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Exocytosis in the Dissociated Pancreatic Acinar Cells of the Guinea Pig Directly Visualized by VEC-DIC Microscopy

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To elucidate the detailed process of exocytosis at the highest possible accuracy, we dissociated the pancreatic acinus of the guinea pig and observed zymogen granules under a video-enhanced contrast differential interference contrast (VEC-DIC) microscope. The preparation was thin enough to resolve each zymogen granule with the best clarity. When acinar cells were stimulated with ACh (20 μM), many zymogen granules near the lumen showed an abrupt light intensity change. For a period of 10 s immediately before exocytosis, zymogen granules neither shifted their position nor altered their shape within an accuracy of 38 nm. The time required for individual granules to change the light intensity (the releasing time) ranged from 0.15 to 0.70 s. After each response, the granule maintained its altered contrast for a few seconds until it was retrieved to a planar membrane. No compound exocytosis including granule-granule fusion was observed. We concluded that the exocytosis is not directly initiated by any supramolecular change but by a purely molecular event.

According to electron microscopic studies (1–4), the exocrine pancreas secretes digestive enzymes by exocytosis of zymogen granules. Exocytosis is assumed to involve several steps (5): (i) transport of a zymogen granule towards apical pole, (ii) docking of the granule to an appropriate membrane site, (iii) fusion of the granule membrane with the cytoplasmic membrane, (iv) release of granule contents to the luminal space, and (v) retrieval of fused membrane after exocytosis. In electron microscopy, however, fixed preparations were examined to reconstruct only a possible sequence, so that exact dynamics of exocytosis such as the time interval between these steps remained unclear. Especially, in agonist-induced exocytosis, the timing of change in granule shape or position immediately before their exocytosis (6, 7) was not clear. Electrochemical measurements of secretion (8) did not exactly report the release of enzyme and mucus themselves. Therefore, the light microscopies are necessary at their best resolution in living cells.

A direct visualization of granules under a VEC-DIC (video-enhanced contrast differential interference contrast) microscope assures their normal dynamics which would often be modified by probes for visualization like fluorescent dyes (9). We have visualized exocytosis in diced tissues, and found no direct link of vesicle trafficking to exocytosis of pancreatic acini (10). The preparations remained in a natural configuration, but their thickness was rather large for ideal light microscopy. To achieve the highest possible resolution, thin preparations are advantageous. In the present study, we made the pancreatic acinus as thin as possible without loosing its physiological activity. A group of cells in a loose complex of the acinus were thin enough to achieve the best resolution without any staining, thus allowing us to trace a single granule immediately before, during and after exocytosis at a nanometer scale in real time.

METHODS

Pancreatic tissues were excised from the guinea pig anesthetized with pentobarbital sodium (50 mg/kg, ip.). They were cut into small pieces (<1 mm^3) and dissociated further by incubation with collagenase (Type-I, Sigma, St Louis, MO) for 20 min at 37°C. Acini dissociated from the tissue were placed on the chamber of which bottom was made of a coverslip. The acini were superfused continuously on the microscope stage with standard medium containing (in mM): NaCl 115; KCl 3; $CaCl_2$ 2; $MgCl_2$ 1; glucose 5; KH_2PO_4 1; $NaHCO_3$ 20

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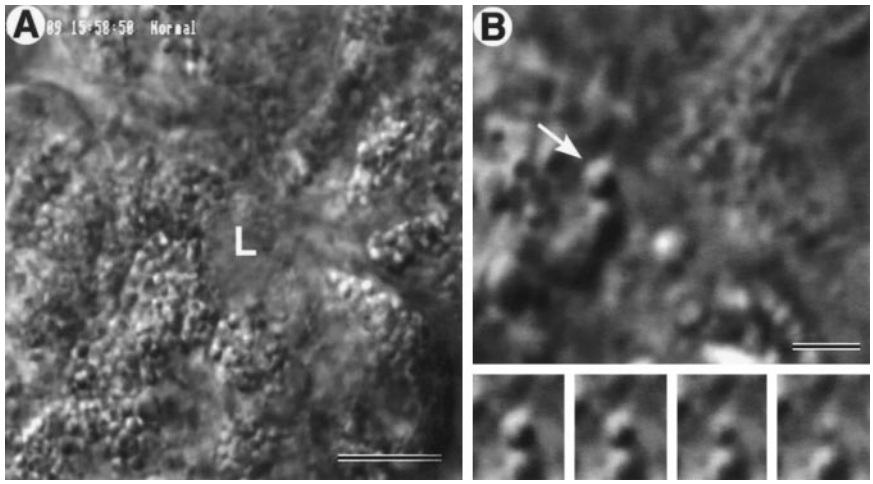

FIG. 1. VEC-DIC microscopic image of exocrine pancreatic cells in a dissociated acinus. (A) Low magnification view of the acinus before stimulation. L, lumen of the acinus. Scale bar, 10 μm. (B) High magnification view of the same acinus. Scale bar, 2 μm. When the acinus was stimulated by superfusion with medium containing 20 μM ACh, a granule (arrow) underwent an exocytotic response as shown in the bottom panel. The interval between each frame in the bottom panel was 100 ms.

and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid 16 (pH 7.4). The medium was warmed to 33°C and oxygenated while it was passed through a thin silicone tubing jacketed with an oxygen-containing vessel. Apparatus used for VEC-DIC microscopy was similar to that described earlier (11, 12). Briefly, images obtained under an inverted microscope (Axiovert 10, Zeiss, Obercochen) were captured with a $\frac{1}{2}$ inch CCD video camera (IT-23A, NEC, Tokyo), and the video signal was processed with an image processor (PIP-4000, ADS, Osaka) to enhance contrast and resolution. The processed image was observed on a 14 inch monitor screen (VM-1221, Hitachi, Tokyo) mostly at a magnification of 4400. The video images were continuously recorded in an S-VHS videotape. The tape was played back later to analyze the position and the light intensity of a single granule with the same image processor.

RESULTS AND DISCUSSION

Dissociated pancreatic acini were thin enough to observe each zymogen granules clearly. The width of acinar lumen and the accumulation of zymogen granules near the lumen were well preserved as signs for intactness of polarized cells (Fig. 1A). Most granules ranged from 0.3 to 1.0 μm in diameter (Fig. 1B). At a resting state, zymogen granules fluctuated very slowly within a region of their diameter, and showed no change in shape for more than 30 min. When acinar cells were stimulated by superfusion of ACh-containing medium, many zymogen granules near the lumen disappeared after showing an abrupt light intensity change (Fig. 1B, lower panel). The secretory activity of individual cells could be measured precisely by counting the number of such responses representing exocytosis (Fig. 2). Only in the period of stimulation (horizontal bar in Fig. 2), exocytotic responses were observed. The response before the stimulation was totally absent. Since all of these responses were completely suppressed by 1 μM atropine, they are the receptor-mediated secretory response.

When the focus of the DIC microscope was adjusted exactly on a secretory granule, it appeared as a half bright and half dark object. The bright-dark direction in reference to the DIC axis indicated that the granule had a refractive index higher than the cytoplasm surrounding it. The light intensities in the bright part and the dark part of a single granule were measured for a period of its exocytosis (Fig. 3). Upon exocytosis, the bright part became dark and the dark part became bright, suggesting a large decrease in refractive index inside the granule (11). In most granules, time required for this light intensity change ranged from 0.15 to 0.70 s. When the limiting membrane of a zymogen granule fuses with the apical membrane, the granule

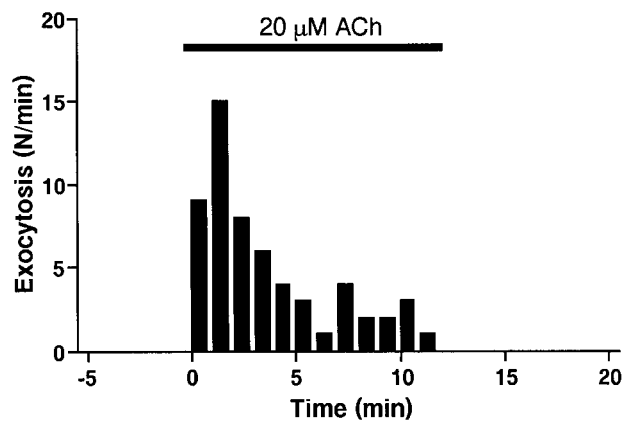

FIG. 2. Frequency histogram of exocytotic responses in an acinus stimulated with 20 μM ACh. Stimulation was applied during the period indicated by horizontal bar. The ordinate represents the number of exocytotic responses counted every minute.

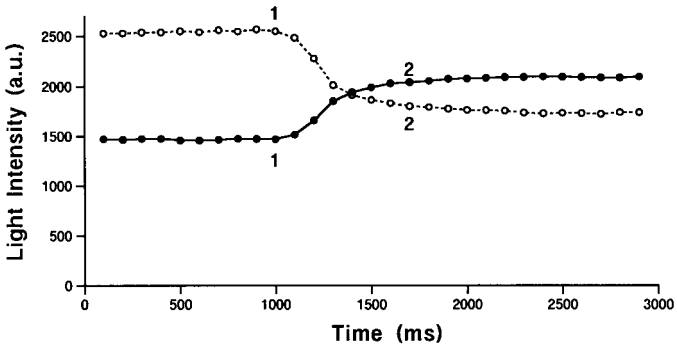

FIG. 3. Time course of the light intensity change of a single granule during its exocytotic response. The interval between each point was 100 ms. Open circles represent the light intensity in the bright part of the granule, filled circles that of the dark part. The ordinate represents the light intensity expressed in the sum of pixel values (8 bit scale) measured in a square area (8 × 10 pixels) covering the bright or dark area. The time required for a transition of the light intensity (90%) was determined in this example as 700 ms from dot 1 to dot 2. Two curves crossed at 400 ms after the initiation of exocytosis (dot 1).

contents diffuse out to the luminal fluids. If the granule membrane remains for a while even after the release of granule contents, the luminal fluid fills the granule. Then, the refractive index inside the remaining granule transiently becomes smaller than the cytoplasm. This process accounts for the crossing of the light intensity curves for bright part and dark part of a granule. Later, it took a few seconds at least, for the two curves to merge. This is the time required for the fused granule membrane to form a planar membrane (retrieval time). The retrieval time was much shorter than that in the colonic goblet cells (13), and it was short enough that other granule always fused with the planar plasma membrane. There was no tendency for granules to respond on the same site of the apical membrane. We never observed that a granule fused with another granule in the cytoplasm. In accordance with all these findings, we did not observe the compound exocytosis or such an invaginating apical membrane as often formed in the colonic goblet cells (13). A difference of granule contents between zymogen granules and goblet cell granules may account for these differences in the retrieval time and the exocytosis mode of both types of cell.

The dynamic aspect of granule changes before and during exocytosis was studied in stimulated cells. A granule showed an abrupt light intensity change in a period of several video frames till it disappeared (Fig. 4A). For a period longer than 10 s immediately before exocytosis, the granule showed neither drifting movement nor shape change. For detailed analysis, differential images were made by subtracting a video frame from the previous one, and average intensities of the differential images were projected as profiles (Figs. 4B and 4C). They showed that the granule fluctuated only slightly till the moment of its exocytotic fusion. For the sake of comparison, an image of granule was shifted by one pixel (38 nm) and then the resultant image was subtracted from the original image. The differential image and its average intensity profile (1-1*) clearly showed that the effect of the pixel shift was larger than any contrast appeared in differential images or intensity profiles naturally observed before exocytosis. Therefore, zymogen granules were shifted in position or altered in shape not more than 38 nm immediately before exocytosis. These findings suggest that zymogen

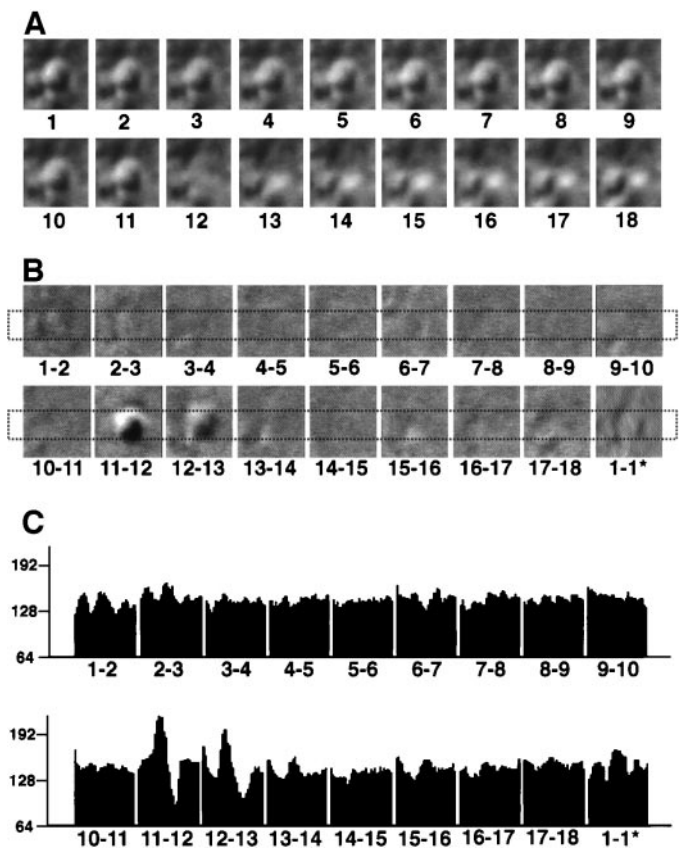

FIG. 4. Lack of movement of zymogen granule immediately before and during its exocytosis. (A) Sequential images of a granule that underwent exocytosis during stimulation. The interval between each frame was 1 s. The granule showed a light intensity change in images 11 through 13, and then disappeared. (B) Differential images obtained by subtracting frames in A from its previous one. (C) Projected intensity profiles of the areas indicated by dotted line in B (the ordinate is expressed in an 8 bit scale). The numbers in B and C correspond to those in A. The frames denoted as 1-2 in B and C are a difference image and its intensity profile is made from frames 1 and 2 in A. The frames denoted as 1-1* in B and C were made by shifting image 1 horizontally by one pixel (38 nm in distance) and subtracting it from the original frame 1. Patterns of differential images and their intensity profiles were faintly varied with time, suggesting a possible fluctuation of the granule before exocytosis. However, the fluctuation was estimated to be smaller than 38 nm, if any, because the each subtracted frame before exocytosis shows a pattern of contrast smaller than that in the image 1-1* and the projection 1-1*. The lumen was on the right side of the granule.

granules are closely docked to an appropriate site on the apical membrane for quite a long time before exocytosis, and that no translocation of granules occurs immediately before exocytosis. There was no morphological sign prior to exocytosis. This suggests that the fusion of granule with the plasma membrane is initiated totally by a molecular process as proposed by Xu *et al.* (14). Supramolecular structural changes such as swelling of granules and loss of actin barriers are not involved in the initiation mechanism directly leading to regulated exocytosis. The findings also suggest that the compound exocytosis is rare in pancreatic acinar cells under the physiological condition.

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