Spatiotemporal analysis of exocytosis in mouse parotid acinar cells

Ying Chen,1 Jennifer D. Warner,1 David I. Yule,2 and David R. Giovannucci1
1Department of Neurosciences, Medical College of Ohio, Toledo, Ohio; and 2Department of Pharmacology and Physiology, University of Rochester Medical Center, Rochester, New York

Submitted 5 April 2005; accepted in final form 29 June 2005

The complex and highly controlled process governing the exocytotic release of cargo molecules from the secretory granules of exocrine, endocrine, or neuronal cells involves the interaction, fusion, and dynamic reorganization of internal and surface membranes. Although there is substantial insight into the molecular mechanisms directing the exocytosis of neurotransmitter or hormone (13, 25), there is currently an insufficient understanding of the controlled secretion of protein from the exocrine cells of the digestive system. A notable exception has been the use of electrophysiological and optical methods to probe Ca2+ handling and the exocytosis of digestive enzyme from isolated or small clusters of pancreatic acinar cells (2, 6, 8, 16–18, 30, 32, 33). In a recent study, however, we demonstrated (7) that although apparently morphologically and functionally similar, mouse pancreatic and parotid acinar cells exhibit important functional divergence in their Ca2+ signaling properties, and thus pancreatic cells alone may not be universally applicable for understanding other exocrine cells. For example, in parotid and pancreatic acinar cells, fluid and protein secretion is regulated by both parasympathetic and sympathetic input. In the parotid, cholinergic or β-adrenergic receptor activation evokes rises in cytosolic Ca2+ concentration ([Ca2+]i) and cAMP concentration, respectively, and both messengers can act as intracellular messengers to induce amylase release (34). A primary difference between parotid and pancreatic acinar cells is that robust exocytosis in the parotid can be stimulated by increased cytosolic levels of cAMP. According to the current rubric of excitation-secretion coupling, this secretory activity is believed to occur without a change in [Ca2+]i. Moreover, biosynthetic labeling analysis has shown that the Ca2+- or cAMP-mobilizing agonists carbachol (CCh) and isoproterenol (Iso) induced trafficking of α-amylase-containing vesicles in parotid acini via a process that involves both major and minor regulated secretory pathways (3, 5). Indeed, a number of candidate proteins for controlling protein secretion have been identified in parotid acinar cells by molecular, protein biochemical, and immunocytochemical methods (3, 9, 10). However, the cellular and molecular mechanisms by which Ca2+ and cAMP mediate exocytosis in the parotid have not been clarified.

In the present study we sought to address this gap in our understanding by using time-differential imaging analysis in combination with highly time-resolved membrane capacitance (Cm) measurements to image the secretory activity of single mouse parotid acinar cells in real time after agonist-induced or direct photolytic elevation of Ca2+. Moreover, optical methods were successfully applied to a parotid gland organotypic slice preparation. Our observations revealed that there are two apparent Ca2+-dependent vesicle pools. After moderate elevation of Ca2+, one pool was rapidly induced to fuse with the plasma membrane. Although imaged with time-resolved Cm methods, single exocytotic events could not be resolved either electrically or optically. The other vesicle pool underwent exocytosis after a delay, was resolvable by both electrophysiological and optical methods, and most likely corresponded to the individual fusion events of dense core zymogen-containing secretory granules (ZSGs). ZSG fusion events produced persistent postfusion structures, were often found to coincide at similar subcellular sites or “hot spots,” and produced a spatial pattern of exocytotic activity that progressed outward from apical or lateral aspects of the cell toward the cell interior when robustly stimulated. These observations suggest that there are
multiple classes of secretory vesicles in parotid acinar cells that differ in their size, Ca\(^{2+}\) sensitivity, kinetics of exocytosis, and membrane retrieval mechanisms. Thus exocytic activity in parotid acinar cells exhibits characteristics both similar to and divergent from those of the better-studied pancreatic acini.

**MATERIALS AND METHODS**

Preparation of mouse parotid gland acinar cells and salivary gland slices. Animal study protocols were approved by the Medical College of Ohio Institutional Animal Care and Use Committee. Single acinar cells or small groups of acinar cells were isolated from parotid glands that were freshly dissected from 18- to 25-g NIH Swiss Webster mice (Charles River). The glands were minced with fine scissors and subjected to stepwise digestion in minimum essential medium (Eagle’s Spinner modification) containing either 1% BSA, 0.01% trypsin, or 0.5 mM EDTA (step 1) or 1% BSA, collagenase P (0.04 mg/ml) (Boehringer Mannheim), and protease (bacterial) type XIV (1 mg/ml; Sigma, St. Louis, MO) (step 2). Digestion was performed in a water bath shaking at 1 Hz for 10 min (step 1) and twice for 20 min each (step 2) at 37°C under continuous 95% O\(_2\)-5% CO\(_2\). After gentle trituration, single acinar cells were collected by centrifugation at 70 g, resuspended in BSA-free basal Eagle medium supplemented with 2 mM glutamine and penicillin-streptomycin, filtered through 80-μm nylon mesh, and attached onto poly-L-lysine-coated glass coverslips.

Cells were maintained in DMEM in a tissue culture incubator and used within 1–4 h after plating.

For slice preparation, 2 ml of a 3% (wt/vol) low-temperature gelling point agarose solution (Sigma type VIIA) was prepared in physiological salt solution (PSS) that contained (in mM) 140 NaCl, 10 HEPES-NaOH, 4.7 KCl, 1.13 MgCl\(_2\), 1 CaCl\(_2\), and 10 n-glucose, pH 7.3, and warmed to 90°C. The agarose solution was then maintained at ~35°C, and the block was trimmed so that minimal agarose remained around the tissue. The block was then glued to the tissue stand of a vibratome, and the chamber was filled with ice-cold nominal-Ca\(^{2+}\) PSS. Parotid gland tissue prepared by this method was cut into 40- to 50-μm-thick sections. The slices were collected and kept at 25°C until recording. Experiments monitoring Ca\(^{2+}\) or secretory dynamics in isolated cells or slices were performed with PSS at 25°C 1–4 h after slice preparation.

**Time-resolved \(C_m\) measurements.** \(C_m\) measurements were performed with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster, CA), an IT-C 16 digital interface (Instrutech, Port Washington, NY), and an Apple G4 computer running IGOR Pro (Wavemetrics, Lake Oswego, OR) and a software-based phase-sensitive detector (Pulse Control software, Dr. Richard Bookman, University of Miami Medical School, Coral Gables, FL) as previously described (8). The standard intracellular recording/caged-Ca\(^{2+}\) solution contained (in mM) 110 CsMeSO\(_4\), 20 CsCl, 10 HEPES-Tris, 10 NP-EGTA, 5 CaCl\(_2\), 3.2 MgCl\(_2\), 2 Tris-ATP, 0.2 GTP, and 0.075 Oregon Green BAPTA-5N, pH 7.2.

**Flash photolysis, digital imaging, and time-differential imaging analysis.** Caged compounds were photoconverted with a pulsed xenon arc flash lamp system (TILL Photonics, Eugene, OR) ported to a Nikon TE2000 microscope equipped with differential interference contrast (DIC) optics through a fiber-optic guide and epifluorescence condenser. High-intensity UV light flashes (0.1–1 ms) were focused onto the image plane with a DM400 diachoic mirror and a Nikon SuperFluor ×40 oil-immersion objective. Fluorescence or transmitted light images were obtained with a TILL Photonics monochrometer-based high-speed digital imaging system. For measurement of [Ca\(^{2+}\)], cells were illuminated with 340-, 380-, or 488-nm light and fluorescence was collected through a 525 ± 25-nm band-pass filter (Chroma Technologies, Brattleboro, VT). The numerical apertures of the objective and condenser for time-differential imaging analysis were 1.3 and 0.52, respectively. Transmitted light optical resolution was estimated to be ~350 nm. For experiments in which alternating bright-field and fluorescent images were collected, a high-speed Uniblitz VS35 optical shutter (Vincent Associates, Rochester, NY) was placed in the tungsten lamp illumination path. Pairs of bright-field and fluorescence images (30- and 50-ms exposure, respectively) were obtained at 5 Hz. Time-differential images were generated by subtraction of each brightfield frame by its preceding frame. Time-differential imaging has been used and validated previously in pancreatic acinar cells and other cell types as a method of visualizing individual secretory granule fusions (2). Parotid acinar cells were amenable for this approach, likely because of the large size and phase-dark core of their ZSGs. Exocytotic fusion created a change in optical density that was detected as a spot in the difference image. Average spot diameter after calibration with 1-μm beads (Molecular Probes) as standards was estimated to be 890 nm. Only those spots that appeared and disappeared within one or two frames were considered to be exocytotic events.

**Data analysis.** Fluorescence and time-differentiated images were generated, analyzed, and represented with TillVisiION (TILL Photonics) and ImageJ (W. S. Rasband, National Institutes of Health, Bethesda, MD) software packages. For statistical analysis, all numerical values of the data are expressed as means ± SE, where n is the number of cells examined. Statistical significance was determined where appropriate by Student’s t-test with Prism3 software.

**RESULTS**

**Exocytosis induced by cAMP and Ca\(^{2+}\) photorelease.** In pancreatic acinar cells, \(C_m\) measurements have been used to demonstrate that application of a cholinergic agonist or artificial Ca\(^{2+}\) elevation can induce changes in cell surface area (8, 15, 16, 22). In the current study we determined whether this technique could be used to monitor membrane surface dynamics of parotid acinar cells. To achieve this, we patch-clamped acutely isolated parotid cells that largely retained polar distribution of ZSGs and monitored changes in \(C_m\) induced by cAMP elevation. In contrast to the rapid, smooth rises in \(C_m\) evoked by Ca\(^{2+}\) elevation in pancreatic acinar cells that we and others have previously reported, cAMP-raising agonists produced staircase-like changes in \(C_m\) that persisted over several minutes of treatment. As shown in Fig. 1A, local bath application of 10 μM Iso, a β-adrenergic receptor agonist previously shown to effectively induce amylase release in the parotid, induced stepwise increases in \(C_m\) that we interpreted to reflect the exocytotic fusion of individual or multiple ZSGs. Similar results were obtained whether 10 μM forskolin or 1 mM dibutyryl cAMP was used to elevate \(C_m\) levels. As shown in Fig. 1B, individual events in cells treated with cAMP-raising agonists ranged in magnitude from 5 to 270 IF. The majority of events were clustered around 20, 40, or 60 IF (20 IF was estimated to be the amount of membrane equivalent to the addition of 1 ZSG, given that the average diameter of these organelles is ~0.8 μm). Correspondingly, transmitted light images of the patch-clamped acinar cells before and after Iso treatment for several minutes revealed an apparent reduction in the number of phase-dark ZSGs from the cells’ apical regions. The average cumulative rise in \(C_m\) over an ~5-min interval after cAMP elevation was 351 ± 190 fF (n = 9), corresponding to the exocytosis of ~18 ZSGs. As \(C_m\) measurements are a net measure of cell surface dynamics, the staircase-like profile of the \(C_m\) change indicated that little apparent endocytosis was activated over the time that the cells were monitored. In contrast to the \(C_m\) responses evoked by 10

**AJP-Cell Physiol • VOL 289 • NOVEMBER 2005 • www.ajpcell.org**
μM Iso, treatment with 1–3 μM Iso had no effect on resting Cm or induced a slow, smooth increase or decrease in Cm in patch-clamped single cells (n = 5). Occasionally, one or two stepwise events were seen superimposed on the smooth change in Cm. The absence of Cm steps was surprising because 1 μM Iso has been shown to efficiently induce amylase release from parotid acini (34) and may indicate that adrenergic signaling is compromised in single cells or that another vesicle population whose fusions cannot be resolved as individual events can be induced by lower concentrations of agonist to release amylase.

Because the stepwise changes in Cm occurred in cells in which [Ca2+]i was buffered at ~170 nM by the patch pipette dialysis solution, we tested whether Ca2+ played a role in the Iso-induced response. In cells in which Ca2+ was clamped below 30 nM by addition of BAPTA to the pipette, no events were observed after Iso treatment, suggesting that despite the inability to detect a volume-averaged change in cytosolic levels, Ca2+ was required for cAMP-induced exocytotic activity (n = 4). Therefore, we next determined the exocytotic response evoked by elevation of Ca2+ in the absence or presence of cAMP. To achieve this, we used xenon flash lamp-based UV photolysis of caged Ca2+ to apply controlled, spatially uniform Ca2+ challenges. As shown in Fig. 1Ca, flash-evoked photorelease of Ca2+ induced a rapid, smooth change in Cm similar to the exocytotic burst evoked in pancreatic acinar cells (8, 15, 16). This type of Cm change contrasted with the stepwise changes induced by Iso treatment. The evoked rise in Cm had a maximum amplitude that averaged 594 ± 63 fF (n = 10) and was best fit by an exponential line with multiple time constants. Because the exocytotic burst response exhibited a smooth Cm profile that lacked the stepwise increases observed after application of Iso, we hypothesized that the rapid, artificial elevation of Ca2+ with flash photorelease evoked multiple, overlapping fusion events that were not individually resolvable. To test this we applied a continuous, low-level intensity photolytic stimulus to induce a more gradual “ramplike” increase in Ca2+ (7). Surprisingly, in another cell pretreated for 3 min with 3 μM Iso (blue bar, e) is also shown. D: maximal Cm change and corresponding estimate of zymogen-containing secretory granule (ZSG) fusions induced within 5 min after photorelease of Ca2+ or cAMP elevation under different experimental conditions.

![Fig. 1. Time-resolved membrane capacitance (Cm) measurements of membrane dynamics in isolated patch-clamped parotid acinar cells. A: application of 10 μM isoproterenol (Iso, horizontal bars) induced stepwise increases in Cm (a), whereas 3 μM Iso was much less effective at inducing stepwise changes (b). Resting intracellular Ca2+ concentration ([Ca2+]i) was buffered at ~170 nM (traces a and b) or <10 nM by addition of BAPTA to the pipette solution (trace c). B: time dependence and amplitudes of exocytotic events induced by treatment with low-level photolysis of caged Ca2+ (black symbols) or 10 μM Iso (red symbols). C: high-intensity flash photorelease of Ca2+ (a) evoked a rapid increase in Cm, whereas low-intensity flash (b) or continuous low-level photorelease (c, dotted line) induced 2 apparent phases of exocytosis. Symbols denote high (●), or low (○), intensity UV flash application. Note longer time base in trace c. Comparison between low-level photolysis-evoked increases in Cm in a control cell (d) and another cell pretreated for 3 min with 3 μM Iso (blue bar, e) is also shown. D: maximal Cm change and corresponding estimate of zymogen-containing secretory granule (ZSG) fusions induced within 5 min after photorelease of Ca2+ or cAMP elevation under different experimental conditions.](http://ajpcell.physiology.org/)

---

*AJP-Cell Physiol • VOL 289 • NOVEMBER 2005 • www.ajpcell.org*
pared with control (614 ± 80 fF vs. 951 ± 207 fF; n = 9). Figure 1D shows the average maximal cumulative amplitude induced by Iso, Ca²⁺ release, or Ca²⁺ release in the presence of elevated cAMP.

Optical measurements of secretory dynamics. The observations with time-resolved $C_m$ measurements suggested that distinct vesicle populations of differing sizes might underlie the two apparent phases of activity induced by Ca²⁺ elevation. To test whether ZSG fusion was linked to one or both of these phases of exocytosis, we applied time-differential imaging methods to DIC images of patch-clamped acinar cells to visualize exocytotic fusion dynamics at the single-cell level. These events are detected as changes in optical density as phase-dark core material is extruded from the ZSG on exocytotic fusion. This method has been previously applied to image single exocytotic events in pancreatic acinar cells (1, 2, 11) and submandibular salivary gland cells (26). Initially, to determine applicability of time-differential imaging in the parotid, we used 1–3 μM Iso treatment for 3 min before high-intensity flash photorelease of Ca²⁺ to activate robust exocytotic activity in acinar cells. As shown in Fig. 2, application of a high-intensity flash to an acinar cell loaded via the patch pipette with caged Ca²⁺ evoked a burst of ZSG fusions after a delay of ~400 ms. On average the latency between the flash and the

Fig. 2. Sequential montage of exocytotic events evoked by high-intensity flash photorelease of caged Ca²⁺ in a single patch-clamped parotid acinar cell. The first 100 time-differential image frames (10 s total duration) after flash (caught in frame 1) demonstrate the detection of single or multiple ZSG fusions (red arrowheads). The cell was treated for 3 min with 3 μM Iso before the photorelease of Ca²⁺ to induce a robust exocytotic response. A map of the evoked fusions is shown in cell 1 in Fig 3.
first exocytotic event was 600 ± 144 ms (n = 6). This delay was significantly longer than the rapid activation of increases in membrane surface area measured by the \( C_m \) method. The highest rates of exocytosis occurred within the first 10–20 s after flash but persisted, generally at a lower rate for much longer periods (see Fig. 4C). An advantage of the time-differential imaging analysis method over the \( C_m \) method is the ability to create a spatial map of exocytotic activity. For example, as shown in Fig. 3B, ZSG fusions induced by flash photolysis of caged \( \text{Ca}^{2+} \) began at the apical border of the cell, and subsequent events occurred in progressively deeper regions of the cell. Thus the burst of ZSG fusions generally initiated in the apical region and proceeded sequentially into deeper regions of the cell interior. These events appeared and disappeared within 1–2 frames and had an average spot diameter of 1.2 ± 0.03 μm (estimated from pixel numbers and before calibration with bead standards; n = 160). In addition, larger events with a more complex profile were occasionally observed. These events appeared to reflect the multivesicular fusion of two to four ZSGs. As shown in Fig. 3C, a Gaussian fit to a histogram describing the distributions of diameter sizes of events that could be resolved as discrete spots indicated that events were distributed around 1.14 ± 0.04 μm. Spot diameter after correction by calibration with bead standards was estimated to be 890 nm, comparable to the average size of a single parotid ZSG determined from electron micrographs. Moreover, ZSG fusions often occurred in clusters or exocytotic hot spots that were largely confined to the apical or lateral granule-containing regions of the cells (1). In many cases where exocytotic activity was robust, ZSG fusions at these hot spots occurred over several successive image frames. This sequential activity suggested that the fusion of one ZSG can recruit or coordinate the subsequent fusion of other granules. An example of this coordinated behavior is shown in Fig. 4B.

In addition to the direct visualization of ZSG exocytosis, a remarkable feature regarding the fate of fused ZSGs was observed. In contrast to neurons or endocrine cells, where secretory vesicles either transiently fuse or fully collapse into the surface membrane so that they may be rapidly retrieved or recycled, the release of the phase-dark cores of the ZSGs after fusion produced stable, phase-bright structures that remained for tens to hundreds of seconds. Similar structures have been observed in pancreatic acinar cells and alveolar septal cells with multiphoton or confocal microscopy (14, 18, 28). Relative intensities from regions of interest (ROIs) encompassing sites of individual exocytotic events selected from corresponding time-differential images from cell 1 in Fig. 3A were quantified, and some examples are shown in Fig. 3D. (Line traces of the time derivative of these events are shown in Fig. 3E.) It should be noted that in some cases, multiple fusions were detected within the same ROI, consistent with the observation of hot spots of exocytotic activity. The average lifetime of phase-bright structures obtained from single fusion events from several cells that initiated within the first 20 s after flash were grouped as either short lived (<100 s) or long lived (>100 s). Short-lived event intensity returned to baseline with an average duration of 41 ± 11 s (n = 7). Long-lived events had an average duration of at least 228 ± 18 s (n = 11). However, considering that some movement of the patched cell during long periods of imaging sometimes occurred and that many of these events either remained stable for the duration or occurred close to the end of the recording interval, these lifetime values should be considered a lower-end estimate of the stability of these structures. An example of the postfusion structures observed in parotid acinar cells is shown in Fig. 4, A and B. To
follow the lifetime of these structures, transmitted light images were expressed as $\Delta T/T_0$, where $T_0$ is the average optical density of 10 frames before stimulation and changes in optical density ($\Delta T$) are relative to the preflash image.

Estimates of exocytotic rates with time-differential imaging analysis. Differential imaging analysis was used to investigate the Ca$^{2+}$ dependence of ZSG exocytosis. Events evoked after activation of exocytosis were counted and expressed as the rate of ZSG fusion (events/min). As shown in the histogram in Fig. 4C, the delay following flash photorelease of Ca$^{2+}$ for the first 10 fusion events/cell was generally $\sim$14 s. These data demonstrated that Ca$^{2+}$-activated ZSG fusion follows a biphasic relationship in which the majority of events are evoked in the first 20 s after Ca$^{2+}$ elevation. Average rates of ZSG fusions under different experimental conditions are shown in Fig. 4D. The rates of exocytotic activity in single patch-clamped cells after attainment of whole cell configuration with pipettes containing no added Ca$^{2+}$ and BAPTA, 60 nM Ca$^{2+}$, or 600 nM Ca$^{2+}$ were $0.25 \pm 0.25, 2.1 \pm 1.5$ and 4 events/min ($1 \leq n \leq 4$), respectively. In contrast, the instantaneous elevation of Ca$^{2+}$ to micromolar levels by UV flash photorelease evoked an average response of 63 events/min ($n = 2$). To assess the magnitude of the Ca$^{2+}$-induced exocytotic response in the presence of elevated cAMP, acinar cells were treated with 3 μM Iso for $\sim$3 min before flash photolysis. The average response to the photolytic release of Ca$^{2+}$ after Iso treatment was $165 \pm 13$ events/min ($n = 6$). The rate of exocytotic events was augmented nearly threefold compared with control cells. Furthermore, in contrast to the enhancement estimated by $C_m$ measurements, the total number of ZSG fusions determined by imaging over a 30-s interval after flash photolysis was nearly fivefold that of control. These data indicate that elevated cAMP levels greatly enhanced the number of ZSGs available for Ca$^{2+}$-evoked exocytotic release. The mismatch between estimates of the cAMP-mediated enhancement obtained with $C_m$ measurements and optical methods suggests that $C_m$ measurements may not properly track changes in surface area due to sequential compound exocytosis, perhaps because of space clamp errors.

Exocytotic rates in parotid slices. Enzymatically dispersed single acinar cells or small clusters of three to eight acinar cells (small acini) have been used extensively as models to study exocrine secretion and calcium dynamics, and their study has resulted in a wealth of information regarding these processes. However, some reports have debated whether dissociated acinar cells can exhibit altered morphology and function (1, 20). To address this possibility, we developed an organotypic slice preparation of the mouse parotid gland, using a vibratome that
circumvented the need to use enzymes. As shown in Fig. 5A, this preparation largely preserved the normal three-dimensional arrangements of cell types in this tissue as indicated by F-actin localization. Moreover, as shown in Fig. 5, B–E, parotid slices were readily imaged with conventional digital brightfield and fluorescence microscopy. For example, an intact acinus from a slice (Fig. 5B) was treated with 1 μM Iso and imaged with transmitted light and time-differential analysis. A projection of nearly 1,000 frames of the exocytotic events (image inverted for clarity) induced over 8 min of continuous Iso application is shown in C. Image map was constructed by projection of time-differentiated images, and resulting image was inverted. D: montage of Ca\(^{2+}\) dynamics induced by 300 nM carbachol (CCh) treatment in a small acinus (~3 cells) of a parotid gland slice. Slice was loaded with 5 μM fura-2 AM for 1 h and imaged at 2 Hz. E: fluorescence ratio changes expressed as ratio units (ru) from ROIs placed on 1 cell of the acinus in D as indicated. Red line is recorded from the apical region, and black line is from ROI placed in the basal region.

Using the slice preparation, we quantified the rates of exocytosis induced by adrenergic or cholinergic activation in the semi-intact gland with time-differential imaging analysis methods. Spontaneous ZSG fusions in slices had a relatively low frequency of occurrence. Basal rates of exocytosis were similar between slices and single cells (0.35 ± 0.17 and 0.25 ± 0.25 events/min, respectively; \(n = 4\)). Application of 0.3, 3, or 10 μM CCh evoked concentration-dependent increases in ZSG exocytosis (1.0 ± 0.5, 1.4 ± 0.5, and 4.4 ± 1.7 events/min,
respectively; $4 \leq n \leq 7$). As shown in Fig. 6, A and B, 10 µM CCh activated ZSG fusions that largely mapped to apical or lateral regions of cells within an acinus, mirroring the distribution of ZSGs, similar to that demonstrated in single cells. Similar results were also obtained after 1 µM Iso treatment. The total number of events per acinus over 6 min in the presence of CCh or Iso was 137 and 121, respectively. To determine the kinetics of exocytosis, events of individual cells of acini were counted in 10-s intervals, binned, and plotted as shown in Fig. 6, C and F. These experiments revealed that CCh application induced a biphasic response in which the rate of exocytosis was initially 7 fusions/min but decreased rapidly thereafter. In contrast, Iso treatment produced a lower initial rate but a persistent level of exocytotic fusions. The overall rates of exocytosis over a 6-min interval for these CCh- and Iso-treated acini were 1.7 and 4.4 events/min for Iso and CCh, respectively. On average, the overall rates of exocytosis were similar between 1 µM Iso- and 1 µM CCh-treated acini. The average rate of exocytosis induced under different experimental conditions is shown in Fig. 6G.

Interestingly, in contrast to isolated acinar cells, in which 1–3 µM Iso was largely unable to induce obvious ZSG fusions, 1 µM Iso was an effective secretagogue in semi-intact gland slices. To test whether Ca$^{2+}$ was necessary for the Iso-induced exocytosis, slices were treated with 100 µM dimethyl-BAPTA-AM (DMB), a high-affinity cell-permeant Ca$^{2+}$ buffer ($K_d = 40$ nM). Incubation for 1 h at room temperature with DMB decreased the average fura-2 resting ratio value from 0.25 to 0.18 ratio units ($n = 4$). As shown in Fig. 6G, treatment with DMB significantly reduced the Iso-evoked exocytotic response (2.8 $\pm$ 0.8 events/min to 1.2 $\pm$ 0.6 events/min; $n = 5$ and 8, respectively). However, DMB treatment was unable to completely abolish the response as introduction of a strong exogenous Ca$^{2+}$ buffer did in patch-clamped acinar cells.

**DISCUSSION**

In this study, we applied electrophysiological and optical methods to investigate ZSG fusion activated by direct or agonist-induced elevation of Ca$^{2+}$ and cAMP in mouse parotid acinar cells and compared our findings with recent observations regarding exocytosis in pancreatic acinar cells. The findings indicate that parotid and pancreatic acinar cells exhibit
Comparison of time-resolved $C_m$ measurements and time-differential imaging analysis of membrane dynamics. The rate of exocytosis induced by continuous low-level photolysis and determined by counting stepwise events from $C_m$ records roughly matched that of measured by differential imaging analysis ($\approx 4$ events/min). However, when absolute rises in $C_m$ rather than steps were counted, the estimate of exocytotic activity was two- or threefold greater. This apparent discrepancy could largely be accounted for if a significant number of the stepwise increases observed reported compound exocytotic events. Consistent with this idea, many of the $C_m$ steps were indeed multiples of 20 fF (the amount of membrane estimated for 1 ZSG fusion). Similarly, with the understanding that the time resolution was significantly slower than $C_m$ measurements (100 ms vs. 10 ms), examination of time-differentiated images often indicated what appeared to be multivesicular fusion events. On the other hand, high-intensity flash photorelease of Ca$^{2+}$ produced an exponential rise in $C_m$ that rapidly reached a new steady state and suggested the nearly simultaneous, unresolved fusion of $\approx 30$ ZSGs. These estimates indicated a greater number of individual fusion events than that observed by direct visualization of fusion events by optical methods. Moreover, the increase in $C_m$ initiated within a few milliseconds after cessation of the flash, in contrast to the delay (600 ms on average) between the flash and the initial fusion event detected by differential imaging. Indeed, the shortest delay observed with optical methods was at least 200 ms. This observation suggested that, in addition to ZSG fusion, $C_m$ measurements also detected the rapid fusion of another vesicle type. The idea that there are multiple vesicle types that differ in size, Ca$^{2+}$ sensitivity, and kinetics of exocytosis is not without precedence. Moreover, multiple pathways of amylase release have been identified in parotid acinar cells (4). Constitutive and minor regulated pathways account for the majority of release of $\alpha$-amylase under basal conditions. The majority of evoked release is via regulated fusion of ZSGs in response to adrenoceptor and muscarinic cholinoreceptor receptor activation. However, recent studies have demonstrated that the minor regulated pathway is functionally linked to granule exocytosis. Exocytotic fusion of the minor regulated pathway is apically directed, precedes granule exocytosis, and can be selectively discharged under low-level stimulus conditions (i.e., 1 $\mu$M Iso) (3, 5). It has been proposed that the minor pathway can recruit syntaxin 3 to the apical membrane (or remove via endocytosis) and modulate the number of docking sites for exocytotic fusion of ZSGs (3). The initial phase of $C_m$ increase we observed exhibits features that are qualitatively similar to the minor regulated pathway in the parotid. The rise in $C_m$ was continuous, suggesting fusion of a population of vesicles that were too small to be resolved by either $C_m$ or optical methods. The initial, sometimes biphasic $C_m$ change was induced by low-level stimulation and was abolished by strong intracellular Ca$^{2+}$ buffering with BAPTA. In addition, BAPTA treatment and block of the initial phase of exocytosis abolished the secondary stepwise increases in exocytosis. Conversely, pretreatment with low concentrations of Iso enhanced the secondary phase of ZSG fusion evoked by low-level photolysis of caged Ca$^{2+}$. Thus it is tempting to speculate that the initial phase of exocytosis induced by Ca$^{2+}$ ramps identified in the present study reflects recruitment of the minor pathway. Indeed, ultrastructural studies of parotid and submandibular salivary glands have demonstrated the presence of small-diameter vesicles (<150 nm) at the subliminal face of apical membrane or on or around ZSGs (23, 27). However, it is also possible that transient fusion of ZSGs, delayed fusion pore expansion, or retention of the phase-dense material underlies the initial phase reported by $C_m$ measurements. Accordingly, as differential imaging detects the loss of phase-dark material, we would not be able to visualize ZSG fusions faster than the time required to extrude core material. Indeed, we did occasionally detect a fusion event that projected over two image frames (100 ms/frame), indicating that this process may require 0.1–0.3 s to complete. Therefore, additional study specifically addressing this will be needed for a complete understanding of the relationship between these two phases of exocytosis.

Comparison of ZSG exocytosis in parotid and pancreatic acinar cells. In part because $C_m$ measurements distinguish neither the specific site nor the identity of a vesicle that fuses with the cell surface, we used time-differential imaging methods pioneered by others to optically assess parotid ZSG fusion dynamics in real time. We found that parotid cells had a relatively low rate of basal exocytosis but that high rates of ZSG fusion could be evoked after the controlled photorelease of caged Ca$^{2+}$ with a minimum delay of ~200 ms. In fact, the average delay between the flash application and the first ZSG fusion (600 ms) was more than an order of magnitude shorter in parotid cells than the delay reported for photolysis-evoked fusion in pancreatic cells (10 s). In general the kinetics of the responses were similar to those reported with the time-differential imaging analysis of pancreatic acinar cells. We found that increases in cytosolic Ca$^{2+}$ by direct elevation or CCh application produced a biphasic response characterized by an initial burst of exocytosis with rates that decreased over the subsequent 25–30 s and then persisted at a lower rate over minutes. (Interestingly, Iso application produced a largely monotonic, but persistent, level of fusion events that lasted throughout the stimulus application.) However, whereas the overall rates of exocytosis and total numbers of fusion events induced by agonist treatment were comparable between parotid cells and those reported for pancreatic cells, the rate and number of events evoked by flash photolysis were much greater. Furthermore, under strong stimulation, ZSG fusion proceeded from apical or sometimes lateral borders toward the cell interior. We interpreted this activity as the sequential compound fusion of ZSGs that proceeded from the primary sites of fusion at the cell surface into successively deeper regions of the cell interior (see below). Consistent with this idea, ZSG fusions were shown coordinate subsequent fusions at so-called hot spots. Indeed, our observations and interpretations match well with those of Campos-Toimil et al. (2), with the exception that sequential fusion at hot spots was much more rapid in parotid than in pancreatic acinar cells. For example in pancreatic cells, the delay between events at defined hot spots was ~50 s. In the parotid, frame-to-frame analysis showed that multiple events at hot spots could occur over several successive frames (~100 ms of each other). Moreover, unlike the pancreatic cells, a number of events appeared to be comprised of multivesicular fusions.

Although we concede the possibility that under strong stimulus conditions primary fusions might be induced at distal sites
on the cell surface, our interpretations are largely consistent with recent observations on the exocytotic process in pancreatic acinar cells. Recently, several groups have applied multiphoton and confocal methods to directly visualize exocytotic activity in pancreatic acinar cells. An emerging consensus regarding the process of ZSG fusion in pancreatic acinar cells is that after activation by Ca\(^{2+}\) levels >1 \(\mu\)M, a subpopulation of ZSGs fuse initially at the apical membrane, and additional granule-granule fusions proceed in a sequential fashion as they “daisy-chain” on each other (18). It is believed that sequential compound exocytosis is an adaptation to increase the efficiency of protein secretion where access to the subluminal apical domain is limited. Importantly, after primary fusion events, additional ZSG fusions appear to proceed independently of receptor activity or Ca\(^{2+}\) gradients (19). Therefore, it is hypothesized that sequential compound exocytosis requires the redistribution of a surface membrane protein to sites on the newly fused granules that in turn act as nuclei for subsequent granule-granule fusions (18, 21). Thus the recruitment of secondary or tertiary granules is believed to be limited by the diffusion of protein factor(s) from the plasma membrane to the granular membranes without the mixing of plasma membrane and granule membrane lipids (18, 28). Consistent with this idea, ZSG fusions in parotid acinar cells were shown to produce persistent, phase-clear structures that generally lasted for 220 s. The lifetimes of these events were similar to the F-actin-stabilized postfusion structures, or secretory granule “ghosts,” produced in pancreatic acinar cells shown by multiphoton microscopy (19, 29, 31).

Monitoring exocytosis in parotid gland slice. In contrast to those reported for pancreatic acinar cells, many of the exocytotic events in single parotid cells appeared to comprise multivesicular ZSG fusions. To ascertain whether this was a relevant characteristic of parotid cells or a consequence of patch-clamping isolated cells and artificial stimulus conditions, we treated parotid organotypic slices with agonist and optically monitored ZSG fusions. Numerous multivesicular fusions were also observed in response to Iso or CCh treatment in slices, and thus this mode of secretory activity likely represents a real physiological property of these cells. In contrast, we found that isolated cells did not respond to low levels of Iso or CCh with ZSG fusions, whereas cells in acini in semi-intact slices responded to 1 \(\mu\)M Iso with robust secretory activity. Therefore, although isolated cells were a good model for studying mechanistic aspects of Ca\(^{2+}\) and secretory activity, enzymatic treatment may alter the amount or integrity of surface receptors and reduce the exocytotic response. Thus this indicates that some caution in using isolated cells is warranted and that the parotid slice preparation may provide a useful system to address questions regarding these processes in a less invasive and more physiologically relevant context. It is also important to note that the overall rate of secretion, as measured by time-differential image analysis, in parotid slices is lower than that reported for pancreatic acini. However, the total mass of zymogen release may be similar because the percentage of multivesicular fusions appears greater in parotid. Whether the lower rate of exocytosis reflects a true difference in the magnitude of secretory activity remains to be determined.

In conclusion, despite remarkable similarity in morphology and general function between these two cell types, parotid cells demonstrate secretory kinetics that are more rapid and perhaps subject to more dynamic regulation than pancreatic cells. These differences may reflect the multifunctional role for and the need to rapidly and dynamically adjust saliva output in processes such as mastication, digestion, oral health, and speech.

GRANTS
This work was supported by National Institute of Dental and Craniofacial Research Grant DE-014756-03 (to D. I. Yule and D. R. Giovannucci).

REFERENCES


