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Secretory vesicles in live cells are not free-floating but tethered to filamentous structures: A study using photonic force microscopy

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Abstract

It is well established that actin and microtubule cytoskeletal systems are involved in organelle transport and membrane trafficking in cells. This is also true for the transport of secretory vesicles in neuroendocrine cells and neurons. It was however unclear whether secretory vesicles remain free-floating, only to associate with such cytoskeletal systems when needing transport. This hypothesis was tested using live pancreatic acinar cells in physiological buffer solutions, using the photonic force microscope (PFM). When membrane-bound secretory vesicles (0.2–1.2 μm in diameter) in live pancreatic acinar cells were trapped at the laser focus of the PFM and pulled, they were all found tethered to filamentous structures. Mild exposure of cells to nocodazole and cytochalasin B, disrupts the tether. Immunoblot analysis of isolated secretory vesicles, further demonstrated the association of actin, myosin V, and kinesin. These studies demonstrate for the first time that secretory vesicles in live pancreatic acinar cells are tethered and not free-floating, suggesting that following vesicle biogenesis, they are placed on their own railroad track, ready to be transported to their final destination within the cell when required. This makes sense, since precision and regulation are the hallmarks of all cellular process, and therefore would hold true for the transport and localization of subcellular organelles such as secretory vesicles.

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Keywords: Cell secretion; Photonic force microscopy; Live secretory cells; Secretory vesicle tether

1. Introduction

It has become increasingly clear that the movement of organelles in cells can be attributed to two groups of motile systems, one based on microtubules, and the other based on actin. Microtubules have been recognized as the railroad for movement of organelles over long distances within the cell (>1 μm), where as the actin system is responsible for transport over shorter distances, typically from tens to a few hundred nanometers. Thus, microtubule-dependent motors such as kinesin and kinesin-related proteins, and the superfamily of actin-dependent myosin motors, have all been implicated in intracellular organelle transport [1,2]. Myosin motors include the conventional myosin (myosin II) and a large group of unconventional myosins (myosin I, III, V, and VI). In recent years, the prime candidate for secretory vesicle transport in cells has been reported to be the class V of myosin motors [3–5]. Myosin V is composed of two heavy chains that dimerise via a coiled-coil motif, located in the stalk region of the heavy chain [6]. The heavy chain contains an amino-terminal actin-binding motor domain [6], followed by a neck region where up to six regulatory light chains can bind. The carboxy-terminus globular domain of the heavy chain is thought to mediate organelle-binding specificity [7]. Interaction between the actin and the microtubule transport system, seems to be a requirement for the correct delivery of intracellular cargo such as secretory vesicles [8–10].

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Although some information is available on the transport system of secretory vesicles, little is understood about the timing of association of these vesicles with the transport system. The present study was undertaken to determine whether secretory vesicles in live cells remain free-floating, only to associate with the transport systems following a secretory stimulus, or whether they are always tethered. Isolated live pancreatic acinar cells were used in the present study. Our results demonstrate that all secretory vesicles within live pancreatic acinar cells are tethered and not free-floating. Nocodazole and cytochalasin B disrupt much of this tether. Immunoblot analysis of isolated secretory vesicles, further determines the association of actin, myosin V, and kinesin to them. These studies demonstrate for the first time that secretory vesicles in live pancreatic acinar cells are tethered and not free-floating, suggesting that following vesicle biogenesis, they are placed on their own specific railroad track, ready to be transported to their final destination when required. This makes sense, since precision and regulation are the hallmark of all cellular process, and therefore would also hold true for the transport and localization of subcellular organelles within the cell.

2. Materials and methods

2.1. Isolation of pancreatic acinar cells

Isolation and preparation of acinar cells for photonic force microscopy (PFM) were performed using minor modifications of a published procedure [11]. 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 0.5-mm^3 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 $CaCl_2$, 1.2 mM $MgCl_2$, 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 μm Spectra-Mesh (Spectrum Laboratory Products, Saint Paul, MN, USA) 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, USA) glass cover slips or mica An hour after plating, cells were used in our studies, before and after exposure to 10 μM nocodazole and 20 μM cytochalasin B for 1 min, followed by six washes (10 vol/wash) in buffer A at room temperature (RT).

2.2. Isolation of Zymogen granules

Zymogen granules (ZG) were isolated according to a minor modification of a published procedure [12]. The pancreas from male Sprague–Dawley rats was dissected and diced into 0.5-mm^3 pieces before being suspended in 15% (wt/vol) ice-cold homogenization buffer (0.3 M sucrose, 25 mM Hepes, pH 6.5, 1 mM benzamidine, 0.01% soybean trypsin inhibitor) and homogenized using 3 strokes of a Teflon-glass homogenizer. The homogenate was centrifuged for 5 min at $300 \times g$ at 4 °C. The supernatant fraction was mixed with 2 vol of a Percoll–Sucrose–Hepes buffer (0.3 M sucrose, 25 mM Hepes, pH 6.5, 86% Percoll, 0.01% soybean trypsin inhibitor) and centrifuged for 30 min at $16{,}400 \times g$ at 4 °C. Pure ZGs were obtained as a loose white pellet at the bottom of the centrifuge tube, and used in the study.

2.3. Isolation of synaptic vesicles

Synaptic vesicles (SV) were prepared from rat brains using minor modification of published procedure [13,14]. Whole rat brain from Sprague–Dawley rats (100–150 g) were isolated and placed in ice-cold buffered sucrose solution (5 mM Hepes pH 7.4, 0.32 M sucrose) supplemented with protease inhibitor cocktail (Sigma, St. Louis, MO) and homogenized using Teflon-glass homogenizer (8–10 strokes). The total homogenate was centrifuged for 3 min at $2500 \times g$. The supernatant fraction was further centrifuged for 15 min at $14{,}500 \times g$, and the resultant pellet was resuspended in buffered sucrose solution, which was loaded onto 3–10–23% Percoll gradients. After centrifugation at $28{,}000 \times g$ for 6 min, the enriched synaptosomal fraction was collected at the 10–23% Percoll gradient interface. To isolate SV, the isolated synaptosomes were diluted with 9 vol of ice-cold H_20 (hypotonic lysis of synaptosomes to release SV) and immediately homogenized with three strokes in Dounce homogenizer, followed by a 30 min incubation on ice. The homogenate was centrifuged for 20 min at $25{,}500 \times g$, and the resultant supernatant (enriched SV preparation) were used in our studies.

2.4. Photonic force microscopy

In case of the PFM [15] the mechanical cantilever of the atomic force microscope (AFM) is replaced by the 3-D trapping potential of a laser focus. In the PFM, a nano- to micrometer-sized particle, such as latex, glass or metal bead is used as a tip. In the present study, however, 0.2–1.2 μm isolated secretory vesicles in buffer, or secretory vesicles within live pancreatic acinar cells, were trapped by the 3-D trapping potential of the laser focus of the PFM, and pulled in different directions. The difference in the refractive index between the medium and the trapped particle, the diameter of the particle, the laser intensity and the intensity profile in the focal volume, determine the strength of the trapping potential and in this way the forces that can be applied. The trapped vesicle can be pulled in various directions at forces of several tens of pN, and the movement can be observed using a video camera mounted on the microscope. Furthermore, the 3-D position of the trapped vesicle with respect to the laser focus can be

determined with a spatial resolution of about one nanometer, and a temporal resolution of 10 μs using a quadrant photodiode positioned at the back-focal plane of the microscope consider [16] allowing to determine the forces acting on the vesicle.

2.5. Immunoblot analysis

ZG from the exocrine pancreas, and SV from brain tissue, were isolated and used in this study. Actin and Myosin V antibodies were purchased from Sigma (St. Louis, MO), and Kinesin antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). For immunoblot analysis, solubilized ZG or SV proteins were resolved using 12.5% SDS-PAGE, followed by electro-transferred to nitrocellulose. The nitrocellulose membrane with transferred proteins were incubated for 45 min at 4 °C in blocking buffer (5% non-fat milk in PBS containing 0.1% Tween 20), and immunoblotted for 1 h at RT with the specific antibody in PBS-Tween (PBS containing 0.1% Tween 20) buffer. Resolved ZG proteins were probed using actin and kinesin antybodies, at a dilution of 1:400, and the myosin V at a dilution of 1:3,000 in PBS-Tween. Similarly, for resolved SV proteins, actin and Myosin V were used at a dilution of 1:1,000. The immunoblotted nitrocellulose sheets were washed three times in PBS-Tween, followed by incubation for 30 min at RT in horseradish peroxidase-conjugated secondary antibody (1:3,000) in PBS-Tween. The immunoblots were then washed three times in the PBS-Tween, processed for enhanced chemiluminescence and photographed using a Kodak Image Station 414.

3. Results and discussion

Secretory vesicles in isolated live pancreatic acinar cells in near physiological buffer were trapped using the 3-D trapping potential of the PFM laser focus. When attempts were made to move the vesicles in live cells by moving the laser trap of the PFM, they resisted movement in all directions. However, when the same live cells were exposed to 10 μM nocodazole and 20 μM cytochalasin B for 1 min at RT and washed, resulting in limited dissociation of microtubules and actin, vesicles trapped in the PFM laser focus could now be moved, for example, towards the plasma membrane of the cell, as depicted in Fig. 1. On close examination, a tether (red arrow head) appearing to originate at the supranuclear region of the cell (the Golgi complex), is found attached to the trapped vesicle (yellow arrow head) (Fig. 1). As previously reported in neurons and neuroendocrine cells [3–5], this study suggests that secretory vesicles in the exocrine pancreas are also transported on actin and microtubule systems to their destination at the cell plasma membrane following their biogenesis at the Golgi complex. On immunochemical examination of isolated secretory vesicles of the exocrine pancreas and neurons, actin, myosin V and kinesin, were found associated (Fig. 2), further confirming the involvement of both the actin and microtubule systems in intracellular transport of secretory vesicles in cells. Although, the presence of the motor proteins myosin and kinesin in secretory vesicles of the exocrine pancreas made sense, the presence of actin was puzzling. One possibility for the association of actin in such isolated secretory vesicles could be the broken-off remnant pieces of actin filaments associated with myosin V at the vesicle membrane, following cellular rupture and vesicle isolation. Alternately, actin may associate with secretory vesicles for

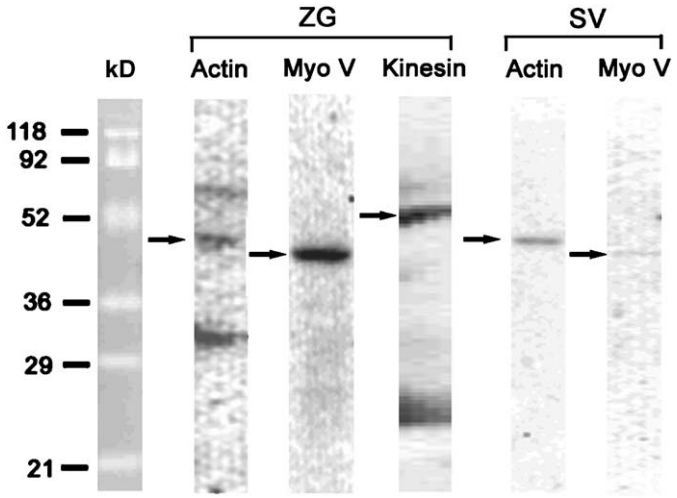

Fig. 2. Actin and microtubule motors are associated with secretory vesicles. Immunoblot analysis of zymogen granules (ZG) and synaptic vesicles (SV) demonstrate the presence of actin and myosin V. Kinesin, the microtubule motor, is also found in ZG.

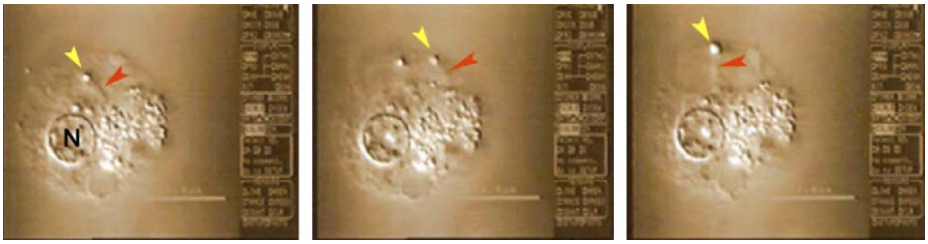

Fig. 1. Secretory vesicles (yellow arrowhead) are tethered and not free-floating in live pancreatic acinar cells. Note vesicle tether (red arrowhead) originating from the supranuclear region (above the nucleus, N) of the cell. Pretreatment of a live pancreatic acinar cells with nocodazole and cytochalasin, allows individual secretory vesicles to be pulled, when trapped in the laser focus of the PFM.

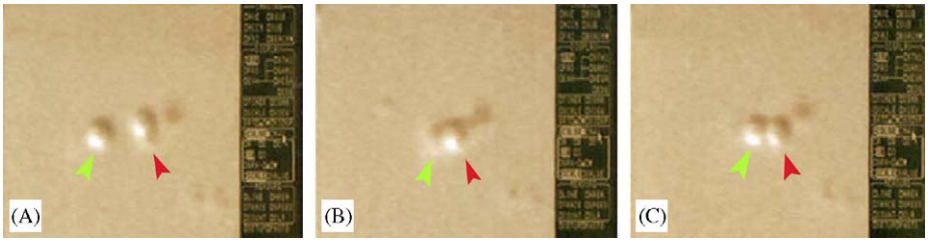

Fig. 3. Isolated secretory vesicles do not bind each other. (A) When an isolated ZG (red arrowhead) trapped at the PFM laser focus is brought in contact with a fixed isolated ZG (green arrowhead)using the maximum force possible with the PFM (B), they fail to exhibit any binding interaction and can be pulled apart easily (C).

other unknown functions, such as the interactions between secretory vesicles. To test this hypothesis, the interaction between isolated secretory vesicles was examined. When isolated secretory vesicle trapped at the PFM laser focus was pressed against another fixed secretory vesicle, no binding interactions were observed between them (Fig. 3). This study demonstrates that no detectable interactions exist between isolated vesicles, hence there is no interaction between the actin of one vesicle with the myosin of another. It is very likely that during isolation of secretory vesicles, actin filaments associated with myosin V at the vesicle membrane, break-off and remain associated with the vesicle via myosin V. These studies demonstrate for the first time that secretory vesicles in live cells are tethered and not free-floating, suggesting that following vesicle biogenesis, it is placed on its own railroad track, ready to be transported to its final destination within the cell when required. This makes sense, since precision and regulation are the hallmark of any cellular process, and therefore would hold true for the transport and localization of subcellular organelles in cells.

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