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Structure and Dynamics of the Fusion Pores in Live GH-secreting Cells Revealed Using Atomic Force Microscopy

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ABSTRACT

Earlier studies in live pancreatic acinar cells identified new cellular structures at the cell plasma membrane called 'pits' and 'depressions', where membrane-bound secretory vesicles dock and fuse to release vesicular contents. In the current study, using atomic force microscopy we identify similar structures at the plasma membrane of GH-secreting cells of the pituitary and implicate their involvement in hormone release. Pits containing 100-200 nm in diameter depressions or fusion pores were identified in resting GH-secreting cells. Following stimulation of secretion the size of depression enlarged and gold-tagged GH antibody were found to bind to the pit structures in the stimulated GH cells. This study documents for the first time the presence of these structures and their involvement in secretions in a neuroendocrine cell.

Introduction

Until recently (1 and unpublished data), our understanding of secretory vesicle fusion at the cell plasma membrane was obtained from morphological (2-5), electrophysiological (6-11), and biochemical studies (2, 12-15) all suggesting the presence of 'fusion pores' at the cell plasma membrane. Using atomic force microscopy (AFM), the structure and dynamics of the 'fusion pore' were first revealed and examined in live pancreatic acinar cells (1 and unpublished data). In live resting pancreatic acinar cells, 'pits' measuring 0.5-2 μm and containing 3-20 'depressions' of 100-180 nm diameter were identified only at the apical region of these cells where membrane-bound secretory vesicles are known to dock and fuse. Following stimulation of secretion, only 'depression' enlarged and returned to resting size following completion of secretion. Exposure of acinar cells to cytochalasin B, a fungal toxin that inhibits actin polymerization, resulted in a 50-60% loss of stimulable amylase secretion. A significant decrease in depression diameter was also observed in acinar cells exposed to the fungal toxin. To determine if similar structures are present in neuroendocrine cells, the GH-secreting cell of the pig pituitary was studied using AFM. Results from this study demonstrate the presence of pits and depressions in GH-secreting cells of the pituitary and their involvement in hormone release.

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Material and Methods

Experimental animals

Yorkshire pigs, raised at the Iowa State University Animal Nutrition Farm, were used for these experiments. Newborn pigs, 1-8 days of age, were killed with electricity and decapitated. Pituitary glands were immediately removed and collected in cold sterile EBSS solution (4 °C). Posterior lobes were discarded and anterior lobes were transferred to a sterile cold (4 °C) MEM-0.1% BSA medium. Animal care and experimental protocols were in accordance with the guidelines and approval of the Iowa State University Committee on Animal Care.

Isolation and stimulation of GH-secreting cells

The protocol used for establishing primary cell cultures from pig anterior pituitary gland is a modification of procedure used for establishing neuronal cultures (16). In brief, tissue was incubated with 2.5% papain solution for 40 min at 37 °C. After the incubation, tissue was mechanically dispersed in a presence of 1 ml of culture medium by triturating through a 1-ml fire-polished glass pipette 8-10 times and plated on the poly-L-lysine (1 mg/ml, MW 100,000; Sigma) coated dishes. Cells were incubated overnight at 37 °C in a humidified 5% CO_2/95% air atmosphere to allow them to adhere. Culture medium consisted of Eagle's MEM supplemented with 10% FBS and 40 mM glucose, 2 mM L-glutamine, 1 mM pyruvate, 14 mM sodium bicarbonate and penicillin/streptomycin. Cells in PBS, were stimulated with 20 μM of the GH secretagogue L-692,585.

Assay of GH-secretion using Reverse Hemolytic Plaque Assay

Reverse Hemolytic Plaque Assays (RHPA) were performed using a minor modification of the method of (17). Staphylococcus protein A was coupled to ovine red blood cells using 0.1% $CrCl_3$. Isolated pituitary cells were incubated for 48 h to allow cells to acclimate. Cells were then treated with trypsin (2.5 mg trypsin in 10 ml MEM-0.1% BSA) to detach the cells from the culture dish. After gentle trituration using a fire-polished glass pipette, cells were separated by centrifugation (1500 × *g* for 10 min), washed twice in MEM-0.1% BSA. A cell concentration of 2.5 x 10^5 cells/ml was used in RHPA experiments. A 1:1 pituitary cell to 50% protein A-labeled erythrocyte solution were mixed, and infused into Cunningham slide chambers. The chambers with the cell infusion were preincubated for 50 min at 37 °C in 5% CO_2-95% air. Slides were pretreated with poly-L-lysine (0.1 mg/ml; MW 100,000), to ensure cell adhesion. After incubation, the chambers were rinsed with DMEM-0.1% BSA, followed by addition of anti porcine GH raised in monkey (1:60 dilution in DMEM-0.1% BSA), in the presence and absence of 100 nM L-692,585. After 3 h incubation, plaque formation was initiated by infusing guinea pig complement (1:40 in DMEM-0.1% BSA). The reaction was terminated after 50 min by infusion of 1% glutaraldehyde. The secretion index is represented by the size of the plaque. These isolated cells were used in our AFM studies.

Atomic Force Microscopy

'Pits' and 'depressions' at the plasma membrane in live and fixed GH secreting cells (n = 24) in PBS pH 7.5, were imaged by the AFM (Bioscope III, Digital Instruments) 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 $N\,m^{-1}$, and an imaging force of <400 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.

Immunogold AFM

Following stimulation of secretion using 20 μM L-692,585, live GH secreting cells of the pituitary were exposed to 1:200 dilution of Growth Hormone-specific antibody , and 30 nm gold conjugated secondary antibody, washed in PBS, prior to AFM imaging in PBS at room temperature.

Cell fixation and immunogold localization

Following stimulation of secretion using 20 μM L-692,585, live GH secreting 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 Growth Hormone-specific antibody, and 30 nm gold conjugated secondary antibody, washed in PBS, prior to AFM imaging in PBS at room temperature.

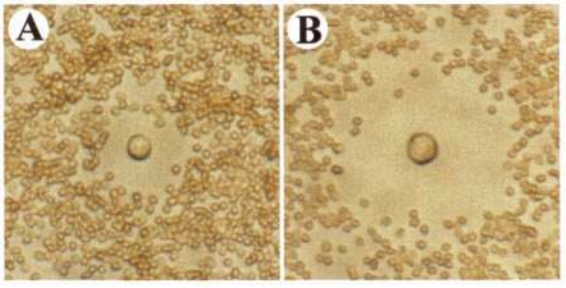

Fig. 1. Light microscopy revealing the extent of growth hormone release from isolated GH cells of the pituitary gland, in a typical Reverse Hemolytic Plaque Assay. Larger the plaque (clear area), more the release. Note the presence of a small plaque in a resting GH cell (A), compared to a L-692,585 stimulated cell (B).

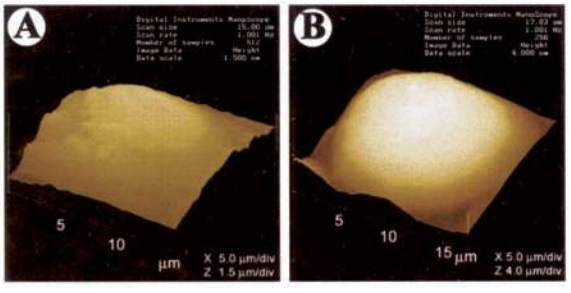

Fig. 2. Atomic Force Microscopy (AFM) performed on whole GH cells. AFM micrograph of a live (A) and fixed (B) GH secreting cells, imaged in fluid (PBS).

Results and Discussion

Reverse hemolytic plaque assay on isolated GH secreting cells of the pig pituitary demonstrated GH release following exposure to L-692,585 (Fig. 1). Examination of resting and stimulated GH cells demonstrates no detectable changes following fixation (Fig. 2). Examination of resting GH cells revealed the presence of 'pits' and 'depressions' at the plasma membrane. Depressions in resting cells measure 154 ± 4.5 nm (mean ± SE). However, following exposure of GH cells to the secretagogue L-692,585, a 40% increase in the size (215 ± 4.6 nm; $P < 0.01$) of 'depressions' is demonstrated (Fig. 3, 4). When stimulated live cells were exposed to 30 nm-gold-tagged GH-antibody, gold particles were found to decorate 'pit' and 'depression' structures (Fig. 3). Aldehydes fixation of biological samples is known to result in decreased elasticity and increased hardness (18, 19 and *personal observation*). Since force spectroscopy is used to image objects using the AFM, less elastic samples are better resolved. From studies using that pancreatic

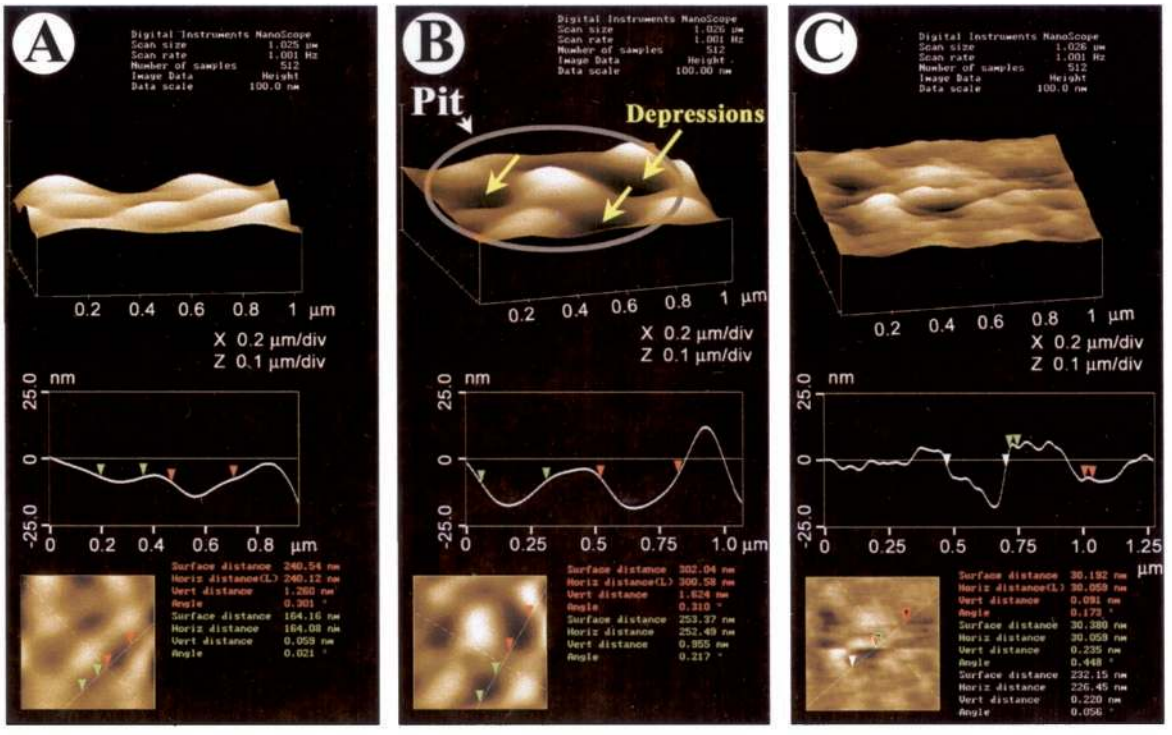

Fig. 3. High resolution Atomic Force Microscopy (AFM) performed on live GH secreting cells, showing a single 'pit' with 3-4 'depressions' (*top panel*). The scan line in the *middle panel* depicts the relative height and depth between arrows, and the *bottom panel* is section analysis of two 'depressions' showing their relative depth and diameter. (A) in the resting cell and (B) when the same cell is GH-secretagogue stimulated, and (C) following exposure to 30 nm-gold-tagged GH-antibody following the stimulation. Note the enlargement of the 'depressions', following stimulation of secretion [(A) compared with (B)] and decoration of the 30 nm-gold-tagged GH-antibody (C) at the edges and one within a 'depressions'.

acinar cells (unpublished), it was determined 1 h treatment of ice-cold 2.5% paraformaldehyde in phosphate buffered saline, pH 7.5, was ideal in retaining the structural integrity of 'pits' and 'depressions'. No detectable changes were identified in live cells following fixation. To obtain high resolution images of the cells, and the 'pits' and 'depressions' and to determine the distribution of 30 nm GH-immunogold labeling, stimulated GH cells were fixed following immunogold labeling. In conformation with our observation in live GH cells, AFM images of the immunolabeled fixed cells, demonstrate specific localization of immunogold at 'depressions' (Fig. 4), implicating them to be secretory sites at the cell plasma membrane. In agreement with earlier studies in pancreatic acinar cells, the GH-secreting neuroendocrine cells of the pituitary demonstrate the presence of 'pits' and 'depressions' at the plasma membrane, where secretory vesicles dock and fuse to release vesicular contents. The presence of 'pits' and 'depressions' in both exocrine and neuroendocrine cells suggests that these structures may be universal to secretory cells, where exocytosis occurs (Fig. 5).

Acknowledgments

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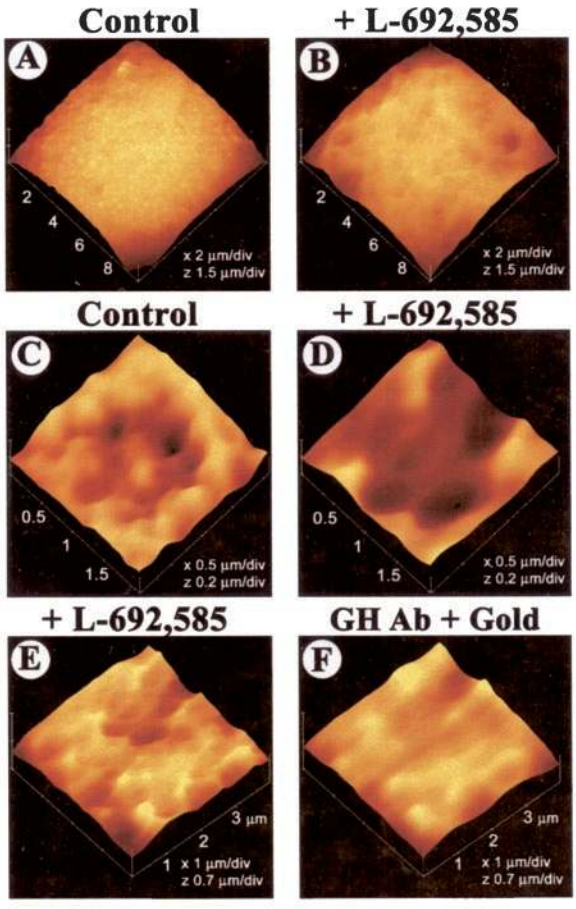

Fig. 4. High resolution Atomic Force Microscopy (AFM) performed on resting and stimulated GH secreting cells, after fixation. Note a large area of a cell surface in resting (A) and in a stimulated (B) cell. AFM micrographs of a 'pit' with 'depressions' in resting cell (C), again clearly demonstrating the enlargement of 'depressions' or fusion pores following stimulation of secretion (D). Exposure of 'pits' in a stimulated cell (E) to 30 nm gold-tagged GH-antibody, results in binding of released growth hormone at the site to 30 nm gold-tagged GH-antibody (F). Note the loss of fusion pores due to large amounts of gold-tagged antibody binding at these sites.

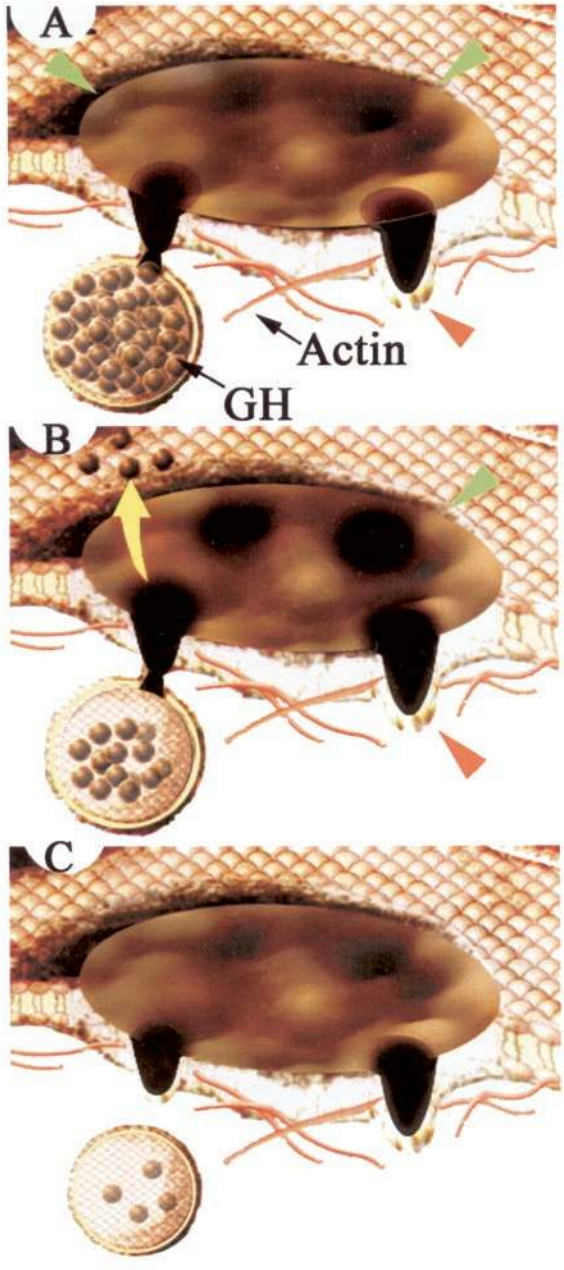

Fig. 5. Schematic diagrams depict cross sectional views at cell plasma lipid membrane of a 'pit' (green arrow head) and 'depressions' (red arrow head) as well as a vesicle which contains hormone (GH), within the cell cytoplasm. Immediately following GH-secretagogue stimulation, the secretory vesicle containing hormone docks and fuses with the 'depression' (A) and then releases hormone (B) through the fusion pores of the 'depression' (yellow arrow). After hormone release from the vesicle (C), the 'depression' becomes smaller and the vesicle returns to the cellular cytoplasm.

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