaging microscopy equipped with a microspectrofluorometer in a single cell. The results suggest that Ca\(^{2+}\) enters from the extracellular space at some restricted regions of the plasma membrane presumably through apical surface membrane, and that Ca\(^{2+}\) releases from the store(s) in the endoplasmic region(s).

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