Cellular secretion studied by force microscopy

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Abstract

Using the optical microscope, real adventures in cellular research began in earnest in the latter half of the nineteenth century. With the development of the electron microscope, ultramicroscopy, and improved cell staining techniques, significant advances were made in defining intracellular structures at the nanometer level. The invention of force microscopy, the atomic force microscope (AFM) in the mid 1980s, and the photonic force microscope (PFM) in the mid 1990s, finally provided the opportunity to study live cellular structure-function at the nanometer level. Working with the AFM, dynamic cellular and subcellular events at the molecular level were captured in the mid 1990s, and a new cellular structure '*the porosome*' in the plasma membrane of all secretory cells has been defined, where specific docking and fusion of secretory vesicles occur. The molecular mechanism of fusion of the secretory vesicle membrane at the base of the porosome membrane in cells, and the regulated release of intravesicular contents through the porosome opening to the extracellular space, has been determined. These seminal discoveries provide for the first time a molecular mechanism of cell secretion, and the possibility to ameliorate secretory defects in disease states.

Introduction

Microscopy has played a pivotal role in both defining cellular structure and in elucidating dynamic cell processes. Historically, cellular microscopy had its origin in 1665 when Robert Hook, using a primitive optical microscope, described the cellular structure of cork. This was followed twenty years later when Antonie

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van Leeuwenhoek identified bacteria using a single lens optical instrument. However, it took improvements in optical microscopes and almost two hundred years before it became apparent that all plant and animal tissues were composed of cells. This led Matthias Schleiden and Theodor Schwann, in 1839, to propose the cell doctrine that proclaimed that all life forms are composed of one or more cells; cells arise from existing cells; and the cell comprises the smallest unit of life. Thus, modern cell biology was born.

The adventure into cellular research using the optical microscope began in earnest in the latter half of the nineteenth century. Since cells are colorless, a variety of stains were developed to enhance image contrast in the optical microscope. This allowed for the identification of different cell types and some intracellular structure including the nucleus, chromosomes, and Golgi. As optical microscopes became more sophisticated the spatial resolution of light microscopy, limited by the wavelength of visible light, was reached. This imaging plateau allowed for intracellular structure to be resolved only down to the level of mitochondria. In 1933, Ernst Ruska built the first transmission electron microscope that extended resolution beyond that obtainable by light microscopy. In 1986 the Nobel Prize in Physics was awarded to Ernst Ruska for his invention of the electron microscope. With the development of electron microscope technology, improved staining techniques, and the ultramicrotome for cutting thin sections of cells, significant advances were made in defining intracellular structure to the nanometer level. Cellular and nuclear membranes were identified along with the rough and smooth endoplasmic reticulum, ribosomes, zymogen granules, and the fine structure of mitochondria. Concurrently with the structural information gained through electron microscopy, the application of differential centrifugation [1, 2] and cell fractionation procedures [3] allowed cellular components to be isolated and functional information to be equated to structure. In 1974, the Nobel Prize in Physiology or Medicine was awarded to Albert Claude, Christian DeDuve, and George E. Palade for their discoveries concerning the structural and functional organization of the cell.

In the Palade Nobel Lecture "Intracellular Aspects of the Process of Protein Synthesis," published in 1975 [4], a great deal of the lecture was devoted to the synthesis and secretion of proteins by the pancreatic exocrine cell. The overall goal of research, that Palade and others at the Rockefeller Institute for Medical Research conducted, was to define the role that ribosomes, endoplasmic reticulum, Golgi complex, and other cellular components played in the synthesis of proteins and the secretory process that ended when proteins were expelled from the cell through the plasma membrane. Working with Lucien Caro, using electron microscopic autoradiography, and pulse labeled proteins, a good approximation of the route and timetable for the entire process of protein synthesis to the expulsion of proteins from the cell was determined [5]. Out of this work, and with subsequent work involving cell fractionation, electron microscopy, autoradiography, and other techniques, a model for the overall process for cell secretion was proposed that still stands today [4].

Cell secretion is a dynamic process that occurs in all living cells and involves transport of intracellular products to the exterior of cells. In order to address fundamental questions regarding dynamic processes, new methodology and instrumentation to record dynamic events would need to be used. One unanswered question concerned the association of the secretory vesicle with the plasma membrane. Early on it was established that secretory vesicles discharged their contents into the glandular lumina by a process originally called "membrane fusion" [6] and later called exocytosis [7]. In his Nobel lecture, Palade pointed out that exocytosis was probably a general mechanism which all cells used to discharge macromolecular products. He also observed that there was a high degree of specificity indicating that the process was not a random encounter of the secretory vesicle with the plasma membrane. He noted that the vesicles fuse only with the plasmalemma although there are other membranes proximal to the vesicles. More specifically, in exocrine cells vesicle fusion is limited to the apical or luminal domain of the plasmalemma. These observations led Palade to propose that there are likely complementary recognition sites in both the vesicle and the plasma membrane that are involved in binding prior to fusion [4]. Atomic force microscope (AFM), an instrument capable of imaging dynamic processes on cell surfaces, allowed identification of a new membrane structure, '*the porosome',* first in the apical plasma membrane of pancreatic acinar cells and subsequently in neurons, where secretory vesicles specifically dock and fuse [8]. We now know that Palade's prediction has been realized.

The porosome discovered

Transmission electron microscopic studies on a variety of cells in the 70s and 80s of 20th century have provided evidence that secretory vesicles fused with the plasma membrane [9–12]. After stimulation of secretion, high-resolution electron microscopy on freeze-fractured mast cells identified 100 nm depressions or pores in the plasma membrane. These pores make contact and fuse with the secretory vesicle effectively forming a channel connecting the interior of the vesicle with the extracellular space [10]. A similar study on freeze-fractured frog neuromuscular junctions, following nerve stimulation, showed the formation of 30–150 nm diameter holes at the plasma membrane [13]. Although at the time it was commonly believed that the secretory vesicle membrane after fusion was totally incorporated into the plasma membrane, this could not be demonstrated [10]. Instead, observations of the existence of empty or partially empty secretory vesicles after secretion would indicate a transient fusion of the vesicle with the plasma membrane and partial release of the vesicle contents. This observation was further supported by electro-physiological and patch clamp studies in mast cells showing that secretory granules transiently fused with the plasma membrane and release part of their contents [11, 12, 14–16]. Capacitance measurements on stimulated mast cells showed the expulsion of material from secretory vesicles that did not completely fuse with the plasma membrane [17]. These studies, combined with images obtained with the electron microscope, formed the basis for a new model where the fusion pore was formed by regulatory proteins to create a depression in the plasma membrane proximal to a secretory vesicle to form a 'hemi-fusion' intermediate. An aqueous channel between the secretory vesicle and the plasma membrane was formed following opening of the membranes at the point of fusion, allowing the contents of the vesicle to be expelled from the cell [18]. In 1997, using the atomic force microscope, the hypothetical fusion pore was identified, for the first time, as an entirely new cellular structure '*the porosome*' present at the apical cell plasma membrane of pancreatic acinar cells (Fig.1). The structure was described as circular
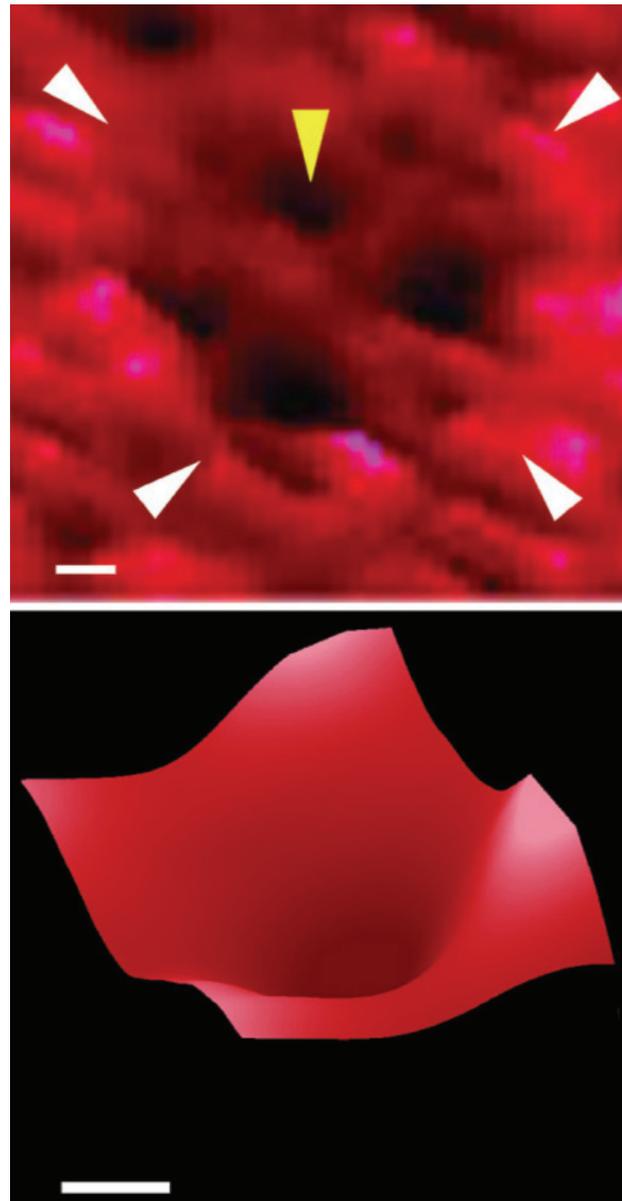

Fig. 1 An AFM image of a porosome on the apical plasma membrane of an isolated live rat exocrine pancreas cell. In the top image a pit (white arrowhead) containing four porosomes (yellow arrowhead) are identified. In the bottom image a high-resolution AFM image of an isolated porosome is shown [51]. Scale = 100 nm [51]. *Image courtesy of Dr. Bhanu P. Jena.*

pits measuring 400 to 1200 nm containing 3 or 4 depressions within each pit that measured 100 to 180 nm in diameter and 15–35 nm in depth [8]. The depressions were later named as 'porosomes' or fusion pores, and their structure, composition, and reconstitution determined [19–21].
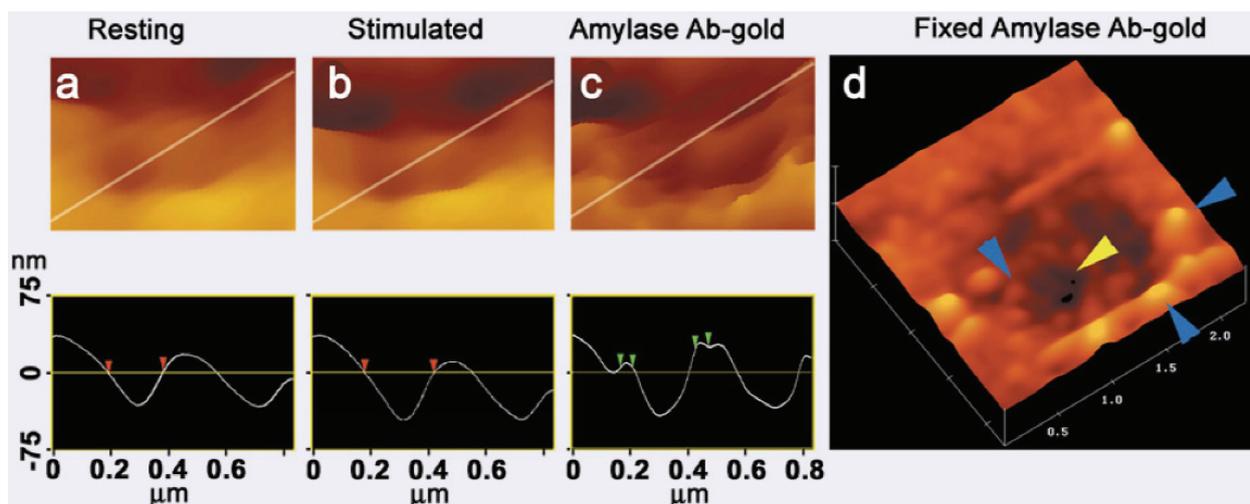
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Fig. 2 AFM images (**a** and **b**) show dilation of the porosome to allow expulsion of vesicular contents. Section analysis through one of the porosomes in (**a**) and (**b**) show enlargement of the porosome following stimulation of secretion. (**c**) Exposure of live pancreatic cells to gold conjugated-amylase antibody, results in specific localization of gold to the edge of the porosome. (**d**) AFM micrograph of a stimulated, and fixed, pancreatic cell showing a pit (yellow arrowhead) with immunogold localization of amylase specific antibody (blue arrowhead) associated with the porosome [19]. *AFM images courtesy of Dr. Bhanu P. Jena.*

Porosome structure, function and dynamics using AFM

Further experiments using AFM concentrated on defining the porosome as a specific organelle and showing how it allowed for the docking of secretory vesicles and the expulsion of their contents. Upon exposure of the pancreatic acinar cells to mastoparan or other secretagogues that stimulate amylase secretion, dynamic AFM image sequences showed that the diameter and depth of the depressions within the pits increased and returned to normal upon cessation of secretion [8, 19–21]. Conversely, when the cells were exposed to cytochalasin B, which inhibits actin polymerization, a significant loss in amylase secretion and a concurrent decrease in size of the depressions resulted [8]. This evidence strongly suggested that individual depressions were in fact porosomes where secretory vesicles could transiently dock and release their contents outside the cell [8, 19–21]. Porosomes have also been identified in AFM studies on chromaffin cells in the adrenal medulla [22], the GH-secreting cells of the pituitary gland [23], neurons, and mast cells [24, 25]. Confirmation that depressions were docking points for secretory vesicles came when gold-labeled antibody to amylase was imaged by AFM and found to selectively associate with depres-

sions (Fig. 2) following stimulation of secretion in pancreatic acinar cells [26]. A similar study in somatotrophs of the pituitary gland also found gold-labeled GH-specific antibody localized at depressions following stimulation of secretion [23]. Porosome is now considered a dynamic structure critically acting in cell secretion allowing membrane opening and expulsion of secretory vesicle content [27].

AFM imaging has further been used to determine the morphology of the porosome on the cytoplasmic side of the membrane in both neurons [25, 28] and in the exocrine pancreas [20, 28]. Isolated plasma membranes from pancreatic cells were immobilized on freshly cleaved mica and imaged in buffer (Fig. 3). Circular disks of 500 to 1000 nm were imaged, that corresponded to the depressions found on the apical cell surface. Within the disks were one or several inverted cup-shaped structures that were 10–15 nm in height. These structures were of the expected morphology on the cytoplasmic compartment of the depressions seen on the apical membrane. Frequently, zymogen granules (ZG) of 400 to 1000 nm in diameter were found associated with the cup shaped structures, providing further evidence that the circular disks were the cytoplasmic compartment of the pits observed on the apical membrane surface, and that the cup
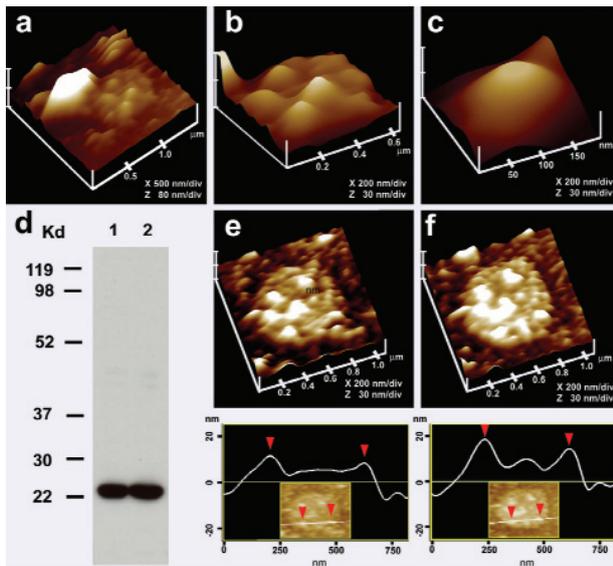
Fig. 3 The Morphology of the cytosolic side of the porosome in AFM studies on isolated pancreatic plasma membrane (PM) preparations. (**a**). AFM image of isolated PM reveals the cytosolic side of a pit with inverted cup-shaped structures, (porosome). Note the 600 nm diameter zymogen granule at the left hand corner of the pit. (**b**). Higher magnification of the same pit showing clearly the 4–5 porosomes on the cytosolic side of the membrane. (**c**). A single porosome imaged from the cytosolic side of the membrane. (**d**). Immunoblot analysis of 10 µg and 20 µg of pancreatic plasma membrane preparations, using SNAP-23 antibody, demonstrates a single 23 kDa immunoreactive band. The AFM image in (**e**) shows cytosolic side of the plasma membrane showing a pit with a number of porosomes. The same pit is shown in (**f**) following addition of the SNAP-23 antibody. Note the increase in height of the porosome cone base revealed by section analysis (bottom panel), demonstrating localization of SNAP-23 antibody at the base of the porosome [20]. *AFM images courtesy of Dr. Bhanu P. Jena.*

shaped structures were the cytoplasmic side of the fusion pores or porosomes [20, 28].

Membrane fusion has been observed to be highly specific for cellular processes, including enzyme release, hormone secretion, and neurotransmission. This implied that some inherent recognition event existed between the cell membrane and the vesicle. SNAP-25 and syntaxin (t-SNAREs) proteins associated with the plasma membrane and secretory vesicle-associated protein (v-SNAREs) have been identified as essential elements for vesicle fusion with the plasma membrane [29, 30]. As expected, t-SNARE

proteins were found at the base of the cup-shaped structures, demonstrating them to be porosomes [20].

Discovery of SNARE-induced membrane fusion by AFM

AFM studies on reconstituted lipid bilayers showed that ring structures formed when v-SNARE reconstituted lipid vesicles were exposed to t-SNARE reconstituted membranes supported on a mica surface [31]. Electrophysiological studies on t-SNARE reconstituted membrane further demonstrated that on addition of v-SNARE reconstituted lipid vesicles there was an increase in capacitance and conductance through the membrane. The ring structures did not form when v-SNARE protein alone was added to the t-SNARE lipid membrane, and an increase in conductance was not observed. These observations supported the notion that proteins must reside in separate opposing lipid bilayers for the formation of the t-/v-SNARE ring complex, leading to the establishment of continuity between opposing bilayers [31]. Ring structures ranging in size from 15 to 300 nm in diameter formed when t-SNARE reconstituted membranes were incubated with v-SNARE lipid vesicles. This was likely due to vesicles of different sizes since vesicle curvature would dictate ring size. By using extrusion filters of different sizes, homogeneous populations of both t-SNARE and v-SNARE liposomes were produced [32, 33]. Vesicles sizes of 40–50, 150–200, and 800–1000 nm were produced by different extrusion filters and examined by AFM. When small (40–50 nm) t-SNARE or v-SNARE vesicles were allowed to interact, ring structures of about 20 nm were imaged by AFM (Fig. 4). When the diameter of the t-/v-SNARE proteoliposomes was increased, a corresponding increase in the complex size was seen by AFM demonstrating a strong relationship between vesicle diameter and ring size [34]. In this reconstituted system, like the natural system, vesicles are soft and when fusion occurred the vesicles flattened and the contact area increased so that smaller vesicles produced smaller ring structures and larger vesicles produced larger ring structures. Deformation of the soft vesicles caused an increase in the contact
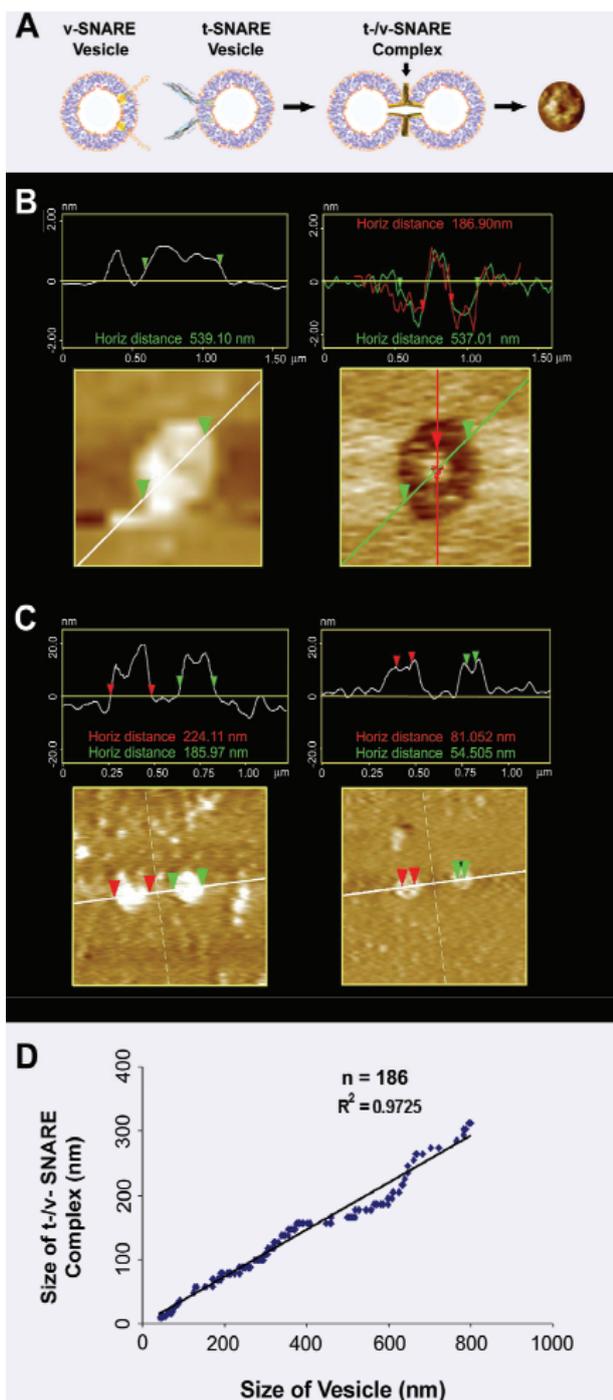
area between vesicles leading to an increase in the Gibbs free energy so that spontaneous fusion between opposing bilayers becomes less likely [35–37]. In principle, this could explain why slow secretory cells, like those of the exocrine pancreas, have large secretory vesicles for slower fusion and release of digestive enzymes while neurons that need fast fusion and fast release have smaller vesicles [34].

After fusion and the expulsion of their contents [38], secretory vesicles release from the cell membrane. Soluble N-ethylmaleimide-sensitive factor (NSF), an ATPase in the presence of ATP, has been implicated in the disassociation of the t-SNARE/v-SNARE complex [29, 39]. Disassembly of the t-/v-SNARE complex was investigated in a reconstituted system using t-SNARE and v-SNARE vesicles (ranging in size from 0.2 to 2 µm in diameter) and purified NSF. Right angle light scattering was used to monitor the association and disassociation of t-/v-SNARE reconstituted liposomes in solution both with and without NSF, and ATP being present [33]. After allowing for the formation of the t-/v-SNARE complex, the addition of ATP and NSF resulted in an increase in intensity of light scattering attributed to the dissociation of the SNARE complex. When NSF alone was added to the t-/v-SNARE complexes without ATP, or when ATP was added without NSF, no dissociation occurred. This suggested that dissociation is both an enzymatic and energy-driven process [40]. Using AFM dynamic studies, the disassembly of the t-/v-SNARE complex was monitored in real time. When assembled complexes were exposed to NSF and ATP, they were seen to disassemble confirming the results from right angle light scattering experiments (Fig. 5, 6). These studies confirmed that NSF and ATP were both required for the disassembly of t-/v-SNARE complexes [40].

Fig. 4 Reconstituted v-SNARE and t-SNAREs liposomes interact in a circular array to form conducting pores. The size of the SNARE complex is directly proportional to the size of the liposomes and the vesicle diameter. Schematic diagram showing the interaction of t-SNARE and v-SNARE reconstituted vesicles. At the extreme right, (**A**) is a single t-/v-SNARE complex imaged by AFM. In the (**B**) figure are AFM images of vesicles before and after removal by the AFM cantilever tip, exposing the t-/v-SNARE complex. In (**C**) Interacting t-SNARE and v-SNARE vesicles imaged by AFM at low (<200 pN) and high forces (300–500 pN). Note, that at low imaging forces, in the left image only the vesicle profile is imaged. However, at higher forces, in the right image the soft vesicle is flattened, allowing the SNARE complex to be imaged. In (**D**) a plot of vesicle diameter *vs*. SNARE complex size. Note the high correlation coefficient ($R^2 = 0.9725$) between vesicle diameter and the size of the SNARE complex [34]. *Figure courtesy of Dr. Bhanu P. Jena.*

AFM and PFM on secretory vesicle dynamics

AFM allowed scientists to elucidate some subtle steps in the molecular mechanism of cell secretion accompanied by vesicle swelling and its involvement in regulated expulsion of intravesicular contents [24, 28]. Therefore, it was demonstrated that the extent of vesicle swelling is directly proportional to the amount of intravesicular contents expelled. Similarly, it has been reasonably well established that the movement of organelles within cells is not random but directed by two groups of transport systems. Movement over long distances within cells has been attributed to the microtubule-kinesin and kinesin-related proteins, while actin-myosin systems are thought to be responsible for movement over shorter distances [41, 42]. The actin-class V myosin motors have been implicated as the primary movers of secretory vesicles [43–45]. However, evidence seems to require some interaction between the actin and microtubule transport systems for movement of organelles, including secretory vesicles within cells [46–48]. One of the questions that has recently been addressed concerns whether the secretory vesicles are free-floating and only become associated with the transport system after stimulation of secretion. Recent studies using the photonic force microscope (PFM) demonstrated that in live cells, secretory vesicles are not free floating but tethered [49]. The PFM, an instrument similar to the AFM, operates by using the trapping potential of a laser focus, instead of using a sharpened tip on the end of a micro-cantilever to sense the surface. A latex sphere, or other small bead, trapped in the laser beam is monitored in
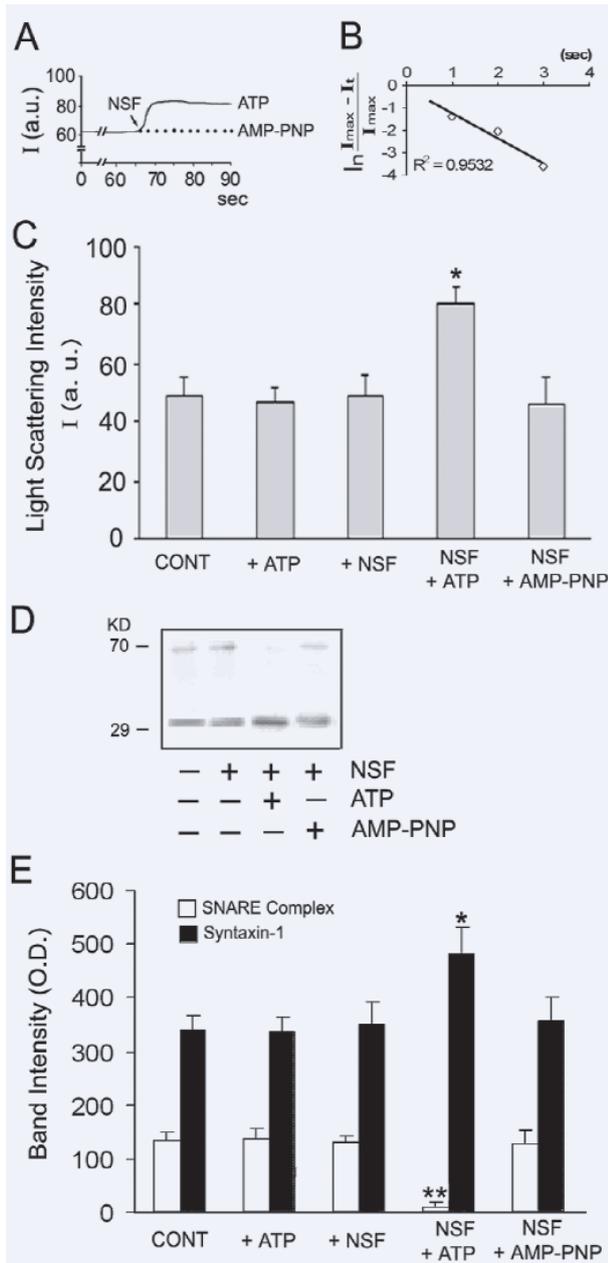
Fig. 5 The NSF-ATP induced dissociation of t-SNARE and v-SNARE associated liposomes. In (**A**) Real-time light scattering profiles of interacting t-SNARE and v-SNARE vesicles in solution in the presence and absence of NSF (depicted by arrow). In presence of ATP, NSF rapidly disassembles the SNARE complex and dissociates SNARE-vesicles represented as by a rapid increase in light scattering. No change in light scattering is observed when ATP is replaced with a non-hydrolyzable analog AMP-PNP. (**B**) Shows the kinetics of NSF-induced dissociation. The graph depicts first-order kinetics of vesicles dissociation elicited by NSF-ATP. (**C**) NSF requires ATP to dissociate vesicles. NSF in the presence of ATP dissociates vesicles ($p < 0.05$, n = 4, Student's *t*-test). However, NSF alone or NSF in the presence of AMP-PNP had no effect on the light scattering properties of SNARE-associated vesicle ($p > 0.05$, n = 4, Student's *t*-test). (**D**) When t- and v-SNARE vesicles are mixed in the presence or absence of ATP, NSF, NSF+ATP, or NSF+AMP-PNP, and resolved by SDS-PAGE followed by immunoblots using syntaxin-1 specific antibody, t-/v-SNARE disassembly was found to be complete only in the presence of NSF-ATP (**E**). Densitometric scan of the bands reveals significant changes in SNARE complex and syntaxin-1 reactivity only when NSF and ATP were included in reaction mixture ($p < 0.05$, n = 3; and $p < 0.01$, n=3, Student's *t*-test) [40]. *Data courtesy of Dr. Bhanu P. Jena.*
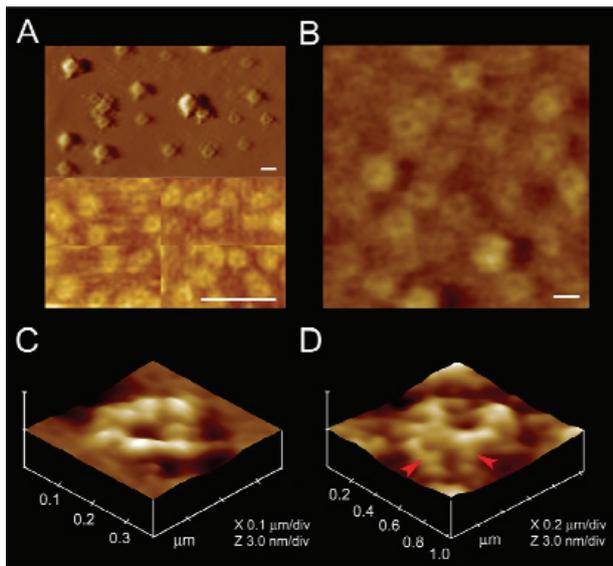
Fig. 6 AFM images of NSF-ATP induced disassembly of the t-/v-SNARE ring complex. Representative AFM micrograph of t-/v-SNARE complexes formed when large (top panel **A**) or small (bottom panel **A**) t-/v-SNARE ring complexes are formed due to the interaction of large and small v-SNARE reconstituted vesicles interacting with a t-SNARE reconstituted lipid membrane. Bar = 250 nm. (B) Disassembly of large t-/v-SNARE complex. Bar = 250 nm. (C) High resolution of a t-/v-SNARE ring complex, and (D) a disassembled complex [40]. *Images courtesy of Dr. Bhanu P. Jena.*

found that the vesicles resisted movement in all directions (Fig. 7). Conversely, when cells were exposed to nocodazole and cytochalasin B, which leads to a limited dissociation of the microtubule-actin network, movement of the secretory vesicles, trapped in the laser focus, did occur [50]. This work was further complemented by immunochemical examination and finding that actin, kinesin, and myosin V were associated with isolated vesicles from the exocrine pancreas and neurons. This further confirmed the involvement of the actin-microtubule network in the active intracellular transport of vesicles. Using the dynamic properties and sensitivities of a scanning probe microscope this work demonstrated for the first time that secretory vesicles in living cells are tethered and not free floating [49].

Conclusion

Scanning probe microscopes (SPM) due to their unique capabilities of nanoscale resolution, pico-Newton sensitivity, and the ability to image living cells in physiologically friendly environments have become a welcome resource for biological research. These unique capabilities of SPM have been successfully exploited to physically identify the porosome as a new cellular organelle. The skillful application of SPM and other technologies has given the scientific community new insight into the travels of secretory vesicles to the porosome and the ultimate expulsion of their contents to the outside of the cell. Physical evidence that secretory vesicles are not free floating
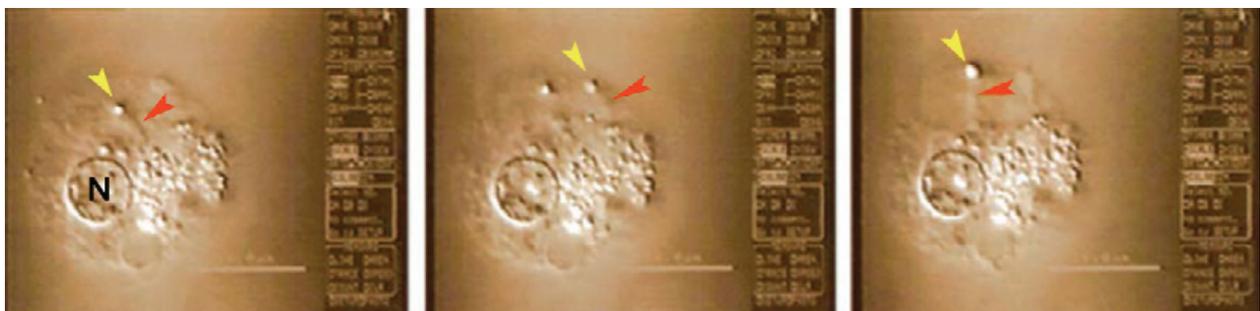
3-D as it is moved over the surface resulting in a computer generated topographic map of the surface [50]. By trapping secretory vesicles (0.2 to 1.2 μm) at the laser focus in live pancreatic acinar cells, it was

Fig. 6 Secretory vesicles are tethered and not free-floating in live pancreatic acinar cells. Photonic Force Microscope Video images of a live pancreatic acinar cell pretreated with nocodazole and cytochalasin, allows individual secretory vesicles (yellow arrowhead) to be pulled, when trapped at the laser focus of the PFM. Note vesicle tether (red arrowhead) originating from the supranuclear region (above the nucleus, N) of the cell [49]. *Images courtesy of Dr. Bhanu P. Jena.*

but are conveyed to the porosome by the actin-micro-tubule network has been documented using the photonic force microscope. Identification of the porosome and of secretory vesicles docking on the cytoplasmic side of isolated plasma membranes has been imaged by AFM. Imaging of the t-/v-SNARE dependent docking of secretory vesicles to membranes has been established and the enzymatic (NSF) and energy (ATP) dependent requirement for the disassociation of secretory vesicles from membranes has been physically established by AFM imaging.

Microscopy has played a significant role in our understanding of cell secretion. Electron microscopy has been used to broadly define the path from the synthesis of proteins within the cell to exocytosis. The dynamic imaging aspects of scanning probe microscopy, using both atomic force microscopy (AFM) and photonic force microscopy (PFM), have been eloquently exploited to reveal a new cellular structure '*the porosome*' as the docking point for secretory vesicles and has further elucidated the mechanics of cell secretion [52].

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