Exocytosis and movement of zymogen granules observed by VEC-DIC microscopy in the pancreatic tissue en bloc

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Received 10 July 1998; accepted in final form 10 May 2000

Ishihara, Yukio, Takashi Sakurai, Taizou Kimura, and Susumu Terakawa. Exocytosis and movement of zymogen granules observed by VEC-DIC microscopy in the pancreatic tissue en bloc. Am J Physiol Cell Physiol 279: C1177–C1188, 2000.—The dynamic aspects of exocytosis, especially in the normal acinar tissue en bloc, have remained unclear. We visualized exocytosis directly in the tissue of the exocrine pancreas of rodents by video-enhanced contrast-differential interference contrast (VEC-DIC) microscopy to investigate various exocytosis-related rates and the relationship between the movement of granules and exocytotic responses. Stimulation of the tissue with bethanechol or cholecystokinin caused many of the zymogen granules in the apical pole to disappear abruptly. The exocytotic transients of individual granules were completed in 0.48–0.65 s. Granules destined to participate in the exocytotic response moved randomly at velocities of ~0.06 μm/s or less during stimulation. In the tissue preparation, granules located far from the apical pole frequently moved back and forth for 1–7 μm without showing exocytosis. Colchicine suppressed this movement and the late phase of the secretory response. Real-time (VEC-DIC) observation of granule dynamics revealed that the initial step of exocytosis was not coupled directly with the microtubule-dependent translocation but with a continuous, slow Brownian fluctuation of granules.

granule movement; pancreas; video microscopy; video-enhanced contrast-differential interference contrast microscopy

THE EXOCRINE PANCREAS SECRETES fluid by channel activity and enzymes by exocytotic membrane fusion. In contrast to channel dynamics, the details of fusion dynamics are still unclear because of a lack of the means to study them in real time. The secretory process of acinar cells has long been studied morphologically by examining fixed preparations (12, 26). The exocytosis of secretory granule in the time domain has been analyzed by capacitance measurements of the plasma membrane with a patch pipette (19, 24). However, this technique is rather difficult, is inapplicable to cells surrounded by an intact tissue, and is complicated by problems caused by membrane retrieval and other morphological changes of the cell. The secretory process of catecholamine-containing vesicles has been investigated by amperometry, that is, by measuring the oxidation current with a thin carbon-fiber electrode. This has been applied to the exocrine pancreas charged with exogenous serotonin (36). The spatial resolution is insufficient to pinpoint the site of exocytosis and to trace back the origins of the corresponding granules.

McCuskey and Chapman (20) attempted to visualize the secretory process at the single granule level using a bright-field light microscope combined with a cinephotographic camera. However, it was not possible to detect exocytosis in living cells without amplification of image intensity. Anderson and McNiven (2) used phase-contrast microscopy to detect exocytosis in pancreatic acinar cells. However, the cells used in their study were derived from a clone that had lost the acinar structure; therefore, exocytosis was not convincingly captured, and the spatial aspects of exocytosis in reference to cellular polarization remained ambiguous and controversial. Recently, Terakawa and colleagues (14, 27, 28, 32–35) used video-enhanced contrast-differential interference contrast (VEC-DIC) microscopy for the dynamic analysis of exocytosis. The DIC lens, with a high numerical aperture, yields an image of a thin optical section of an intact tissue, and the enhancement of the contrast with a video system amplifies a discrete change in the image of the granule. This technique has advantages over the patch-clamp technique or carbon-fiber amperometry in its ability to reveal the spatial details of the secretory response in a single cell in normal tissue.

Knowledge of the mechanisms responsible for the release of digestive enzymes from zymogen granules is important for understanding disorders of the exocrine pancreas, including pancreatitis. In this study, we have exploited the advantages of VEC-DIC microscopy, to visualize the exocytosis of individual granules in pancreatic tissues with the normal acinar structure, and tracked the movements of secretory granules in the regions of synthesis and secretion. This allowed us to quantitate the secretory activities induced by various modes of stimulation and provided us with an opportunity to analyze explicitly the dynamic parameters related to exocytosis as well as the relationship...
between exocytosis and the movements of secretory granules in exocrine cells.

MATERIALS AND METHODS

Preparation. The main body of the pancreas was excised from the male guinea pig (Hartley strain) or rabbit (Japanese White) anesthetized with pentobarbital sodium (50 mg/kg ip for guinea pig, 40 mg/kg iv for rabbit). The tissue was cut immediately into small pieces (~1.0 × 1.0 × 0.5 mm³) with the use of razor blades. A few pieces of tissue were placed in a chamber, the bottom of which was made of a coverslip. The tissues were superfused continually at 33°C with standard medium containing (in mM) 115 NaCl, 3 KCl, 2 CaCl₂, 1 MgCl₂, 5 glucose, 1 KH₂PO₄, 20 NaHCO₃ and 16 HEPES (pH 7.4, adjusted with NaOH). The medium was oxygenated with a slow supply of medicinal gas into a vinyl jacket surrounding silicone tubing for superfusion.

VEC-DIC microscopy. The instruments used for light microscopic observation were similar to those described previously (33). Briefly, acinar cells of the pancreas were observed under an inverted DIC microscope (Axiovert 10, Zeiss, Ober-cohen, Germany) equipped with a ×100 objective lens (Plan-Neofluar, numerical aperture = 1.3, oil immersion) and a ×2.5 insertion lens. The DIC microscope produced an image of the tissue optically sectioned to a slice of about 1 μm thick. The DIC image was captured with a 0.5-in. charge-coupled device video camera (TI-23A, NEC, Tokyo, Japan) and then digitized and processed with an image processor (ARGUS-20, Hamamatsu Photonics, Hamamatsu, Japan) for real-time enhancement of the contrast (VEC). The processed images were observed on a video monitor screen (14 in., black and white, Panasonic, Osaka, Japan) at a final magnification of ×4,400 or ×11,000 and recorded on videotape with an S-VHS format video cassette recorder (AG-7500, Panasonic). All pictures and data in this study were reproduced from these video records. For reproduction, video frames were digitized and arranged using picture-handling software (Photoshop 3.0J, Adobe, Mountain View, CA). Figures 1–3 and 9–11 were printed with a digital printer (UP-D7000, Sony, Tokyo, Japan).

Stimulation of the pancreas. The pancreatic tissues were stimulated by adding bethanechol (BCh; 1, 10, 50, and 100 μM) or cholecystokinin octapeptide (CCK-8; 5 nM) to the standard medium. In some cases, Na⁺ (65 mM) in the standard medium was replaced with K⁺. Ca²⁺-chelated medium was prepared by adding EGTA (1 mM) and an aliquot of NaOH to the Ca²⁺-omitted standard medium.

Assessment of exocytotic response by light intensity change. Release of highly condensed substances from the granules was assumed to give rise to a change in the refractive index, which would appear as a discrete light intensity change under a DIC microscope. Therefore, we considered that an abrupt change in light intensity of a granule was an exocytotic response. To study the dynamic aspects of such an exocytotic response, we measured the light intensity changes of a single granule quantitatively. Sequential video images of a single granule were stored in multiframe memories of the tissue optically sectioned to a slice of about 1 μm thick. The DIC image was captured with a 0.5-in. charge-coupled device video camera (TI-23A, NEC, Tokyo, Japan) and then digitized and processed with an image processor (ARGUS-20, Hamamatsu Photonics, Hamamatsu, Japan) for real-time enhancement of the contrast (VEC). The processed images were observed on a video monitor screen (14 in., black and white, Panasonic, Osaka, Japan) at a final magnification of ×4,400 or ×11,000 and recorded on videotape with an S-VHS format video cassette recorder (AG-7500, Panasonic). All pictures and data in this study were reproduced from these video records. For reproduction, video frames were digitized and arranged using picture-handling software (Photoshop 3.0J, Adobe, Mountain View, CA). Figures 1–3 and 9–11 were printed with a digital printer (UP-D7000, Sony, Tokyo, Japan).

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Results of all granules appearing in the focal plane (about 1 μm in thickness) were clearly captured, and those located about 1 μm off the focal plane in both sides were also captured, although the latter images were blurred. The precise numbers of exocytotic responses every minute were counted to quantitate the secretory activity at the cellular level. This number would reflect roughly one-half of the total number of exocytotic events that occur in a spherical region of 4–5 μm in a single acinus.

 Movements of zymogen granules. To examine the movements of zymogen granules, the x-y coordinates of the centers of a zymogen granule on the monitor screen were measured. A single granule was tracked in every video frame for a few seconds. The granule movements were measured as the differences of the coordinates between two frames. The velocities of granule movements were calculated by dividing the distance by the intervals between frames. The granules that underwent exocytosis near the lumen (at the apical membrane) were chosen for analysis for periods of 3.7–5.7 s immediately before exocytosis. For the purpose of comparison, the movements of granules chosen randomly from the same sequence of video records that did not undergo exocytosis and that were located far from the apical membranes (central region in the cell) were analyzed. The data in the resting states and the stimulated states were compared. The resolution for a positional measurement by the VEC-DIC system was about 40 nm in space (7) and 33.3 ms in time.

Amylase activity. Several pieces of acinar tissue prepared for microscopic observations were incubated in test tubes (0.5 ml) in the absence or presence of BCh (10 μM, 100 μM, and 1 mM) for 10 min at 37°C. The amylase activities in the extracellular medium (~200 μl) in the test tubes were assayed by Special Reference Laboratory (Tokyo, Japan) enzymatically by use of 2-chloro-4-nitrophenyl-d-maltolheptaose as a substrate. The wet weights of the tissues in each tube were measured, and the activities were normalized to the weights.

Statistical analyses. Statistical analyses were performed by using the unpaired t-test. Significance was attributed at P < 0.01. All data were calculated and analyzed with a personal computer (Power Macintosh 8100/80, Apple Computer, Dallas, TX) using application software (StatView 4.1, Abacus Concepts, Berkeley, CA). Data are expressed as means ± SE.

Results

General view of the acini. Each acinus of the exocrine pancreas (guinea pig or rabbit), consisting of 8–10 acinar cells, was clearly visible at a magnification of ×4,000 (Fig. 1). Exocrine acini were easily distinguished from endocrine islets by several characteristics. Acinar cells were pyramidal or trapezoidal in shape; apicals assembled together to form a narrow void space presumably an acinar lumen. These cells had nuclei with a very vague outline in the basal side. In contrast, islet cells showed a rather round shape and a clear nucleus. Usually, the lumens of most acini in the resting state were shrunken; occasionally, some wide lumens with a gap width of 1–2 μm were seen. The zymogen granules in each acinar cell were clearly visible. They were 0.3–1.5 μm in diameter and were concentrated near the apical pole of each acinar cell. They fluctuated very slowly within an area comparable to their diameter in the region crowded with granules.
The granules located outside the crowd and in the perinuclear region showed a similar fluctuation. However, in addition to the fluctuation, these granules sometimes moved quite rapidly in a straight manner for a distance three times their diameter or much longer. Application of 100 μM colchicine suppressed these longer movements significantly, but not the slower fluctuations (see Movement of zymogen granule below).

Occasionally, after inappropriate preparation procedures, insufficient oxygenation, or excessive stimulation, large vacuoles formed inside the acinar cells. We found vacuoles also in cells stimulated with 1 mM BCh for 30 min. The vacuoles were characterized by a refractive index lower than the surrounding area, a spherical shape, and an amorphous content. In these cells, no further physiological responses were observed. Therefore, care was taken to prevent such morphological disorders. These preparations were discarded.

**Single exocytotic responses.** Stimulation of acinar cells by superfusion with a medium containing BCh (1, 10, 50, and 100 μM) or CCK-8 (5 nM) caused many zymogen granules to show abrupt changes in light intensity and to disappear sequentially. The positions of individual granules did not change significantly until they disappeared (see Movement of zymogen granule below). In a single cell, the intervals between such responses were short at the beginning and then became longer by 1–2 min. The intervals between individual responses seemed to be quite random throughout. Such responses were always found in the region near the lumen (Fig. 2). The images of response were sharper and clearer in the rabbit than in the rat.

In an acinus, each acinar cell showed similar responses to the stimulants. No responses were observed 1–2 min after removal of the stimulants from the medium. There was a decrease in the number of spontaneously responding granules at 3–5 min. The acini were slightly shrunken during the initial few seconds of 100 μM BCh stimulation. After vigorous releases of substances from many granules, the outlines of the luminal space became vague.

**Time courses of light intensity changes of a single granule during exocytosis.** The changes in image and light intensity of some zymogen granules in acinar cells stimulated with BCh were analyzed by using the digital image processor. Immediately before the occurrence of the changes of light intensity, the zymogen granules showed no discernible change in shape and size. For example, the diameter of a granule remained in a range of 0.62–0.67 μm for more than 100 ms (frames 1–4 in Fig. 3). The changes in light intensities were assumed to indicate the secretory responses. In most granules, the initial light intensity was quite stable; the light intensity then suddenly changed to another level and reached a plateau. When this transition was completed, the granules disappeared. The dynamics of these secretory responses in many granules were analyzed (Fig. 4). The dynamics among exocytotic responses induced by different concentrations of BCh (1, 10, and 100 μM) were compared in histograms (Fig. 5). They were not significantly different (P < 0.01). The average transition times in exocytosis (releasing time) in the cells stimulated with these concentrations of BCh ranged from 0.48 to 0.65 s.

**Secretory activity measured from the frequency of exocytosis.** To quantify the secretory activity, frequency histograms were produced by playing back the videotape and counting the numbers of exocytotic responses in a single acinus during every minute. When the acini were stimulated by addition of BCh (1, 10, 50, and 100 μM) to the superfusion medium, exocytotic responses were observed in almost all preparations (Fig. 6). A high concentration of BCh (hyperstimulation; 50 and 100 μM) always induced the exocytotic responses at a high frequency during an initial period of a few min-

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**Fig. 1.** Video-enhanced contrast-differential contrast (VEC-DIC) microscopic images of an acinus of the guinea pig obtained before (A) and 30 min after stimulation with 50 μM betahanechol (BCh; B). After stimulation, acinar lumen was slightly enlarged and the number of zymogen granules was decreased. Scale bar = 5 μm.
utes. During continued application of BCh for about 30 min, the response frequency fell, and the responses tended to disappear. When 1 mM BCh was used, secretory activities were similar to those shown with 100 μM, but large vacuoles appeared inside many acinar cells. Sometimes, after long stimulation (over 30 min) with 100 μM BCh, similar vacuoles were formed. At lower concentrations of BCh (1 and 10 μM), the frequencies of exocytotic responses were much lower, and the responses did not display a peak even in the initial phase (Fig. 6). These responses ceased 1–2 min after acini were returned to the standard medium.

The secretory activity, measured as the frequency of exocytosis in the dose-response curve, increased with concentrations of BCh from 1 to 100 μM (Fig. 7A). A similar dose-response relationship was found for the

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Fig. 2. VEC-DIC microscopic images of the acini of the rabbit. A: low-magnification view of the tissue. B: high-magnification view of an acinus. Scale bars = 5 μm. C: schematic representation of the sites where exocytotic responses occurred in the initial 20 s of stimulation with 20 μM ACh in the acinus shown in B. Circular marks were traced from video images made by processing the original video tape through the time differential mode. All the changes in images that occurred during a period as short as 33 ms were captured. Solid curves indicate the longitudinal axes of the luminal space. Exocytotic responses were always found in the region near the lumen.

Fig. 3. Sequential images of a granule undergoing exocytosis produced by 50 μM BCh. Interval between each frame was 67 ms. Frames 4–11 show the process of an exocytotic response. The granule disappeared in the latter images. Note that there was no visible change from frame 1 to frame 4. The diameters of the granule in frames 1–4 ranged from 0.62 to 0.67 μm.
release of amylase from acinar tissues measured under similar conditions (Fig. 7B). The BCh-induced responses were blocked almost completely in the presence of 1 μM atropine (Fig. 8A). Elevation of the K\(^+\) concentration (to 65 mM) in the medium did not induce an exocytotic response. However, in the same preparation, stimulation with CCK-8 (5 nM) 10 min after reduction of the K\(^+\) concentration to the normal level induced a large burst of exocytotic responses (Fig. 8B). The BCh-induced exocytotic responses were also suppressed strongly and reversibly when lanthanum chloride (10 μM) was added to the medium (Fig. 8D).

Fig. 4. Time course of the light intensity changes of the granule during its exocytotic response in the guinea pig. Ordinate represents the light intensity expressed in percentage of the initial value. Light intensity changed in 967–1,333 ms. Time required for the light intensity to change by 95% of the maximum was defined as the release time (solid line), which was 0.37 s (from point 1 to point 2).

Fig. 5. Histograms of the release times for the exocytotic responses induced by different concentrations of BCh (100 μM in A, 50 μM in B, 10 μM in C, and 1 μM in D) and those found without stimulation (spontaneous release; D) in the guinea pig. Abscissa represents the time required for the light intensity to change (release time). Average values of the release times ranged from 0.48 to 0.65 s. There was no significant concentration dependence of the release time (P < 0.01). N, number of responses; m, mean time; n = no. of cells.

Fig. 6. Frequency histograms of exocytotic responses in a single acinus stimulated with BCh in the guinea pig. Different concentrations of BCh (100 μM in A, 50 μM in B, 10 μM in C, and 1 μM in D) were applied during the periods indicated by the horizontal bars. Ordinate represents the number (N) of exocytotic responses counted every minute. The secretory activity increased with the concentration of BCh.
Movement of zymogen granules. To study the movement of a granule immediately before its exocytosis, the traveling path (trajectory) of a single granule that underwent exocytosis was traced retrospectively by playing back the videotape. For a period of 5 s immediately before the exocytosis, the average velocity of granule movement was 0.06 μm/s (Table 1). During this period, the average maximal distance of granule movement was 0.16 μm. In contrast, the average velocity of granules that did not undergo exocytosis, because they were located in the central region of the cell, was 0.11 μm/s. The maximal distance of movement of these granules was 0.29 μm. To compare these data with those of granules in the resting state, granules of the same acinus were also measured before stimulation. Groups of granules were chosen from the area near the lumen and from the central region in the cell. The average velocity and the maximal distance of granule movement were, respectively, 0.08 μm/s and 0.17 μm near the lumen and 0.10 μm/s and 0.21 μm in the central region. These values were not significantly different ($P < 0.01$) from those obtained in the stimulated state. Actually, these movements appeared on the monitor screen as very slow fluctuations as described above. A typical example of the movement of a granule that underwent exocytosis is shown in Fig. 9. Many granules underwent exocytosis without any spatially directed drift in the apical region, similarly to this example.

Occasionally, granules in the central region showed a linear, long movement (>1 μm). Granules frequently

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**Fig. 7.** Dose-response curves for secretory activity measured by counting the frequency of exocytotic events (A) or by assaying the enzymatic activity (B). In A, the numbers of exocytotic events were counted for an initial period of 5 min in the tissues stimulated at 3 different concentrations of BCh (1, 10, and 100 μM). Numbers of preparations measured were 4, 6, and 5, respectively. In B, dose-response curves are for amylase released from several pieces of tissue to the medium, measured in test tubes. Tissues were stimulated with BCh at 10, 100, and 1,000 μM. Activities were normalized for the wet weight of acinar tissues and expressed in IU/mg. The numbers of samples were 3, 4, 5, and 3, respectively, for each of the points shown.

**Fig. 8.** Effects of various reagents on the secretory activity (guinea pig). A: atropine (1 μM; Atr). B: high concentration of K+ (65 mM) and cholecystokinin octapeptide (CCK-8; 5 nM). C: Ca2+-free medium (1 mM EGTA). D: lanthanum (La; 10 μM). Ordinate represents the number of exocytotic events counted every minute. A–D represent data obtained from different preparations.
glided 5–10 μm in 3 s at a stretch. Some moved from the nuclear region to the back row of the granule accumulation in the apical pole (Fig. 10). Others moved in a reverse direction along a similar track. Such long movements (traveling or gliding) were not observed in the luminal region. These movements were thought to be of different nature and thus were excluded from the analyses of the central region, displayed in Table 1, and were analyzed separately. A typical case of the linear and long-distance movement of a granule is shown in Fig. 11. To ascertain the nature of the long movements of granules, the effects of colchicine and BCh on the numbers of granules that moved over 1 μm were examined. The numbers of traveling granules were decreased by 100 μM colchicine and were increased by 20 μM BCh (Fig. 12). Neither colchicine nor BCh affected the smaller fluctuating movements. Such long-distance traveling of granules never led directly to exocytosis in the central region. The presence of 100 μM colchicine (for 10 min before BCh application) failed to suppress the secretory activity, as measured by the frequency of exocytosis (Fig. 12A). The presence of 100 μM colchicine (for 20 min before BCh application) slightly suppressed the number of exocytotic responses (Fig. 12B). This suppression was stronger in the later phase of the secretory response than in the initial phase (compare with Fig. 12A).

**DISCUSSION**

In this study, exocytosis of zymogen granules in the pancreatic tissue was visualized directly in real time.

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**Table 1. Average velocity and maximal distance of movement of granule**

<table>
<thead>
<tr>
<th>Region</th>
<th>Resting</th>
<th>Stimulated</th>
<th>n</th>
<th>Resting</th>
<th>Stimulated</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal</td>
<td>0.08 ± 0.04</td>
<td>0.06 ± 0.02*</td>
<td>10</td>
<td>0.17 ± 0.05</td>
<td>0.16 ± 0.07*</td>
<td>10</td>
</tr>
<tr>
<td>Central</td>
<td>0.11 ± 0.05</td>
<td>0.11 ± 0.04*</td>
<td>11</td>
<td>0.21 ± 0.06</td>
<td>0.29 ± 0.10*</td>
<td>11</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of granules. Values represent granules that underwent exocytosis. Stimulation was applied with bethanechol (50 μM). During stimulation, average velocity and maximal distance were significantly different (*P < 0.01) between granules that underwent exocytosis and those that remained without exocytosis.
by VEC-DIC microscopy. The fine movements of zymogen granules in individual cells with normal acinar structure could be analyzed in both the absence and presence of exocytosis. Thus granule movements in the resting secretory states were compared. In the resting state, secretory granules showed frequent bidirectional gliding movements between the perinuclear region and the subapical region, but granules near the apical pole showed only very slow movements. Stimulation induced exocytosis at the apical membrane and produced a slight decrease or no change in the fluctuation movements and a slight increase in the gliding movements of granules in the perinuclear region. Therefore, we conclude that the result of the directed movements in the middle part of the cell was to cause secretory granules to accumulate in the apical pole and that the granules were driven to the final docking site only by Brownian movement.

Exocytosis visualized by VEC-DIC microscopy. The exocytotic response of a single granule was clearly visualized by the present technique as an abrupt change in light intensity followed by the disappearance of the granule. This made it possible to quantitate and analyze the secretory process dynamically at a subcel-

Fig. 10. A: DIC image of an acinus of the rabbit. L, luminal space. Scale bar = 5 μm. B: schematic representation of the directions and loci of granules, showing long-distance movements (>1 μm) of the acinus shown in A. Some moved from the nuclear region to the back row of the granule accumulation region in the apical pole. Others turned backward. C–E: sequential images of a granule showing a basoapical gliding followed by a reverse gliding movement. Each position of granule marked by the arrow corresponds to c, d, and e in B. Scale bar = 3 μm.
The abrupt light intensity changes of a granule under a VEC-DIC microscope were generally considered to be exocytotic responses for the following reasons:
1) the responses of granules were induced only when cells were stimulated with specific agonists;
2) the responses were always followed by the disappearance of the granule;
3) the responses could be suppressed by addition of a specific antagonist or by removal of Ca$^{2+}$ from the medium; and
4) similar optical changes of vesicles in chromaffin cells were associated with the release of substances oxidizable with a carbon-fiber electrode at 1500 mV (34). In the present experiments, the exocytotic responses appeared exclusively when the tissue was stimulated with BCh (10–100 μM) (Fig. 6) and only in the vicinity of the lumen (Fig. 2C). In accordance with the similar responses in other secretory cells, the granules in pancreatic acinar cells also disappeared after such responses (Fig. 3). In addition, the dose dependence of the secretory activity, measured as the frequency of exocytotic events, was similar to that measured for the release of amylase activity (Fig. 7). Therefore, it was concluded that the optical responses we observed were due to exocytosis.

The responses were completely suppressed by 1 μM atropine (Fig. 8), suggesting that muscarinic receptors are involved in triggering the response (6). The BCh-induced exocytotic responses were significantly suppressed by removal of Ca$^{2+}$ from the external medium (Fig. 8). The exocytotic responses were also strongly suppressed when lanthanum, known to block Ca$^{2+}$ channels on the cytoplasmic membrane, was added to the medium. These results are in full accordance with the properties of the intracellular Ca$^{2+}$ response and amylase secretion measured earlier (37). Furthermore, these results are compatible with other studies of exocytosis performed by VEC-DIC microscopy (14, 27, 28, 32–35).

In pancreatic acini prepared by other methods, the dose-response curves for amylase release induced by muscarinic agonists showed some inhibitory effects at concentrations in the range of 50–100 μM (10). These preparations showed receptor desensitization (38) as well as a rapid decay of both the Ca$^{2+}$ transients (21) and amylase releases (36) at high doses. In salivary glands, water secretion, which provides a vehicle for amylase, also shows reduced efficiency during muscarinic stimulation at high doses, whereas the energy consumption is enhanced at the same doses (22, 23). One of the unique aspects of our preparation was that the basal release observed without stimulation was
Before exocytosis, no increase in granule diameter of more than 50 nm were observed (Fig. 3). This absence of significant enlargements of the granules observed in the present preparation seems to exclude the possibility that one granule fused with another before exocytosis. However, one granule may fuse with another when one had fused with the apical membrane. This occurs frequently in colonic goblet cells, resulting in a large invagination or cavity in the apical pole of the cells after prolonged stimulation (35). In pancreatic acinar cells, formation of the apical invagination was rare, indicating that the membrane retrieval after an exocytotic response was sufficiently fast (~1 s).

The discrete light intensity changes of a single zymogen granule reflected the release of highly condensed substances into the extracellular space. The time required for this optical change reflected the process from the initial phase of fusion to the complete release of its contents (releasing time). According to our measurements, the average times required for BCh-induced exocytosis ranged from 0.48 to 0.65 s (Fig. 5). In the case of vesicles containing catecholamines, the estimates of the time required for the contents to diffuse out (releasing time) from measurements of the oxidation current by means of a carbon-fiber electrode ranged from 1 to 100 ms (3, 5, 39, 42). It is reasonable to assume that the larger the granule size, the slower the dynamics. Our video microscopic measurement of zymogen granules gave a release time compatible for larger granules (35).

Other factors that affect the dynamics of the exocytotic process might include the nature of the granule membrane, solubility of granule contents, density of substances in the acinar lumen, kinds of stimulants, and so on. It is also possible that water secretion, depending on the concentration of BCh, alters the luminal environment. However, the dynamics in the presence of different concentrations of BCh (1, 10, and 100 μM) were very similar. This suggests that the release time depends more on the granule itself than on the luminal environment.

Movement of a single granule before exocytosis. Agonist-induced exocytosis has been assumed to involve the following sequential steps (9, 12, 26): 1) movement of zymogen granules toward the apical pole, 2) docking of the granule to an appropriate membrane site, 3) fusion of the granule membrane with the cytoplasmic membrane, and 4) release of granule contents into the luminal space. These processes were assumed to occur mainly as a result of studies of stationary images obtained by electron microscopy. Although the movements of granules in several types of secretory cells were studied (1, 11, 15, 16, 30), the relationship between the movement and exocytosis remains unclear because the properties of both processes were not observed simultaneously.

Anderson and McNiven (2) claimed that they detected exocytosis in pancreatic acinar cells under a phase-contrast microscope. However, the clonal cells they used had lost the acinar structure; therefore, neither exocytosis nor cellular polarization was convincingly determined. Because it was difficult to quan-
titrate secretory activity at a single granule level, the
movement of granules they observed could be irrele-
vant to exocytosis. In fact, no directed shift of granules
toward the cell membrane has been reported in mast
cells (43), goblet cells (35), and chromaffin cells (33)
stimulated with agonists. Therefore, a question still
remained as to when and how granules translocated
from their site of synthesis to the site of exocytosis.

The normal basoapical polarities of the acinar cells
were maintained intact in the present study. The side
views indicated that long-distance movements of gran-
ules occurred frequently in the central region of the
cells; these were found not to be directly related to
exocytosis (Fig. 11). The suppression of these move-
ments by colchicine (Fig. 12) suggests that granules
are transported along the microtubule track (4, 29, 40).
Thus granules synthesized in the perinuclear region
are transported rapidly toward the apical pole by the
microtubule-mediated gliding mechanism. After the
transport, most granules stay in the region of granule
accumulation, but some leave by the retrograde trans-
port mechanism. The probability of granule transport
to the region of granule accumulation is always higher
than that of the retrograde transport away from it.
Because of these dynamics, granules accumulate near
the apical pole even in the unstimulated state. This
view is in disagreement with the findings described in
a recent report in which granules flocked after stimu-
lation (2). However, the increase in overall transport
during stimulation (Fig. 12) is in accordance with a
report in which the association of kinesin with zymo-
gen granules increased during stimulation (18).

The finding that application of colchicine suppressed
exocytosis more strongly in the later phase of the
secretory response than during the initial 1-min period
(Fig. 11B) supports the view that the microtubule-
mediated supply of granules to the apical pole is one of
the limiting processes for the secretion only during the
persistent phase. Our direct observation of granule
transport provides an explanation of why colchicine
inhibited the amylase release by only 25–30% (12).
In anterior pituitary cells, colchicine also suppressed
exocytosis by a similar degree sometime after inhibition
of granule trafficking (our unpublished observation).

Zymogen granules moved <0.16 μm in the 5-s period
immediately before exocytosis. The average velocity
of the granule movement was 0.06 μm/s. The slowness
of this Brownian movement near the apical membrane
was probably due to restriction by cytoskeletal network
(particles of a similar size show much faster Brownian
motion in water). In fact, the actin cytoskeleton in the
vicinity of the apical membrane in the pancreatic acini
of the rat is dense (17). However, granules seem to
move through the network, since individual actin fila-
ments can bend and swing quite readily (42). Although
granule movements were very small, they clearly con-
tinued to move until they underwent exocytosis. This
suggests that granules are docked to the plasma mem-
brane in a form of a dynamic equilibrium that allows
some reversibility.

None of the granules that underwent exocytosis
moved faster in the stimulated state than in the rest-
ing state (Table 1). The granules actually tended to
slow down their movement. These findings suggest
that granules in front of the region of accumulation
may bind to and unbend from the apical membrane in
a form of dynamic equilibrium irrespective of the pres-
ence or absence of stimulation. The decrease of move-
ment would reflect a slight shift of the equilibrium
from the unbound to the bound state. Stimulation
triggers the chemical step necessary to induce exocy-
tosis only for those granules that happen to be in the
bound state so that the fusion of granules with the
apical membrane proceeds.

We are grateful to Dr. Charles Edwards for critical reading of the
manuscript.

The video sequences of exocytosis and movement of zymogen
granules are accessible at http://www.hama-med.ac.jp/w3s/photon.

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