Exocytosis, dependent on Ca\(^{2+}\) release from Ca\(^{2+}\) stores, is regulated by Ca\(^{2+}\) microdomains

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Summary
The relationship between the cellular Ca\(^{2+}\) signal and secretory vesicle fusion (exocytosis) is a key determinant of the regulation of the kinetics and magnitude of the secretory response. Here, we have investigated secretion in cells where the exocytic response is controlled by Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores. Using live-cell two-photon microscopy that simultaneously records Ca\(^{2+}\) signals and exocytic responses, we provide evidence that secretion is controlled by changes in Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) in relatively large-volume microdomains. Our evidence includes: (1) long latencies (>2 seconds) between the rise in [Ca\(^{2+}\)] and exocytosis, (2) observation of exocytosis all along the lumen and not clustered around Ca\(^{2+}\) release hot-spots, (3) high affinity (K\(_d\)=1.75 \(\mu\)M) Ca\(^{2+}\) dependence of exocytosis, (4) significant reduction in exocytosis in the presence of cytosolic EGTA, (5) spatial exclusion of secretory granules from the cell membrane by the endoplasmic reticulum, and (6) inability of local Ca\(^{2+}\) responses to trigger exocytosis. These results strongly indicate that the control of exocytosis, triggered by Ca\(^{2+}\) release from stores, is through the regulation of cytosolic [Ca\(^{2+}\)] within a microdomain.

Key words: Exocytosis, Calcium, Epithelial, Zymogen, Pancreatic, Two photon

Introduction
Ca\(^{2+}\)-dependent exocytosis is an essential and widespread process (Sudhof, 2004). An increase in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) triggers secretory vesicle fusion with the plasma membrane leading to the release of vesicle cargoes, such as neurotransmitters and proteins, for example, hormones and enzymes. In excitable cells, Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels (Rizzuto and Pozzan, 2006) is the major route to elevate cytosolic [Ca\(^{2+}\)] and trigger exocytosis. In some excitable cells, Ca\(^{2+}\) channels and exocytic sites are closely apposed; they are positioned within volumes of nanometre dimensions that are called nanodomains (Adler et al., 1991; Bucurenciu et al., 2008; Stanley, 1993) and enable fast, efficient regulation of the secretory response (Stanley, 1993). In other excitable cells, clusters of Ca\(^{2+}\) channels provide a localized ‘cloud’ of Ca\(^{2+}\), triggering exocytosis across microdomains (Beaumont et al., 2005; Borst and Sakmann, 1996; Chow et al., 1994). In the latter case, the secretory response is slower, but precise control of [Ca\(^{2+}\)] within the microdomain is used to fine-tune the secretory output (Chow et al., 1994).

The role of Ca\(^{2+}\) release from intracellular stores in triggering exocytosis is less well understood. In cells with voltage-gated Ca\(^{2+}\) channels, Ca\(^{2+}\) release can modulate exocytic responses (Dyachok and Gylfe, 2004; ZhuGe et al., 2006) but in many cell types Ca\(^{2+}\) release is the exclusive source of increase of cytosolic [Ca\(^{2+}\)] (Matthews et al., 1973; Tse et al., 1997). How the sites of Ca\(^{2+}\) release from stores are related to the sites of exocytosis and control secretion in these cells is not known.

A good example of secretion regulated by Ca\(^{2+}\) release from Ca\(^{2+}\) stores is in exocrine acinar cells. Here, exocytosis of enzyme containing granules (Chen et al., 2005; Nemoto et al., 2001) is dependent on Ca\(^{2+}\) release through inositol trisphosphate receptors (InsP\(_3\)R) on the endoplasmic reticulum (ER) Ca\(^{2+}\) store (Futatsugi et al., 2005; Ito et al., 1997). This Ca\(^{2+}\) response has complex characteristics in space, time and amplitude (Fogarty et al., 2000a; Kasai and Augustine, 1990; Kasai et al., 1993; Thorn et al., 1993). Through the use of Ca\(^{2+}\) buffers (Kidd et al., 1999) and high-speed imaging (Fogarty et al., 2000b; Kidd et al., 1999) it has been shown that there is one hot spot of Ca\(^{2+}\) release that can act alone, giving a local response or act to initiate larger, global Ca\(^{2+}\) signals (Fogarty et al., 2000b; Shin et al., 2001). This hot spot is likely to represent a site of enrichment of the Ca\(^{2+}\) release apparatus, possibly with more-sensitive isoforms of the InsP\(_3\)R (Futatsugi et al., 2005; Lee et al., 1997; Nathanson et al., 1994; Park et al., 2008) or with a greater density of IP\(_3\)Rs (Callamaras et al., 1998). How these complex Ca\(^{2+}\) signals are employed to regulate the exocytic response is not known.

Here, we test the hypothesis that control of exocytosis in acinar cells is through local Ca\(^{2+}\) release that targets high [Ca\(^{2+}\)] to close-by sites of exocytosis within nanodomains. We employ high-speed two-photon microscopy to simultaneously measure cytosolic Ca\(^{2+}\) (with Fura-2 and Fura-FF) and exocytosis (with extracellular aqueous dyes) in response to the endogenous agonist cholecystokinin (CCK) and the photoliberation of Ca\(^{2+}\) from o-nitrophenyl (NP) tagged to EGTA (NP-EGTA). Our results show that events of exocytosis are not clustered around hot spots of Ca\(^{2+}\) release and we conclude that Ca\(^{2+}\) release from Ca\(^{2+}\) stores regulates exocytosis through the control of a microdomain. In cells that do not require a rapid secretory response we speculate that fine-tuning of Ca\(^{2+}\) levels within the microdomain gives precise control of secretory output.

Results
Simultaneous recording of cytosolic Ca\(^{2+}\) signals and exocytic responses
Apical-to-basal Ca\(^{2+}\) waves, owing to release of Ca\(^{2+}\) from intracellular stores, are a characteristic feature of responses of
pancreatic acinar cell to agonists (Kasai and Augustine, 1990). Fig. 1 shows an example of such a response recorded in freshly isolated tissue fragments loaded with Fura-2 and stimulated with a physiological concentration of 20 nM CCK.

The image sequence in Fig. 1A shows the Ca2+ response in four cells on the edge of the tissue fragment (composed of 10–50 cells); the relative time point of each image is indicated with roman numerals (see Fig. 1B, upper graph). The Fura-2 fluorescence signal was converted into ratios and plotted in pseudocolor in Fig. 1A (upper images). To observe exocytic responses the tissue was bathed in sulforhodamine B (SRB), a fluorescent probe that surrounds the cells and diffuses into the lumens between the cells (Fig. 1A, lower images, colored red) (Nemoto et al., 2001; Thul and Falcke, 2004). Upon granule fusion SRB enters the granules, which seen as the sudden appearance of small spherical objects (~0.8 μm diameter) at the apical pole of the cells (Fig. 1A, lower sequence). The average Fura-2 response in each cell is plotted in Fig. 1B (upper graph) and the time-course of the exocytic responses measured as normalized SRB changes – within regions of interest (ROIs) centered on each exocytic granule are shown in Fig. 1B (lower graph).

These CCK-induced global Ca2+ responses occur asynchronously (Yule et al., 1996); they originate in the apical region spreading across the cell to the basal pole. Fig. 1C shows the image sequence and a graph of average Fura-2 ratio against time of the Ca2+ response for the lower left cell of Fig. 1A. The graph plots average changes in three ROIs spread across the cell and shows the apical to basal spread of the Ca2+ wave. We calculated the velocity of the Ca2+ wave to be 10.5±0.77 μm/second (mean ± s.e.m., n=17), which is comparable to previously published data (Larina and Thorn, 2005).

Response to an exogenous Ca2+ signal – spatial organization of exocytosis

To initially characterize the stimulus-secretion relationship in the absence of the spatial complexities of the agonist-evoked response we induced a cytosolic increase of [Ca2+] by uncaging of Ca2+ from the photolabile Ca2+ buffer NP-EGTA. This method elevates Ca2+ uniformly across the cell. The image sequences in Fig. 2 (and in supplementary material Movie S1) show three cells at the edge of a pancreatic fragment loaded with NP-EGTA (AM ester) and Fura-2. The upper panel in Fig. 2A shows the ratiometric pseudocolor Fura-2 response to a 100-ms UV flash. The lower panel in Fig. 2A shows the induced exocytic response recorded by the entry of SRB into individual granules. The graph of the Fura-2 ratio over time shows a large, rapid rise in [Ca2+] after the UV flash that triggers exocytic activity (Fig. 2B, upper panel), observed as increases in SRB fluorescence in ROIs at each site of exocytosis (Fig. 2B, lower panel).

Fig. 1. Ca2+ responses within an acinus to CCK stimulation. Cells were loaded with Fura-2 and stimulated by the addition of 20 nM CCK to the bathing solution. (A) Within an acinus, individual cells asynchronously show a rise in [Ca2+]. (Top and middle rows) Top row: Fura-2 response (F0-F/F0 in pseudocolor) at individual time points (i–vii, indicated on the graph in B) throughout the response (blue, low [Ca2+], to red, high [Ca2+]). The cell in the upper left responds first, followed sequentially by the other three cells in the group. Middle row: SRB fluorescence (red) that surrounds the cells and diffuses into the lumen between cells. Individual exocytic fusion events, observed as SRB dye enters the granules are seen in the last three images (v–vii). The boxed area in the first image of the middle row indicates the area that is shown enlarged in the four black and white images (a–d). These images show individual exocytic events at time points a–d (arrowheads), indicated on the bottom graph in B. (B) Exocytic events follow the Ca2+ signals. (Top graph) Average Fura-2 ratiometric response in ROIs within each of the cells (color-coded for each cell, shown in image I in A). (Bottom graph) Time-course of the SRB dye signals in ROIs placed over each individual granule that undergoes fusion (dots above the graph indicate the times of the peaks of each exocytic event). (C) Within a single cell, the Ca2+ response is seen as a wave spreading from apical to basal regions. The enlarged images show a time sequence of images (0.2 second time intervals) from the lower left-hand cell (shown in A). The Ca2+ wave initiates in the apical region (red circle) and then spreads to the basal region (green circle). Scale bar: 10 μm. The graph shows average ratiometric fluorescence changes in each ROI (red, yellow, green) plotted against time. The Ca2+ signal rises first in the apical region.
We then used these Ca\(^{2+}\) responses that were induced by the uncaging process to map sites of exocytosis and to determine whether they are clustered along the lumen. Cells were loaded with NP-EGTA and stimulated with a 100-millisecond UV flash. We then measured granule-to-granule distances along the lumen from one granule to all other exocytic granules in the same cell. A frequency histogram showed no evidence of clustering around short granule-to-granule distances (Fig. 3A). However, granule-to-granule distances would be affected by the lengths of lumen in each cell. Therefore, for each cell a scatter plot of granule-to-granule distances was plotted against the lumen length (Fig. 3B). The predicted line – if the granule-to-granule separation were random – shows a close approximation to our data; consistent with a lack of preferential sites of exocytosis along the lumen (Fig. 3C). These data therefore indicate that all regions along the lumen are equally capable of exocytosis.

We applied the same clustering analysis to the exocytic response to 20 μM CCK with similar frequency distributions of granule-to-granule distances (supplementary material Fig. S1A). Since it is known that compound exocytosis (granule-to-granule) fusion is prevalent in this cell type, we extended this analysis to identify the location of primary granules (those fusing directly with the plasma membrane) and secondary granules (those fusing with primary granules). Supplementary material Fig. S1B shows that the frequency plot of granule-to-granule distances is very similar for primary and secondary granules.

Response to an exogenous Ca\(^{2+}\) signal – exocytosis has a Ca\(^{2+}\) K\(_d\) of 1.75 μM

We also used the responses induced by uncaging Ca\(^{2+}\) to determine the Ca\(^{2+}\) dependence of exocytosis. Here, we varied the duration of the UV flash over a range from 5 ms to 200 ms and, for each duration flash, measured the maximal response to Fura-FF (a low-affinity Fura-2 derivative with a measured in vivo K\(_{eff}\) of 1.84 μM for Ca\(^{2+}\), see Materials and Methods) (n=309 cells). The duration of each flash was then calibrated as a Ca\(^{2+}\) change, plotted against the number of exocytic events per cell (by using entry of SRB extracellular dye into fused vesicles). The graph shows a sigmoid relationship with an estimated K\(_d\) of 1.75 μM for the Ca\(^{2+}\) dependence of exocytosis (Fig. 4). This is similar to the K\(_d\) of 2 μM Ca\(^{2+}\) that is found for enzyme release in these cells (Knight and Koh, 1984). Our K\(_d\) value is also comparable to endocrine cells, such as chromaffin cells, where the calculated K\(_d\) is 1.6 μM (Augustine and Neher, 1992). In all further experiments, we employed a 100-ms UV flash (3.4 μM Ca\(^{2+}\)) to induce maximal exocytic responses.

In summary, experiments where Ca\(^{2+}\) was uncaged from NP-EGTA show that, in principle, exocytosis can occur all along the lumen and that the exocytic process is relatively sensitive to cytosolic Ca\(^{2+}\). We next set out to determine the spatial relationship between the agonist-evoked Ca\(^{2+}\) signal and the triggered exocytic responses.

Agonist-evoked initiation sites of Ca\(^{2+}\) signals are distant from sites of exocytosis – morphometric analysis

We used first derivatives and region mapping of the Ca\(^{2+}\) response to determine the precise point of origin of the Ca\(^{2+}\) responses to
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Ca²⁺ nanodomains control exocytosis. Instead, they indicate the further distances. These data, therefore, do not support the idea that any events of granule fusion at the origin of the Ca²⁺ signal. Response initiation and the sites of granule fusion (Fig. 6) did not occur in advance of surrounding regions (Fig. 5C). In a small number of cells (<10%) the Ca²⁺ signal appeared simultaneously occurring in ROIs in single cells. Each fluorescent response was calibrated according to our in-vitro assay of the Fura-FF Kᵥ. Changes in the duration of the UV flash varied the amount of Ca²⁺ liberated. The exocytic response was recorded as the number of fused granules per cell and data are given as the mean ± s.e.m. (n=309 cells, minimum of seven cells) of the exocytic responses plotted against the change in [Ca²⁺] induced by the UV flashes. The calculated Kᵥ for the Ca²⁺ sensitivity of exocytosis was 1.75 µM.

CCK (Fig. 5, n=22 cells). These initiation hot spots of the Ca²⁺ signal probably correspond to the local enrichment of InsP₃Rs in regions below the apical plasma membrane as previously described by Kidd and colleagues (Kidd et al., 1999). Here, we measured changes of Fura-2 intensity in 3×3 µm ROIs, which robustly identified the origin of the Ca²⁺ responses. In most cases, a single discrete origin was identified (Fig. 5C,D) with the Ca²⁺ rise occurring in advance of surrounding regions (Fig. 5C). In a small number of cells (<10%) the Ca²⁺ signal appeared simultaneously across a broader region. We interpret these latter responses to be indicative of the Ca²⁺ origin lying outside the plane of the two-photon cross-section and did not include these experiments in further analysis.

The measured spatial relationship between the site of Ca²⁺-response initiation and the sites of granule fusion (Fig. 6) did not show any events of granule fusion at the origin of the Ca²⁺ signal. The numbers of exocytic events increased to a maximum at a distance of 3 µm from the Ca²⁺ signal origin and then decreased at further distances. These data, therefore, do not support the idea that Ca²⁺ nanodomains control exocytosis. Instead, they indicate the importance of larger-volume microdomains and even suggest a distinct separation of sites of Ca²⁺ release from sites of exocytosis.

Ca²⁺ initiation sites are distant from sites of exocytosis – block of exocytosis by EGTA
EGTA, a Ca²⁺ chelator with a slow on-rate for binding Ca²⁺, is often used as an indicator of the spatial extent of a Ca²⁺ response (Beaumont et al., 2005; Borst and Sakmann, 1996; Bucurenciu et al., 2008; Chow et al., 1994; Stanley, 1993). Since EGTA is unable to act as an effective Ca²⁺ buffer within nanodomains, a Ca²⁺ target that is close (<200 nm) to a Ca²⁺ source will be unaffected by the presence of EGTA (Bucurenciu et al., 2008; Thul and Falcke, 2004). By contrast, EGTA is an effective buffer when the Ca²⁺ source is further away from the Ca²⁺ target (<1 µm). We loaded the cells with EGTA-AM and recorded the Ca²⁺ and the exocytic responses (Fig. 7). The Fura-2 responses decreased with increased duration of EGTA-AM loading, which is as expected because EGTA will compete with Fura-2 for cytosolic Ca²⁺. The exocytic response was significantly reduced within 30 minutes of EGTA-AM loading (Fig. 7). These data support the idea that exocytosis is regulated by Ca²⁺ microdomains.

Crowding limits access of secretory granules to the apical plasma membrane
To further investigate the ultrastructural relationship of organelles in the apical region of acinar cells we next used thin-section transmission electron microscopy. The ER surrounds secretory granules in the apical region (Bolender, 1974). We now established that these ER projections extend right up to the apical plasma membrane. Fig. 8 shows examples of typical electron micrographs of the region surrounding the lumen of an acinar endpiece. In all of our electron microscopy sections we found evidence of rough ER lying immediately under the apical plasma membrane, sterically blocking granule access to the apical cell membrane, and measured the distances between the apical plasma membrane and the centre point of the first layer of secretory granules under the membrane. If the granules were tightly packed against the cell membrane then the distance to the centre point of each granule should equal the granule radius. We measured the mean granule diameter as 748.6 ± 11.1 nm (mean ± s.e.m., n=230) and, with tight packing, a

![Fig. 4. Exocytic response following uncaging of Ca²⁺ from NP-EGTA. The peak Ca²⁺ response to the UV photolysis of NP-EGTA was measured with Fura-FF within ROIs in single cells. Each fluorescent response was calibrated according to our in-vitro assay of the Fura-FF Kᵥ. Changes in the duration of the UV flash varied the amount of Ca²⁺ liberated. The exocytic response was recorded as the number of fused granules per cell and data are given as the mean ± s.e.m. (n=309 cells, minimum of seven cells) of the exocytic responses plotted against the change in [Ca²⁺] induced by the UV flashes. The calculated Kᵥ for the Ca²⁺ sensitivity of exocytosis was 1.75 µM.](image)

![Fig. 5. Identification of the site of origin of the Ca²⁺ wave. Cells stimulated with 20 pM CCK show the Ca²⁺ signal starting at the apical pole and then spreading out across the cell. (A) Fura-2 responses (F₀/F₀ ratio in pseudocolor) from a CCK-evoked Ca²⁺ response in a single cell within a pancreatic fragment. (B) Full sequence of still images from a movie (five frames per second) taken as the Ca²⁺ response spreads across the cell. (C) Same cell as in B, enlarged (at the very first movie frame that shows the start of the Ca²⁺ response) with four ROIs of 3×3 µm (boxed areas) centered around the origin of the Ca²⁺ response. (D) The main graph is the magnification of the gray-shaded area in inset graph, showing the average fluorescence within each of the ROIs shown in C. The red ROI rises earlier and faster than the surrounding ROIs; for this cell, this region is therefore identified as the point of origin of the Ca²⁺ response.](image)
normal distribution centered on 374 nm (the radius) is therefore expected, with a further peak at 1122 nm (reflecting another layer of granules). Instead, the frequency-distance plot shows a first peak at 500 nm, indicating that granules are further away from the plasma membrane than predicted (Fig. 8B), and no further peak at greater distances. We therefore conclude that the granules are relatively loosely packed and are separated from each other and from the cell membrane by structures such as the ER.

Local Ca\(^{2+}\) responses fail to trigger exocytosis

A characteristic of the physiological response to CCK is that the global Ca\(^{2+}\) responses (as focused on in this study) are interspersed with fast local Ca\(^{2+}\) responses that remain within the apical region (Kasai et al., 1993; Thorn et al., 1993). These local responses are thought to represent Ca\(^{2+}\) release from Ca\(^{2+}\) hot spots that fail to propagate across the cell (Thorn et al., 1993). Cytosolic Ca\(^{2+}\) levels within the nanodomain of the hot spot region are transiently expected to be high (>50 \(\mu\)M) (Thul and Falcke, 2004), and sufficient – in principle – to elicit exocytosis within this region. In six independent experiments we compared local and global cytosolic Ca\(^{2+}\) responses in 20 cells stimulated with 10–12 pM CCK. A total of 66 Ca\(^{2+}\) events were observed, 25 of which were local. All global Ca\(^{2+}\) signals induced exocytosis. By contrast, no local Ca\(^{2+}\) signals induced exocytosis (Fig. 9). We therefore conclude that local Ca\(^{2+}\) responses are not sufficient to drive exocytosis, which further supports the notion that nanodomains are not important in the regulation of secretion and that sites of Ca\(^{2+}\) release are further away from sites of exocytosis.

**Discussion**

Our study describes the spatial relationship between sites of Ca\(^{2+}\) release and sites of exocytic fusion in a cell type where secretion is

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**Fig. 6.** Exocytic events are excluded from the site of origin of the Ca\(^{2+}\) wave. (A) Shown is the same cell as in Fig. 5. Left image: The frame showing the first sign of a Ca\(^{2+}\) response and the 3×3 \(\mu\)m region identified as the site of origin of the Ca\(^{2+}\) response. Middle and right image: First (showing the lumen) and last SRB images (showing the lumen and all exocytic events, respectively). The location of each exocytic event in the cell of interest was then measured by referring to the centre of the 3×3- \(\mu\)m region of the origin Ca\(^{2+}\). (B, C) Graphs of these distances are shown for all the cells analysed (n=22) (B) and for this particular cell (C).

**Fig. 7.** Cytosolic EGTA blocks exocytosis. Cells loaded with EGTA-AM were stimulated with 50 pM CCK. Both the Ca\(^{2+}\) response recorded by using Fura-2 and the exocytic response recorded by using extracellular SRB decreased as a function of the EGTA-AM loading time. Comparisons between Fura-2 AM loading and Fura-2 loaded through a patch pipette (Thorn et al., 1993) indicate that, at 30 minutes, intracellular EGTA concentrations reach a concentration of >200 \(\mu\)M. Data were obtained from 3–6 independent experiments per set of data (**P<0.01 compared with controls measured using Student’s t-test).

**Fig. 8.** Organelles spatially compete with granules in the sub-apical region. (A) Electron micrographs of the apical region of acinar cells with the lumen (containing microvilli) and the sub-apical region packed with zymogen granules. In all sections the sub-apical region also contained other organelles – such as rough ER – which lay between granules and the apical plasma membrane. (B) Frequency histogram of the distances between the apical membrane and granule centres is not consistent with tight packing of granules against the cell membrane. Instead the data suggest that intercalated organelles, such as the ER, spatially separate granules from each other and from the cell membrane.
dependent on Ca\(^{2+}\) release from Ca\(^{2+}\) stores. On the basis of previous work, with cells dependent on Ca\(^{2+}\) entry to trigger exocytosis, we expected a close apposition between hot spots of Ca\(^{2+}\) release and sites of granule fusion. However, our findings indicate no sites of clustered exocytosis. Instead, we show that exocytosis occurs all along the luminal membrane and is in fact excluded within a region of ~3 \(\mu\)m around the hot spot of Ca\(^{2+}\) release, suggesting that the ER, required for Ca\(^{2+}\) release, is locally obstructing granule access to the plasma membrane. Combined with the observations that EGTA profoundly inhibits exocytosis and that local Ca\(^{2+}\) responses do not trigger exocytosis we conclude that secretion, dependent on Ca\(^{2+}\) release from Ca\(^{2+}\) stores, is controlled by cytosolic Ca\(^{2+}\) microdomains. We suggest that this regulation of the microdomain sacrifices speed of secretory control for precision; small adjustments in [Ca\(^{2+}\)] fine tune the secretory output.

### Ca\(^{2+}\)-release-dependent triggering of exocytosis – a model

The evidence presented here supports the idea that Ca\(^{2+}\) release controls an apical cytosolic microdomain of Ca\(^{2+}\) that in turn triggers exocytosis. Within this microdomain the Ca\(^{2+}\) concentration reflects the activity of many ion channels and will be regulated both by the ensemble control of the Ca\(^{2+}\) release channels and the mechanisms of Ca\(^{2+}\) clearance. In turn this tight regulation of the Ca\(^{2+}\) concentration leads to a precise control of secretion.

By contrast, nanodomain control is found in some neurons; the very local delivery of Ca\(^{2+}\) through ion channels tightly couples the Ca\(^{2+}\) stimulus to secretory output with very short latencies. The limitation of this mechanism is that the stochastic opening and closing of the ion channels produces rapid and extreme local changes in Ca\(^{2+}\) in the cytosolic nanodomain beneath the Ca\(^{2+}\) channel. Active and passive mechanisms of Ca\(^{2+}\) clearance in this nanodomain mean that closure of a Ca\(^{2+}\) channel leads to a rapid drop in [Ca\(^{2+}\)]. Thus, the delivery of sufficient Ca\(^{2+}\) levels to trigger exocytosis is dependent on the probabilistic opening of the Ca\(^{2+}\) channel and this can lead to failures to drive exocytosis (Stanley, 1993). Dependence on nanodomains is, thus, necessary to drive fast processes (e.g. neuromuscular control of muscle contraction) but leads to unpredictability in controlling the number of exocytosis events.

So how is the microdomain of Ca\(^{2+}\) regulated in the acinar cells? We have previously measured how much Ca\(^{2+}\) is released during responses in acinar cells and have shown that local Ca\(^{2+}\) responses are comparable (slightly larger) to the puffs seen in oocytes (Fogarty et al., 2000b; Xun et al., 1998). Modeling of cytosolic free [Ca\(^{2+}\)] suggests that, in the immediate vicinity of a puff site, cytosolic Ca\(^{2+}\) can rise to >50 \(\mu\)M but drops off dramatically further away from the puff site (Thul and Falcke, 2004) where, at distances of 1 \(\mu\)m, the cytosolic Ca\(^{2+}\) is predicted to be <1 \(\mu\)M (Thul and Falcke, 2004). With our estimate of a K_d of 1.75 \(\mu\)M Ca\(^{2+}\) for exocytosis of a secretory response driven solely by Ca\(^{2+}\) release from the hot spot would need to have the exocytic sites within 1 \(\mu\)m of the Ca\(^{2+}\) hot spot. Since we show here that no clustering of exocytosis is evident around hot spots of Ca\(^{2+}\) release, local Ca\(^{2+}\) responses cannot induce exocytosis and EGTA can effectively block exocytosis, a more realistic model is that the explosive Ca\(^{2+}\) release from Ins\(P_2\)Rs within the hot spot recruits further Ins\(P_2\)Rs along the ER (Fogarty et al., 2000a). The summed Ca\(^{2+}\) release from these Ins\(P_2\)Rs thus collectively contributes to microdomain Ca\(^{2+}\) levels that, in turn, control exocytosis.

We also suggest that the regulation of [Ca\(^{2+}\)] within a microdomain is the key to integrating convergent cell stimuli on the control of cell secretion. This way, the regulation of Ins\(P_2\)Rs via the CCK and acetylcholine cell-surface receptors is the major mechanism of control (Kasai and Augustine, 1990; Nathanson et al., 1992). This will be modulated by triggering Ca\(^{2+}\) release from ryanodine receptors (Nathanson et al., 1992) through other signaling pathways, such as the secretin-dependent cAMP regulation of Ins\(P_2\)Rs (Giovannucci et al., 2000) and through the regulation of mechanisms of Ca\(^{2+}\) clearance (Camello et al., 1996). Divergent cell stimuli are thus integrated by converging on the regulation of [Ca\(^{2+}\)] within the microdomain, which then precisely controls secretory output of the cell.

### Mechanisms of regional targeting of the Ca\(^{2+}\) release system and the exocytic machinery

Despite our conclusion that the hot spots of Ca\(^{2+}\) release are only loosely coupled with sites of exocytosis it nevertheless should be recognized that both the Ca\(^{2+}\) release system and the exocytic machinery are precisely localized within the cell by mechanisms that are poorly understood.

**Fig. 9. Local Ca\(^{2+}\) spikes do not induce exocytosis.**

(A–B) Upper row of images in A: Fura-2 Ca\(^{2+}\) responses (F_{0} – F/F_{0}) following stimulation with CCK taken at different time points (i–v; indicated in B). Shown are two consecutive, short-lasting Ca\(^{2+}\) spikes that are locally restricted to the apical region (red circle). Lower row of images in A: SRB fluorescence (red). Throughout the occurrence of these local Ca\(^{2+}\) spikes there is no change in the SRB signal and no indication of any exocytic events. (B) The large Fura-2 ratiometric changes in the cell from a region of interest placed in the apical pole (red) compared with very little change in a region placed in the basal pole (green). (C) The full sequence of images (and the associated graph of average fluorescent changes) taken over the first local Ca\(^{2+}\) response clearly shows the spatially restricted nature of the signal.
It is well established that InsP$_3$Rs are enriched beneath the apical plasma membrane of polarized epithelia (Lee et al., 1997; Nathanson et al., 1994; Yule et al., 1997); as shown by immunohistochemistry, the receptors colocalize with apical markers such as the F-actin apical web (Waterman-Storer and Salmon, 1998), and tight-junction markers such as ZO-1 (Larina et al., 2007; Turvey et al., 2005). The mechanisms of this localization are not well understood but disruptions of both the microtubular system (Colosetti et al., 2003) and the F-actin network perturb the generation of Ca$^{2+}$ signals (Turvey et al., 2005). It has been proposed that the microtubular system acts to position the ER (Fogarty et al., 2000c) and that F-actin is part of a complex that specifically anchors InsP$_3$Rs (Foskett et al., 2007). Functional work has proven that the whole of the ER within these cells forms a single continuous network (Park et al., 2000).

Electron microscopy of the sub-plasmalemmal region under the apical pole has shown that it is enriched in secretory granules with interspersed ER elements (Bolender, 1974) (Fig. 8). Although there is no evidence for close association (docking) of granules at the cell membrane it is clear that there must be mechanisms that move the granules to the apical region and retain them there. Again, these mechanisms probably depend on the cytoskeleton, a suggestion that is supported by earlier reports that movement of granules depends on kinesin (Marlowe et al., 1998) and myosin 1 (Poucell-Hatton et al., 1997), and also recently that, in situ, granules are tethered in the apical region (Abu-Hamdah et al., 2006). Most recently a proteomic analysis has shown that myosin Vc is present in zymogen granules (Chen et al., 2006). In terms of the molecular components of exocytosis in non-excitable cells our knowledge lags behind excitable cells. So, whereas soluble NSF attachment protein receptors (SNARE) proteins on the cell membrane and granule membrane have been identified (Cosen-Binker et al., 2008; Gaisano et al., 1996; Hansen et al., 1999), it is still unclear which SNARE proteins are actually involved in exocytosis of zymogen granules at the apical plasma membrane.

**Comparison of other measures of secretion**

The two-photon method we used here has been proven to reliably identify exocytosis of zymogen granules (Larina et al., 2007; Nemoto et al., 2001; Thorn et al., 2004). Using cell capacitance measurements as a read-out for exocytosis, changes are detected that might not be directly associated with fusion of a zymogen granule. The expectation that fusion of a zymogen granule should lead to rapid, large-step increases in capacitance is seen in parotid cells (Chen et al., 2005) but has rarely been observed in pancreatic acinar cells, in which slower increases are usually seen (Ito et al., 1997; Maruyama and Petersen, 1994). Ito et al. kinetically separated these capacitance signals, and suggested a fast component possibly owing to processes other than the secretion of amylase (fusion of zymogen granules) (Ito et al., 1997). The underlying mechanism used by this fast component is unknown but it might explain why capacitance changes have been seen with local Ca$^{2+}$ responses (Maruyama and Petersen, 1994); and yet we find no evidence that exocytosis of zymogen granules is induced by local Ca$^{2+}$ spikes (Fig. 9).

**Conclusions**

Ca$^{2+}$ release from Ca$^{2+}$ stores is either the exclusive regulator of, or a component in, the regulation of exocytosis in many different cell types. Our work described here shows that exocytosis is precisely controlled by regulating Ca$^{2+}$ release in a microdomain. We further show that, in response to physiological stimuli, exocytosis is only driven by global Ca$^{2+}$ signals.

**Materials and Methods**

**Cell preparation**

Mice were humanely killed according to local animal ethics procedures. Isolated mouse pancreatic tissue was prepared by a collagenase digestion method in normal NaCl-rich extracellular solution (Thorn et al., 1993) that was modified to reduce the time in collagenase and to limit mechanical disruption. The resultant preparation was composed of pancreatic lobules and fragments (50–100 cells). In the indicated experiments pancreatic fragments were loaded with 2 μM Fura-2 (or Fura-2FF) acetoxyethyl ester (AM) for 30 minutes at 30°C. Fragments were then washed and placed onto poly-L-lysine-coated glass coverslips and used within 3 hours of isolation from the animal. In experiments using NP-EGTA the NP-EGTA-AM (1 μM) was loaded together with Fura-2-AM.

**Live-cell two-photon imaging**

We used a custom-made, video-rate, two-photon microscope employing a Sapphire-Ti laser (Coherent), with a 60× oil immersion objective (NA 1.42, Olympus), providing an lateral resolution (full width, half maximum) of 0.26 μm and a z-resolution of 1.3 μm (Thorn et al., 2004). We imaged exocytic events using Sulforhodamine B (SRB, 20 μg/ml, Sigma), as a membrane-impermeant fluorescent extracellular marker excited by continuous laser pulses at 800 nm, with fluorescence emission detected at 550–700 nm. Fura-2 was excited at the same wavelength with fluorescence emission detected at 450–550 nm. The Fura-2 signal was analysed using the following formula: Fluorescence ratio = (resting fluorescence – signal fluorescence) – resting fluorescence, where the resting fluorescence is taken from an average of frames before stimulation. Images, with a resultant capture rate of six frames/second (resolution of 10 pixels/μm, average of five video frames), were analysed with the Metamorph program (Molecular Devices Corporation). Kinetins of exocytic events were measured as changes in SRB fluorescence from ROIs (0.78 μm$^2$, 100 pixels) centered over granules. Traces were rejected if extensive movement was observed. All data are shown as the mean ± s.e.m.

**Photoliberation of Ca$^{2+}$ from NP-EGTA**

An epifluorescent mercury light source provided high-intensity ultraviolet (UV) light to uncage Ca$^{2+}$ from o-nitrophilidin (NP-EGTA) in a 30–μm diameter field at the image plane. The duration of exposure to UV light was limited by a computer-controlled shutter (Prior) and was varied between 5 and 200 ms.

Fura-FF was calibrated in vivo by loading the cells with 2 μM Fura-FF-AM (for 30 minutes) and then permeabilizing with 500 nM ionomycin in the presence of a NaCl-rich extracellular solution (Thorn et al., 1993) that was modified to reduce the NaCl content. Mouse pancreatic tissue was prepared by a collagenase digestion method in normal NaCl-Ringer solution (Thorn et al., 1993) modified to reduce the NaCl content. The mouse pancreatic tissue was prepared by a collagenase digestion method in normal NaCl-Ringer solution. Cells were then loaded with 2 μM Fura-2 (or Fura-2FF) acetoxyethyl ester (AM) for 30 minutes and then washed and placed onto poly-L-lysine-coated glass coverslips and used within 3 hours of isolation from the animal.

**Image analysis**

All morphometric analysis was performed using the Metamorph imaging suite. Individual cells were readily identified by the outline that is apparent in the extracellular SRB stain. However, some examples had cells lying on top of one another and we used the Fura-2 signal to aid in the identification of single cells. Here, the asynchronous Ca$^{2+}$ response in each individual cell (as described in Fig. 1) supported unambiguous identification of single cells.

When measuring distances between granules (Figs 3, 4, 6), we identified the approximate center of all fused granules labeled with SRB and measured granule-to-granule distances parallel to the length of the lumen. The two-photon z-thickness of 1.3 μm approximates to the diameter of the lumens between the cells. Experimentally, we focused through each tissue fragment and selected image planes to optimise the length of lumen observed (because this is where exocytosis exclusively occurs). Most images, therefore, have lumens much longer than the diameter of individual granules, making it simple to measure inter-granule distances parallel to the lumen. By contrast, where the lumen is complex, errors are possible in our estimates of inter-granule distance. However, this did not bias our data analyses because errors of above and below the estimate are equally probable. In calculating the expected granule-to-granule distances (Fig. 3) we assumed to have triggered the maximal exocytic response and a granule diameter of 1 μm, which were to give us the simple linear relationship of: $y = 0.4x$, where $x$ is the granule-to-granule distance and $y$ is the length of the lumen.
In our estimates regarding the focal point of origin of a Ca2+ signal, our experimental approach was – again – to select image planes with long lumen. Knowing that InsP3Rs are located along the lumen (Lee et al., 1997; Nathanson et al., 1994; Yule et al., 1997), we were likely to image the origin of the Ca2+ signal in most recordings. By using the maximal increase of the Fura-2 signal in ROIs along the lumen we believe that, in most cases, were able to identify a single point of origin for a Ca2+ signal. In the few instances where the rate of increase of the Fura-2 signal appeared diffuse along the lumen – indicating a Ca2+-signal-origin outside the plane of focus – records were rejected.

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