



## STRUCTURE AND DYNAMICS OF THE FUSION PORE IN LIVE CELLS

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Atomic force microscopy reveal pit-like structures typically containing three or four, ~150 nm in diameter depressions at the apical plasma membrane in live pancreatic acinar cells. Stimulation of secretion causes these depressions to dilate and return to their resting size following completion of the process. Exposure of acinar cells to cytochalasin B results in decreased depression size and a loss in stimuable secretion. It is hypothesized that depressions are the fusion pores, where membrane-bound secretory vesicles dock and fuse to release vesicular contents. Zymogen granules, the membrane-bound secretory vesicles in exocrine pancreas, contain the starch digesting enzyme, amylase. Using amylase-specific immunogold labeling, localization of amylase at depressions following stimulation of secretion is demonstrated. This study confirms depressions to be the fusion pores in pancreatic acinar cells. High-resolution images of the fusion pore in live pancreatic acinar cells reveal the structure in much greater detail than has previously been observed.

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### INTRODUCTION

The fusion of membrane-bound secretory vesicles at the cell plasma membrane and consequent expulsion of vesicular contents are fundamental cellular processes regulating neurotransmission, enzyme secretion or hormone release. Following secretory stimuli, secretory vesicles dock and fuse at defined plasma membrane locations. Electrophysiological studies on mast cells suggest the presence of fusion pores at the cell plasma membrane, which become continuous with the secretory vesicle membrane following stimulation of secretion (Monck *et al.*, 1995). Atomic force microscopy (AFM) performed at nanometer resolution on live pancreatic acinar cells further reveals the presence of a group of pore-like structures at the apical plasma membrane

(Schneider *et al.*, 1997). The morphology and dynamics of the pore-like structures suggest that they may be the fusion pores reported earlier from electrophysiological studies (Monck *et al.*, 1995).

Pancreatic acinar cells are polarized secretory cells, where membrane-bound secretory vesicles called zymogen granules (ZG) located primarily at the apical end of the cell, dock and fuse with the apical plasma membrane to release vesicular contents (Jena *et al.*, 1994). The apical end of live pancreatic acinar cells, when imaged at near nanometer resolution using AFM (Binnig *et al.*, 1986), have circular structures called pits of ~500 nm in diameter and 25–35 nm in depth (Schneider *et al.*, 1997). Within each pit, several depressions or pore-like structures, each measuring 100–180 nm in diameter and 15–25 nm in relative depth, are seen (Schneider *et al.*, 1997). No similar plasma membrane structures are detected at the basolateral end of pancreatic acinar cells. Following stimulation of secretion, there is ~30–35% increase in diameter of depressions, and a

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consequent 129% increase in amylase release (Schneider *et al.*, 1997). No change in size of pits is seen at any time following stimulation of secretion. On completion of secretion, the dimensions of depressions return to their resting values. The time-course of increase and decrease in depression size strongly correlates with amylase release. Furthermore, when live acinar cells are exposed to cytochalasin B, a fungal toxin that inhibits actin polymerization (Bauduin *et al.*, 1975) a 22% decrease in the resting diameter of 'depressions' and a consequent inhibition of amylase secretion is observed. Cytochalasin B-treated cells when stimulated, exhibit a 50–60% loss in amylase release, compared to untreated controls (Schneider *et al.*, 1997). Cytochalasin B-treated cells exhibit only a modest (5%) increase in depression diameter following stimulation of secretion. These studies suggest depressions to be the fusion pores or porosomes, where membrane-bound secretory vesicles may dock and fuse to release vesicular contents (Schneider *et al.*, 1997).

Further delineation of the role of depressions as the fusion pore required a direct determination of the release of secretory products at these sites. Initial attempts to determine release at depressions were unsuccessful due to low signal to noise ratios in experiments utilizing AFM's capability to detect physical disturbance at depressions during expulsion of vesicular contents in exocytosis. Therefore in this study, immunogold labeling of amylase at the plasma membrane of pancreatic acinar cells were examined following secretion. Results from this study demonstrate depressions to be the fusion pores of live pancreatic acinar cells, where membrane-bound secretory vesicles dock and fuse to expel vesicular contents.

## MATERIALS AND METHODS

### *Western blot analysis*

Immunoblot analysis was performed on pancreatic fractions. Protein in pancreatic fractions was estimated by the Bradford method (Bradford, 1976). Pancreatic fractions were boiled in Laemmli reducing sample preparation buffer (Laemmli, 1970) for 5 min. Equal loads of protein were resolved in a 12.5% SDS-PAGE, followed by electrotransfer to 0.2 m nitrocellulose sheets. The nitrocellulose was incubated for 1 h at room temperature in blocking buffer (5% non-fat milk in PBS containing 0.1% Triton X-100 and 0.02%  $\text{NaN}_3$ ), and immunoblotted for 2 h at room temperature with either

affinity-purified  $\alpha$ -amylase antibody (StressGen Biotechnologies Corp, San Diego, CA, U.S.A.). The primary antibody was used at a dilution of 1:10,000 in blocking buffer. The immunoblotted nitrocellulose sheets were washed in PBS containing 0.1% Triton X-100 and 0.02%  $\text{NaN}_3$ , prior to incubation for 1 h at room temperature in horseradish peroxidase (HRP)-conjugated secondary antibody at a dilution of 1:2000 in blocking buffer. The immunoblots were washed in PBS containing 0.1% Triton X-100 and 0.02%  $\text{NaN}_3$ , processed for enhanced chemiluminescence and exposure to X-OMAT-AR film. The exposed films were then developed and photographed.

### *Isolation of pancreatic acinar cells*

Isolation and preparation of acinar cells for secretion experiments, Light Microscopy, AFM and electron microscopy (EM), were performed. Pancreatic acinar cells and hemi-acini were isolated using a minor modification of our published procedure (Jena *et al.*, 1991). For each experiment, a male Sprague Dawley rat weighing 80–100 g was euthanized by carbon dioxide inhalation. The pancreas was excised and chopped into three 0.5-mm pieces, which were mildly agitated for 10 min at 37°C in a siliconized glass tube with 5 ml of oxygenated buffer A (98 mM NaCl, 4.8 mM KCl, 2 mM CaCl, 1.2 mM  $\text{MgCl}_2$ , 0.1% bovine serum albumin, 0.01% soybean trypsin inhibitor, 25 mM Hepes, pH 7.4) containing 1000 units of collagenase. The suspension of acini was filtered through a 224  $\mu\text{m}$  Spectra-Mesh (Spectrum Laboratory Products, Saint Paul, MU, U.S.A.) polyethylene filter to remove large clumps of acini and undissociated tissue. The acini were washed six times, 50 ml per wash, with ice-cold buffer A. Isolated rat pancreatic acini and acinar cells were plated on Cell-Tak-coated (Collaborative Biomedical Products, Bedford, MA, U.S.A.) glass coverslips. Two to three h after plating, cells were imaged by the AFM before and during stimulation of secretion. Isolated acinar cells and small acinar preparations were used in the study because fusions of regulated secretory vesicles at the cell plasma membrane in pancreatic acini are confined to the apical region and are impossible to image by the AFM in whole tissue or large acinar preparations.

### *Light and immunoelectron microscopy*

Following isolation, rat pancreatic acini and acinar cells were resuspended in oxygenated buffer A and placed on a Cell-Tak-coated glass

slide. Live pancreatic acinar cells were observed and photo-graphed using a Zeiss Axiovert 200 microscope, with a 100 $\times$  objective. For electron microscopy, purified acinar cells in buffer A were centrifuged at 2000  $\times g$  for 2 min and the acinar pellet was resuspended in fixative. Cells were fixed in 2.5% buffered formaldehyde (PFA) for 30 min, and the cell pellets were embedded in unicryl, sectioned, the sections were transferred to coated specimen grids, and immunostained. The immunostained sections on EM grids were then dried in the presence of uranyl acetate and methylcellulose, and examined in a transmission electron microscope.

#### *Amylase measurement following exposure of acinar cells to mastoparan*

Exocytosis from acinar cells was measured by determining the percentage of total cellular amylase release, following exposure of cells to a secretagogue. Amylase, one of the major contents of ZGs, was measured by the procedure of Bernfeld (1955). In a typical amylase assay, rat pancreatic acini dissociated as single cells and clumps of 2–6 cells were used. Fifty to 75 cells in 200 ml of total reaction mixture (buffer A) in the presence or absence of 20 M mastoparan were incubated at room temperature. Following incubation, the cells were centrifuged at 2000  $\times g$  for 2 min in an Eppendorf microcentrifuge. The supernatant containing the secreted amylase was assayed. The cells in the remaining 100  $\mu$ l of incubation mixture were sonicated, and the sonicate was diluted and assayed for amylase. From the above measurements, the total cellular amylase and percent release from the cells were calculated. Five microliters of the supernatant or lysed cell fractions was added to 95  $\mu$ l of ice-cold amylase assay buffer (10 mM NaH<sub>2</sub>PO<sub>4</sub>; 10 mM Na<sub>2</sub>HPO<sub>4</sub>; 20 mM NaCl) placed in 12  $\times$  75 mm glass tubes in an ice bath. The reaction was started by adding 100  $\mu$ l of a 10 mg/ml potato starch in amylase assay buffer solution. The mixture was vortexed and incubated for 15 min at 37°C. Following the incubation, the mixture was cooled in an ice bath and 400  $\mu$ l of a color reagent (44 mM 3,5-dinitrosalicylic acid, 200 mM KOH, and 20 mM sodium potassium tartarate) was added. The mixture in glass tubes was covered and lowered into a boiling water bath for 25 min followed by cooling and the addition of 1.4 ml of distilled water. The mixture was then brought to room temperature and transferred to a plastic cuvette, and absorbance at 530 nm was measured with a

spectrophotometer (Beckman DU-530, Fullerton, CA, U.S.A.).

#### *Immunogold AFM*

Immunogold localization in live pancreatic acinar cells, after 5 min. following stimulation of secretion using 10  $\mu$ M mastoparan. The live pancreatic acinar cells were exposed to 1:200 dilution of  $\alpha$ -amylase specific antibody (Biomedica Corp. Foster City, CA, U.S.A.) and 30 nm gold conjugated secondary for 1 min, washed in PBS, prior to AFM imaging in PBS at room temperature. Pits and depressions at the apical end of live pancreatic acinar cells in PBS pH 7.5, were imaged by the AFM (Bioscope III, Digital Instruments, Santa Barbara, LA, U.S.A.) using both contact and tapping mode. All images presented in this manuscript were obtained in the 'tapping' mode in fluid, using silicon nitride tips with a spring constant of 0.06 N  $\cdot$  m<sup>-1</sup>, and an imaging force of <200 nN. Images were obtained at line frequencies of 1 Hz, with 512 lines per image, and constant image gains. Topographical dimensions of pits and depressions at the cell plasma membrane were analyzed using the software nanoscopeIIIa4.43r8 supplied by Digital Instruments.

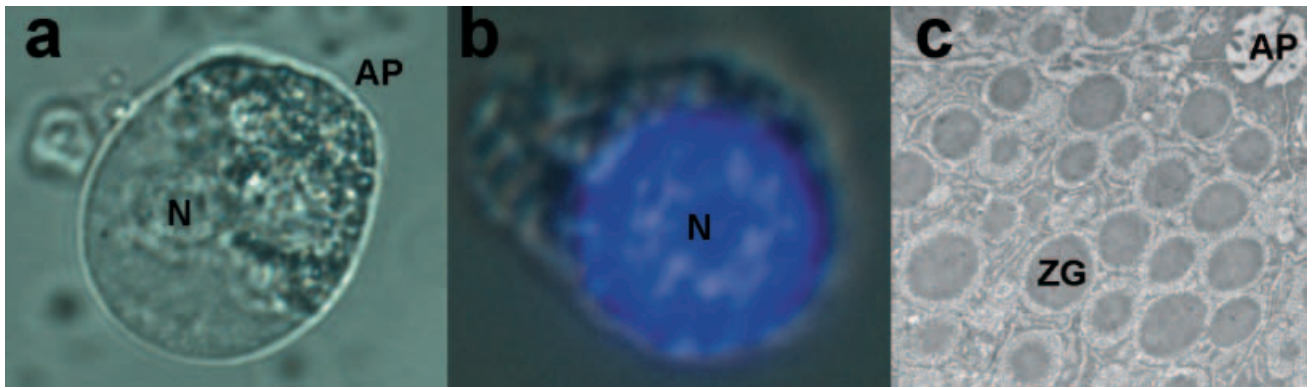
#### *Cell fixation and immunogold localization*

Following stimulation of secretion using 10  $\mu$ M mastoparan, the live pancreatic acinar cells were fixed for 30 min using ice-cold 2.5% paraformaldehyde in PBS. Cells were then washed in PBS, followed by labeling with 1:200 dilution of  $\alpha$ -amylase-specific antibody (Biomedica Corp., Foster City, CA, U.S.A.) and 10 nm gold conjugated secondary for 15 min, fixed and washed in PBS, prior to AFM imaging in PBS at room temperature.

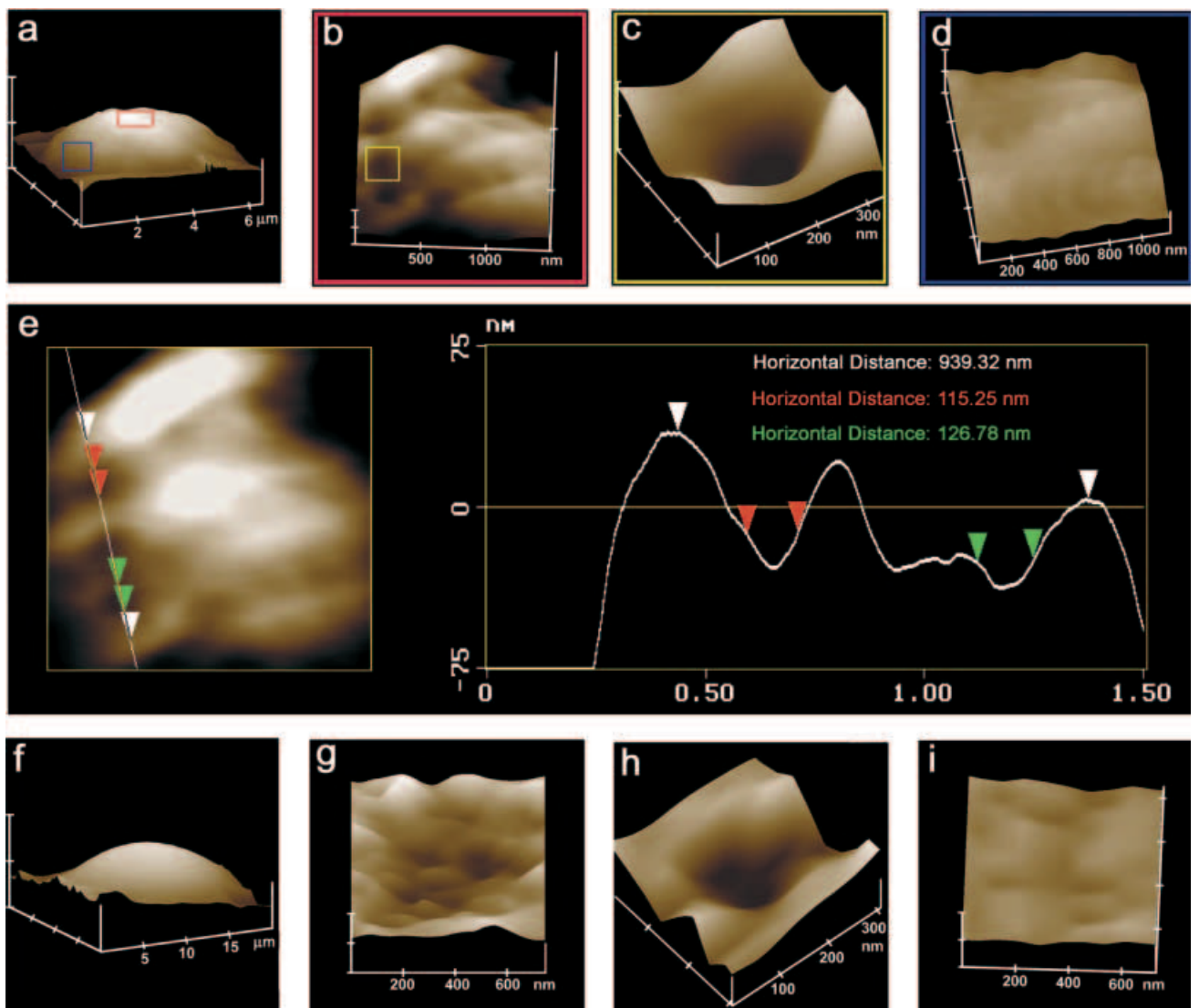
## RESULTS

#### *High-resolution images of live pancreatic acinar cells using the AFM*

To obtain high-resolution AFM images of live pancreatic acinar cells, and of pits and depressions at the apical plasma membrane in these cells, care and caution was exercised in their isolation, and tight adhesion to Cell-Tak-coated glass coverslips. Isolated cells were first observed using light microscopy (Fig. 1), prior to examination using the AFM (Fig. 2). Images of whole live cells in



**Fig. 1.** Light and electron micrographs of isolated pancreatic acinar cells (a–c). Note the presence of zymogen granules (ZG) at the apical end (AP) of the cell, and the nucleus (N) at the basolateral end. A DAPI-stained nucleus is shown in b.



**Fig. 2.** Atomic Force Microscopy (AFM) performed on live and fixed pancreatic acinar cells demonstrate the presence of fusion pores (depressions) at the apical plasma membrane. AFM micrograph of a live pancreatic acinar cell (a). High resolution AFM image of the rectangular area enclosed by red lines at the apical end of the cell, demonstrates the presence of pits and depressions (b). Further magnification of one of the depressions (yellow square), is shown in (c). The basolateral end of the live cell (purple rectangle, in (a) when imaged at high resolution (d), shows little structure. No pits and depressions are seen at the basolateral end of the cell. Figure (e) is a zoom in of (b), with section analysis demonstrating the size of two depressions. The bottom panel f–i, are similar structures in fixed cells, demonstrating fixation protocol is able to retain pit and depression structures.

physiological buffer obtained using light, fluorescent, and AFM provide orientation and identification of their apical and basolateral aspects. Following imaging by AFM, pancreatic acinar cells were stained using DAPI (4'-6-diamino-2-phenylindole-2 HCl) to confirm orientation of the nucleus, and subsequently with trypan blue to assess cell viability. Trypan blue staining confirmed cells to be intact and viable following AFM studies (not shown).

Light and fluorescent microscopic examination of isolated pancreatic acinar cells attached firmly to Cell-Tak-coated glass coverslips, reveal the presence of a prominent nucleus at the basolateral end of the cell (Fig. 1a,b). Isolated pancreatic acinar cells were found to vary in size from 7 to 22  $\mu\text{m}$ . Electron micrographs of the pancreatic acinar cells (Fig. 1c) demonstrate the presence of electron-dense ZG, the membrane-bound secretory vesicles located primarily at the apical end of the cell, where they dock and fuse at the apical plasma membrane to release vesicular contents during exocytosis. Furthermore, live acinar cells in physiological buffer that were lying on their sides and were firmly attached to Cell-Tak-coated glass coverslips could be imaged by the AFM using higher force, revealing the apical location of ZG of various sizes. To confirm that apically-located structures were ZG, the size-distribution of isolated ZG in near physiological buffer were imaged by the AFM. The purity of isolated ZG preparation was determined by electron microscopy. These studies reveal the presence of ZG, 200 to 1200 nm in diameter, in rat pancreatic acinar cells, as previously reported (Jena *et al.*, 1997), confirming structures at one end (the apical end) of acinar cells to be ZG.

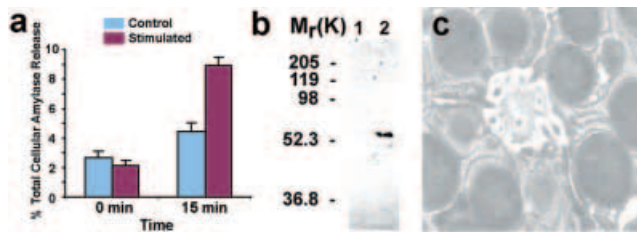
AFM studies on both live (Fig. 2a) and fixed (Fig. 2f) pancreatic acinar cells in physiological buffer revealed the presence of pits, 0.5–1  $\mu\text{m}$  in diameter (Fig. 2b,g) and  $\sim$ 125–185 nm in diameter and 19–25 nm in depth depressions (Fig. 2c,h). No such structures were found at the basolateral surface of the cell (Fig. 2d,i). The size of depressions was similar in pancreatic acinar cells of different sizes. These studies reveal for the first time the structure of pits and depressions in much greater detail. To determine the role of pits and depressions in pancreatic acinar cells, the structure and dynamics of depressions in live acinar cells in physiological buffer, were imaged by the AFM before, during, and after stimulation of secretion. One of the major contents in ZG is the starch digesting enzyme amylase. Following stimulation of secretion, the percent of total amylase released from pancreatic acinar cells can be biochemically

estimated (Fig. 3) and the percent exocytosed of ZG contents calculated. Stimulation of live pancreatic acinar cells with 20  $\mu\text{M}$  of the secretagogue mastoparan, resulted in secretion. As previously shown, dilation of depressions following stimulation of secretion is confirmed from these studies (Fig. 4a,b,d). AFM section analysis of depression diameter demonstrates that following stimulation of secretion, the diameter of depressions increases by 25% from  $185.54 \pm 8.38$  nm to  $231.34 \pm 13.35$  nm ( $P < 0.001$ ). Similarly, the relative depth of depressions increased from  $19.16 \pm 1.54$  nm to  $23.56 \pm 2.67$  nm following stimulation of secretion. No change in the size of pits is observed (data not shown) following stimulation of secretion, as previously reported (Schneider *et al.*, 1997).

To identify amylase-release sites at the acinar cell surface, an antibody specific to  $\alpha$ -amylase was used. This antibody was selected due to its high specificity for rat pancreatic amylase, as assessed using immunoblot analysis and immunoelectron microscopy (Fig. 3b,c). Using this amylase-specific antibody, immunoblot assay of 1  $\mu\text{g}$  of total pancreatic homogenate and purified ZG proteins respectively, resolved using SDS-PAGE, demonstrates an enrichment of the 54 kDa amylase immunoreactive band in the ZG fraction (Fig. 3b). Immunoelectron microscopy on pancreatic acinar cells using the  $\alpha$ -amylase-specific antibody, further confirms immunolocalization of stored amylase within ZG (Fig. 3c). Immunogold was found localized primarily in ZG, with little labeling elsewhere. The  $\alpha$ -amylase specific antibody in combination with 30 nm gold-conjugated secondary, was therefore chosen to be used in the detection of amylase release at the cell surface of live as well as fixed pancreatic acini using the AFM.

#### *Localization of amylase-specific immunogold at pits and depressions in live pancreatic acinar cells*

For AFM studies, live acinar cells were stimulated with the secretagogue mastoparan, followed by exposure to the amylase-specific antibody and protein A-gold. These AFM studies performed in near physiological buffer on live cells, reveal specific localization of immunogold at pits and depressions (Fig. 4c). High-resolution images of single pits with their depressions in live pancreatic acinar cells at resting, following stimulation, and subsequent exposure to the  $\alpha$ -amylase antibody and protein A-conjugated 30 nm gold, reveal the dilation of depressions following stimulation of secretion (Fig. 4b), and immunogold decorating the margins of

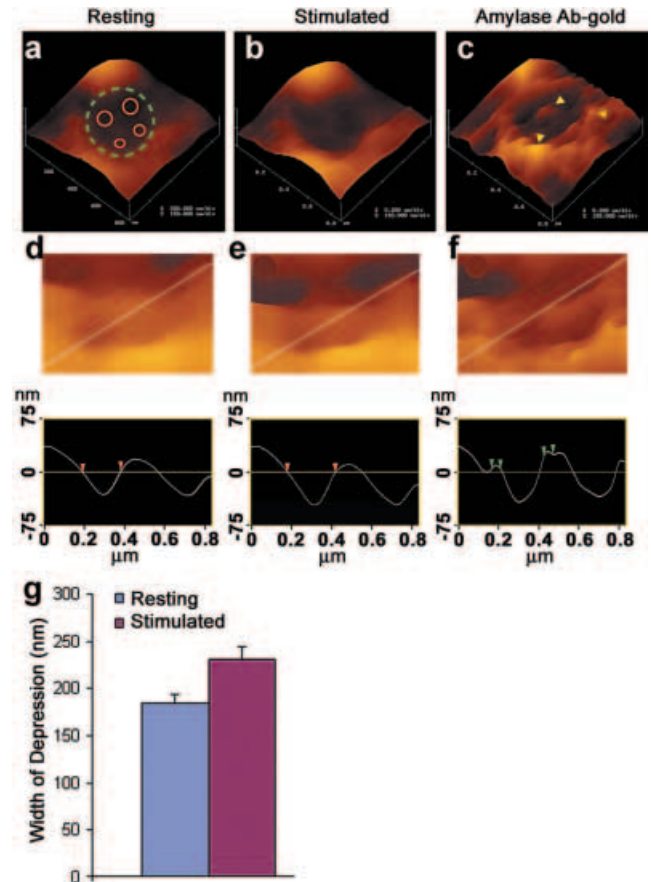


**Fig. 3.** Mastoparan stimulated amylase release from isolated pancreatic acinar cells. An amylase specific antibody recognizes amylase in zymogen granules. Stimulation of isolated pancreatic acinar cells at room temperature for 15 min using 20  $\mu$ M mastoparan, results in a significant increase in amylase release (a). One microgram of protein each taken from total pancreatic homogenates (b1) and from isolated zymogen granules or ZG (b2), when resolved using SDS-PAGE and electro-transferred to nitrocellulose, followed by immunoblot analysis using the  $\alpha$ -amylase-specific antibody, results in detection of a single band (54 kDa) in the ZG fraction (b2). Immunoelectron microscopy using the  $\alpha$ -amylase-specific antibody and a secondary gold, demonstrates specific labeling of ZGb.

pits and depressions (Fig. 4d). This was clearly revealed by section analysis of the structure (Fig. 4, middle and lower panels). In several studies, immunogold was also detected within pits and depressions, demonstrating amylase at these sites. Little gold is found decorating other areas of the acinar cell surface. Since depressions were the only structures that dilated following stimulation of secretion (Schneider *et al.*, 1997), the presence of amylase at depressions following stimulation of secretion demonstrate for the first time that these sites are the fusion pores at the cell plasma membrane of pancreatic acinar cells, where membrane-bound secretory vesicles dock and fuse to release vesicular contents to the cell exterior.

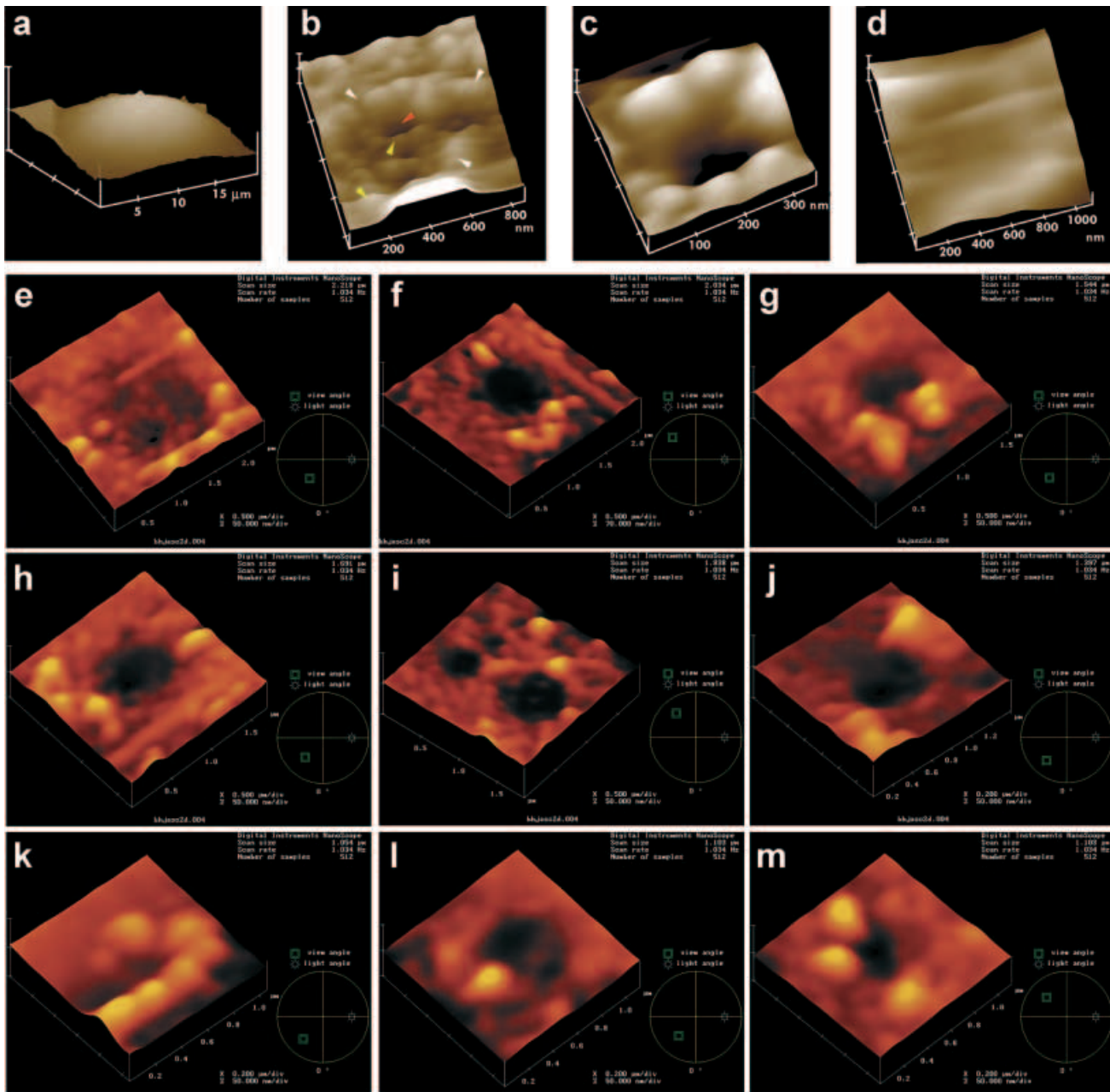
#### *Localization of amylase-specific immunogold at pits and depressions in fixed pancreatic acinar cells*

Fixation of biological samples using aldehydes, is known to result in decreased elasticity and increased sample hardness (Wu *et al.*, 1998; Chen and Moy, 2000; and unpublished observations). Since AFM uses force spectroscopy to image objects, harder and less elastic samples are better resolved. After testing various fixatives, it was found that 1 h treatment of ice-cold 2.5% paraformaldehyde in phosphate-buffered saline, pH 7.5, was best in retaining the structural integrity of pits and depressions. No detectable changes were observed in live cells following fixation. Therefore, the above fixation recipe was chosen in this part of the study. To obtain high-resolution images of



**Fig. 4.** Depressions dilate to allow the expulsion of vesicular contents. Three-dimensional AFM micrographs of pits (a,d) (green broken circle) and depression (red circles), show an enlargement of depressions (b, e) and localization of amylase-specific immunogold (c, f) (yellow arrowhead) at pits and depressions in live pancreatic acinar cells, following stimulation of secretion. Exposure of stimulated cells to amylase-specific antibody followed by 30 nm gold-protein A, results in localization of gold at the edges of depressions, demonstrating the release of amylase at these sites. Section analysis (middle and lower panel) depicting depressions within pits (d) enlarge, (e) to allow release vesicular contents to the cell exterior, as depicted by amylase-specific immunogold localization at pits and depressions (f). Note the increase in width of 'depressions' following stimulation of secretion (g).

the cell surface and associated structures and to determine the distribution of amylase-specific immunogold labeling, stimulated acinar cells were fixed following immunogold labeling procedures as described (Materials and Methods). In conformation with our observation on live acinar cells, high resolution AFM images of the immunolabeled fixed cells further demonstrate the specific localization of immunogold at depressions (Fig. 5), implicating them to be secretory sites at the cell plasma membrane.

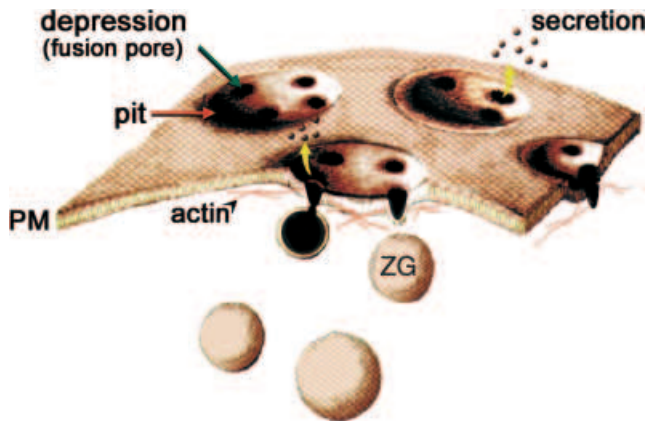


**Fig. 5.** AFM micrographs of fixed cells demonstrating amylase-specific immunogold localization at pits and depressions. Following stimulation of secretion, the acinar cell was exposed to amylase-specific antibody followed by 30 nm gold-protein A, and fixed (a). Note gold decorating pit and depressions at the apical end of the cell (b) (yellow arrowheads). A high magnification of one depression is shown in (c), with gold decorating its edges. Similar to live acinar cells, little morphology is seen at the basolateral end of the cell (d), and no gold is localized at this end. Several AFM micrographs of pits and the depressions from a number of experiments depict the specific immunolocalization of amylase-specific immunogold (yellow spots) (c–m).

**DISCUSSION**

Our earlier studies using the AFM revealed a new group of plasma membrane structures in live pancreatic acinar cells, involved in exocytosis (Schneider *et al.*, 1997). In the 1997 study, using isolated pancreatic acinar cells plated on Cell-Tak

coated mica, the surface topology of the plasma membrane before, during and after stimulation of secretion in live acinar cells, was obtained using the AFM. In resting acinar cells, pits measuring 0.5–2 μm and containing typically 3–4 depressions measuring 100–180 nm in diameter were identified only at the apical region of these cell where



**Fig. 6.** Schematic diagram depicting pits and depression at the cell plasma membrane, where secretory vesicles dock and transiently fuse to release vesicular contents.

membrane-bound secretory vesicles were known to dock and fuse. Following stimulation of secretion, dynamic size changes occur only in depressions. No change in pits or the topology of the plasma membrane surface at the basolateral end of the cell were detected. Following stimulation of secretion, a 35% increase in depression diameter was observed, that correlated with an increase in measured enzyme release. Thirty minutes following stimulation of secretion, a 20% decrease in depression size and no further increase in enzyme secretion was observed. Exposure of acinar cells to cytochalasin B, a fungal toxin that inhibits actin polymerization, results in a 50–60% loss of stimutable amylase secretion. A significant decrease in depression diameter was also observed following treatment of acinar cells with cytochalasin B. These earlier studies outlined above, enabled us to identify for the first time the presence of a new group of plasma membrane structures at ultra high resolution in live pancreatic acinar cells and suggested their dynamic involvement in exocytosis. The present study demonstrates the release of vesicular contents at pits and depressions, confirming depressions to be the fusion pores. Since zymogen granules range in size from 200 to 1200 nm in diameter (Jena *et al.*, 1997), the total incorporation of a secretory granule membrane at a 'depression' would distend the structure much more than what is observed in the present and earlier published studies (Schneider *et al.*, 1997). It can be concluded, therefore, that ZG in pancreatic acinar cells dock and fuse not

completely but transiently at depressions, to release vesicular contents to the cell exterior. The involvement of depressions as transient docking and fusion stations at the cell plasma membrane, for membrane-bound secretory vesicles, is schematically depicted in Figure 6.

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