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Cell-to-cell propagation of intracellular signals fluorescently visualized with acridine orange in the gastric glands of guinea pigs

Yasuko Fukushi, Takashi Sakurai [1], Susumu Terakawa *

Medical Photonics Research Center, Hamamatsu University School of Medicine, Hamamatsu, Japan

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ABSTRACT

Secretion from the gastric gland involves the activation of various types of cells in a coordinated manner. In order to elucidate the mechanisms underlying the coordination of secretion, we studied live fluorescence images of guinea pig gastric glands stained with acridine orange (AO). On 2 μM AO staining, individual cells were characterized by metachromatic colors and various intensities of fluorescence. When the gland was stimulated with 100 μM of histamine, green fluorescence was transiently increased in parietal cells and intermediate cells and propagated along the gland for a long distance over many cells. Local stimulation in a couple of cells with histamine in the presence of suramin also induced propagation. However, the fluorescence response was suppressed by the addition of H-89, a protein kinase A inhibitor. These findings suggest that a cAMP-dependent signal propagates intercellularly through a variety of cells to induce coordinated secretion in the entire gastric gland.

1. Introduction

A variety of cells, including parietal cells, mucous neck cells, chief cells and enterochromaffin-like (ECL) cells, in the gastric gland secrete different substances, such as acid, mucus and enzymes, in order to achieve digestion without destroying the host's own tissue. To this end, there must be a mechanism of coordination between individual cells. In order to elucidate the mechanisms underlying coordinated secretion, the location of individual cells, particularly parietal cells, should be identified, and the signaling activities of these cells should be examined in relation to the responses of other cells. Parietal cells can be distinguished by their triangular shape and relatively large size. Although the distribution of these cells in various positions in the stomach has occasionally been described [1], the actual distribution, in fact, differs in each gastric gland. Moreover, identifying the location of the cells in their living state is difficult, as immunostaining and scanning electron microscopic (SEM) observations require fixation of the gastric gland. Some researchers use the gastric gland model [2]; however, coordinated secretion in living gastric glands has rarely been studied [3]. In order to determine the types of cells that participate in group activities, we developed a simple method to identify parietal cells in living gastric glands and demonstrate the coordinated activity of these cells.

The presence of gap junctions in gastric glands has been demonstrated [4–6]. Gap junctions consist of membrane proteins and connexin, which form molecular pores approximately 1–2 nm in diameter large enough to allow for the permeation of molecules smaller than 1,000 Da, such as cAMP [7,8], through the cell membrane. Gap junctions in the gastric mucosa, which are primarily formed by connexin32 [9], are important for the host defense system [6], while those present in glands play a role in the regulation of acid secretion [4]. However, signal transmission through these gaps in the gastric gland has not yet been physiologically demonstrated. In the present study, we describe fluorescence images that captured histamine-induced intracellular signals propagating from cell to cell in living glands.

2. Materials and methods

2.1. Isolation of the gastric gland

Male guinea pigs of the Hartley strain (4 weeks old, 250–300 g in weight) fasted for 18–20 h were used. The gastric glands were isolated according to Berglindh's and Holton's methods with a slight modification [10,11]. Small pieces of mucosal tissue were obtained from the isolated stomach. These pieces suspended in a storing medium were pipetted and centrifuged at 700 rpm. The

* Corresponding author. Address: Medical Photonics Research Center, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan. Fax: +81 53 435 2392.
E-mail address: terakawa@hama-med.ac.jp (S. Terakawa).
[1] Present address: Toyohashi University of Technology, Japan.

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composition of the storing medium was as follows (in mM): NaCl, 133.5; KCl, 5.0; $CaCl_2$, 1.0; $MgSO_4$, 1.2; glucose, 11.1; and HEPES, 0.5 (pH 7.4). During the experiment, the medium was replaced with a perfusion medium containing the following (in mM) (which has been shown to promote secretion): NaCl, 110.0; KCl, 5.0; $CaCl_2$, 5.0; $MgCl_2$, 1.2; glucose, 16.7; and $NaHCO_3$, 26 (pH 7.4) [11].

2.2. Immunostaining of parietal cells

The isolated gastric glands were fixed with 4% paraformaldehyde for 3 h, then rinsed with PBS. The glands were treated with 0.1% Triton-X100 for 20–24 h at room temperature (RT) to permeabilize the cell membrane. After rinsing with PBS, the specimens were treated with methanol containing 3% hydrogen peroxide for 15 min with 10% goat serum for 10 min. Murine anti H^+, K^+-ATPase α subunit monoclonal IgG1 (Fitzgerald Industries International, USA) was diluted (1:500) in PBS, and the specimens were incubated for 2 h at RT. The specimens were rinsed with PBS then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG2b as a secondary antibody for 30 min. After rinsing with PBS, the cells were visualized using color development with 3,3-diaminobenzidine (DAB) solution.

2.3. Fluorescence microscopic observation

Acridine orange (Invitorogen, Japan), Fluo-4 AM (Dojindo Molecular Technologies, Inc., Japan) or LysoSensor™ Yellow/Blue DND-160 (Invitrogen, Japan) were used. The gastric glands were loaded with 0.1–100 μM AO in the suspension medium for 5–10 min at RT. The glands were also loaded with Fluo-4 AM and LysoSensor (LS) at 4–8 μM and 10 μM, respectively, for 10–15 min at RT. Following dye loading, the gland was placed on a coverslip attached to a silicone frame measuring 80 μm in thickness (as a spacer) and covered with another coverslip. A medium saturated with 5%CO_2 + 95%O_2 gas was perfused through the space between the coverslips at a flow rate of 150 μl/min and a solution temperature of 30 °C. The gastric glands were observed under an upright microscope (Eclipse FN1, Nikon, Japan) equipped with a cooled digital color camera (ORCA-3CCD C7780, Hamamatsu Photonics, Japan) that acquired images at 2–5 s intervals for 10–20 min. The excitation wavelength was 450–490 nm for both AO and Fluo-4 and 360–370 nm for LS (bandpass filter). The emission wavelength for these probes (longer than 520 nm) was used for fluorescence imaging. The fluorescence intensity was normalized to the initial intensity.

2.4. Stimulation

For bulk stimulation, histamine (Sigma–Aldrich, USA) was used at a concentration of 100 μM, common to previous investigations [3,12,13], as a high concentration (750 μM) has been estimated to be necessary to stimulate rat gastric glands [14]. The stimulant was added to the perfusion medium 1 min after the onset of perfusion and illumination. The flow rate of the perfusion medium was maintained at 150 μl/min with a peristaltic pump (MINIPULS Evolution, GILSON, USA). The average time to initiate both Ca^{2+} and AO responses was defined as the time of half of the maximum intensity (28 cells per six glands and 26 cells per six glands were used for the calculations, respectively). For local stimulation, a perfusion medium containing 500 μM of histamine and 4.4 mM of Lucifer yellow (LY) was supplied from a micropipette. LY served as a label indicating the flow of the stimulant under the fluorescence microscope. The micropipette was made using a pipette puller (PN-3, Narishige Scientific, Japan) to form a sharp tip measuring approximately 5 μm in diameter. The gastric gland was placed on a framed coverslip that was kept open to introduce the micropipette. The tip of the micropipette was placed at a distance of approximately 20–40 μm from a target cell in the gland. A hydrostatic pressure (20 cm H_2O) was applied to the pipette by operating a valve electrically. In order to avoid leakage of the stimulant to unwanted areas, the bath was bulk perfused with a stimulant-free, suramin-containing medium at a flow rate of 2 ml/min, which was high enough to create a laminar flow in the histamine-containing medium near the target cell, thereby removing ATP and other extracellular substances released from the cells and blocking any purinergic receptors on cells neighboring the stimulated site.

2.5. AO fluorescence spectra measurement

The fluorescence spectra of AO diluted in the medium were measured with a spectrofluorometer (FP-777, JASCO Co., Japan). The concentrations of the AO solution were 2–100 μM, 1 mM and 10 mM. The excitation wavelength was fixed at 490 nm, and the measurement wavelength varied from 400 to 700 nm.

3. Results

3.1. Immunostaining and AO staining of gastric cells

Parietal cells express H^+, K^+-ATPase. This enzyme was visualized in the gastric glands using immunostaining, as shown in Fig. 1A. Although the distribution of parietal cells showed a variety of patterns, the typical distribution involved localization in two-thirds of the basal side of the gland (Fig. 1A). Fig. 1B shows the results of the 2 μM AO staining of the same gland shown in Fig. 1A. Comparing the AO staining in Fig. 1B with the distribution pattern of the immunostaining in Fig. 1A, we found that the cytoplasm of mucous neck cells and surface mucous cells was stained in a bright green color, whereas the cytoplasm of parietal cells was stained in either a pale green color or no color. The cytoplasm of the intermediate cells between the parietal cells was stained in a brighter color than that of the parietal cells. Many vesicles in the intermediate cells were stained in a red color, resulting in an orange appearance (Fig. 1B). In addition, Fig. 1D presents the superimposed images of the 2 μM AO staining in Fig. 1C and mapping of the Ca^{2+} response (an increase in Fluo-4 fluorescence is expressed in blue (pseudo color)) induced by histamine stimulation. Since the pale green and dark cells stained with AO were consistent with the cells exhibiting a Ca^{2+} response, these cells were identified as parietal cells. Based on the reproducible results, 2 μM AO was used as a negative marker of parietal cells.

Meanwhile, when the gastric glands were stained with 100 nM AO, both the granules and cytoplasm exhibited a green color (Fig. 1E). In opposite cases of 100 μM AO staining, red fluorescence in the cytoplasm and green fluorescence in the nucleus appeared, irrespective of the cell type (Fig. 1F). Fig. 1G shows the concentration-dependent spectra of AO fluorescence. The peak of the spectrum shifted from 520 (green) to 610 nm (red) depending on the AO concentration.

3.2. Histamine-induced intracellular signals observed with AO, Ca^{2+}-dye and LS

The intracellular calcium responses of glandular cells sporadically started approximately 4–6 min after the onset of 100 μM histamine stimulation. These cells included parietal cells, mucous neck cells and many unidentified cells in the entire gland (Fig. 2A–D). The Ca^{2+} responses visualized with Fluo-4 in some cells appeared to propagate only to one or two neighboring cells.

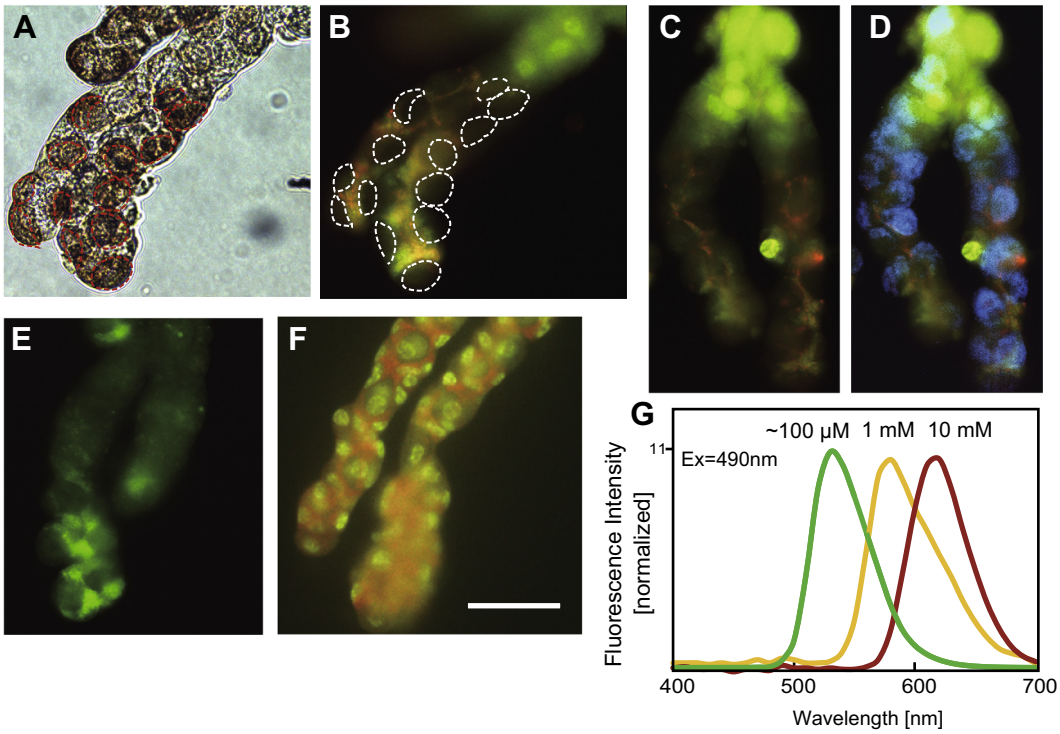

Fig. 1. Immunostaining of parietal cells in the guinea pig, and its comparison with the live fluorescence staining with AO. A: Parietal cells in a gland were visualized by immunostaining with the H^+, K^+-ATPase antibody and DAB. B: The same gastric gland as shown in Fig. 1A was stained with 2 μM AO. Dotted lines indicate parietal cells identified in Fig. 1A. C: Gastric glands were stained with 2 μM AO. D: Superimposed image of 2 μM AO staining in Fig. 1C and a newly obtained image of 4 μM Fluo-4 staining after 100 μM histamine stimulation. Blue color is the pseudo color of Fluo-4. E: A gastric gland stained with 100 nM AO. F: A gastric gland stained with 100 μM AO. G: AO fluorescence spectra observed by excitation at 490 nm varied with the AO concentration. Scale bar, 40 μm (A-F).

On the other hand, the AO responses induced by 100 μM histamine stimulation were different from the Ca^{2+} responses. The green fluorescence of a cell (Cell 1 in the dashed square in Fig. 2E and H, for example) increased at 1.5 min after the onset of stimulation, after which that of many neighboring cells sequentially increased (Fig. 2E–G, H–J, K–M and N–P), resulting in the propagation of a fluorescent wave along the longitudinal axis of the gland. The fluorescence intensity of the initial cell reached a peak, temporarily highest in all cells, and the peak position propagated from one cell to another for approximately two minutes. Finally, the fluorescence level of all cells decreased to the original level (Supplement 1 provides a video image of the propagation). The propagation of fluorescence signal was observed irrespective of the direction of gland axis in reference to the flow of perfusion solution. The time course of the fluorescence signal along a gland axis is shown in Fig. 2Q. When the gland was not stimulated with histamine, no changes in AO fluorescence, other than slow photobleaching due to illumination, were observed for a long period. The effects of photobleaching were very small, and the AO response was the result of physiological stimulation of the gastric gland. The AO response reached its half height at 2.5 min on average, while the Ca^{2+} response reached its half height at five minutes on average after the onset of histamine stimulation (Fig. 2Q).

The images of the pH distribution obtained using LS were compared with the distribution pattern of the parietal cells observed on immunostaining (Fig. 2R). The regions of low pH stretched primarily in the lumen of the gland. Even before histamine stimulation, a certain amount of HCl appeared to have already been secreted to the lumen following mechanical stimulation during preparatory handling of the gland (Fig. 2S). Following 100 μM histamine stimulation, HCl was secreted toward the lumen then moved to the surface of the gland (Fig. 2T–U). In contrast, an AO response was observed in the cell body region (Fig. 2E–P), indicating that the AO response did not represent pH changes in the gland.

We examined the pharmacological sensitivity of the propagating fluorescence signal. In the presence of H-89 (50 μM), an inhibitor of PKA, the gastric gland stained with AO was stimulated with 100 μM histamine. The time course of the fluorescence change revealed that a small number of cells slightly responded; however, most of the cells simply exhibited a decrease in fluorescence intensity over time as a result of photobleaching (Fig. 2Q). These findings indicate that the AO response is related to the PKA signal pathway.

3.3. Local stimulation

Cell–cell communication is possible via intracellular signal passage or the extracellular paracrine pathway involving purinergic agonists, for example. In order to elucidate the mechanisms underlying the propagation of the AO fluorescence signal from cell to cell, we applied local stimulation to one end of the gland. When a stimulant was supplied from a glass pipette, the solution marked with LY formed a stream (approximately 40 μm in width) covering 2–3 cells only (Fig. 3D). Following local stimulation with 500 μM histamine at one end of the gland (near the gastric surface side), parietal cells dimly stained with AO in the bottom half of the gland demonstrated a clear fluorescence response (Fig. 3A–C). The very faint green fluorescence of AO in the cytoplasm of several parietal cells increased, then propagated to neighboring cells sequentially (Fig. 3A–C and Supplement 2 video). Fig. 3E–I show enlarged images of the findings of Fig. 3A–C. The green fluorescence of AO increased at one minute in Cell 1 and 2 (Fig. 3F) and at 3.5 min in Cell 3 (Fig. 3H); the fluorescence in all cells then returned to the original level (Fig. 3I). The propagation of the AO signal was equally observed in the presence of suramin (100 μM), a blocker

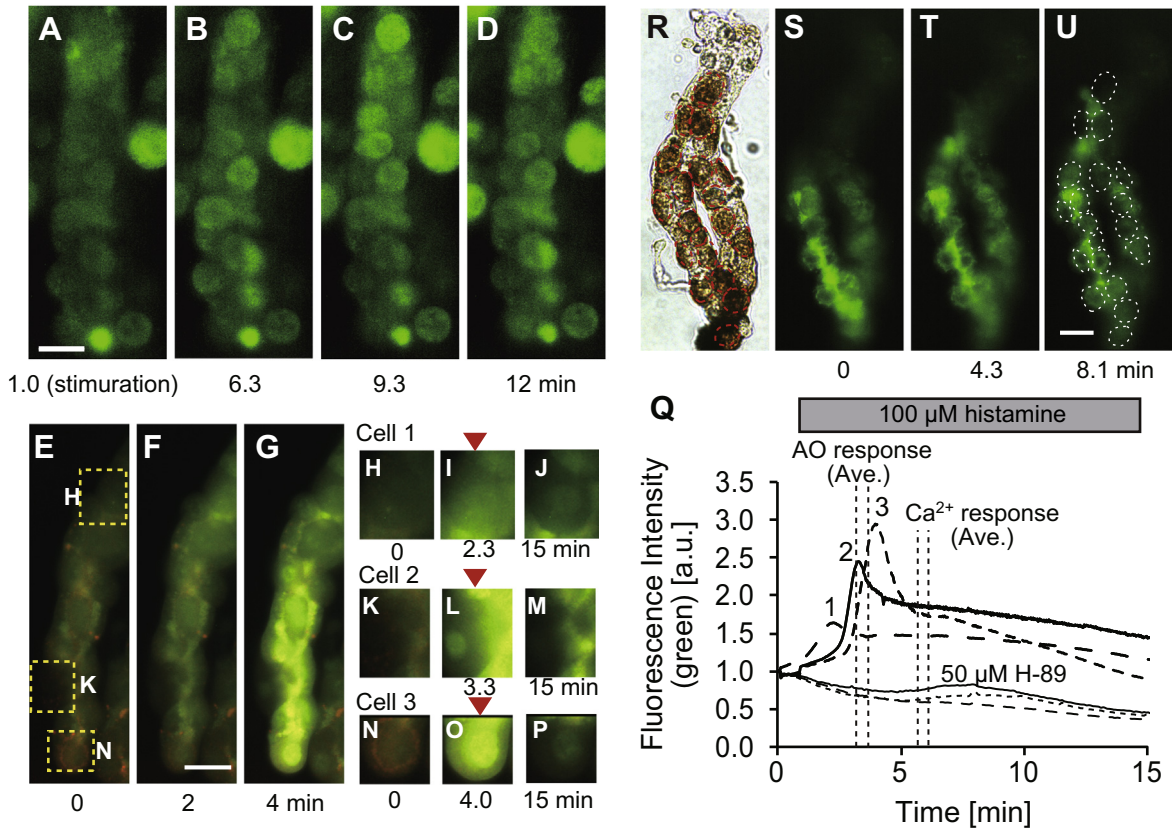

Fig. 2. Ca^{2+} responses, the propagation of green fluorescence of AO and pH images induced by 100 μM histamine. The bulk stimulation started at 1 min and continued later on. A-D: Sequential images of Fluo-4 fluorescence increases before and after the stimulation. E-P: AO fluorescence changes before and after the stimulation. H-J, K-M and N-P: Sequential images of AO fluorescence changes shown in H-J (Cell 1), K-M (Cell 2) and N-P (Cell 3). Arrowheads indicate the time when the fluorescence intensity of the initial cell reached a peak. Q: The time courses of AO fluorescence changes of Cell 1, 2 and 3 (indicated with dashed squares of H, K and N). The average initiation time of AO responses and Ca^{2+} responses with their standard error are indicated with dashed lines. (26 cells, 6 glands for AO and 28 cells, 6 glands for Ca^{2+} responses). Error bars,±SEM. The time courses of AO intensity changes of 3 cells induced by the histamine in the presence of 50 μM H-89, a PKA inhibitor. R: Parietal cells in a gland were visualized by immunostaining with the H^+, K^+-ATPase antibody and DAB. S-U: Sequential images of LS before and after the stimulation. Dotted lines indicate parietal cells identified in Fig. 2R.

of purinergic receptors, in the extracellular medium. The time courses of the fluorescence intensity changes are shown in Fig. 3J. These results suggest that the fluorescence signal propagates through the intracellular pathway.

4. Discussion

AO has the metachromatic property of appearing in a fluorescence color of green or red depending on its form (monomer or dimer) [15]. A solution containing AO at a concentration less than 100 μM exhibits green fluorescence, as it contains mostly the monomer form, while approximately 10 mM AO solution shows red fluorescence due to the presence of the dimer form (Fig. 1G). The gastric gland demonstrated metachromatic staining: a green color on 100 nM AO staining (Fig. 1E) and a red color on 100 μM AO staining (Fig. 1F). Nuclei generally exhibit a green color because AO stains DNA via the intercalation of monomer molecules between bases. Therefore, the fluorescence patterns of AO-stained cells are significantly variable. Berglindh et al. observed a fluorescence response related to acid secretion in the rabbit gastric gland using 100 μM of AO [13]. The same response was not observed in the guinea pig. Instead, 2 μM staining revealed the long distance propagation of an intracellular signal. We noticed that parietal cells could be distinguished from other cells when the gastric glands were stained with 2 μM AO. The cytoplasm of the parietal cells exhibited quite dark or pale green fluorescence, whereas the cytoplasm of mucous neck cells displayed bright green fluorescence and some intermediate cells between the parietal cells (Fig. 1B and C) demonstrated a bright orange color. It is noteworthy that these distinctions between individual cells disappeared when 100 μM AO was used for staining (Fig. 1F). More interestingly, both intracellular and intercellular signals were captured only by 2 μM AO staining of the gastric glands (Fig. 2E-P). The AO response was blocked by a PKA inhibitor, H-89 (Fig. 2Q). These findings indicate that the AO response does not reflect HCl secretion itself, but rather an upstream event of intracellular signaling leading to HCl secretion.

The pathway for the propagation of the AO signal is either intracellular or extracellular. The presence of purinergic receptors in exocrine cells is a candidate for the pathway via the extracellular paracrine release of ATP, as reported in the intestines and salivary glands [16,17]. We examined the effects of local stimulation in the presence of suramin, a blocker of purinergic receptors, and observed no suppressive effects on the propagating signal. The presence of gap junctions between gastric gland cells is well known [4–6] and a possible candidate as an intercellular signal pathway; however, the actual propagation of dynamic signals of any sort through this pathway has not been demonstrated to date. There are various blockers of gap junction channels, none of which have a specificity high enough to draw any reliable conclusions [8], as these compounds also block the paracrine pathway by blocking the panx1 and P2X7-activated channels. We demonstrated that the AO signal travels for a long distance, as shown in Fig. 3 and the

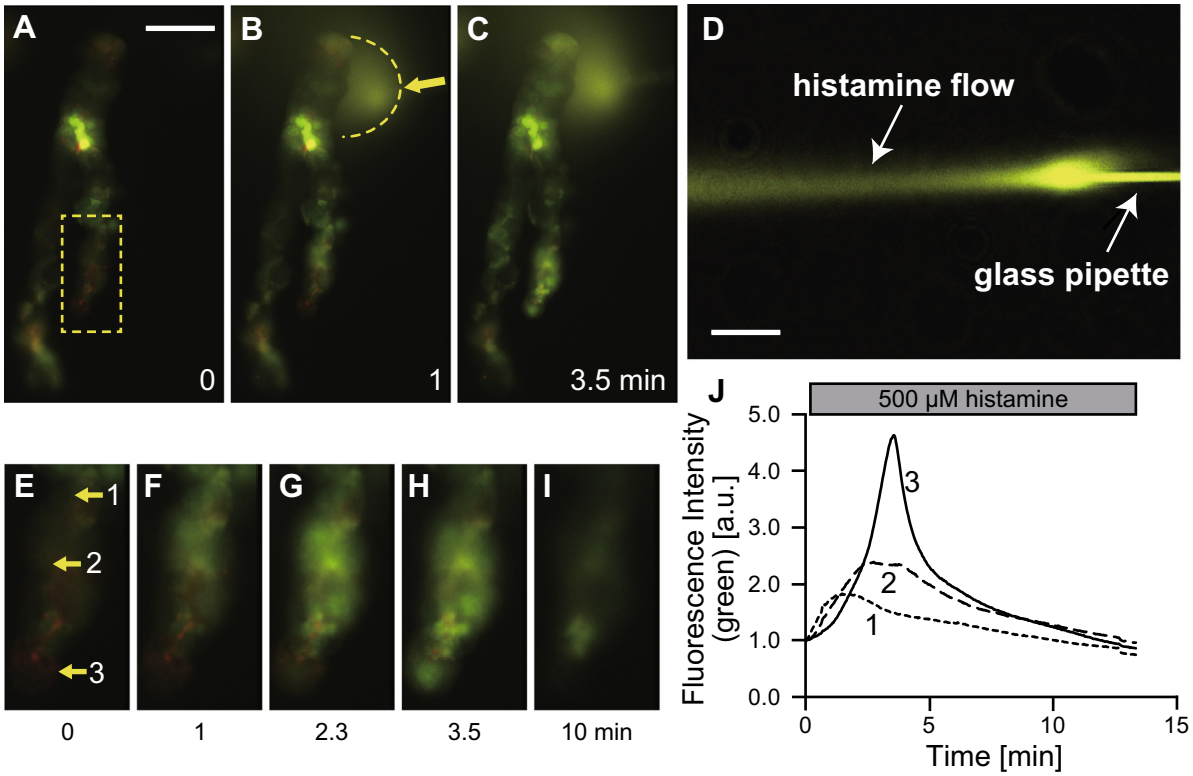

Fig. 3. AO fluorescence signal propagation induced by local stimulation with a 500 μM histamine-containing solution colored by LY. A-C: Sequential images of AO fluorescence changes before and during the local stimulation. The stimulation was applied from 0.03 to 13 min. A: Image observed before stimulation. B-C: Images observed during the local stimulation (it was supplied as a stream from a glass pipette as indicated by the arrow and dashed lines). D: A stream of stimulating solution containing histamine and LY flowing from a glass pipette. E-I: Sequential images enlarged from the area indicated by dashed square in A. J: The time course of AO fluorescence changes of Cell 1, 2 and 3 in E. Scale bars, 40 μm (A-D).

Supplement 2 video. Histamine may not directly stimulate chief or ECL cells, although it may possibly stimulate sensitive cells elsewhere, while an increase in cAMP propagates from cell to cell through the gap junction in order to coordinate secretion throughout the entire gland. Chief cells exhibit a Ca^{2+} response coupled with an increase in the cAMP concentration [18,19]. Therefore, even if a chief cell is not directly stimulated by histamine [20], the Ca^{2+} response occurs when the cAMP level rises intracellularly as the signal propagates through the gap junction. Similarly, with respect to ECL cells, an intracellular cAMP signal or Ca^{2+} response [21,22] is induced by the gap junction pathway, which allows molecules smaller than 1,000 Da, such as cAMP, to move directly from cytoplasm to cytoplasm [7,8]. It is likely that the cAMP signal propagates for a long distance along the axis of the gland in order to evoke a secretory activity in most cells. However, it is still puzzling why the propagation occurred with a limited number of cells responding only as initiators. One idea is that parietal cells exhibit differences in maturity. For example, they are known to be produced in the isthmus region then gradually move to the basal region of the gland as they grow older. Older parietal cells in the basal region display functional heterogeneity [12,23]. These cells may have a lower sensitivity to histamine, despite an ample pathway of gap junctions.

The Ca^{2+} response did not demonstrate clear propagation along the axis of the gland, but rather started individually in each cell. We suspect that the Ca^{2+} responses in individual cells were produced with variable delay following AO signal propagation. Alternatively, as the Ca^{2+} responses began at a significantly later time than the AO responses, the gap junctions between many cells may be closed at later times. These findings agree with those of a report showing that the Ca^{2+} response induced by histamine stimulation is not directly related to HCl secretion [24].

In conclusion, cell-to-cell propagation of intracellular signaling was revealed using 2 μM AO staining of the gastric gland in guinea pigs. The signal propagated through most cells, except for mucous neck cells. The propagation of a signal along a gland reflects the coordinated activation of a combination of many secretory cells in the gastric gland. The dynamic responses described in the present paper appeared only when the entire gastric gland was observed as a tissue.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.095.

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