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*Review*

# Histamine Increased the Uptake of Rhodamine 123 in Mitochondria of Living Parietal Cells in Cultured Gastric Glands from Starved Guinea Pigs

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**We previously found that parietal cells in guinea pigs starved for a few days contained giant mitochondria while the cells of animals starved and then injected with histamine mostly did not. To test whether mitochondria change their size and activity according to energy demands for acid secretion, we examined the dynamics of mitochondria stained by rhodamine 123 in living parietal cells in gastric glands under a confocal microscope. The glands were isolated from guinea pigs starved for 60–72 hr. Because mitochondria were closely packed in the cytoplasm, we failed to observe the morphological changes of each mitochondrion in parietal cells. However, we successfully observed that $10^{-5}$ M histamine induced an increase in the fluorescence intensity (the concentration of rhodamine 123) in mitochondria in subpopulations of parietal cells; the fluorescence intensity increased sharply within minutes after the histamine administration in some cells, while it gradually increased from just after the administration in other cells. We also found that a subpopulation of mitochondria within a parietal cell responded to the secretagogue. The findings suggest that parietal cells exist as a heterogeneous population in gastric glands and contain heterogeneous mitochondria in terms of their mitochondrial response to histamine.**

**Key words: confocal microscopy, histamine, mitochondria, parietal cells, rhodamine 123**

## I. Introduction

Mitochondria are ubiquitous cytoplasmic organelles numerous in cells that generate and expend large amounts of energy such as hepatocytes, cardiomyocytes, renal tubule cells and gastric parietal cells They are an excellent morphological indicator of the state of a cell's activity and health Unique about them is the appearance of giant mitochondria Giant mitochondria have been found in a variety of human diseases, in experimental animals subjected to nutritional manipulation, the administration of reagents and metabolic injury, and in hepatocytes and hepatocyte-derived cell lines treated with chemicals generating free radicals [13, 22, 24, 31, 39] Few papers exist on giant mitochondria in normal cells in a physiological state [23, 37, 40] in spite of the large number of studies on giant mitochondria under pathological conditions

Gastric parietal cells show unique behavior corresponding to their state of gastric acid secretion, and will dramatically transform their membrane system from a resting to an acid-secreting type In response to the stimulation of acid secretion, parietal cells expand the apical canalicular membrane by forming developed microvilli at the expense of an extensive intracellular membrane network called tubulovesicles, which is the characteristic feature of the resting-type parietal cells Both membranes are rich in the gastric proton pump (H, K-ATPase) Many investigators have thus

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focused on the mechanism of the morphological transformation because the nature of the tubulovesicles is ambiguous, and controversy exists as to whether the tubulovesicles are a distinct membrane compartment [9, 25] or continuous with the apical membrane [29] To the best of our knowledge, there are no reports showing that mitochondria in parietal cells, which supply a large amount of energy for the acid secretion, change their morphology corresponding to the membrane transformation In our previous study, we found that resting parietal cells in the stomach of guinea pigs starved for 60–72 hr contained giant as well as normal-sized mitochondria while acid-secreting parietal cells contained mitochondria homogeneous in size and structure in the animals fed freely Moreover, giant mitochondria were rarely found in gastric parietal cells in the animals starved and then injected with histamine [26] We also morphometrically demonstrated mitochondrial enlargement in the resting state under starvation and a return to normal dimensions in parietal cells of guinea pigs stimulated with histamine following the period of starvation [27] We therefore hypothesized that mitochondria change their size and activity according to the energy demand for acid secretion in parietal cells In this review, we described the dynamics of mitochondria stained by rhodamine 123 after the stimulation of acid secretion with histamine in living parietal cells in gastric glands from fasted animals under a confocal microscope This may be the first study to clearly monitor the functional state of mitochondria in individual living parietal cells in gastric glands *in situ*

## II. Preparation for Confocal Microscopy

Hartley guinea pigs of either sex weighing 300–500 g were used and there were no sex differences in the present study The animals were maintained on a 12-hr light/dark cycle and had access to a standard diet (REQ, Charles River Japan Inc , Tokyo) and water *ad libitum* before the experiment They were fasted for 60–72 hr before the following processing Animal experimentation protocols were approved by the university's Animal Research Committee

The isolation procedure for gastric glands was that of Berglindh and Obrink [5] with some modifications Under sodium pentobarbital anesthesia, the animals were perfused with oxygenated 20 mM HEPES-buffered Hanks' balanced salt solution containing $10^{-4}$ M cimetidine and $10^{-7}$ M atropine under high pressure The stomach was excised, cut open along the lesser curvature and emptied The cardial and antral regions were then discarded The corpus was rinsed several times in Dulbecco's PBS The mucosal surface was blotted with filter paper to remove adhering mucus and surface mucus cells The mucosa was scraped vigorously with a scalpel to free the glandular mucosa from the muscular layer and then minced into small pieces with a pair of scissors The pieces were incubated under gentle stirring with 50 ml of oxygenated collagenase (1 mg/ml, Type IA, Sigma-Aldrich Japan K K , Tokyo)-20 mM HEPES-buffered Eagle's minimum essential medium containing $10^{-4}$ M cimetidine, $10^{-7}$ M atropine, rabbit albumin (1 mg/ml, Fraction V, Sigma-Aldrich Japan K K ) and trypsin inhibitor (0 2 mg/ml, Nissui Pharmaceutical Co , Ltd , Tokyo) at 37°C for 90 min After the incubation, the suspension was filtered through nylon mesh (60G) into a 15 ml test tube In the test tube, intact glands rapidly sedimented to the bottom while the free or damaged cells remained in suspension, and thus isolated glands could be washed without centrifugation After washing three times, the viability of parietal cells in the glands was assessed using the trypan blue-exclusion test the viability was more than 95% in the glands

Isolated gastric glands were suspended in 20 mM HEPES-buffered Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 (DMEM/F12, JRH Biosciences, Lenexa, KS, USA) containing heat-inactivated 10% newborn calf serum (Equitech-Bio, Inc , Kerrville, TX, USA) at a concentration of 0 5–1×$10^6$ cells/ml The suspension was diluted twofold with a collagen solution (2 16 mg/ml type I and 0 24 mg/ml type IV collagen in HEPES-buffered DMEM/F12) or EHS-tumor matrix gel solution (Asahi Techno Glass Co , Ltd , Funabashi, Japan) at 4°C Fifty μl of the suspension was dropped into the well (10 mm in diameter) of a glass-bottom culture dish (35 mm in diameter, MatTek Co , Ashland, MA, USA) and allowed to polymerize for 30 min at room temperature Then 2 5 ml of the culture medium was added to the dish

We used rhodamine 123 to examine the structure and functional state of mitochondria in parietal cells because it is of low toxicity, quickly reaches an equilibrium between cells and medium and can be used to reveal the energy state of mitochondria (reviewed in [2, 3]) Rhodamine 123 (Sigma-Aldrich Japan K K ) was added to the medium at a concentration of 1 μg/ml (2 6×$10^{-6}$ M) Gastric glands in the gel were maintained at 30°C or 37°C on a microscope stage in a handmade chamber equipped with a temperature controller Mitochondria in living cells were imaged using a confocal inverted microscope (CLSM, Leica, Wetzlar, Germany) fitted with (1) a 2–50 mW argon ion laser beam generator and (2) a 10× objective lens (NPL Oil Fluotar, NA, 0 45) at a pinhole diameter of 80 μm (7 7 μm at Z-axis resolution) and a 40× objective lens (NPL Oil Fluotar, NA, 1 30) at a pinhole diameter of 180 μm (0 81 μm at Z-axis resolution) Filter combinations were BP488 excitation and LP515 emission for rhodamine 123 Images (8–16 frame average) were acquired at a constant power of the laser beam and a constant voltage of the photomultiplier at 15-min intervals and stored as a size of 512×512 pixels with 256 gradients After the uptake of rhodamine 123 into mitochondria was saturated (the intensity of the fluorescence became constant), histamine was added to the medium at a concentration of $10^{-5}$ M To examine the correlation between the concentration of rhodamine 123 and the fluorescence under a confocal microscope, we measured the intensity of the fluorescence from standard samples made at concentrations of 1, 5, 25, 50, 100 and 250 μM rhodamine 123 in a Permafluor mounting medium designed for fluorescence microscopy

## III. Mitochondrial Dynamics in Parietal Cells

The intensity of the fluorescence was highly correlated with the concentration of rhodamine 123 in the standard samples under the confocal microscope (Fig 1)

Living parietal cells were easily identified in gastric glands because of the large number of mitochondria stained by rhodamine 123 in the cells The membrane integrity (trans-membrane potential) is necessary for the accumulation of rhodamine 123 and mitochondria rapidly released rhodamine 123 when they received membrane injuries from radiation over a prolonged period with a strong laser beam Thus it was easy to identify damaged parietal cells having damaged, e g swollen, mitochondria under the confocal microscope We aborted the observation if the parietal cells abruptly lost fluorescence As we found that mitochondria increased in number in parietal cells under an electron microscope after the stimulation by histamine [27], we tried to observe the division of giant mitochondria in living parietal cells It may occur within 60 min after the administration of histamine [26, 27] However, we could not observe each mitochondrion separately in living parietal cells in the matrix gel even under the confocal microscope because the mitochondria were closely packed together in the cytoplasm (Fig 2) Moreover, we could not follow up masses of mitochondria as an identifiable source of fluorescence in the living cells at 37°C at 5-min intervals, in part because at 37°C parietal cells quickly changed in morphology and mitochondria moved in all directions (three-dimensionally due to culture in the gel) within a few min Thus we failed to observe the morphological changes of each mitochondrion in living parietal cells after the stimulation of acid secretion Histamine induced the membrane transformation of parietal cells in gastric glands from the resting to acid-secreting type at 30°C in 30 min under an electron microscope (Fig 3) Therefore, we tried to follow the intensity of the fluorescence from an identifiable mass of mitochondria at 30°C at 15-min intervals after the administration of histamine, after the saturation of rhodamine 123 uptake (within 60 min), the voltage of the photomultiplier was lowered to avoid a saturation of the intensity and then histamine was added

Histamine induced an increase of fluorescence intensity (the amount of rhodamine 123 in mitochondria) in a subpopulation of parietal cells in gastric glands from guinea pigs starved for 60–72 hr Figure 4 gives a typical example of time-lapse images taken with a 10× objective lens (oil, NA, 0 45) showing an increase in the intensity of the fluorescence in parietal cells after the administration Parietal cells were roughly divided into three types based on the changes in the intensity in type one cells, the fluorescence intensity increased sharply within min (usually at 30 or 45 min) after the administration, in type two cells, the fluorescence intensity gradually increased from just after the administration, while in type three cells, it remained nearly constant after the administration (Fig 4B) The former two types were located generally in the upper part of the gastric glands and the latter type chiefly in the lower part In gastric glands without histamine administration (control), the fluorescence intensity did not increase after the saturation of rhodamine 123

Histamine induced an increase in fluorescence intensity in a subpopulation of mitochondria Figure 5 gives an example of time-lapse images taken with a 40× objective lens (oil, NA, 1 30) showing an increase in the intensity of the fluorescence in a mass of mitochondria after the administration In the same cell, histamine clearly induced an increase in fluorescence intensity from one mass of mitochondria but not from another (Fig 5). Mitochondria that responded to histamine were generally located in the basolateral region of parietal cells, but not in the apical region We do not think that this was due to an artifact of the permeability of rhoda-

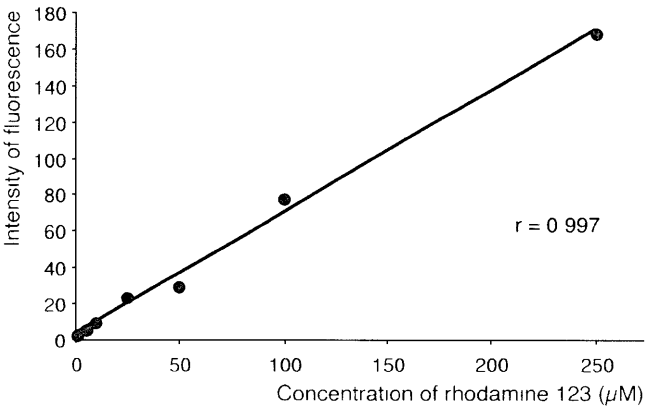


**Fig. 1.** The fluorescence intensity of standard samples at a concentration of 1, 5, 25, 50, 100, and 250 μM rhodamine 123 in Permafluor as a mounting medium The intensity was recorded under a confocal microscope with a 10× objective lens (oil, NA, 0 45) at a pinhole diameter of 80 μm The intensity was highly correlated with the concentration of rhodamine 123 (correlation coefficient=0 997)

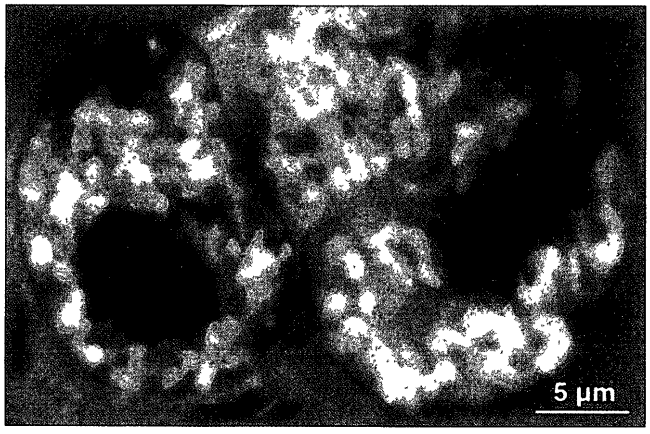


**Fig. 2.** Living parietal cells of gastric glands stained with rhodamine 123 A large number of mitochondria are closely packed in the cytoplasm Individual mitochondria cannot be separately observed in the living cells in matrix gel on an optically sliced plane, 0 81 μm in thickness, even under a confocal microscope with a 40× objective lens (oil, NA, 1 30) Bar=5 μm

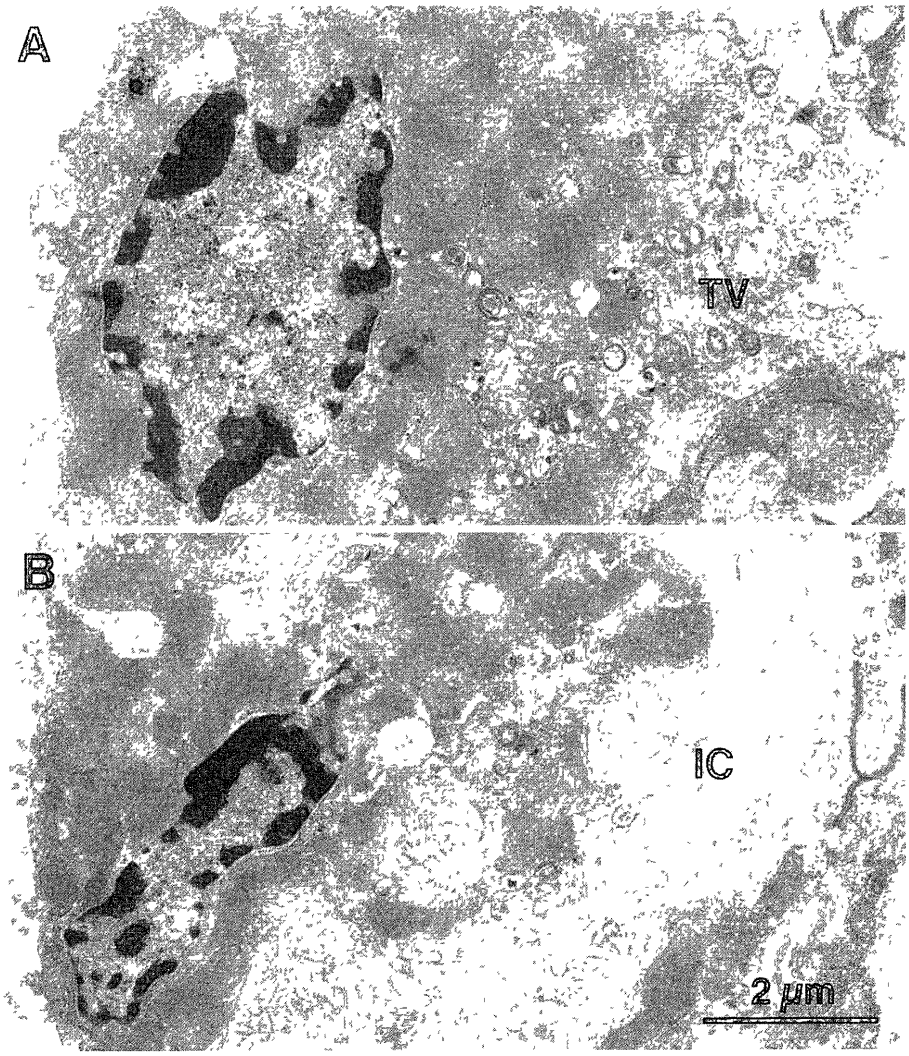


**Fig. 3.** Typical electron micrographs of parietal cells in isolated gastric glands from guinea pigs starved for 60 hr (**A**; control) and in the isolated glands of the starved animal that were incubated with $10^{-5}$ M histamine at 30°C for 30 min (**B**) The parietal cell treated with histamine possesses developed microvilli and normal-sized mitochondria, whereas the parietal cell in the control possesses developed tubulovesicles (TV) and large mitochondria IC intracellular canaliculi Bar=2 μm

mine 123 through the cell membrane because in the control gastric glands the fluorescence intensity was similar among mitochondria in parietal cells (as seen in Fig 2)

## IV. Methodological Validity and Nature of Mitochondria in Parietal Cells

Gastric parietal cells can be morphologically divided into two types an acid-secreting type and a resting type In the previous study, we found that starvation for 60–72 hr significantly induced mitochondrial enlargement in gastric parietal cells and a secretagogue for acid secretion histamine significantly decreased mitochondria in size to the normal dimensions found in the animals fed *ad libitum* within one hr in the cells of starved guinea pigs, mitochondrial enlargement was mostly found in resting-type cells of the animals fasted, some giant mitochondria consisted of several segments on a plane of a single section suggesting the fusion of normal-sized mitochondria in the formation of giant mitochondria [26, 27] Recent studies using fluorescence microscopy demonstrated that mitochondria form a largely interconnected and dynamic network in living cells [1, 7, 30] Using a three-dimensional reconstruction based on serial electron micrographs, Duman *et al* [9] showed mitochondrial interconnection in the gastric parietal cells suggesting a mitochondrial organization as an extensive reticular network throughout the cytoplasm However, the cytochemical properties of mitochondria in the gastric parietal cells were heterogeneous [32, 34] suggesting mitochondria exist as a particle element To reveal the nature of mitochondria in parietal cells, we examined the dynamics of mitochondria stained by rhodamine 123 We selected a culture of gastric glands mounted in matrix gel instead of an isolated cell culture because parietal cells in gastric glands much more

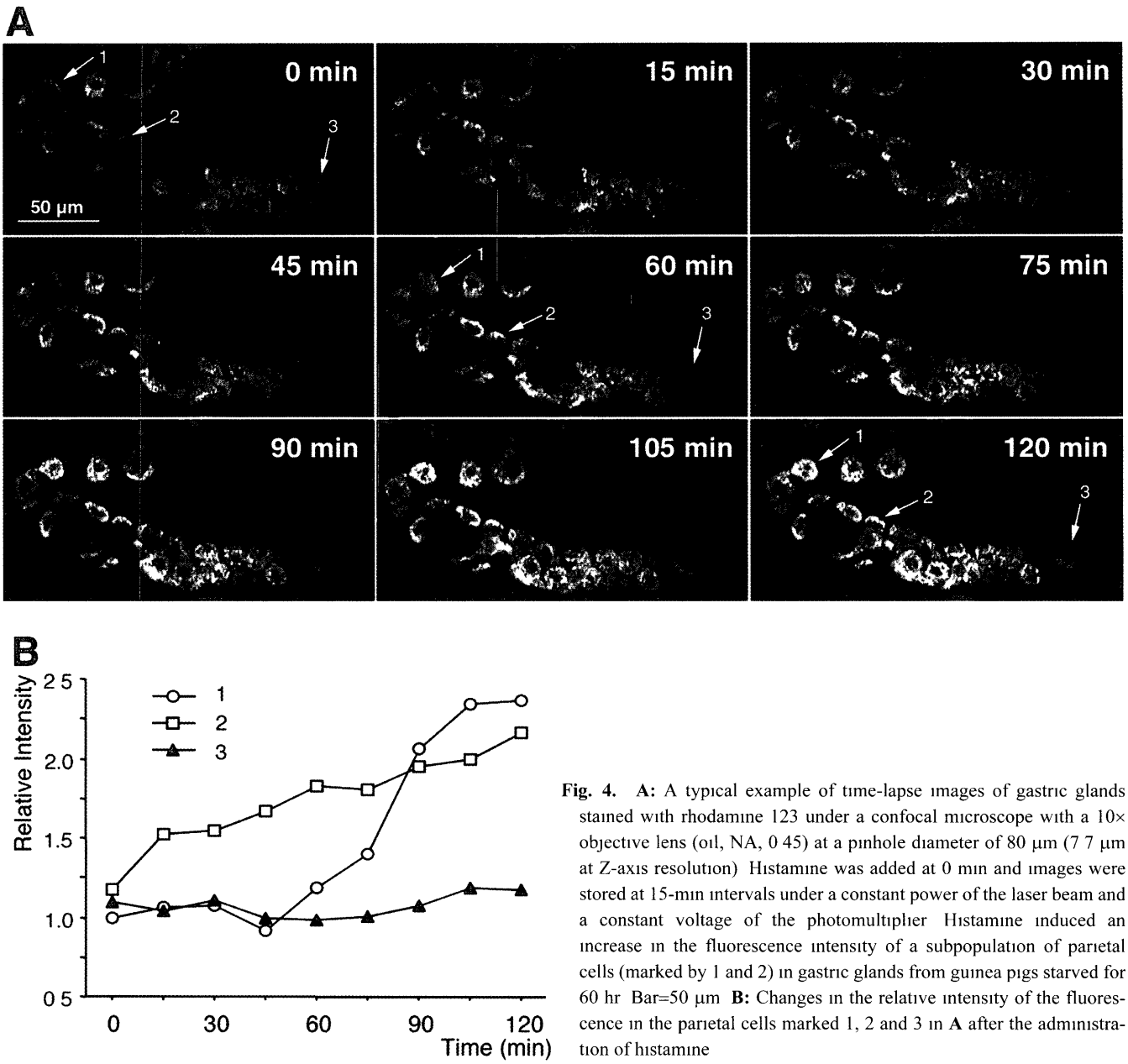


**Fig. 4.** **A:** A typical example of time-lapse images of gastric glands stained with rhodamine 123 under a confocal microscope with a 10× objective lens (oil, NA, 0.45) at a pinhole diameter of 80 μm (7.7 μm at Z-axis resolution). Histamine was added at 0 min and images were stored at 15-min intervals under a constant power of the laser beam and a constant voltage of the photomultiplier. Histamine induced an increase in the fluorescence intensity of a subpopulation of parietal cells (marked by 1 and 2) in gastric glands from guinea pigs starved for 60 hr. Bar=50 μm. **B:** Changes in the relative intensity of the fluorescence in the parietal cells marked 1, 2 and 3 in **A** after the administration of histamine.

closely resemble the situation *in vivo* and the latter has certain disadvantages when examining the nature of mitochondria *in situ*. first, isolated parietal cells frequently lose their normal membrane polarity [15], and second, cultured epithelial cells at or near the periphery or leading edge of the outgrowth accumulate significantly more rhodamine 123 than do cells deeper in the outgrowth [18].

We succeeded in following the intensity of the fluorescence from a mass of mitochondria in an optically sliced plane after the stimulation of acid secretion, histamine induced an increase in the fluorescence intensity of rhodamine 123 in a subpopulation of parietal cells. Johnson *et al* [18] suggested that the functional state of mitochondria was reflected by the magnitude of the transmembrane potential that paralleled the concentration of rhodamine 123 in mitochondria, i.e., the intensity of the fluorescence. Moreover, they showed that cells using ATP faster had a higher mitochondrial potential suggesting that the elevation in membrane potential reflected a requisite increase in the rate of mitochondrial ATP synthesis. Studies demonstrated that the onset of acid secretion increased the level of glycolytic intermediate in the citric acid cycle and of oxygen consumption in gastric parietal cells, indicating the activation of oxidative phosphorylation in mitochondria [4, 5, 11, 33]. Thus the findings by confocal microscopy may directly indicate that the onset of acid secretion increased the functional state of mitochondria in parietal cells *in situ*. The use of potential-dependent probes in conjunction with fluores-

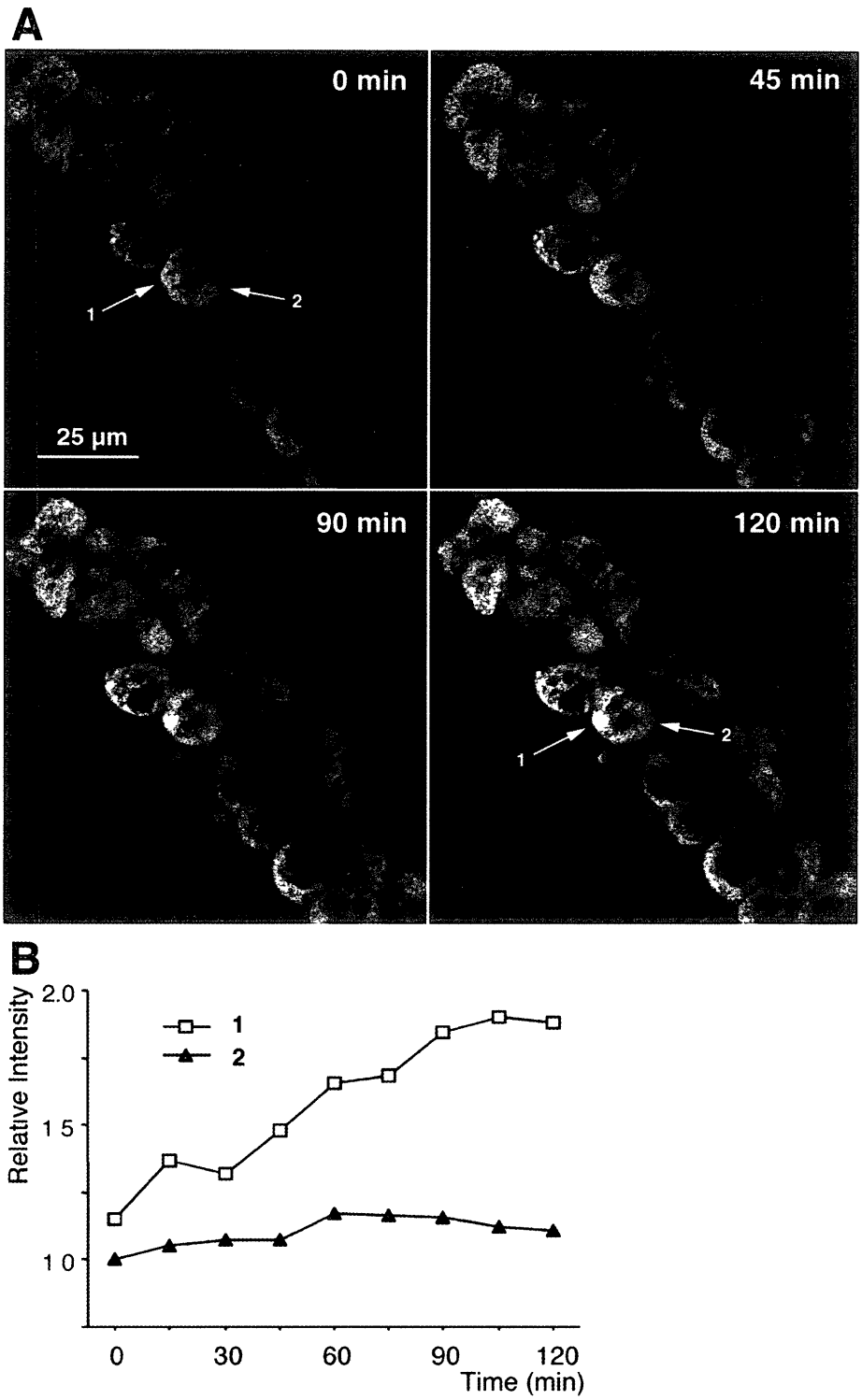


**Fig. 5.** **A:** An example of time-lapse images of gastric glands stained with rhodamine 123 under a confocal microscope with a 40× objective lens (oil, NA, 1.30) at a pinhole diameter of 180 μm (0.81 μm at Z-axis resolution). Histamine was added at 0 min and images were stored at 15-min intervals under the same conditions. Histamine induced an increase in the fluorescence intensity in the mass of mitochondria marked by 1. Bar=25 μm. **B:** Changes in the relative intensity of the fluorescence in the mass of mitochondria marked by 1 and 2 in the same parietal cell in **A** after the administration of histamine.

cence confocal microscopy has the advantage over spectrometric techniques of allowing the examination of individual cells The present study may be the first to clearly monitor the functional state of mitochondria *in situ* in individual living parietal cells

We demonstrated that histamine activated the functional state of mitochondria in a population of parietal cells generally located in the upper part (isthmus and neck) of gastric glands but the mitochondrial response to the secretagogue was heterogeneous among parietal cells Cytochemical studies suggested that acid secretion is more active in parietal cells in the superficial part of gastric glands [6, 8, 16] Our finding agrees with these studies Permeant cationic fluorescent probes stained all mitochondria with a similar fluorescence intensity within a given cell suggesting that the functional state of mitochondria is maintained uniformly by all mitochondria of an individual cell [1, 7, 18, 17, 20–22], whereas Smiley *et al* [36] showed two populations of mitochondria in some cell lines These findings may indicate mitochondria as a single network entity in various types of cells We found intracellular heterogeneity of mitochondria monitored with rhodamine 123 in response to histamine in a single parietal cell This may indicate that mitochondria are not organized as a single reticular network Therefore, the present result is inconsistent with the finding (mitochondria as an extensive reticular network in parietal cells) by Duman *et al* [9] using the three-dimensional reconstruction of serial sections Since the serial sections covered only a thin segment of a parietal cell, this is insufficient to show the overall organization of mitochondria in the parietal cell Because an extremely large number of mitochondria are closely packed in the cytoplasm, it is impossible to precisely follow the continuity of mitochondria in living parietal cells even under a confocal microscope due to the limits of resolution Three-dimensional reconstruction using ultrathin serial sections may elucidate the morphological nature of mitochondria in parietal cells, although it is very difficult to make serial sections to cover a whole parietal cell Another approach may be necessary to address the morphological entity of mitochondria in parietal cells, that is, whether they exist as a particle element or a single network element

Recent studies demonstrated that free radical inducers such as hydrazine and hydrogen peroxide induced the formation of megamitochondria in cultured hepatocytes, and that free radical scavengers countervailed the formation by the inducers [20–22, 41] This indicates that oxidative stress causes the formation of megamitochondria We found that megamitochondria were present in resting parietal cells of starved animals, but not in acid-secreting parietal cells of animals fed *ad libitum* This may indicate that the formation of megamitochondria in gastric parietal cells occurs under low oxidative stress It is generally accepted that superoxide dismutase protects cells against free radicals, and a certain amount of manganese- and copper, zinc-superoxide dismutase exists in the mitochondria and cytoplasm of gastric parietal cells, respectively [10, 28, 35] Thus the mechanism of formation may be different between the two cells It is necessary to examine the effect of free radical inducers on the formation of giant mitochondria in gastric parietal cells

H2-receptor antagonists and H, K-ATPase inhibitors are widely used as antiulcer drugs against gastric acid secretion Morphological studies showed that an administration of H2-receptor antagonists induced the membrane transformation of gastric parietal cells from an acid-secreting type to a resting type [19, 38] They did not focus on ultrastructural changes of mitochondria, but simply reported an increase in condensed mitochondria as well as swollen mitochondria in dead parietal cells Studies also showed that H2-receptor antagonists decreased mitochondrial activity in gastric glandular cells and hepatocytes [12, 14] Thus the confocal microscopy procedure introduced here may be a good method of analyzing the effects of certain drugs on gastric parietal cells *in vitro*

## V. Concluding Remarks

We found that histamine induced an increase in the fluorescence intensity (the concentration) of rhodamine 123 in mitochondria in subpopulations of parietal cells in gastric gland and in subpopulations of mitochondria within a parietal cell The results suggest that parietal cells exist as a heterogeneous population in gastric glands and contain heterogeneous mitochondria in terms of their mitochondrial response to histamine The present study may be the first to clearly monitor the functional state of mitochondria in individual living parietal cells in gastric glands after the stimulation of acid secretion

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