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Visualization of exocytotic secretory processes of mast cells by fluorescence techniques

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Secretory processes via exocytosis in rat peritoneal mast cells were visualized by two complementary fluorescence techniques; one staining pre-exocytotic granules with a basic probe and the other staining post-exocytotic granules with acidic probes. Granules within mast cells were selectively stained with acridine orange and emitted orange yellow fluorescence. Upon stimulation with compound 48/80, release of acridine orange from granules was observed both in population and single cell measurements. This release was seen in some localized area of mast cells. Opening of pores between plasma membranes and granule membranes was monitored using acidic fluorescence probes such as 6-carboxyfluorescein or lucifer yellow CH. Not only granules located at peripheral region, but also granules near the core region participated in exocytosis. The existence of junctions between these granules was suggested. TMA-DPH, a lipophilic membrane probe, which was localized at plasma membrane before stimulation, diffused into granule membranes after stimulation. This shows that after stimulation, some constituents of plasma and granule membranes were mixed. Even after extensive degranulation, mast cells extruded acidic probes, indicating the plasma membranes still play a role of barrier. Activation of lateral motion of granules preceding to exocytosis was not observed. It was concluded that the visualization of secretory processes by fluorescence and image processing techniques will be useful for the study of molecular mechanisms underlying exocytosis.

Introduction

Secretion mediated by exocytosis is one of the fundamental phenomena in many biological systems. Its mechanism is supposed to have large similarities with the release of neurotransmitters at synaptic sites. One hypothesis for the mechanism of secretion is that this process is composed of movement of secretory granules in cytoplasm, adhesion to cell membranes and fusion of granules with cell membranes [1]. Although, this hypothesis is widely accepted, this is not enough proved experimentally and its molecular mechanism is still unclear. For the further elucidation of this mechanism, direct observation of this process is one of the most promising.

Internal pH of many secretory granules is known to be around 5 [2,3]. Weakly basic fluorescent molecules are accumulated within granules as a result [4]. Fluorescence intensity is often quenched in high concentration of fluorescent molecules. Some fluorescent molecules show changes in fluorescence spectra due to stacking (metachromagy). If such molecules are accumulated in secretory granules and then released, both fluorescence intensity and spectra will change due to dilution of chromophores. Consequently, real time monitoring of these parameters will enable us to visualize secretory process in living cells.

Acridine orange is one of the chromophores which assume the properties mentioned above and is used as a pH indicator [5,6]. It emits green fluorescence (emission maximum at 520 nm) in monomer, and orange yellow fluorescence (emission maximum at 600 nm) in oligomer. Quantum efficiency of orange yellow fluores-

Abbreviation: TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene.

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cence is much smaller than that of the green one. If we observe green fluorescence, the release process can be detected, and if we observe orange-yellow fluorescence, location, internal pH and motion of granules can be detected.

Using acidic fluorescent probes, complementary observation to that using basic probes can be performed. Acidic probes are rejected from cells due to negative charges of plasma membranes. Exocytosis will be visualized by inflow of acidic probes to opened granules. Optically sectioned image using confocal imaging technique will enable real time monitoring of this process.

Based on these principles, we attempted to establish a technique to visualize the secretory process in living cells using fluorescence techniques.

Materials and Methods

Preparation and culture of cells. Mast cells were prepared from peritoneal cavity of Wistar rats by the method of Kuno et al. [7]. Isolated cells were plated on cover slips coated with poly(D-lysine) for microscope observations, or were suspended in Lock's solution for population measurements. Cells were used within a day.

Preparation of chromaffin granules. Bovine adrenal chromaffin granules were prepared by the methods of Meyer and Burger [8].

Staining with fluorescence probes. Mast cells were stained with 1 μM of acridine orange for 10 min at 31°C in phosphate buffered saline supplemented with 10% fetal calf serum. They were washed by centrifugations and perfusion. Chromaffin granules were stained with 0.5 to 5 μM of acridine orange in Lock's solution for 10 min at 31°C. Then, they were washed with the same buffer once or twice.

Mast cells were stained with 0.5 mM of lucifer yellow CH or 40 μM 6-carboxyfluorescein in Lock's solution. Mast cells were mixed with the probes 1 min before measurements and they were left unwashed during confocal measurements.

Mast cells were stained simply by mixing with 1 μM of TMA-DPH in Lock's solution at 23°C by perfusion 2 to 4 min before measurement and the cells were used in unwashed state.

Fluorescence observation and imaging. Mast cells were first mounted on a cover slip coated with poly(D-lysine) and left stand for 5 to 10 min in a moisture box and the cover slip were mounted on a perfusion chamber. On poly(D-lysine)-coated cover slips, more than 80% of the cells were morphologically intact and showed a response to compound 48/80. The chamber is sealed with a cover slip and a gasket and is continuously perfused with Lock's solution through syringe needles inserted through the gasket. Stimulation of the cells with compound 48/80 was performed by perfusion. Fluorescence images were observed with a Zeiss axiophoto microscope equipped with a silicon intensified target TV camera (Hamamatsu Photonics C1000) or CCD camera (Hamamatsu Photonics C3077), a image processor (Hamamatsu Photonics ARGUS-10), and a video tape recorder (Sony Umatic VO5600). Recorded data were further processed by another image processor (Hamamatsu Photonics ARGUS-100). Fluorescence images were also recorded in 35-mm films (Fuji-film, Fujichrome-1600 or Neopan-400). Optically sectioned fluorescence images were observed by a confocal microscope (Bio-Rad MRC-600 equipped to Zeiss axioplan microscope) and were recorded to magnetooptic disks (Sony NWP MO disk driver and Sony EDM-1DA1 disks).

For observation of the secretory process using basic and acidic fluorescence probes, a sharp cut filter (half-transmission wavelength 510 nm) or a band pass filter (peak-transmission wavelength 530 nm) was used for the emission side. For observation of motion of granules, a sharp cut filter (half-transmission wavelength 560 nm) was used. For acridine orange, decline of fluorescence intensity due to photobleaching was less than 10% for 3 min of irradiation. For observation of TMA-DPH fluorescence, no filter was used for the emission side in order to collect as many photons as possible. Differential interference imaging was also used for observation of motion.

For population measurement of release of acridine orange from mast cells, a fluorometer (Shimadzu RF502) was used.

All measurements were performed at room temperature (20–26°C).

Histamine release. Histamine release was measured fluorometrically by the method of Shore et al. [9] and Draberova [10].

Scoring deformed cells. Fraction of deformed cells after stimulation with compound 48/80 was determined by counting cells on differential interference photographs. Partly deformed cells were classified into deformed cells. 50 to 100 cells were counted.

Reagents. Acridine orange, lucifer yellow CH, 6-carboxyfluorescein and TMA-DPH were purchased from Poly Science, Kodak, Sigma and Dojin, respectively. All other reagents were from commercial sources.

Results

Visualization of release process in isolated chromaffin granules

At first, we tried whether the release of contents of a single granule can be visualized with acridine orange. As shown in Fig. 1, isolated granules from bovine adrenal chromaffin cells accumulated acridine orange. When these granules were irradiated by intense excit-

ing light, they occasionally broke due to photochemical reaction or thermal expansion of their membranes. Fig. 1 shows fluorescence images of the photoradiation-evoked-release of entrapped acridine orange from isolated chromaffin granules. Super-nova like bursts were clearly demonstrated. These transient changes are interpreted as follows. Just after the break of granules, acridine orange molecules were released out of the granules and the stacked molecules changed to monomers resulting in the increase in 530 nm fluorescence intensity. Then these molecules diffused away and 530 nm fluorescence intensity decreased. Indeed, with the naked eye, transient changes in fluorescence color from orange-yellow to green were observed when a filter which transmits both green and orange-yellow light was used. This supports that acridine orange can be used to monitor the secretory process in secretory cells.

Fluorescence images and monitoring of release process of mast cells stained with acridine orange

Since the results of a single granule indicated a possibility to visualize secretory processes in vital cells using acridine orange, we measured the release of acridine orange from peritoneal mast cells. As shown in Fig. 2, in mast cells, acridine orange was selectively accumulated in granules. Yellow to orange-yellow fluorescence indicates that these granules are acidic. Homogeneous green fluorescence may originate mainly from chromophores bound to negatively charged molecules such as nucleic acids.

The time course of acridine orange fluorescence intensity at 530 nm of a single mast cell stimulated with compound 48/80 is shown in Fig. 3. Here, increase in fluorescence intensity was observed. The time course of this fluorescence intensity was studied in detail. If acridine orange molecules accumulated in granules are

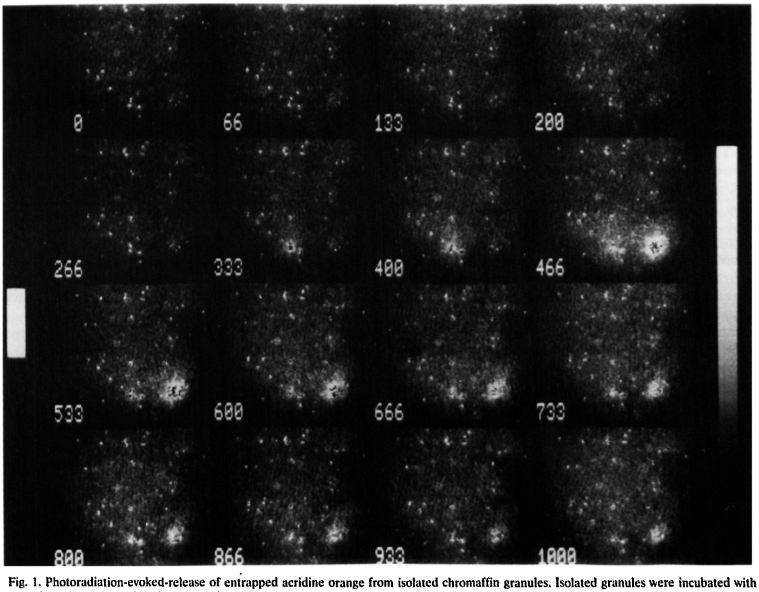


Fig. 1. Photoradiation-evoked-release of entrapped acridine orange from isolated chromaffin granules. Isolated granules were incubated with 1 μM of acridine orange for 10 min at 31°C and washed three times. These granules were irradiated with blue light (maximum at 434 nm) from a ultra high pressure mercury lamp at energy of 6 $\mu W/\mu m^2$. A sharp cut filter with half-transmission wavelength 510 nm was used for emission side. Granules adhered to glass surface were selected. Under this condition, both orange-yellow and green fluorescence can be observed. After 20 s of irradiation, the granules began to burst. The process starts from top left to right. Every 66 ms, a picture was recorded from the sequential images, with a time resolution of 33 ms. In the lower part of the images, release of acridine orange from chromaffin granules is seen as transient increase in fluorescence intensity. The vertical bar at the left represents 10 μm.

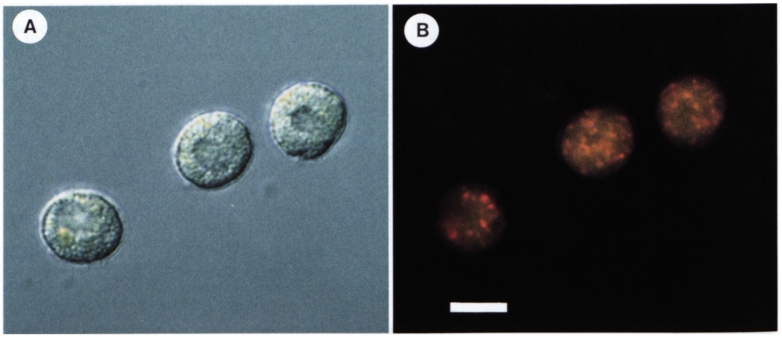


Fig. 2. Peritoneal mast cells stained with acridine orange. Rat peritoneal mast cells were stained with 1 μM of acridine orange for 10 min at 31°C and washed by a centrifugation and perfusion. (A) Differential interference image. (B) Fluorescence image. Granules are selectively stained as yellow dots in the fluorescence image. The horizontal bar represents 10 μm.

released upon stimulation, fluorescence intensity at 530 nm will increase followed by the decrease just like chromaffin granules shown in Fig. 1. This was just the case as shown in Fig. 4. Within 1 s after stimulation, fluorescence intensity increased, and after several seconds, it began to decrease. The decrease in fluorescence intensity is not ascribed to photobleaching, because fluorescence intensity of acridine orange from non-responding cells (macrophages and lymphocytes) remained constant under this experimental condition (less than 10% of decline after 3 min of irradiation). Interesting point is that this time course was heterogeneous within a cell. In the present cell, central region showed quick rise and peripheral region showed slower rise. Decaying process was similar in both regions. Detailed time profiles and spatial heterogeneity were different from cell to cell. However, qualitatively, similar tendency was observed in most of the cells which responded to compound 48/80. In conventional fluorescence microscopes, this local heterogenecity may be obscured due to optical interference of out-of-focus regions. Optical sectioning of fluorescence images using a confocal fluorescence microscope is expected to sensitively detect this local heterogeneity. The results is shown in Fig. 5E. Here, dramatic disappearance of acridine orange in some restricted regions of this mast cell was demonstrated. Judging from the size of granules, multiple granules must be broken in these regions around 5.5 s. With the time resolution of 1.1 s, individual release was partly detected. Comparing the fluorescence and differential interference images (Figs. 5A–D), it is shown that the regions of diminished fluorescence correspond to deformed regions of the cell.

Result of population measurement using a fluorometer of release of acridine orange from mast cells is shown in Fig. 6. Upon stimulation with compound 48/80, dramatic rise in 530 nm fluorescence intensity was seen. Based on the principle of metachromagy of acridine orange, we think this rise is a reflection of the release process. However, compared to histamine release, the time course of the fluorescence intensity of acridine orange showed lag time of 15 s at 20°C, and this was rather similar to that of the increase of deformed cells.

Granules stained with acridine orange did not show any lateral motion both before and just after stimulation. Only after cell morphology changes (more than 1 min after stimulation when deformation of cell envelopes is clearly observed by differential interference microscope), large lateral motion of some granules was detected.

Some granules were leaked out of mast cells without releasing acridine oranges.

Fluorescence images of mast cells stained with lucifer yellow CH

Since plasma membranes assume negative charges, they are impermeable to acidic fluorescent molecules such as lucifer yellow CH or 6-carboxyfluorescein. When these molecules exist in extracellular medium, these molecules flow into secretory granules at the instance of exocytosis. This process cannot be observed by conventional fluorescence microscopes because strong fluorescence from out-of-focus molecules interferes. Using a confocal fluorescence microscope, this interference is largely diminished due to the sharp

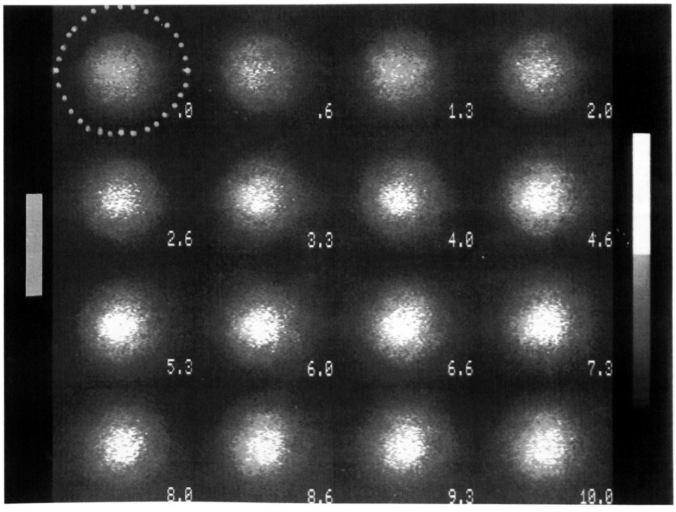
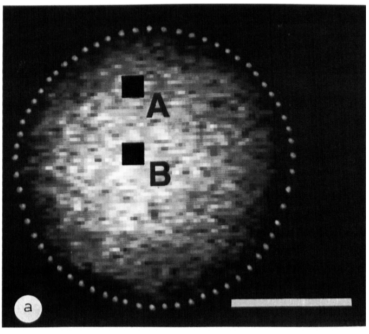


Fig. 3. Release of acridine orange accumulated in a mast cell stimulated with compound 48/80. Mast cells were stained with 3 μM of acridine orange for 10 min at 31°C and washed by a centrifugation and perfusion. The cells were stimulated with 3 μg/ml of compound 48/80 5 s before the first image. Fluorescence at 530 nm was measured. Numbers at the bottom right of each figure represent time after introduction of compound 48/80. Intensity of exciting light was reduced to less than 0.4% of the intensity used in the experiments shown in Fig. 1. At this range, no effects of photoradiation on exocytosis was seen. Decrease in fluorescence intensity due to photobleaching was less than 10% of the initial value after 3 min of irradiation. Note that the gray scale is not directly proportional to absolute fluorescence intensity. (In order to know the absolute value, refer to the graph of Fig. 4) Within 10 s or so after stimulation, fluorescence intensity increases, and around 10 s, it almost saturates. The dotted line indicates the cell envelope. The vertical bar at the left represents 10 μm.



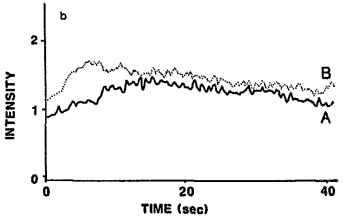


Fig. 4. Time course of fluorescence intensity of acridine orange stained to a mast cell stimulated with compound 48/80. Fluorescence intensity of acridine orange at 530 nm is shown. The cell is the same as that shown in Fig. 3. Fluorescence intensity at two areas surrounded by the square wells is shown. At cell peripheral region, A, fluorescence intensity increases for 10 s followed by gradual decrease. At B, initial increase is rather rapid and decrease begins at 7 s. The dotted line indicates cell envelope. The horizontal bar represents 5 μm.

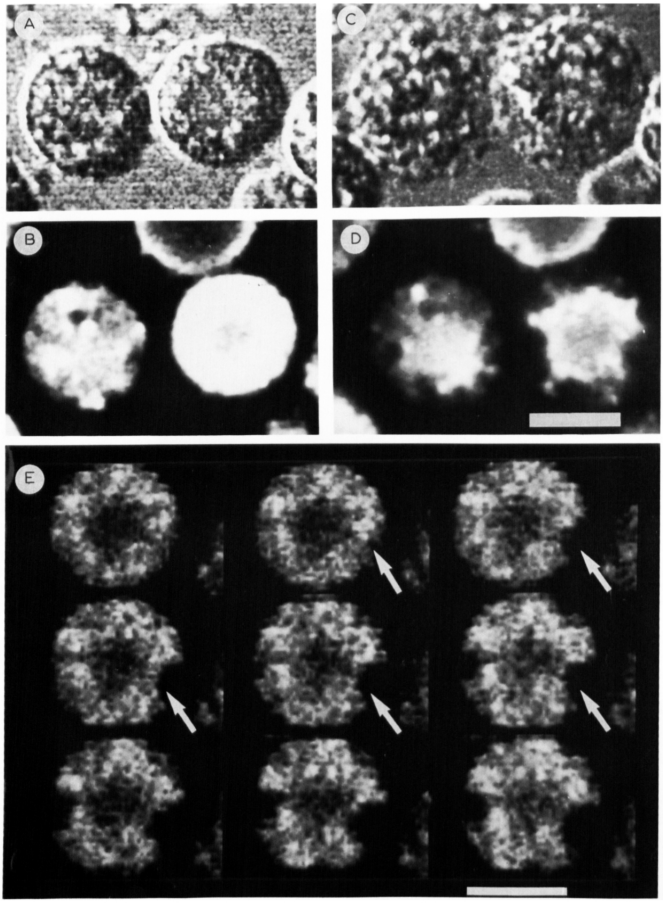


Fig. 5. Optically sectioned fluorescence image of mast cells stained with acridine orange and stimulated with compound 48/80. Fluorescence image of mast cells at 530 nm was optically sectioned using a confocal microscope. Focal depth was 1 μm. The cell was stained with 3 μM of acridine orange for 10 min at 31° C and washed by a centrifugation and perfusion. The cell was stimulated with 3 μg/ml of compound 48/80. A and B: differential interference and fluorescence images before stimulation. C and D: images after stimulation (after about 150 s). The horizontal bar represents 10 μm. E: Time course. The right cell in A–D was selected. The process starts from top-left to right. The cell was stimulated with 3 μg/ml of compound 48/80 about 10 s before the first image. Every 5.5 s a picture was recorded from the sequential images, with a time resolution of 1.1 s. Large scale exocytosis is seen at several sites within 10 s (indicated by arrows). Heterogeneity is more clearly shown than in Fig. 3 where a conventional fluorescence microscope was used. Almost all the cells which responded to compound 48/80 exhibited the similar pattern shown here. The horizontal bar represents 10 μm.

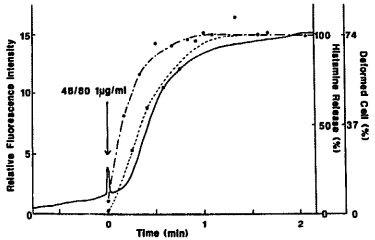


Fig. 6. Monitoring of release process of mast cells by population measurements using acridine orange fluorescence. Mast cells were stained with 1 μM of acridine orange and washed three times. They were suspended in Lock's saline in a quartz cuvette. Fluorescence was measured by a fluorometer with excitation at 488 nm and emission at 525 nm at 20 °C. ———, fluorescence intensity of acridine orange; ·–·–·, histamine release; o------o, fraction of deformed cells. Note that upon stimulation of compound 48/80, fluorescence intensity of acridine orange at 525 nm increased. This time course is similar to that of deformed cells. However, it shows a lag of 15 s compared to the time course of histamine release. According to the property of acridine orange, this rise can be interpreted as to represent release process of mast cells.

optical sectioning and exocytotic process is expected to be visualized. This technique is just complementary to the one using acridine orange. The result is shown in Fig. 7.

Here, cells were seen as black shades indicating lucifer yellow CH does not flow into the cells. According to Fig. 7E, upon stimulation with compound 48/80, inflow of lucifer yellow CH was seen within the time resolution of confocal imaging (1.1 s). This means that the opening time of pores and the diffusion of lucifer yellow CH is much faster than 1.1 s. Interestingly, granules near core region were also subjected to exocytosis with the delay time of several seconds. Since most of these granules were next to granules already junctioned to extracellular medium and they were far away from plasma membranes, it can be speculated that these granules make junctions with the granules already subjected to exocytosis. According to Fig. 7, cytoplasm was still dark even after considerable exocytosis accompanying degranulation and deterioration. This means that plasma membranes are still sealed. According to fluorescence and differential interference images, upon stimulation, some granules were thrown out of cells keeping preloaded acridine orange inside (data not shown). In this case, plasma membranes must be broken. However, inflow of lucifer yellow CH to cytoplasm through this broken area during the initial 2 min was not observed. Therefore, some special mechanisms other than simple collapse of plasma membranes may happen upon degranulation.

Long after washing of external lucifer yellow CH (more than 5 min), stained granules within cells remained unchanged (data not shown). Therefore, opened pores may have been sealed again leading to recycling.

6-Carboxyfluorescein gave similar results as lucifer yellow CH. However, mast cells were more easily damaged by photodynamic reaction in the presence of 6-carboxyfluorescein.

Fluorescence images of mast cells stained with TMA-DPH

According to Fig. 7, granules within mast cells seem to be successively junctioned to external medium. This means that the properties of granule membranes change after exocytosis resulting in fusion between granules. Which molecules are involved in this change? As shown in Fig. 8, TMA-DPH diffused into granule membranes after stimulation. This result indicates that some molecules in plasma membranes diffuse into granule membranes after stimulation. These diffused molecules may be candidates to induce membrane fusion. Concanavalin A bound to plasma membranes, on the other hand, did not diffuse into granule membranes.

Discussion

In the present study, we demonstrated that secretory processes can be visualized in peritoneal mast cells by fluorescence imaging techniques. Basic fluorescence chromophores visualized pre-exocytotic granules and acidic ones visualized post-exocytotic granules. Although this method is fundamentally single cell analysis, the results obtained were seen in most of mast cells which responded to compound 48/80.

Among the weakly basic fluorescent chromophores we tried, acridine orange gave the clearest change when cells were challenged with secretagogues. Breckenridge and Almers [11] used quinacrine as a probe to monitor exocytotic process of mast cells. They used a mutant which has giant granules with diameters more than 1 μm. As far as normal rat peritoneal mast cells are concerned, we found that acridine orange is more sensitive than quinacrine. This may be due to the fact that acridine orange is easy to stack together resulting in changes both in fluorescence intensity and spectrum. On the other hand, quinacrine does not stack due to its two positive charges located opposite sides, and only weakening of fluorescence can be detected upon exocytosis by single cell analysis. The advantage of acridine orange was clearly seen in population measurements (see Fig. 6). Quinacrine gave little change in fluorescence intensity upon stimulation with compound 48/80 in population measurements. However, larger leakage of loaded chromophores from cells were observed with acridine orange than with quinacrine.

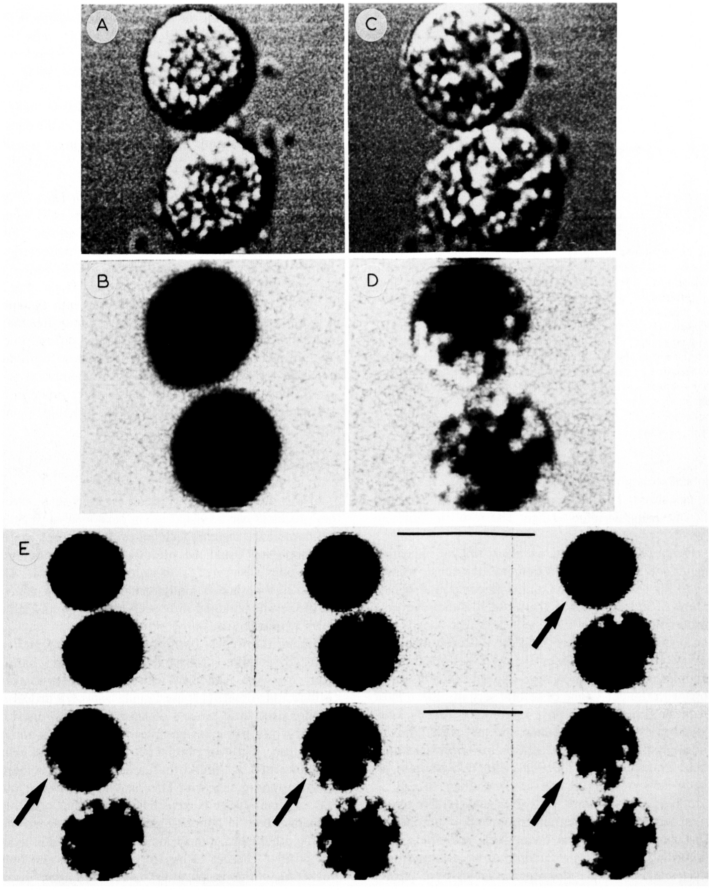


Fig. 7. Inflow of lucifer yellow CH to mast cells stimulated with compound 48/80 represented by optically sectioned images. Mast cells were mixed with 0.5 mM of lucifer yellow CH. Then they were stimulated with 1 μg/ml of compound 48/80 and the following process was observed by a confocal fluorescence microscope. Focal depth was 0.4 μm. A and B: differential and fluorescence images before stimulation. C and D: images after stimulation (after about 80 s). The horizontal bar represents 15 μm. E: time course. The process starts from top-left to right. Every 6.6 s a picture was recorded from the sequential images, with a time constant of 1.1 s. Each picture is an average of five images. Compound 48/80 was applied about 10 s before the first image. Cells are seen as dark shadows because intact cells extrude lucifer yellow. From the second image, inflow of lucifer yellow CH is pictured as white bulges in dark shadows. Note that at the initial stage, inflow was seen at cell peripheries and then inflow into inner granules was seen (indicated by arrows). Sometimes, exocytosized granules exhibited stronger fluorescence than the extracellular medium. Almost all the cells which responded to compound 48/80 exhibited the similar pattern shown here. The horizontal bar represents 15 μm.

We also tried two acidic probes; lucifer yellow CH and 6-carboxyfluorescein. They gave similar results. Points of difference were that lucifer yellow requires higher concentration (0.2 to 1 mM compared to 10 μM for 6-carboxyfluorescein), is more resistant to photodynamic damage, and shows over-accumulation in granules.

Heterogeneous distribution of exocytotic region in peritoneal mast cells were clearly shown in Figs. 4, 5 and 7. Comparing these results, it is clear that confocal imaging is very sensitive to detect the heterogeneity. At these defined regions, many secretory granules seem to be involved in exocytosis. The points to be noticed are (1) at first, granules at cell peripheries were involved in

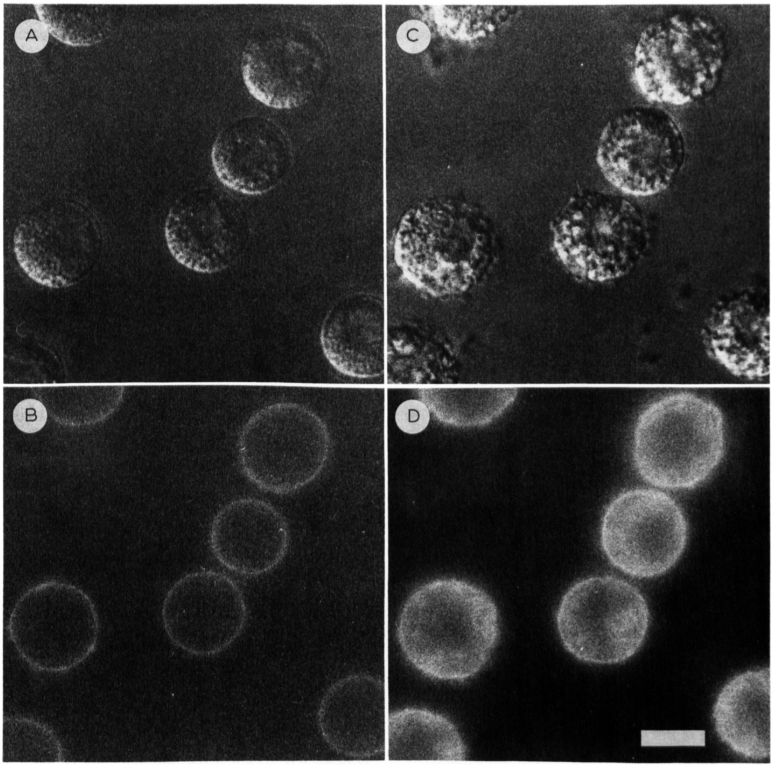


Fig. 8. Diffusion of TMA-DPH from plasma membranes to granule membranes after stimulation with compound 48/80. Mast cells were stained with 1 μM of TMA-DPH in Lock's solution at 23°C by perfusion 4 min before recording. Recording was performed by a conventional fluorescence microscope. A and B: differential interference and fluorescence images before stimulation. C and D: images after stimulation (after 80 s). Note that after stimulation, inner membranes became bright. This indicates that diffusion of plasma membrane components into granule membranes occurs. The difference between contrast before and after stimulation comes from minimum exposure before stimulation in order to reduce the photodynamic effect. The horizontal bar represents 10 μm.

exocytosis, (2) after several seconds, granules in inner regions followed, (3) granules involved in exocytosis in inner regions were very often next to granules already subjected to exocytosis (see Fig. 7E). Mast cells have a spherical shape with diameters around 15 μm and in Fig. 7, central sections of mast cells were measured with a focal depth of 0.4 μm. Therefore, granules in inner region are not ones near plasma membrane far off the measured section. Under a differential interference optical microscope, no clear canals which directly connect granules to cell membranes were observed. Therefore, the most possible mechanism is that granules make junctional pores between them when outer granules were subjected to exocytosis. As shown in Fig. 7, several seconds were required to make junctions between granules. At present we don't know which mechanism is acting to induce the fusion. Inflow of constituents of plasma membranes and/or extracellular medium into granules may induce some physicochemical and structural changes which successively induce fusion with inner granules (see Fig. 8). Such diffusion was also reported in mouse bone-marrow macrophages [12,13]. All these processes may take several seconds. It is reported that compound exocytosis occurs in mast cells, groblet cells and parotid acinar cells [14]. This compound exocytosis may correspond to the heterogeneous and sequential exocytosis shown in Figs. 5 and 7.

Active participation of cytoskeletal components was reported [15–18]. In exocytotic area indicated in the present study, dynamic change in cytoskeleton is considered to be responsible for the heterogeneity. Gradients of cytosolic Ca^{2+} concentration reported in pancreas exocrine cells [19] may be another reason.

In population measurements of secretory process using acridine orange (Fig. 6), almost 1 min was required. While secretion of histamine took 45 s. This difference is ascribed mainly to initial lag in acridine orange measurement. While, the time course of degranulation was close to that of acridine orange. How can these results be explained? The following is one possible explanation. Within granules, acridine orange molecules are highly stacked as explained before. Besides, fluorescent molecules are easy to adhere to proteins and membranes. In order to diffuse out through fusion pores, larger diameter is required for stacked or adhered acridine orange compared to histamine. 15 s may be required to make large pores Formation of this large pores may be be accompanied by structural deformations which can be detected by differential interference images. Breckenridge and Almers reported a similar delay in the response of quinacrine compared to the formation of pores between plasma and granule membranes [11]. In spite of this delay, we think that monitoring the release process of mast cells using acridine orange provides us with a very convenient, stable and continuous method.

By the present technique, lateral motion can be detected. Activation of lateral motion of secretion has been postulated to be one of the essential steps for secretagogue-evoked exocytosis [1,15,16,18,20]. However, this activation was not observed in mast cells. Terakawa et al. (personal communication) got similar results in chromaffin cells. We think, therefore, that granules involved in exocytosis are located in stand-by position just beneath plasma membranes and some inner granules are subjected to exocytosis by fusing with outer granules.

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