Pepsinogen secretion: coupling of exocytosis visualized by video microscopy and \([\text{Ca}^{2+}]_i\) in single cells

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Tao, Chie, Masao Yamamoto, Hiroshi Mieno, Masaki Inoue, Tsutomu Masujima, and Goro Kajiyama. Pepsinogen secretion: coupling of exocytosis visualized by video microscopy and \([\text{Ca}^{2+}]_i\) in single cells. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G1166–G1177, 1998.—Conventional in vitro studies of pepsinogen secretion have measured secretion into the bulk medium and have demonstrated the critical role of \([\text{Ca}^{2+}]_i\) in the process. The present study was undertaken to obtain further details of the process of secretion and its relation to \([\text{Ca}^{2+}]_i\) changes over very short time periods. The relation between \([\text{Ca}^{2+}]_i\) mobilization and exocytosis in an isolated individual peptic cell of the bullfrog was investigated by a method to measure both intracellular \([\text{Ca}^{2+}]_i\) ([Ca^2+]_i), using a fluorescent \([\text{Ca}^{2+}]_i\) indicator, fura 2, and exocytosis from single cells using a video microscope analyzing system. Bombesin (3.2 \times 10^{-7} \text{M}) and bethanechol (3.2 \times 10^{-7} \text{M}) raised a rapid increase in \([\text{Ca}^{2+}]_i\) (initial peak) and a corresponding high frequency of initial exocytosis. After the initial peak, \([\text{Ca}^{2+}]_i\) was maintained at a somewhat elevated level over the baseline (sustained phase), with a correspondingly low frequency of exocytosis. Both the sustained phase of elevated \([\text{Ca}^{2+}]_i\) and the related exocytosis were eliminated by the depletion of extracellular \([\text{Ca}^{2+}]_i\). Low concentrations of bombesin (3.2 \times 10^{-10} \text{M}) and bethanechol (3.2 \times 10^{-7} \text{M}) caused sustained low-amplitude \([\text{Ca}^{2+}]_i\) oscillations with correspondingly low frequencies but also caused sustained exocytosis. These data show that 1) cellular response differs between high and low concentrations of stimulus, 2) there is a close relation between \([\text{Ca}^{2+}]_i\) and exocytosis, 3) exocytosis follows elevation of \([\text{Ca}^{2+}]_i\), and 4) there is a significant positive correlation between the peak \([\text{Ca}^{2+}]_i\) and the number of exocytoses.

bethanechol; bombesin; oscillation; bullfrog

Although the general relationship between stimulation and pepsinogen secretion is well established from bulk solution studies over time, i.e., an initial peak of intracellular \([\text{Ca}^{2+}]_i\) concentration ([Ca^2+]_i) and a burst of secretion, followed by a sustained lower level of \([\text{Ca}^{2+}]_i\). Increase and sustained secretion (1, 7, 8, 21, 30, 31), the relationship between \([\text{Ca}^{2+}]_i\) in single cells and exocytosis of individual granules in single pepsinogen-secreting cells has not previously been demonstrated. In this study, using recently available imaging techniques with computer enhancement (25, 27), we report such data. These data show, in real time, the relation of granule exocytosis in individual isolated peptic cells to both the concentration of \([\text{Ca}^{2+}]_i\) and to the oscillations in \([\text{Ca}^{2+}]_i\) in response to various doses of cholinergic and peptidergic (bombesin (BB)) stimuli. Before the development of the video-microscope system, time-resolved patch-clamp capacitance measurement was the only technique available for the analysis of exocytosis (11, 12, 17) and its relation to \([\text{Ca}^{2+}]_i\) mobilization (3, 4, 18). Because the patch-clamp method is technically difficult and causes cell injury, it was difficult to clarify the real-time relationships in intact cells. In recent years, much progress has been made in video microscopy techniques using a small-area charge-coupled device (CCD) camera and a multifrane digital image processor (25, 27). These techniques have recently been improved by computer enhancement of digital images. Using this powerful technique it is possible to see a single zymogen granule in the living state, to study the profile of secretory activity in isolated intact peptic cells with high spatiotemporal resolution, and to count individual exocytotic events. Furthermore, it is also possible to measure \([\text{Ca}^{2+}]_i\) in single cells using a silicon-intensified targeting (SIT) camera, a fluorescence microscope, and a multifrane digital image processor controlled by a computer program (Argus 100CA2). Using these techniques, we studied the relation between \([\text{Ca}^{2+}]_i\) events and granule exocytosis in isolated individual peptic cells.

MATERIALS AND METHODS

Materials. Bullfrogs (Rana catesbeiana) (6–7 in.) were obtained from Hiroshima Research Lab Animals (Hiroshima, Japan). EGTA, HEPES, carbamyl-β-methylcholine chloride [bethanechol (BCh)], digitonin, albumin, and poly-L-lysine were purchased from Sigma (St. Louis, MO). Collagenase (type I) was obtained from Worthington Biochemical (Freehold, NJ). Fura 2-acetyloxymethyl ester (AM) and fura 2 were purchased from Wako Chemicals (Osaka, Japan). BB was obtained from the Peptide Institute (Osaka, Japan). Calcium Calibration Buffer Kit 2 was purchased from Molecular Probes (Eugene, OR). All other chemicals were of the highest purity available.

Unless otherwise stated, the standard medium (amphibian Ringer solution) (ARS) contained (in mM) 89.4 NaCl, 4.0 KCl, 1.8 CaCl_2, 0.8 MgSO_4, 18 mM NaHCO_3, 11 glucose, 0.1% BSA, and 10 HEPES-NaOH buffer (pH 7.3). The medium was equilibrated with 95% O_2-5% CO_2. The Ca_2+-free medium contained no CaCl_2 or MgSO_4, which were replaced by NaCl (92.9 mM).

Peptic cell preparation. The peptic glands were prepared as previously described (14, 15, 21). The glands were washed with the ARS and allowed to attach naturally to the bottom of a chamber (50 µl) coated with 0.01% poly-L-lysine hydrobromide. Continuous superfusion (1 ml/min) at 20°C of the ARS through the chamber was maintained while glands were monitored using a ×40 objective lens on a Nikon Diaphot inverted microscope connected to a Spex Fluorolog spectrophotometer system.

Measurement of \([\text{Ca}^{2+}]_i\). To measure changes in \([\text{Ca}^{2+}]_i\), glands were incubated in the ARS containing 5 mM fura 2-AM for 30 min at room temperature and equilibrated with...
95% O2-5% CO2. After the glands were loaded with fura 2, they were washed three times and allowed to attach naturally to the bottom of a chamber (50 µl) coated with 0.01% poly-L-lysine hydrobromide that allowed continuous superfusion (1 ml/min) at 20°C. Fluorescent signals from a single cell were monitored using a 340 objective lens on a Nikon Diaphot inverted microscope connected to a Spex Fluorolog spectrofluorimeter Argus-100 system (Hamamatsu Photonics, Hamamatsu, Japan) and Ca2+ imaging software (CA-2; version 3.70) equipped with a SIT video camera (C2400–08, Hamamatsu Photonics). The emission wavelengths alternated every 2.5 or 5 s from 340 to 380 nm. [Ca2+]i was calculated from the fluorescence intensity ratios (340/380 nm) by fitting the ratios to a calibration curve (13, 16).

Observation of exocytosis in peptic clusters. Peptic cells were placed in the bottom of the chamber and observed under a differential interference contrast (DIC) objective lens, using an inverted Nomarski microscope (Axiovert 135T; Zeiss, Germany). The DIC images were detected with a CCD camera (SSC-M350; Sony, Tokyo, Japan). Then the video signals of the camera were contrast-enhanced with a high-speed digital image processor (Pip4000; ADS, Tokyo, Japan) controlled by a personal computer (PC9800; NEC, Tokyo, Japan) monitored at a magnification of ×1,960–2,940. The images were recorded on a videocassette using an S-VHS video recorder (AG-7355, Panasonic, Osaka, Japan). The clusters were placed on the inverted microscope stage at a temperature of 20–25°C and equilibrated with 95% O2-5% CO2 ARS by continuous perfusion. To make frequency histograms of exocytoses, we counted the number of abrupt changes in the brightness of granules in a peptic cell in unit time on slow playback of the videotape. Furthermore, we used a mode of image processing by which continuous images were converted into continuous time-differential images in real time by subtracting the image obtained at 33 ms from that obtained at 0 ms, the image obtained at 66 ms from that obtained at 33 ms, and so on, using a multiframe image processor (Argus-100 system) to reveal the rapid changes in brightness more clearly.

Freeze-fracture methods. Tissue specimens of peptic glands were fixed with 1% glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.3). After immersion in a 40% vol/vol glycerin solution at room temperature, tissue specimens were placed on a copper stage and frozen with liquid nitrogen. The specimens were fractured at −185°C in a J EOL J FD 7000 freeze-fracture apparatus and shadowed with platinum-carbon without etching and then digested in filtered commercial bleach. The replicas were washed twice in distilled water, mounted on grids, and examined in a Hitachi H-7000 electron microscope. Observation was performed on areas in which there were many large secretory granules in peptic cells.

Data analysis. Each experiment reported here was performed on preparations from at least four different frogs. Data are presented as means ± SD. Statistical analysis was performed using the Mann-Whitney test or Wilcoxon's signed-rank test. Significant difference was set at P < 0.05. All data were calculated and analyzed with a personal computer.
RESULTS

Freeze-fracture studies. Figure 1 shows electron micrographs of typical peptic cells stimulated for 2 min with 320 nM BB (magnification ×4,500). The cells had many secretory granules. The peptic cells showed microvilli projecting into the luminal cavity. Exocytoses were detected on the apical membrane after the stimulation, although exocytosis was not observed before stimulation (not shown).

Morphological changes of peptic clusters by agonists. Figure 2 shows DIC microscopic images of typical peptic clusters (magnification ×1,960). Before the stimulation, peptic clusters had narrow lumens and abundant secretory granules. Each peptic cell was 13–18 µm in diameter. The secretory granules appeared as bright or dark spots 0.2–2.5 µm in diameter (Fig. 2, A and B). When the agonist was applied to the perfusion medium, abrupt changes in the brightness of secretory granules were observed at the apical membrane (Fig. 2, C–F). Figure 2G shows time-differential images obtained by subtracting the preceding 33-ms image and by adding a proper offset value. These brightness changes, representing the exocytotic events (19, 20, 22, 25–27), were recorded on a videotape and converted to frequency histograms. A few seconds after the application, the lumen was widened and filled with cloudy material, and the apical membrane became rough (Fig. 3). Exocytosis seemed to occur on the apical membrane. Rapid movement of granules toward the apical membrane was not observed before exocytosis. Only some of the many zymogen granules exocytosed with stimulation. The final step in secretion is that granules fuse with the apical membrane and discharge their contents. After apical exocytosis, some of the zymogen granules near the basolateral membrane transferred to near the apical membrane. They did not fuse with the apical membrane but remained in the apical cytoplasm.

Frequency histograms and intracellular Ca\(^{2+}\) mobilization. In prior studies of dose-related pepsinogen secretion in isolated acini of frog pepsinogen cells, maximal effective concentrations of BCh and BB were found to be 32 µM and 320 nM, respectively (5). Figure 4 compares the exocytotic responses and Ca\(^{2+}\) mobilization. Figure 4A shows BCh- and BB-mediated Ca\(^{2+}\) mobilization in a medium containing Ca\(^{2+}\) (ARS), with an initial rapid increase followed by a plateau elevation. There were significant statistical differences between basal [Ca\(^{2+}\)] and peak [Ca\(^{2+}\)] (Table 3) and between basal [Ca\(^{2+}\)] and sustained [Ca\(^{2+}\)] (data not shown) stimulated by both BCh and BB. Figure 4B shows BCh-mediated and Fig. 4C shows similar BB-mediated exocytotic histograms in a medium containing Ca\(^{2+}\) (ARS). The pattern of exocytosis closely followed the Ca\(^{2+}\) mobilization pattern seen in Fig. 4A. Exocytosis was always observed 10–20 s after the application of agonists. There were significant statistical differences in lag time from the onset of stimuli (both 32 µM BCh and 320 nM BB) to the peak [Ca\(^{2+}\)] and the peak frequency of exocytosis (Table 1). The lag times between the peak [Ca\(^{2+}\)], and the peak frequency

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Fig. 3. Morphological changes of pepsinogen-secreting cells reproduced from sequential video frames (DIC images). Arrow indicates border of apical membrane. A: pepsinogen-secreting cluster obtained 1 s before application of beethanochol (BCh). B: 1 min after application of BCh (32 µM). Lumen has become slightly wider. C: 8 min after application of BCh. Lumen has become wider and still contains cloudy material. Apical membrane has become rough. D: 15 min after application of BCh. Changes in lumen and apical membrane are more pronounced than in C. Bar = 10 µm.
of exocytosis were 15.5 ± 4.8 s (n = 5) (stimulated by BCh) and 13.6 ± 5.1 s (n = 5) (stimulated by BB). Although there was probable variation in the shape of the histograms, the application of agonists caused a large peak of secretion during an initial period of <2 min and a small sustained secretion after the peak. Sustained secretion continued for >30 min (exocytosis had not ended during the 40 min of the observation). Figure 5 shows the same agonists used with a Ca²⁺-free medium. The sustained phase of Ca²⁺ mobilization was not observed (Fig. 5A), and there was no sustained phase of exocytotic events. However, the initial large
peaks of exocytosis occurred within about the same time frame after the application of the agonists (Fig. 5, B and C; cf. Fig. 4).

Repeated stimulation with BCh and BB. To determine whether BB and BCh release Ca^{2+} from the same pools, sequential stimulation by BB and BCh was applied, and the order was then reversed. Figure 6A shows [Ca^{2+}] mobilization from the pools by sequential stimulation with BB and BCh in Ca^{2+}-free medium containing 1 mM EGTA. Figure 6B shows the exocytotic events under the same conditions. [Ca^{2+}] mobilization and exocytosis were only detected during the period of BB stimulation. BCh did not induce an increase in [Ca^{2+}] or exocytosis after the BB stimula-
tion. When the order was reversed and BCh was applied before BB (Fig. 7A) in a Ca$^{2+}$-free solution containing 1 mM EGTA, both agents elicited peaks of 
\[\text{Ca}^{2+}\] and exocytotic events (Fig. 7) that were limited to 30–60 s.

The data suggest that the larger BB-sensitive Ca$^{2+}$ pool incorporates the smaller BCh-sensitive Ca$^{2+}$ pool. Initial spikes of Ca$^{2+}$ caused by either stimulus produced exocytosis.

Relation between Ca$^{2+}$ oscillation and exocytoses. Figure 8A shows dose-related [Ca$^{2+}$] data from a single cell of frog pepsinogen acini induced by high (32 µM) and low (320 nM) concentrations of BCh. Oscillations were most pronounced with the middle concentrations of 320 nM and 3.2 µM BCh but not at lower (32 nM) or higher concentrations (320 µM) (data not shown). Figure 8B shows exocytotic events induced by high (32 µM) and low (320 nM) concentrations of BCh, which produced different patterns of [Ca$^{2+}$] and corresponding patterns of secretion. Figure 9 shows dose-related [Ca$^{2+}$] data and exocytotic events induced by high (320 nM) and low (320 pM) BB. Oscillations were most pronounced with the lower concentrations of 320 pM and 32 pM (data not shown). At the concentration of stimulus showing the greatest oscillation, there was no initial peak of [Ca$^{2+}$] nor an initial spike of secretion, but low frequency of exocytosis continued for 30–60 min (Figs. 10 and 11) (exocytosis had not ended by the time the 40-min observation was over). There are clearly large differences in the pattern and magnitude of [Ca$^{2+}$] responses at various concentrations of both BB and BCh, which are reflected in the pattern of the exocytosis of pepsinogen. The mechanisms responsible for these differences are not yet clear. Nor is it clear which of these responses represents physiological conditions.

There were significant statistical differences in lag times from the beginning of stimulation (32 µM BCh and 320 nM BB) to the elevation of [Ca$^{2+}$] and the onset of exocytosis. Table 1 shows the lag time from the beginning of stimulation to peak [Ca$^{2+}$] and peak frequency of exocytosis.

<table>
<thead>
<tr>
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<th>Time to Peak [Ca$^{2+}$], s</th>
<th>Time to Peak Frequency of Exocytosis, s</th>
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<tr>
<td>Bethanechol (32 µM)</td>
<td>11.2 ± 2.1</td>
<td>26.7 ± 5.7*</td>
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<tr>
<td>Bombesin (320 nM)</td>
<td>14.6 ± 3.4</td>
<td>28.2 ± 5.3*</td>
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</table>

Values are means ± SD of 5 experiments. *P < 0.05 compared with lag time from beginning of stimulation to peak [Ca$^{2+}$] (Mann-Whitney U test).
Fig. 7. Repeated stimulation of the peptic glands by BCh (32 µM; first stimulation) and BB (320 nM; second stimulation) in Ca²⁺-free medium containing 1 mM EGTA. Despite prior BCh stimulation, BB induced an initial rapid increase in [Ca²⁺] (A) and exocytotic events (B). Trace in A represents means ± SD of 6 cells in 1 cluster, and data in B are representative of experiments with 5 different cell preparations.

Fig. 8. A: pattern of [Ca²⁺], stimulated by BCh at two different concentrations (32 µM and 320 nM). Data are means ± SD of 6 cells (32 µM BCh) and are representative of 5 experiments (320 nM BCh). B: pattern of exocytosis at low (320 nM) and high (32 µM) concentrations of BCh shows the lack of an initial spike of secretion at 320 nM. Data are representative of experiments with 5 different cell preparations.
Fig. 10. A low concentration of BCh (320 nM) produces an oscillatory pattern of \([\text{Ca}^{2+}]_i\) with oscillations of a similar magnitude (A). Moreover, the exocytosis pattern and the frequency of oscillations (B) were also very similar. Data are representative of experiments with 5 different cell preparations each. The lag times from the beginning of stimulation to the elevation of \([\text{Ca}^{2+}]_i\) and the onset of exocytosis were significantly different (Mann-Whitney test) (see Table 2).
of exocytosis (Table 2). The lag times between the elevation of $[Ca^{2+}]_i$ and the onset of exocytosis are $43.9 \pm 20.3$ s ($n = 5$) (stimulated by BCh) and $28.6 \pm 19.2$ s ($n = 5$) (stimulated by BB). The time from the beginning of stimulation to the elevation of $[Ca^{2+}]_i$ and the onset of exocytosis diminishes with higher concentrations of stimuli (Table 1).

**DISCUSSION**

Previous studies have indicated that cholinergic agonists such as BCh and peptidergic agonists such as BB bind their receptors, cause the breakdown of phosphoinositides by activating phospholipase C, mobilize cellular $Ca^{2+}$ (1), and finally induce pepsinogen secretion. These prior studies suggested that initial pepsinogen secretion is apparently independent of the type of stimulus (Fig. 12C).

Table 3. Relationship between peak $[Ca^{2+}]_i$ and number of exocytoses

<table>
<thead>
<tr>
<th>Concentration (M)</th>
<th>$n$</th>
<th>Basal $[Ca^{2+}]_i$ (nM)</th>
<th>Peak $[Ca^{2+}]_i$ (nM)</th>
<th>$n$</th>
<th>No. of Exocytoses</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCh</td>
<td></td>
<td></td>
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<tr>
<td>$3.2 \times 10^{-4}$</td>
<td>6</td>
<td>78.0 ± 0.4</td>
<td>475 ± 21*</td>
<td>3</td>
<td>31.4 ± 4.3</td>
</tr>
<tr>
<td>$3.2 \times 10^{-5}$</td>
<td>6</td>
<td>75.0 ± 0.5</td>
<td>338 ± 19*</td>
<td>5</td>
<td>27.2 ± 4.5</td>
</tr>
<tr>
<td>$3.2 \times 10^{-6}$</td>
<td>6</td>
<td>67.0 ± 0.8</td>
<td>235 ± 18*</td>
<td>3</td>
<td>12.5 ± 2.4</td>
</tr>
<tr>
<td>$3.2 \times 10^{-7}$</td>
<td>6</td>
<td>54.0 ± 0.2</td>
<td>221 ± 24*</td>
<td>5</td>
<td>5.7 ± 1.3</td>
</tr>
<tr>
<td>$3.2 \times 10^{-8}$</td>
<td>6</td>
<td>55.0 ± 0.5</td>
<td>104 ± 25*</td>
<td>3</td>
<td>0 ± 0</td>
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<tr>
<td>Bombesin</td>
<td></td>
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<tr>
<td>$3.2 \times 10^{-6}$</td>
<td>6</td>
<td>75.0 ± 0.5</td>
<td>1054 ± 54*</td>
<td>3</td>
<td>60.6 ± 6.4</td>
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<tr>
<td>$3.2 \times 10^{-7}$</td>
<td>6</td>
<td>72.0 ± 0.5</td>
<td>627 ± 46*</td>
<td>5</td>
<td>51.7 ± 6.1</td>
</tr>
<tr>
<td>$3.2 \times 10^{-8}$</td>
<td>6</td>
<td>75.0 ± 0.3</td>
<td>462 ± 27*</td>
<td>3</td>
<td>33.5 ± 4.1</td>
</tr>
<tr>
<td>$3.2 \times 10^{-9}$</td>
<td>6</td>
<td>58.0 ± 0.2</td>
<td>371 ± 20*</td>
<td>3</td>
<td>21.6 ± 3.4</td>
</tr>
<tr>
<td>$3.2 \times 10^{-10}$</td>
<td>6</td>
<td>59.0 ± 0.3</td>
<td>248 ± 16*</td>
<td>5</td>
<td>12.2 ± 2.5</td>
</tr>
<tr>
<td>$3.2 \times 10^{-11}$</td>
<td>6</td>
<td>58.0 ± 0.5</td>
<td>143 ± 11*</td>
<td>3</td>
<td>0 ± 0</td>
</tr>
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</table>

Values are means ± SD for 3–6 experiments. *P < 0.05 compared with basal $[Ca^{2+}]_i$ each (Wilcoxon signed-rank test).
secretion by these agonists is triggered by mobilization of Ca\(^{2+}\) from internal Ca\(^{2+}\) pools, and sustained secretion is dependent on the influx of Ca\(^{2+}\) (1).

Furthermore, pepsinogen secretion is believed to be mediated by exocytosis, a fusion of secretory granules with the plasma membrane that depends on processes that bind phospholipids in the presence of Ca\(^{2+}\), e.g., via annexins (2), followed by an extrusion of their contents to the extracellular space (6). To further define the process of exocytosis and to elucidate the direct relationship between pepsinogen secretion and Ca\(^{2+}\) mobilization, we used a video microscope system. This system has high spatial resolution of at least 160 nm, compared with resolutions of 150 µm obtained using conventional microscope systems. Additionally, this system has a high time resolution (i.e., images can be taken every 33 ms). These capabilities provide powerful tools for dynamic studies of the secretory process at the level of a single granule in a single cell. This system allowed us to observe "popping" responses (abrupt changes in appearance and in light intensity) of the granules, and we concluded that these popping responses are exocytosis in the peptic cells for the following reasons: 1) These popping responses were induced only when peptic cells were stimulated and were never observed without stimulation. 2) After such responses, granules were not recovered by readjustment of the microscope focus. 3) The popping responses were similar to exocytotic responses found in other secretory cells, namely, chromaffin cells (22), colonic goblet cells (21), salivary acinar cells (20), nasal epithelial goblet cells (9), pancreatic \(\beta\)-cells (19), and neutrophils (22). These images are supported by the freeze-fracture studies (Fig. 1).

The present studies provide a much more detailed mechanistic and temporal picture of the secretion processes of the peptic cell than studies of secretion into bulk solutions over long time periods (5, 8, 21) or even studies such as continuous perfusion of isolated glands, in which observations are made at intervals of 10 s or more (14). In bulk solution studies (5, 8, 21, 31) the temporal relation between cell [Ca\(^{2+}\)] and secretion cannot be directly resolved.

In this study we have been able to define secretion temporally and quantitatively, i.e., by timing and counting exocytosis of secretory granules. These data have been related to measurements of [Ca\(^{2+}\)] in peptic cells from the same preparations studied under identical experimental conditions. There were significant statistical differences in lag times from the onset of stimuli (both BCh and BB) to the peak [Ca\(^{2+}\)] and the peak frequency of exocytosis. This is the first time data have been proved showing the time lag between Ca\(^{2+}\) signaling and exocytosis. About 15 s are required for secretory granules to initiate contact and fuse with the apical membrane when a submaximal dose of stimulation is used, and about 40 s are required when a low-dose stimulation is used. A low concentration of Ca\(^{2+}\) may need more time (about 20 s) to initiate the first exocytosis.

The present data show that in every instance the initial Ca\(^{2+}\) peak derived from intracellular Ca\(^{2+}\) pools

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**Fig. 12.** Relation between peak [Ca\(^{2+}\)] and number of exocytoses stimulated by BCh (A) and BB (B) (data from Table 3). There is a significant positive correlation between peak [Ca\(^{2+}\)], and number of exocytoses stimulated by both BCh and BB (Student's t-test). The threshold [Ca\(^{2+}\)] values for stimulation of exocytosis are >128 nM for BCh and >158 nM for BB. C: overlap data from BCh (○) and BB (●) stimulation of peptic cells (from A and B). Values are means ± SD of 3–6 experiments.
was followed, within seconds, by the onset of a large number of exocytoses. Such a peak elevation of [Ca\(^{2+}\)]

could be induced by sequential stimulation, e.g., BCh followed by BB; BCh and BB appear to act on the same intracellular Ca\(^{2+}\) pool (23). In addition to the relation between initial [Ca\(^{2+}\)] spikes and exocytosis, the data also show a subsequent sustained but lower rate of exocytosis (secretion) that is related to a lower sustained elevation of [Ca\(^{2+}\)], which is dependent on the influx of Ca\(^{2+}\) from the extracellular medium (8, 30, 31). Whereas higher concentrations of stimuli produce spikes of [Ca\(^{2+}\)], followed by lower sustained plateaus, low concentrations of BCh (3.2 \times 10^{-7} M) and BB (3.2 \times 10^{-10} M) induced Ca\(^{2+}\) oscillations that continued for almost 30 min, but without an initial [Ca\(^{2+}\)] peak. These modest oscillatory elevations of [Ca\(^{2+}\)] were associated with a continuous low frequency of exocytosis, but without an initial spike of [Ca\(^{2+}\)]; thus there was also no initial burst of exocytosis. Tsunoda (28) felt that although there was insufficient experimental evidence to define any precise mechanism for the biological effect of Ca\(^{2+}\) oscillation, it was likely that small repetitive signals are as effective as large sustained signals. How the oscillations are generated intracellularly and how they function in Ca\(^{2+}\) signaling remain to be elucidated (29). It has been suggested that the Ca\(^{2+}\) oscillation may be a form of frequency-modulated signaling that enhances signal recognition and avoids desensitization and Ca\(^{2+}\) toxicity (10). We found almost identical Ca\(^{2+}\) oscillation and exocytosis patterns with two different agonists, BCh and BB (Fig. 10), suggesting that such patterns may represent physiological conditions.

Although granule movement relative to the plasma membrane is required for exocytosis and is likely to be an integral part of stimulus-secretion coupling (20), in previous video microscopic studies, granule movement toward the captured substance was observed in neutrophils during phagosome formation (22), and granule movement toward the apical membrane was not detectable in chromaffin cells (25, 26), pancreatic \(\beta\)-cells (19), nasal acinar cells, nasal epithelial goblet cells (9), colonic goblet cells (27), or salivary gland cells (20). These observations and our present observations seem to support the concept that in both endocrine and exocrine glands the granules of unstimulated and stimulated cells maintain relatively fixed positions in the apical cytoplasm. Consequently, Ca\(^{2+}\) may not play an important role in translocation of the secretory granule to the apical membrane.

In contrast, with the depletion of all mucin granules in colonic cells in 30–40 min (27), only the apical zymogen granules of the peptic cell are secreted in the 40 min of observation. This may be explained by the more prolonged maturation time of peptic granules or by rapid replacement of secreted granules, since synthesis is stimulated by secretion and peptic cells are not depleted of pepsinogen even after prolonged secretion.

There was a significant positive correlation between the peak [Ca\(^{2+}\)] and the number of exocytoses stimulated by both BCh and BB. The relation between [Ca\(^{2+}\)], and exocytosis appears to be independent of the stimulus, with a threshold [Ca\(^{2+}\)] of 120–160 nM and a possible plateau of ~2,000 nM.

In conclusion, our studies demonstrate that 1) initial exocytosis is related to the mobilization of Ca\(^{2+}\) from intracellular pools, 2) Ca\(^{2+}\) influx from the extracellular medium is responsible for continuing exocytosis in frog pepsinogen-secreting cells, 3) exocytosis follows elevation of [Ca\(^{2+}\)] by 15–40 s, 4) there is a significant positive correlation between peak [Ca\(^{2+}\)], and the number of exocytoses, independent of the nature of the stimulus, and 5) continued exocytosis at lower levels is related to Ca\(^{2+}\) oscillation, which may be the physiological state.

These conclusions are based on visualization of granule exocytosis in individual cells and related to [Ca\(^{2+}\)] changes after stimulation in real time.

We are grateful to B. I. Hirschowitz for reading an earlier draft of the manuscript. We also thank S. Terakawa for helpful advice, and Dr. E. Suzuki and E. Kawai for technical assistance with microvideomicroscopy analysis.

A portion of these results has been published previously in abstract form (Gastroenterology 106: A193, 1994). Address for reprint requests: C. Tao, Kasumi 1-2-3 Minami ku, Hiroshima 734-8551, Japan.

Received 19 August 1997; accepted in final form 25 February 1998.

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