Cell secretion and membrane fusion

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Abstract

Secretion occurs in all cells of multicellular organisms and involves the delivery of secretory products packaged in membrane-bound vesicles to the cell exterior. Specialized cells for neurotransmission, enzyme secretion or hormone release utilize a highly regulated secretory process. Secretory vesicles are transported to specific sites at the plasma membrane, where they dock and fuse to release their contents. Similar to other cellular processes, cell secretion is found to be highly regulated and a precisely orchestrated event. It has been demonstrated that membrane-bound secretory vesicles dock and fuse at porosomes, which are specialized supramolecular structures at the cell plasma membrane. Swelling of secretory vesicles results in a build-up of pressure, allowing expulsion of intravesicular contents. The extent of secretory vesicle swelling dictates the amount of intravesicular contents expelled during secretion. The discovery of the porosome, its isolation, its structure and dynamics at nm resolution and in real time, its biochemical composition and functional reconstitution into artificial lipid membrane, have been determined. The molecular mechanism of secretory vesicle fusion at the base of porosomes, and vesicle swelling, has also been resolved. These findings reveal the molecular mechanism of cell secretion.

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1. Introduction

Secretion and membrane fusion are fundamental cellular processes regulating ER–Golgi transport, plasma membrane recycling, cell division, sexual reproduction, acid secretion, and the release of enzymes, hormones and neurotransmitters, to name just a few. It is therefore no surprise that defects in secretion and membrane fusion give rise to diseases like diabetes, Alzheimer’s, Parkinson’s, acute gastroduodenal diseases, gastroesophageal reflux disease, intestinal infections due to inhibition of gastric acid secretion, biliary diseases resulting from malfunction of secretion from hepatocytes, polycystic ovarian disease as a result of altered gonadotropin secretion, and Gitelman disease associated with growth hormone deficiency and disturbances in vasopressin secretion, are only a few examples. Understanding cellular secretion and membrane fusion helps not only to advance our understanding of these vital cellular and physiological processes, but in the development of drugs to help ameliorate secretory defects, provide insight into our understanding of cellular entry and exit of viruses and other pathogens, and in the development of smart drug delivery systems. Therefore, secretion and membrane fusion play an important role in health and disease. Studies [1–18] in the last decade demonstrate that membrane-bound secretory vesicles dock and transiently fuse at the base of specialized plasma membrane structures called porosomes or fusion pores, to expell vesicular contents. These studies further demonstrate that during secretion, secretory vesicles swell, enabling the expulsion of intravesicular contents through porosomes [16–18, and unpublished observations]. With these findings [1–18], a new understanding of cell secretion has emerged and confirmed by a number of laboratories [19–23].

Throughout history, the development of new imaging tools has provided new insights into our perceptions of the living world and profoundly impacted human health. The invention of the light microscope almost 300 years ago, was the first catalyst, propelling us into the era of modern biology and medicine. Using the light microscope, a giant step into the gates of modern medicine was made by the discovery of the unit of life,
the cell. The structure and morphology of normal and diseased cells, and of disease-causing microorganisms, were revealed for the first time using the light microscope. Then, in 1938, with the birth of the electron microscope (EM), dawned a new era in biology and medicine. Through the mid 1940s and 1950s, a number of subcellular organelles were discovered and their functions determined using the EM. Viruses, the new life forms were discovered and observed for the first time, and implicated in diseases ranging from the common cold to autoimmune disease (AIDS). Despite the capability of the EM to image biological samples at near nanometer resolution, sample processing (fixation, dehydration, staining) results in morphological alterations, and is a major concern. Then in the mid 1980s, scanning probe microscopy evolved [1,24], further extending our perception of the living world to the near atomic realm. One such scanning probe microscope, the atomic force microscope (AFM), has helped overcome both limitations of light and electron microscopy, enabling determination of the structure and dynamics of single biomolecules and live cells in 3D, at near angstrom resolution. This unique capability of the AFM has given rise to a new discipline of ‘nanobioscience’, heralding a new era in biology and medicine. Using AFM in combination with conventional tools and techniques, this past decade has witnessed advances in our understanding of cell secretion [1–18] and membrane fusion [9,15], as noted earlier and briefly described in this article.

The resolving power of the light microscope is dependent on the wavelength of the light used, and therefore, 250–300 nm in lateral and much less in depth resolution, can be achieved at best. The porosome or fusion pore in live secretory cells are cup-shaped structures, measuring 100–150 nm at its opening and 15–30 nm in relative depth in the exocrine pancreas, and just 10 nm at the presynaptic membrane of the nerve terminal. As a result, it had evaded visual detection until its discovery using the AFM [3–8]. The development of the AFM [24] has enabled the imaging of live cells in physiological buffer at nm to subnanometer resolution. In AFM, a probe tip microfabricated from silicon or silicon nitride and mounted on a cantilever spring is used to scan the surface of the sample at a constant force. Either the probe or the sample can be precisely moved in a raster pattern using a xyz piezo tube to scan the surface of the sample (Fig. 1). The deflection of the

![Fig. 2. On the far left is an AFM micrograph depicting ‘pits’ (yellow arrow) and ‘depressions’ within (blue arrow), at the plasma membrane in live pancreatic acinar cells. On the right is a schematic drawing depicting depressions, at the cell plasma membrane, where membrane-bound secretory vesicles dock and fuse to release vesicular contents [3].](image-url)
Fig. 3. Dynamics of depressions following stimulation of secretion. The top panel shows a number of depressions within a pit in a live pancreatic acinar cell. The scan line across three depressions in the top panel is represented graphically in the middle panel and defines the diameter and relative depth of the depressions; the middle depressions are represented by red arrow heads. The bottom panel represents percent of total cellular amylase release in the presence and absence of the secretagogue Mas 7. Notice an increase in the diameter and depth of depressions, correlating with an increase in total cellular amylase release at 5 min after stimulation of secretion. At 30 min after stimulation of secretion, there is a decrease in diameter and depth of depressions, with no further increase in amylase release over the 5 min time point. No significant increase in amylase secretion or depressions diameter were observed in resting acini or those exposed to the nonstimulatory mastoparan analog Mas 17 [3,11].

Fig. 4. AFM micrograph of depressions or porosomes or fusion pores in live secretory cell of the exocrine pancreas (A and B), the growth hormone secreting cell of the pituitary (C) and in the chromaffin cell (D). Note the ‘pit’ (white arrow heads) with four depressions (yellow arrow head). A high-resolution AFM micrograph is shown in (B). Bars = 40 nm for (A and B). Similarly, AFM micrographs of porosomes in β-cell of the endocrine pancreas (E) and mast cell (F) is shown. Note the 100–130 nm porosomes in the β-cell and the 70–80 nm porosomes in the mast cell. (G) Electron micrograph depicting a porosome (red arrow head) close to a microvilli (MV) at the apical plasma membrane (PM) of a pancreatic acinar cell. Note association of the porosome membrane (yellow arrow head), and the zymogen granule membrane (ZGM) (red arrow head) of a docked zymogen granule (ZG), the membrane-bound secretory vesicle of exocrine pancreas. Also a cross section of the ring at the mouth of the porosome is seen (blue arrow head) [8].
obtained in this mode of AFM operation, sample height information generated may not be accurate since the vertical scanning force may depress the soft cell. However, information on the viscoelastic properties of the cell and the spring constant of the cantilever, enables measurement of the cell height. In tapping mode on the other hand, the cantilever resonates and the tip makes brief contacts with the sample. In the tapping mode in fluid, lateral forces are virtually negligible. It is therefore important that the topology of living cells be obtained using both contact and tapping modes of AFM operation in fluid. The scanning rate of the tip over the sample also plays an important role on the quality of the image. Since cells are soft samples, a high-scanning rate would influence its shape. Hence, a slow tip movement over the cell would be ideal and results in minimal distortion and better image resolution. Rapid cellular events may be further monitored by using section analysis. To examine isolated cells by the AFM, freshly cleaved mica coated with Cel-Tak have also been used with great success [3–8]. Also, to obtain optimal resolution, the contents of the bathing medium as well as the cell surface to be scanned should be devoid of any debris.

2. Porosome: a new cellular structure

Earlier electrophysiological studies on mast cells suggested the existence of fusion pores at the cell plasma membrane (PM), which became continuous with the secretory vesicle membrane following stimulation of secretion [26]. AFM has confirmed the existence of the fusion pore or porosome, and revealed its structure and dynamics in the exocrine pancreas [3,4,7,8], neuroendocrine cells [5,6] and neurons [14] at near nm resolution and in real time.

Isolated live pancreatic acinar cells in physiological buffer, when imaged with the AFM [3,4,7,8], reveal at the apical PM a group of circular ‘pits’ measuring 0.4–1.2 μm in diameter which contain smaller ‘depressions’ (Fig. 2). Each depression averages between 100 and 150 nm in diameter, and typically 3–4 depressions are located within a pit. The basolateral membrane of acinar cells is devoid of either pits or depressions. High-resolution AFM images of depressions in live cells further reveal a cone-shaped morphology. The depth of each depression cone measures 15–30 nm. Similarly, growth hormone (GH) secreting cells of the pituitary gland and chromaffin cells, β cells of the exocrine pancreas, mast cells, and neurons, possess depressions at their PM, suggesting their universal presence in secretory cells. Exposure of pancreatic acinar cells to a secretagogue (mastoparan) results in a time-dependent increase (20–35%) in depression diameter, followed by a return to resting size on completion of secretion [3,4,7,8] (Fig. 3). No demonstrable change in pit size is detected following stimulation of secretion [3]. Enlargement of depression diameter and an increase in its relative depth after exposure to secretagogues correlated with increased secretion. Conversely, exposure of pancreatic acinar cells to cytochalasin B, a fungal toxin that inhibits actin polymerization, results in a 15–20% decrease in depression size and a consequent 50–60% loss in secretion [3]. Results from these studies suggested depressions to be the fusion pores in pancreatic acinar cells. Furthermore, these studies demonstrate the involvement of actin in regulation of both the structure and function of depressions. Analogous to pancreatic acinar cells, examination of resting GH secreting cells of the pituitary [5] and chromaffin cells of the adrenal medulla [6] also reveal the presence of pits and depressions at the cell PM (Fig. 4). The presence of porosomes in neurons, β-cells of the endocrine
pancreas and in mast cells has also been demonstrated (Figs. 4 and 5) [14]. Depressions in resting GH cells measure 154 ± 4.5 nm (mean ± S.E.) in diameter. Exposure of GH cells to a secretagogue results in a 40% increase in depression diameter (215 ± 4.6 nm; P < 0.01) but no appreciable change in pit size. The enlargement of depression diameter during secretion and the known effect that actin depolymerizing agents decrease depression size and inhibit secretion [3] suggested depressions to be the fusion pores. However, a more direct determination of the function of depressions was required. This was achieved by immuno-AFM studies. AFM localization at depressions of gold-conjugated antibody to a secretory protein,
Fig. 6. Depressions are fusion pores or porosomes. Porosomes dilate to allow expulsion of vesicular contents. (A and B) AFM micrographs and section analysis of a pit and two out of the four fusion pores or porosomes, demonstrating enlargement following stimulation of secretion. (C) Exposure of live cells to gold-conjugated amylase antibody (Ab) results in specific localization of immuno-gold to the porosome opening. Amylase is one of the proteins within secretory vesicles of the exocrine pancreas. (D) AFM micrograph of a fixed pancreatic acinar cell, demonstrating a pit and porosomes within, immunogold-labeling amylase at the site. Blue arrow heads point to immunogold clusters and the yellow arrow head points to a porosome [4].

demonstrated secretion to occur through depressions [4,5]. The membrane-bound secretory vesicles in exocrine pancreas called zymogen granules (ZGs), contain the starch digesting enzyme amylase. AFM micrographs demonstrated localization of amylase-specific antibodies tagged with colloidal gold at depressions following stimulation of secretion [4] (Fig. 6). These studies confirm depressions to be the fusion pores or porosomes in pancreatic acinar cells where membrane-bound secretory vesicles dock and fuse to release vesicular contents. Similarly, in somatotrophs of the pituitary, gold-tagged growth hormone-specific antibody is found to selectively localize at depressions following stimulation of secretion [5], again identifying depressions in GH cells as fusion pores or porosomes.

To determine the morphology of the porosome at the cytosolic side of the cell, pancreatic PM preparations were used. Isolated PM in buffer when placed on freshly cleaved mica, tightly adhere to the mica surface, enabling imaging by AFM. The PM preparations reveal scattered circular disks measuring 0.5–1 μm in diameter, with inverted cup-shaped structures within [7]. The inverted cups range in height from 10 to 15 nm. On a number of occasions, ZGs ranging in size from 0.4 to 1 μm in diameter were found associated with one or more of the inverted cups. This suggested the circular disks to be pits, and the inverted cups to be fusion pores or porosomes. To determine if the cup-shaped structures in isolated PM preparations are indeed porosomes, immuno-AFM studies were carried out. Since ZGs dock and fuse at the PM to release vesicular contents, it was hypothesized that if porosomes are these sites, then PM-associated t-SNAREs should localize at the base of porosomes. The t-SNARE protein SNAP-23, has been identified and implicated in secretion from pancreatic acinar cells [27]. A polyclonal monospecific SNAP-23 antibody recognizing a single 23 kDa band in Western blots of pancreatic PM fraction, has been used in immuno-AFM studies. When the SNAP-23-specific antibody was added to the PM preparation during imaging with the AFM, the antibody selectively localized to the base of the cup-shaped structure,
which is the tip of the inverted cup. These results demonstrate that the inverted cup-shaped structures in the isolated inverted PM preparations are the porosomes observed from its cytosolic side [7,8]. Target membrane proteins, SNAP-25 and syntaxin (t-SNARE) and secretory vesicle-associated membrane protein (v-SNARE), are part of the conserved protein complex involved in fusion of opposing bilayers [28,9,15]. Since membrane-bounded secretory vesicles dock and fuse at porosomes to release vesicular contents, suggested t-SNAREs to associate at the porosome complex. It was therefore no surprise that the t-SNARE protein SNAP-23, implicated in secretion from pancreatic acinar cells, was located at the tip of the inverted cup (i.e., the base of the porosome) where secretory vesicles dock and fuse.

The structure of the porosome was further demonstrated using transmission electron microscopy (TEM) [7,8] (Fig. 4). TEM studies confirm the fusion pore to have a cup-shaped structure, with similar dimensions as determined from AFM measurement. Additionally, TEM micrographs reveal porosomes to possess a basket-like morphology, with three lateral and a number of vertically arranged ridges. A ring at the base of the complex is also identified [7]. Since porosomes are found to be stable structures at the cell PM, it was hypothesized that if ZGs were to fuse at the base of the structure, it would be possible to isolate ZG-associated porosomes. Indeed, TEM of isolated ZG preparations reveal porosomes associated with docked vesicles [7,8]. As observed in whole cells, vertical structures were found to originate from within the porosome complex and appear attached to its membrane. As discussed later in this review, studies using full length recombinant SNARE proteins and artificial lipid membranes demonstrated that t- and v-SNAREs located in opposing bilayers interact in a circular array to form conducting pores [9]. Since similar circular structures are observed at the base of the porosome, and SNAP-23 immunoreactivity is found to localize at this site, suggests that the t-SNAREs present at the base of porosomes are possibly arranged in a ring pattern.

3. Porosome: isolation, composition, and reconstitution

In the last decade, a number of studies demonstrate the involvement of cytoskeletal proteins in secretion, and some studies implicate direct interaction of cytoskeleton protein with SNAREs [3,29–33]. Furthermore, actin and microtubule-based cytoskeleton has been implicated in intracellular vesicle traffic [3,31]. Fodrin, which was previously implicated in exocytosis [29], has recently been shown to directly interact with SNAREs [32]. Studies demonstrate α-fodrin to regulate exocytosis via its interaction with t-SNARE syntaxin family of proteins [32]. The c-terminal coiled coil region of syntaxin interacts with α-fodrin, a major component of the submembranous cytoskeleton. Similarly, vimentin filaments interact with SNAP-23/25 and hence are able to control the availability of free SNAP-23/25 for assembly of the SNARE complex [30]. All these findings suggest that vimentin, α-fodrin, actin, and SNAREs may be part of the porosome complex. Additional proteins such as v-SNARE (VAMP or synaptobrevin), synaptophysin, and myosin, may associate when the porosome establishes continuity with the secretory vesicle membrane. The globular tail domain of myosin V contains a binding site for VAMP, which is bound in a calcium independent manner [33]. Further interaction of myosin V with syntaxin requires both calcium and calmodulin. It has been suggested that VAMP acts as a myosin V receptor on secretory
Fig. 7. SNAP-23 associated proteins in pancreatic acinar cells. Total pancreatic homogenate was immunoprecipitated using the SNAP-23-specific antibody. The precipitated material was resolved using 12.5% SDS-PAGE, electrotransferred to nitrocellulose membrane and then probed using antibodies to a number of proteins. Association of SNAP-23 with syntaxin2, with cytoskeletal proteins actin, α-fodrin, and vimentin, and calcium channels β3 and β1c, together with the SNARE regulatory protein NSF, is demonstrated (arrow heads). Lanes showing more than one arrow head suggest presence of isomers or possible proteolytic degradation of the specific protein [7].

vesicles and regulates formation of the SNARE complex [33]. Interaction of VAMP with synaptophysin and myosin V has also been observed [34]. In agreement with these earlier findings, recent studies demonstrate the association of SNAP-23, syntaxin 2, cytoskeletal proteins actin, α-fodrin, and vimentin, and calcium channels β3 and β1c, together with the SNARE regulatory protein NSF, in porosomes [7,8] (Fig. 7). Additionally, chloride ion channels ClC2 and ClC3 were also identified as part of the porosome complex [7,8]. Isoforms of the various proteins identified in the porosome complex, were subsequently demonstrated using 2D-BAC gels electrophoresis [8,14]. Three isoforms each of the calcium ion channel and vimentin were found in porosomes [8]. Using yeast 2-hybrid analysis, recent studies confirm the presence and interaction of some of these proteins with t-SNAREs within the porosome complex [14].

The size and shape of the immunoisolated porosome complex was determined in greater detail when examined using both negative staining TEM and AFM [8]. The images of the immunoisolated porosome obtained by both TEM and AFM were super impossible [8]. To further test whether the immunoisolated supramolecular complex was indeed the porosome, the complex was reconstituted into artificial liposomes, and the liposome-reconstituted complex examined using TEM [8]. Transmission electron micrographs reveal a 150–200 nm cup-shaped basket-like structure as observed of the porosome when co-isolated with ZGs. To test if the reconstituted porosome complex was functional, purified porosomes were reconstituted into lipid membranes in an electrophysiological bilayer setup (EPC9) and
Fig. 8. Lipid bilayer-reconstituted porosome complex is functional. (A) Schematic drawing of the bilayer setup for electrophysiological measurements. (B) Zymogen granules (ZGs) added to the cis side of the bilayer fuse with the reconstituted porosomes, as demonstrated by an increase in capacitance and current activities, and a concomitant time-dependent release of amylase (a major ZG content) to the trans side of the membrane. The movement of amylase from the cis to the trans side of the chamber was determined by immunoblot analysis of the contents in the cis and the trans chamber over time. (C) As demonstrated by immunoblot analysis of the immunoisolated complex, electrical measurements in the presence and absence of chloride ion channel blocker DIDS, demonstrate the presence of chloride channels in association with the complex [8].
challenged with isolated ZGs. Both the electrical activity of the reconstituted membrane as well as the transport of vesicular contents from the cis to the trans compartment, was monitored. Results of these experiments demonstrate that the lipid membrane-reconstituted porosomes are functional supramolecular complexes (Fig. 8) [8]. ZGs fused with the porosome-reconstituted bilayer, as demonstrated by an increase in capacitance and conductance, and in a time-dependent release of the ZG enzyme amylase from cis to the trans compartment of the bilayer chamber (Fig. 8B). Amylase is detected using immunoblot analysis of the buffer in the cis and trans chambers (Fig. 8B), using a previously characterized amylase-specific antibody [4]. As observed in immunoblot assays of isolated porosomes, chloride channel activities are also detected within the reconstituted porosome complex (Fig. 8C). Further, the chloride channel inhibitor DIDS, was found to inhibit current activity in the porosome-reconstituted bilayer. In summary, these studies demonstrate that the porosome in the exocrine pancreas is a 100–50 nm in diameter supramolecular cup-shaped lipoprotein basket at the cell PM, where membrane-bound secretory vesicles dock and fuse to release vesicular contents. Similar studies have now been performed in neurons, demonstrating both the structural (Fig. 5E and F) and functional reconstitution of the isolated neuronal porosome complex. The biochemical composition of the neuronal porosome has also been determined (unpublished).

4. SNARE-induced membrane fusion

As eluded earlier, membrane fusion is mediated via a specialized set of proteins in the secretory vesicles and the plasma membrane. Three soluble N-ethylmaleimide-sensitive factor (NSF)-attachment protein receptors (SNAREs) have been implicated in membrane fusion [28]. Target membrane proteins, SNAP-25 and syntaxin (t-SNARE) and secretory vesicle-associated membrane protein (v-SNARE), are part of the conserved protein complex involved in fusion of opposing bilayers [28]. Although the structure of SNARE complex formed by interacting native [35] or recombinant [36,37] t- and v-SNAREs was known from studies using electron microscopy [35,36] and X-ray crystallography [37], the molecular mechanism of the involvement of SNAREs to bring about membrane fusion remained unknown until 2002 [9].

Fig. 9. Pore-like structures are formed when t-SNAREs and v-SNARE in opposing bilayers interact. (A) Unfused v-SNARE vesicles on t-SNARE-reconstituted lipid membrane. (B) Dislodgement or fusion of v-SNARE-reconstituted vesicles with a t-SNARE-reconstituted lipid membrane, exhibit formation of pore-like structures due to the interaction of v- and t-SNAREs in a circular array. The size of the pores range between 50 and 150 nm (B–D). Several 3D AFM amplitude images of SNAREs arranged in a circular array (C) and some at higher resolution (D), illustrating a pore-like structure at the center is depicted. Scale bar is 100 nm. Recombinant t-SNAREs and v-SNARE in opposing bilayers drive membrane fusion. (E) When t-SNARE vesicles were exposed to v-SNARE-reconstituted bilayers, vesicles fused. Vesicles containing nystatin/ergosterol and t-SNAREs were added to the cis side of the bilayer chamber. Fusion of t-SNARE containing vesicles with the membrane observed as current spikes that collapse as the nystatin spreads into the bilayer membrane. To determine membrane stability, the transmembrane gradient of KCl was increased, allowing gradient-driven fusion of nystatin-associated vesicles [9].
To determine the molecular mechanism of SNARE-induced membrane fusion, the structure and arrangement of SNAREs associated with lipid bilayers were examined using AFM. The bilayer electrophysiological setup allowed measurements of membrane conductance and capacitance, prior to and after t-SNARE- or v-SNARE-reconstitution, and
following exposure of v-SNARE or t-SNARE-reconstituted lipid vesicles. These studies demonstrate that the interaction of t-/v-SNARE proteins to form a pore or channel, is dependent on the presence of t-SNAREs and v-SNARE in opposing bilayers. Addition of purified recombinant v-SNARE to a t-SNARE-reconstituted lipid membrane increased only the size of the globular t-SNARE oligomer without influencing the electrical properties of the membrane [9]. However, when t-SNARE vesicles are added to v-SNARE-reconstituted membrane, SNAREs assemble in a ring pattern (Fig. 9) and a stepwise increase in capacitance and conductance is observed (Fig. 10). Thus, t- and v-SNAREs are required to reside in opposing bilayers to allow appropriate t-/v-SNARE interactions leading to membrane fusion only in the presence of calcium [9,15]. Studies using SNARE-reconstituted liposomes and bilayers [15] demonstrate: (i) a low fusion rate ($\tau = 16$ min) between t- and v-SNARE-reconstituted liposomes in the absence of $Ca^{2+}$; (ii) exposure of t-/v-SNARE liposomes to $Ca^{2+}$, drives vesicle fusion on a near physiological relevant time-scale ($\tau \sim 10$ s), demonstrating an essential role of $Ca^{2+}$ in membrane fusion (Figs. 11 and 12). These results support earlier findings on the role of $Ca^{2+}$ and SNAREs in cortical vesicle fusion in sea urchin eggs [38,39], where $Ca^{2+}$ was found to act downstream of SNAREs. Since the $Ca^{2+}$ effect on membrane fusion in SNARE-reconstituted liposomes is downstream of SNAREs, this suggests a regulatory role for $Ca^{2+}$-binding proteins in membrane fusion in the physiological state [15]. It is further demonstrated from these studies that in the physiological state in cells, both SNAREs and $Ca^{2+}$ operate as the minimal fusion machinery [15]. Native and synthetic vesicles exhibit a significant negative surface charge primarily due to the polar phosphate head groups. These polar head groups produce a repulsive force, preventing aggregation and fusion of apposing vesicles. SNAREs bring opposing bilayers closer to within a distance of 2–3 Å, allowing $Ca^{2+}$ to bridge them [15]. The bound $Ca^{2+}$ then leads to the expulsion of water between the bilayers at the bridging site, allowing lipid mixing and membrane fusion. Hence SNAREs, besides bringing opposing bilayers closer, dictate the site and size of the fusion area during secretion. The size of the t-/v-SNARE complex forming the pore is dictated by the curvature of the opposing membranes, hence depends on the size of t-/v-SNARE-reconstituted vesicles. The smaller the vesicles, the smaller the pores formed (unpublished observation).

Fig. 10. Opposing bilayers containing t- and v-SNAREs, respectively, interact in a circular array to form conducting pores. (A) Schematic diagram of the bilayer-electrophysiology setup. (B) Lipid vesicle containing nystatin channels (red) and both vesicles and membrane bilayer without SNAREs, demonstrate no significant changes in capacitance and conductance. Initial increase in conductance and capacitance may be due to vesicle–membrane attachment. To demonstrate membrane stability (both bilayer membrane and vesicles), the transmembrane gradient of KCl was increased to allow gradient-driven fusion and a concomitant increase of conductance and capacitance. (C) When t-SNARE vesicles were added to a v-SNARE membrane support, the SNAREs in opposing bilayers arranged in a ring pattern, forming pores (as seen in the AFM micrograph on the extreme right) and there were seen stepwise increases in capacitance and conductance (−60 mV holding potential). Docking and fusion of the vesicle at the bilayer membrane, opens vesicle-associated nystatin channels and SNARE-induced pore formation, allowing conductance of ions from cis to the trans side of the bilayer membrane. Then further addition of KCl to induce gradient-driven fusion resulted in little or no further increase in conductance and capacitance, demonstrating that docked vesicles have already fused [9].
5. Regulation of secretory vesicle swelling: involvement in expulsion of vesicular contents

The molecular mechanism of vesicle swelling [16–18] and its involvement in regulated expulsion of intravesicular contents [14] has been established. Secretory vesicle swelling is critical for secretion [40–43]; however, the underlying mechanism of vesicle swelling was largely unknown until recently [16–18]. In mast cells, an increase in secretory vesicle
Fig. 11. Fluorimetric fusion assays demonstrate the ability of Ca\textsuperscript{2+} to induce rapid lipid mixing of plain (AV) and SNARE-associated vesicles. Addition of 5 mM Ca\textsuperscript{2+} to liposomal solution significantly increases the fusion of plain and SNARE-associated vesicles ($P < 0.05$, Student’s $t$-test between AV and AV+ Ca\textsuperscript{2+} or t-/v-SNARE-AV+ Ca\textsuperscript{2+}, $n = 5$). Note the inability of SNAREs in the absence of Ca\textsuperscript{2+} to significantly induce vesicle fusion ($P > 0.1$, Student’s $t$-test between AV and t-/v-SNARE-AV, $n = 5$). Incorporation of t-/v-SNAREs at the vesicles membrane increases the overall yield but does not alter the rate of Ca\textsuperscript{2+}-induced membrane fusion (A). The graph depicts the first-order kinetics of SNAREs vesicle fusion in the presence and absence of Ca\textsuperscript{2+} (B) [15].

Volume after stimulation of secretion has previously been suggested from electrophysiological measurements [44]. However, direct evidence of secretory vesicle swelling in live cells was first demonstrated in pancreatic acinar cells using the AFM [16]. Isolated ZG\textsubscript{S} of the exocrine pancreas and parotid glands, possess Cl\textsuperscript{-} and ATP-sensitive, K\textsuperscript{+}-selective ion channels at the vesicle membrane whose activities have been implicated in vesicle swelling. Additionally, secretion of ZG contents in permeabilized pancreatic acinar cells requires the presence of both K\textsuperscript{+} and Cl\textsuperscript{-} ions. In vitro ZG–pancreatic plasma membrane fusion assays further demonstrate potentiation of fusion in the presence of GTP [43]. G\textsubscript{q11} protein has been implicated in the regulation of both K\textsuperscript{+} and Cl\textsuperscript{-} ion channels in a number of tissues.
Fig. 12. Conductance and capacitance measurements of SNARE-reconstituted lipid bilayers. The EPC9-electrophysiological setup is shown (A). In the presence of 5 mM EGTA, t-SNARE-associated vesicles containing nystatin channels at their membrane (represented as red structures at the vesicle membrane) interact with v-SNARE-reconstituted lipid bilayer without fusing (B). Note no change in conductance or capacitance following exposure of SNARE-associated lipid vesicles to the bilayer. The vesicles fuse, however, when 3 mM KCl is applied, demonstrating fusion of docked vesicles and presence of an intact bilayer (B). The green arrow head indicates when the stirring is switched on to mix the addition. In the presence of 1 mM CaCl$_2$, the t-SNARE-associated vesicles fuse with the v-SNARE-reconstituted bilayer as depicted in a consequent increase in conductance and capacitance. Since a large majority of docked vesicles have fused, addition of 3 mM KCl has no further effect (C). Traces (B and C) are representative profiles from one of five separate experiments [15].

Analogous to the regulation of K$^+$ and Cl$^-$ ion channels at the cell plasma membrane, their regulation at the ZG membrane by a G$_{m3}$ protein has been demonstrated [16]. Isolated ZGs from exocrine pancreas swell rapidly in response to GTP [16]. These studies suggested the involvement of rapid water gating into ZGs following exposure to GTP. Therefore, when the possible involvement of water channels or aquaporins in ZG swelling was explored [17], results from the study demonstrate the presence of aquaporin-1 (AQP1) at the ZG membranes
and its participation in GTP-mediated vesicle water entry and swelling [17]. To further understand the molecular mechanism of secretory vesicle swelling, the regulation of AQP1 in ZGs has been investigated [18]. Detergent-solubilized ZGs immunoprecipitated with monoclonal AQP-1 antibody, co-isolates AQP-1, PLA2, Gα3, potassium channel IRK-8, and the chloride channel CIC-2 [18]. Exposure of ZGs to either the potassium channel blocker glyburide or the PLA2 inhibitor ONO-RS-082, blocked GTP-induced ZG swelling. RBC, known to possess AQP-1 at the plasma membrane, also swell on exposure to the Gα3-agonist mastoparan, and respond similarly to ONO-RS-082 and glyburide, as do ZGs. Additionally, liposomes reconstituted with the AQP-1 immunoisolated complex from solubilized ZGs, were found to swell in response to GTP. Glyburide or ONO-RS-082 abolished the GTP effect in reconstituted liposomes. Furthermore, immunoisolate-reconstituted planar lipid membrane demonstrate conductance, which is sensitive to glyburide and an AQP-1-specific antibody. These results demonstrate a pathway and potassium channel involvement in AQP-1 regulation [18], contributing to our understanding of the molecular mechanism of ZG swelling. Although secretory vesicle swelling is involved in membrane fusion [43], studies demonstrate that its primary role is in the expulsion of vesicular contents during secretion. The extent of swelling is directly proportional to the amount of intravesicular contents expelled ([14], unpublished observation).

6. Molecular understanding of cell secretion

Fusion pores or porosomes are present in all secretory cells examined, from exocrine, endocrine, neuroendocrine, to neurons, where membrane-bound secretory vesicles dock and transiently fuse to expel vesicular contents. Porosomes in pancreatic acinar or GH-secreting cells are cone-shaped structures at the plasma membrane, with a 100–150 nm diameter opening. Membrane-bound secretory vesicles ranging in size from 0.2 to 1.2 μm in diameter dock and fuse at porosomes to release vesicular contents. Following fusion of secretory vesicles at porosomes, only a 20–35% increase in porosome diameter is demonstrated. It is therefore reasonable to conclude that secretory vesicles “transiently” dock and fuse at the site. In contrast to accepted belief, if secretory vesicles were to completely incorporate at porosomes, the PM structure would distend much wider than what is observed. Furthermore, if secretory vesicles were to completely fuse at the plasma membrane, there would be a loss in vesicle number following secretion. Examination of secretory vesicles within cells before and after secretion demonstrates that the total number of secretory vesicles remains unchanged following secretion [10, 23]. However, the number of empty and partially empty vesicles increases significantly, supporting the occurrence of transient fusion. Earlier studies on mast cells also demonstrated an increase in the number of spent and partially spent vesicles following stimulation of secretion, without any demonstrable increase in cell size. Similarly, secretory granules are recaptured largely intact after stimulated exocytosis in cultured endocrine cells [19]. Other supporting evidence favoring transient fusion is the presence of neurotransmitter transporters at the synaptic vesicle membrane. These vesicle-associated transporters would be of little use if vesicles were to fuse completely at the plasma membrane to be compensatorily endocytosed at a later time. In further support, a recent study reports that single synap-
The fusion of secretory vesicles at the cell plasma membrane occurs transiently. Complete incorporation of membrane at the cell plasma membrane would occur when cells need to incorporate signaling molecules like receptors, second messengers or ion channels.

The discovery of the porosome, and an understanding of the molecular mechanism of membrane fusion and the swelling of secretory vesicles required for expulsion of vesicular contents, provides an understanding of secretion and membrane fusion in cells at the molecular level. These findings have prompted many laboratories to work in the area and further confirm these findings. Thus, the porosome is a supramolecular structure universally present in secretory cells, from the exocrine pancreas to the neurons, and in the endocrine to neuroendocrine cells, where membrane-bound secretory vesicles transiently dock and fuse to expel vesicular contents. Hence, the secretory process in cells is a highly regulated event, orchestrated by a number of ions and biomolecules.

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**References**