Stable Golgi-Mitochondria Complexes and Formation of Golgi Ca$^{2+}$ Gradients in Pancreatic Acinar Cells

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We have determined the localization of the Golgi with respect to other organelles in living pancreatic acinar cells and the importance of this localization to the establishment of Ca$^{2+}$ gradients over the Golgi. Using confocal microscopy and the Golgi-specific fluorescent probe 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl)sphingosine, we found Golgi structures localizing to the outer edge of the secretory granular region of individual acinar cells. We also assessed Golgi positioning in acinar cells located within intact pancreatic tissue using two-photon microscopy and found a similar localization. The mitochondria segregate the Golgi from lateral regions of the plasma membrane, the nucleus, and the basal part of the cytoplasm. The Golgi is therefore placed between the principal Ca$^{2+}$ release sites in the apical region of the cell and the important Ca$^{2+}$ sink formed by the peri-granular mitochondria. During acetylcholine-induced cytosolic Ca$^{2+}$ signals in the apical region, large Ca$^{2+}$ gradients form over the Golgi (decreasing from trans- to cis-Golgi). We further describe a novel, close interaction of the peri-granular mitochondria and the Golgi apparatus. The mitochondria and the Golgi structures form very close contacts, and these contacts remain stable over time. When the cell is forced to swell, the Golgi and mitochondria remain juxtaposed up to the point of cell lysis. The strategic position of the Golgi (closer to release sites than the bulk of the mitochondrial belt) makes this organelle receptive to local apical Ca$^{2+}$ transients. In addition the Golgi is ideally placed to be preferentially supplied by ATP from adjacent mitochondria.

The pancreatic acinar cell is the classical model for studies of the secretory pathway. In the pancreatic acinar cell, Palade (1) first described the segregation, transport, and discharge of secretory proteins. Ca$^{2+}$ regulates processing of secretory proteins within (2) and transport along the secretory pathway (3–5). In turn many of the membrane-bound compartments of the secretory pathway serve as a source and a sink for Ca$^{2+}$ (6–9). Ca$^{2+}$ signaling and the secretory pathway are inextricably tied in pancreatic acinar cells (10). Structural and functional polarity is an important aspect of this reciprocal relationship (11–13). Initiation of the secretory pathway occurs at the basolateral part of the cell with protein synthesis and terminates with Ca$^{2+}$-dependent secretion at the apical membrane (14–16).

The endoplasmic reticulum (ER) and nucleus are located in the basolateral part of the cell, whereas the secretory granules (SGs) are located in the apical pole (1, 11, 17). The most striking polarization is that of the mitochondria with three distinct groupings: peri-granular, sub-plasmalemmal, and peri-nuclear (18–22).

In this study we examined the localization of the Golgi with respect to other cellular organelles and the functional consequences of this localization. We found close Golgi-mitochondria contacts that remain remarkably stable over time and during different cellular perturbations. Finally, we visualized Ca$^{2+}$ gradients that are formed over the Golgi as a consequence of relative positioning of the Golgi and mitochondria.

**EXPERIMENTAL PROCEDURES**

Pancreatic Acinar Cell Preparation—Pancreata were obtained from male CD 1 mice (21–30 days old) as previously described (23) in accordance with the Animals (Scientific Procedures) Act, 1986. Undissociated tissue was immobilized and imaged as previously described (24). Single cells and clusters of acinar cells were obtained by brief (10-min) collagenase digestion of the pancreas, followed by gentle agitation with a pipette. For all experiments, isolated cells, pancreatic clusters, or pancreata were suspended in a standard HEPES-buffered physiological salt solution containing 140 mM NaCl, 4.7 mM KCl, 1.13 mM MgCl$_2$, 10 mM HEPES, 10 mM glucose, 1 mM CaCl$_2$, pH adjusted to 7.2 (with NaOH). In studies of Ca$^{2+}$ signaling, cells were stimulated with pressure application of acetylcholine (ACh) (see below).

Confocal Microscopy—Live cells were examined on a Leica TCS SP2 or Leica SP2-AOBS confocal microscope with a 63× water immersion objective lens and a 1.2 NA. SGs were visualized using confocal reflectance (excitation, 545 nm; emission, 535–548 nm). To visualize the Golgi, cells were loaded with 2.5 μM NBD C$_6$-ceramide for 15 min at 4 °C. NBD C$_6$-ceramide was excited by a 476 nm laser line, and emission was collected at 500–550 nm. Cells were loaded with 50 nM Mito-Tracker Deep Red for 15 min at 37 °C to visualize mitochondria. Mito-Tracker Deep Red was excited with a 633 nm laser line, and emission was collected above 650 nm. For ER labeling, cells were incubated with 500 nM BODIPY Texas Red thapsigargin for 15 min at room temperature and excited at 594 nm, and the emission was collected between 600 and 650 nm. Images of intracellular organelles were obtained with a confocal pinhole corresponding to 1 Airy unit. The Ca$^{2+}$ and 650 nm. Images of intracellular organelles were obtained with a confocal pinhole corresponding to 1 Airy unit. The Ca$^{2+}$ and 650 nm.

1 The abbreviations used are: ER, endoplasmic reticulum; ACh, acetylcholine; SG, secretory granule; NBD C$_6$-ceramide, 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl)sphingosine.

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Applications of agonists and analysis to calculate cytosolic Ca$^{2+}$ gradients were performed as described by Gerasimenko et al. (25).

Briefly, ACh stimulation of pancreatic acinar cells was via a pipette filled with a 100 μM ACh solution. The pipette was attached to a pressure injection system (Eppendorf, Hamburg, Germany); this system allowed short (0.1–1-s) applications of ACh. In some experiments local Ca$^{2+}$ signals were produced by stimulation with a low (10 nm) concentration of ACh. The formation of Ca$^{2+}$ gradients across the cells axis with respect to the Golgi was analyzed in the following way: cells in which ACh elicited apically localized Ca$^{2+}$ signal were selected for analysis. Line profiles of Fura Red fluorescence were taken across the cell from the apical to basolateral pole; the signal was then averaged for six such selected lines. The profile at rest was then subtracted from the stimulated profile (during peak of Ca$^{2+}$ transient) giving rise to the $ΔF$ (change in fluorescence) profile reflecting Ca$^{2+}$ changes along the apical to basolateral axis. This trace was then compared with the distribution of NBD C6-ceramide fluorescence recorded along the same line.

**Chemicals:** All fluorescent dyes were obtained from Molecular Probes (Eugene, OR). All other chemicals were from Sigma.

**RESULTS**

**Localization of the Golgi and Its Interaction with the Peri-granular Mitochondrial Belt**—Labeling of the Golgi using the Golgi-specific probe NBD C6-ceramide (26) in live cells reveals a large crescent-shaped organelle also located in the peri-granular part of the cell (Fig. 1A, I and II; supplemental Fig. S1; supplemental Video 1). A similar localization of the Golgi has been described previously (using immunofluorescence labeling) in fixed isolated pancreatic acinar cells (27, 28). Using two-photon microscopy, we also investigated the positioning of the Golgi in cells located within intact (undissociated) pancreatic tissue or large pancreatic clusters and found a clearly resolved crescent-shaped organelle located in the peri-granular region (see supplemental Fig. S1). We were able to disassemble this structure by incubating the cells for 30 min with brefeldin A (5 μg/ml; n = 42; see supplemental Fig. S2), a compound known to disassemble the Golgi (29).

Previous studies have shown a dense belt of mitochondria around the SGs in pancreatic acinar cells (18–22). Positioning of the Golgi in this region suggested a possible overlap of the two organelles and prompted us to investigate their relative positioning. Co-labeling of acinar cells with NBD C6-ceramide (Fig. 1A, II) and MitoTracker Deep Red (Fig. 1A, III) shows that these two organelles are juxtaposed and sometimes interwoven (Fig. 1A, IV). The peri-granular mitochondrial belt is positioned predominantly on the basolateral side of the Golgi (Fig. 1A, IV, representative of 132 cells). Magnification of this part reveals that the Golgi (Fig. 1B, I) has close contacts with the mitochondria from the peri-granular belt (Fig. 1B, II and III). In the regions of contacts, the distance between these organelles is so small that it is beyond the resolution of confocal microscopy (Fig. 1B, III). Thus, the energy-dependent membrane trafficking steps from the ER to the Golgi and on through the Golgi stacks (1) should be well served by the observed close proximity of the Golgi to the ATP-producing mitochondria. The membrane trafficking steps also depend on Ca$^{2+}$ signals (4) and Ca$^{2+}$ gradients (5). Localization of the Golgi on the apical side of the mitochondrial belt could potentially expose the Golgi to frequent Ca$^{2+}$ elevations induced in this cell type by physiologic doses of secretagogues (Refs. 8, 23, and 30; see also “The Golgi Localization and Cytosolic Ca$^{2+}$ Gradients” below).

Another interesting feature of Golgi and mitochondria positioning can be seen in a region adjacent to the apical part of the lateral plasma membrane. The *white dashed line* in Fig. 1C, I–IV, depicts the lateral membrane. It is clear that the mitochondria (Fig. 1C, III), but not the Golgi (Fig. 1C, I, II), extend up to the surface of the lateral membrane (Fig. 1C, IV and supplemental Video 2). These peri-granular mitochondria (Fig. 1C, III) extend past the edge of the Golgi (Fig. 1C, II–IV). Comparison of the distribution of the two organelles (Fig. 1) with the recently reported patterns of mitochondrial Ca$^{2+}$ accumulation suggests that the lateral mitochondria not covered by the Golgi are the mitochondria that participate in the initial Ca$^{2+}$ uptake (20). The region adjacent to the apical part of the lateral membrane was reported to contain Ca$^{2+}$-releasing channels (reviewed in Ref. 31); therefore, the lateral “Golgi-free” mitochondria could potentially be important participants in the signaling events generated in this part the cell.

**The Golgi-Mitochondria Complexes Are Stable Over Time and Resist Cell Perturbation**—In many cell types mitochondria form a dynamic interconnected network (32, 33). We used three-dimensional confocal reconstructions recorded over time to examine the stability of these close contacts formed between Golgi and mitochondria. Three-dimensional images of mitochondria and Golgi were recorded over time and analyzed. Despite small changes in cell shape and some dynamic movement of the two organelles, the regions in which they come into close contact remained stable over time (269 s) (Fig. 2, A and B, representative of 11 cells). No significant changes in Golgi-mitochondria contacts were seen during short (~5-min) stimulation with supramaximal doses of secretagogues, 10 μM ACh (n = 13; supplemental Fig. S3), or 5 nM cholecytokinin (n = 4; data not shown).

To further test the stability of Golgi-mitochondria contacts we induced artificial swelling of the cells by replacing the normal extracellular solution with distilled water (Fig. 2, C and D). The Golgi structures continued to be in close contact with mitochondria up to the point of cell lysis (Fig. 2D, III). At the time point immediately prior to lysis, the cell had swollen by 45 ± 7% (increase in the area of the central optical section; n = 7). During the cell expansion, up to the time of cell lysis, the Golgi and mitochondria moved simultaneously away from their initial location with regions of close contacts still present (n = 7). After cell lysis in distilled water, the mitochondria lost most of their staining, rounded up, and disconnected from the Golgi and other organelles (data not shown).

**Relative Positioning of Golgi and Other Organelles in Live Pancreatic Acinar Cells**—Fig. 3 shows the relative positioning of intracellular organelles. Interestingly, in this cell type, the Golgi apparatus is not immediately adjacent to the nucleus (Fig. 3A). The mitochondria, which occupy a more basal location than the Golgi structures, create a narrow barrier between the Golgi and the nucleus (Fig. 3, A and C).

The bulk of the ER is found basally with respect to the Golgi and the mitochondria (Fig. 3B); however, the thin projections of the ER described previously (17, 34) can be seen in the apical (with respect to Golgi) part of the cells (Fig. 3B). These ER projections account for the apical Ca$^{2+}$ release from the luminal connected ER (34, 35).

The Golgi surrounds the SGs, whereas the peri-granular mitochondrial belt engulfs the Golgi (Fig. 3C) from both the basal and lateral sides. This dense layered packing results in the Golgi occupying a position in the vicinity of the SGs on the apical (with respect to the majority of the mitochondria) part of the cell (Fig. 3C). The Ca$^{2+}$ released from the ER projections in the secretory granule area (Fig. 3B) should therefore first reach the Golgi before the bulk of the mitochondria can come into play and absorb this Ca$^{2+}$ via high capacity mitochondrial uniports (36, 37). We decided to verify this hypothesis by directly mapping Ca$^{2+}$ gradients developed during localized apical Ca$^{2+}$ signals with respect to the position of the Golgi.

**The Golgi Localization and Cytosolic Ca$^{2+}$ Gradients**—By co-loading NBD C6-ceramide and the Ca$^{2+}$-sensitive dye Fura Red, we imaged apically restricted Ca$^{2+}$ signals with respect to the Golgi localization (Fig. 4). The transmitted image of the cell cluster is shown in Fig. 4A. The apical (red circle) and basal...
Fig. 1. The peri-granular mitochondrial belt surrounds the Golgi: close contacts between Golgi and mitochondria. A, I, transmitted light image showing the acinar cell cluster. The fluorescent markers show the localization of the Golgi (A, II, green) and mitochondria (A, III, red). The region in the blue box (see A, I) is enlarged in B, I–III; the part in the red box is enlarged in C, I–IV. Overlay of the two markers (A, IV) shows that the Golgi is located to the apical side of the peri-granular mitochondrial belt. The region in the blue box was enlarged for a more detailed examination of the relationship between the Golgi and mitochondria (B, I–III). Overlay of the Golgi and mitochondrial staining shows that these two organelles are juxtaposed around the apical-basal interface. C, I shows an enlarged part of the transmitted image. The white dashed line indicates the lateral membranes close to the apical region. Labeling of the Golgi (C, II) reveals that the proximal end of the Golgi is some way from the lateral membrane, whereas the peri-granular mitochondrial belt (C, III) encapsulates the Golgi (C, IV) extending right up to the lateral membrane (C, III and IV) and continuing for some distance along the lateral membrane.
the same line is shown on Fig. 4C, II. By measuring the Ca\textsuperscript{2+} level along a line across the cell (Fig. 4A, II) before the stimulation and at the time points of maximal Ca\textsuperscript{2+} response, we could ascertain the Ca\textsuperscript{2+} gradients across the Golgi (n = 5). We found that during apical, physiologically relevant Ca\textsuperscript{2+} signaling (23, 30), the Golgi is exposed to Ca\textsuperscript{2+} gradients that dissipate 1–2 μm past the Golgi (Fig. 4C, II). For comparison, in separate experiments, we stained the Golgi and mitochondria (Fig. 4D) and drew similar line profiles as used in Fig. 4, A–C, to compare the localization of the Golgi and mitochondria (n = 10). The mitochondrial staining profile partially overlaps with the Golgi, but most of the mitochondrial staining was found within a 2-μm region past the Golgi. Uptake of Ca\textsuperscript{2+} by these mitochondria (20) probably accounts for the dissipation of the Ca\textsuperscript{2+} gradient immediately past the Golgi.

The notion that mitochondrial Ca\textsuperscript{2+} uptake plays a critical role in terminating local Ca\textsuperscript{2+} signals and formation of peri-granular Ca\textsuperscript{2+} gradients is supported by our previous studies, in which inhibition of mitochondria with the protonophore carbonyl cyanide m-chlorophenylhydrazone or an inhibitor of the electron transport chain antimycin (or a mixture of antimycin and oligomycin) resulted in conversion of local Ca\textsuperscript{2+} signaling to global Ca\textsuperscript{2+} responses (18). Straub et al. (19) reported that inhibition of mitochondria with carbonyl cyanide 4 trifluoromethoxyphenylhydrazone resulted in globalization of Ca\textsuperscript{2+} spikes induced by inositol 1,4,5-trisphosphate uncaging. Similar results were obtained using Ru360, an inhibitor of the mitochondrial uniporter (38). Direct measurements of mitochondrial Ca\textsuperscript{2+} (20) and indirect evidence (an increase in NADH during brief Ca\textsuperscript{2+} signals) (39) also support the barrier role of mitochondria.

In our current study we repeated experiments with inhibition of mitochondria by a mixture of rotenone and oligomycin (with additional staining for Golgi and visualization of mitochondria). We confirmed the previous results and were able to record the formation of global Ca\textsuperscript{2+} signals appearing in the apical, peri-granular, and basal regions almost simultaneously (supplemental Fig. S4, n = 4; an additional benefit of these experiments is that the position of the mitochondria is revealed.
by NADH autofluorescence, which is clearly seen to surround the Golgi. Because the Golgi is sandwiched between the Ca^{2+} source (release sites in the apical region) and the Ca^{2+} sink (mitochondrial uniporters), Ca^{2+} gradients are formed over this organelle with the Ca^{2+} concentration over the trans-Golgi higher than that over the cis-Golgi. Thus, in pancreatic acinar cells, the Golgi is regularly exposed to elevations in cytosolic Ca^{2+} higher than that over the cis-Golgi. Therefore, the potential not only to benefit from Ca^{2+} signals but also to shape these signals serving either as a Ca^{2+} sink or as the amplifier of the Ca^{2+} responses. The positioning of the Golgi in front of the mitochondrial barrier provides it with useful signaling commodities, Ca^{2+} elevations and gradients, which are required for secretory cargo processing (2) and vesicular trafficking (3-5).

In our previous study we found that the cytosolic Ca^{2+} gradients could reach hundreds of nanomoles per micrometer when measured along the line drawn from the apical to the basal part of the acinar cell (25); in the current work we found that these cytosolic Ca^{2+} signals cross the Golgi and dissipate within the peri-granular mitochondrial belt without spreading into the nucleus and the rest of the basal cytosol.

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FIG. 4. The Golgi localization and cytosolic Ca^{2+} gradients. Cells shown on the transmitted image (A, I) were loaded with Fura Red to measure cytosolic Ca^{2+} changes and NBD C_{6}ceramide (A, II) to visualize the Golgi. B, the time of the pressure application of ACh (1 mM in the pipette solution) from the pipette located close to the cell surface to measure cytosolic Ca^{2+} gradients. C, I, the fluorescence gradient along a line (pictured in A, I; line averaging and polygonal approximation were used to decrease the noise) at the peak of the response was compared with that at rest (time intervals are indicated by dashed lines on B). The gradient is shown in C, I. The Golgi fluorescence along the same line is presented in C, II. The relative distribution of Golgi (green) and mitochondria (black) staining along a line connecting the apical and basal parts of the cell (a different cell from that shown in A–C) is shown in D.
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