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Secretion machinery at the cell plasma membrane

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Summary

Secretion is a fundamental cellular process involving the regulated release of intracellular products from cells. Physiological functions such as neurotransmission, or the release of hormones and digestive enzymes, are all governed by cell secretion. Three critical activities occur at the cell plasma membrane to ensure secretion. Membrane-bound secretory vesicles *dock*, *fuse*, and *expel* their contents to the outside via specialized and permanent plasma membrane structures, called *porosomes* or fusion pores. In recent years, significant progress has been made in our understanding of these three key cellular activities required for cell secretion. The molecular machinery and mechanism involving them is summarized in this article.

Keywords

cell secretion; porosome / fusion pore; SNARE-induced membrane fusion; vesicle swelling and content expulsion

Introduction

Cell secretion has been the subject of intense research for decades, however only recently, the general molecular machinery and mechanism of the process has emerged. In particular, considerable knowledge on cell secretion has been gained from *in vivo* studies complemented by *in vitro* work, using atomic force microscopy (AFM) combined with conventional imaging, biochemical, electrophysiological, and molecular approaches. Understanding the structural assembly and interaction of membrane proteins has always been difficult, especially their crystallization and hence the determination of their native structure-function. Similarly the resolution limit of the light microscope (250 nm in lateral and much less in depth resolution), had precluded determination of live cellular structure-function at the nm scale and in real-time. The AFM has helped overcome these difficulties and limitations in studying membrane proteins and their complexes, especially those involved in the secretory process in cells. Secretion is a precisely regulated and universal cellular process, involving delivery of intracellular products to the extra-cellular space. Cellular cargo destined for secretion are packaged and stored in membranous sacs or vesicles, which on demand dock and fuse at specialized plasma membrane structures called porosomes [1–6]. The establishment of continuity between secretory vesicle membrane and the porosome membrane requires calcium and the assembly of a molecular complex [7] formed when specific proteins [8–11] in opposing bilayers interact. At the nerve terminal for instance, target membrane proteins SNAP-25 and syntaxin (t-SNAREs) associated at the base of porosomes [4], and synaptic vesicle-associated

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protein VAMP or v-SNARE, interact in a circular array [7] to form conducting channels in the presence of calcium [7,12]. During cell secretion, swelling of secretory vesicles result in a build-up of intra-vesicular pressure, required for the expulsion of vesicular contents through porosomes to the outside [13]. The extent of vesicle swelling dictates the amount of vesicular contents expelled [13]. In the past two decades, besides discovery of the porosome [1–6], the discovery of SNAREs [14–16], the molecular assembly [7,17^{••}] and disassembly [18,19[•]] of the membrane-associated t-/v-SNARE complex [7,17^{••},18], in addition to the molecular mechanism of secretory vesicle swelling [20–22] required for the regulated release of intravesicular contents, has been determined using, among other approaches, the AFM.

Porosome: the universal secretion machinery

Electrophysiological measurements of mast cells [23–27] as well as adrenal chromaffin cells [28], suggested the presence of fusion pores at the cell plasma membrane as “dynamic entities”, originating following a secretory stimulus [29]. Results from earlier studies on cell capacitance and conductance measurements, suggested that following stimulation of secretion, the “exocytotic fusion pore” abruptly appears as a 1–2 nm in diameter pore at the cell plasma membrane, with conductance similar to a large ion channel [29]. During cell secretion, the fusion pore either irreversibly expands or closes [24,29–31]. The latter process, where the fusion pore opens allowing secretory vesicles to fuse momentarily and subsequently close, has been referred to as ‘transient fusion’ [24,26,30,31]. Experimental data from these and other studies, and from theoretical considerations, gave rise to a hypothetical working model of the exocytotic fusion pore [29,32] in cells. According to the model, the fusion pore is formed by the regulatory function of a group of proteins responsible for bringing the plasma membrane into a highly curved dimple, close to a tense secretory vesicle membrane [29].

However, the discovery of the porosome (Fig. 1) [1–6] as permanent (not induced) and dynamic structures at the cell plasma membrane, brought to light a molecular understanding of the secretory process in cells [33]. Old questions such as the appearance of partially empty vesicles following cell secretion were answered [33]. Porosomes in pancreatic acinar (Fig. 1A–1D) [4–6] or growth hormone-secreting cells are permanent structures at the cell plasma membrane, with a 100 to 180 nm-diameter opening. Membrane-bound secretory vesicles ranging in size from 0.2 to 1.2 μ m 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 [1–3]. It is therefore reasonable to conclude that secretory vesicles “transiently” dock and fuse at the base of porosomes, instead of complete merger of the secretory vesicle membrane at the site. In contrast to accepted belief, if secretory vesicles were to completely incorporate at porosomes, the plasma membrane structure would distend much greater than what is observed. Furthermore, if secretory vesicles were to completely merge at the cell plasma membrane, there would be a loss in vesicle number following cell secretion. Examination of secretory vesicles within cells before and after secretion demonstrates that the total number of secretory vesicles remains constant following cell secretion. However, the number of empty and partially empty vesicles increases significantly, supporting the occurrence of transient fusion [33]. In agreement, it has been demonstrated that secretory granules are recaptured largely intact following stimulated exocytosis in cultured endocrine cells [33]. Further support of transient fusion is provided by the presence of neurotransmitter transporters at the synaptic vesicle membrane. Synaptic vesicle-associated transporters would be of little use if vesicles were to fuse and merge completely at the cell plasma membrane, to be endocytosed at a later time. Similarly in neurons, 10–15 nm porosomes at the presynaptic membrane (Fig. 1E, 1F) allow for the transient docking and fusion of synaptic vesicles, during neuro transmission. Studies report that single synaptic vesicles fuse transiently and successively without loss of vesicle identity [33].

Over the years, the term “fusion pore” has been loosely referred to plasma membrane dimples that originate following a secretory stimulus, or to the continuity or channel established between opposing lipid membrane during membrane fusion. Hence for clarity, the term “porosome” was assigned to this newly discovered structure at the cell plasma membrane, where secretory vesicles transiently dock and fuse to release their contents. In the past decade, studies demonstrate that porosomes are present in all secretory cells examined, such as acinar cells of the exocrine pancreas, neurons, chromaffin cells, mast cells, growth hormone cells of the pituitary gland, β -cells of the endocrine pancreas [1–6,33], and recently in astrocytes (personal observation). Furthermore, the porosome has been isolated, its composition determined [4,6], and it has been both structurally and functionally reconstituted in artificial lipid membrane [5,6].

As previously discussed, in the exocrine pancreas and neuroendocrine cells, porosomes range in size from 100–180 nm, whereas in neurons and astrocytes, they measure merely 10–15 nm. Results from both AFM and EM studies demonstrate porosomes in every cell type examined (exocrine pancreas, to neuroendocrine cells, to neurons), to possess a cup-shaped basket-like morphology. During cell secretion, the porosome opening dilates 25–45% to facilitate expulsion of intravesicular contents, returning to its resting size following completion of secretion [1]. Porosomes are supramolecular lipoprotein structures, composed of among other proteins [4,6], SNAP-23/25, syntaxin, synaptotagmin, the ATPase *NSF*, cytoskeletal proteins (actin, α -fodrin, and vimentin), calcium channels β 3 and α 1c, chloride ion channels *CIC2* and *CIC3*, and in some cases their isoforms [4]. Recent studies further demonstrate cholesterol to be an integral component of the porosome complex, required for retaining its integrity and intramolecular interactions [34]. Apart from the fact that t-SNAREs and calcium channels are present at the base of porosomes [4], and the likely association of actin at the neck region of the structure [1] to regulate opening and closing to the outside, the localization of other proteins within the porosome complex remains to be determined. The size and complexity of the membrane-associated porosome has precluded an understanding of its atomic structure, which will ultimately provide further structural and functional details about the complex.

Secretory vesicle fusion at plasma membrane-associated porosomes

A primary understanding of membrane fusion in cells was made possible following discovery of a *N*-ethylmaleimide-sensitive factor (*NSF*) [35] and SNARE proteins [14–16], and their involvement in membrane fusion [36,37]. Target membrane proteins, SNAP-25 and syntaxin (t-SNARE) and secretory vesicle-associated membrane protein (*VAMP* or v-SNARE), are part of the conserved protein complex involved in fusion of opposing bilayers [37]. *VAMP* and syntaxin are both integral membrane proteins, with the soluble SNAP-25 associating with syntaxin. Hence, the key to our understanding of SNARE-induced membrane fusion requires determination of the atomic arrangement and interaction between membrane-associated v- and t-SNAREs. Ideally, the atomic coordinates of membrane-associated SNARE complex using x-ray crystallography would provide atomic details of the structure and its involvement in membrane fusion. So far such atomic details of membrane-associated t-/v-SNARE complex has not been possible, primarily due to solubility problems of membrane SNAREs, compounded with the fact that v-SNARE and t-SNAREs need to reside in opposing membranes when they meet, to form the appropriate physiologically relevant SNARE complex. The remaining option has been the use of nuclear magnetic resonance spectroscopy (NMR), however the NMR approach too has not been successful, primarily due to the molecular size limitation of the NMR. Regardless of these set backs and limitations, AFM force spectroscopy has provided for the first time at nm resolution, an understanding of the structure, assembly, and disassembly of the membrane-associated t-/v-SNARE complex in physiological buffer solution [7,12,17^{••},18[•]].

The structure and arrangement of SNAREs associated with lipid bilayers were first examined using AFM [7]. A bilayer electrophysiological assay allowed measurements of membrane conductance and capacitance, prior to and after t-SNARE- or v-SNARE-reconstitution, and following exposure to v-SNARE- or t-SNARE-reconstituted lipid vesicles. Results from the study demonstrate that t-SNAREs and v-SNARE when present in opposing bilayers, interact in a circular array, and in the presence of calcium they form conducting channels (Fig. 2) [7, 17^{••}]. The interaction of t-/v-SNARE proteins to form a conducting channel is strictly 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, results in an increase only in the size of the globular t-SNARE oligomer, and is without influence on the electrical properties of the membrane [7]. However, when t-SNARE vesicles are added to v-SNARE reconstituted membrane (Fig. 2A–2E), SNAREs assemble in a ring configuration (Fig. 2C, 2D), and a stepwise increase in capacitance and conductance is observed, demonstrating the establishment of continuity between the opposing bilayers (Fig. 2E). These results demonstrate that t- and v-SNAREs are required to reside in opposing bilayers (Fig. 2F), to allow appropriate t-/v-SNARE interactions leading to membrane fusion in the presence of calcium [7]. Studies using SNARE-reconstituted liposomes and bilayers [12] demonstrate: (i) a low fusion rate ($\tau=16$ min) between t- and v-SNARE-reconstituted liposomes in the absence of Ca^{2+} ; and (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 Ca^{2+} and SNAREs in combination to be the minimal fusion machinery in cells [12]. 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 opposing vesicles. SNAREs bring opposing bilayers closer, to within a distance of 2–3 Å, [12] enabling Ca^{2+} bridging. The bound Ca^{2+} then leads to the expulsion of water between the bilayers at the bridging site, leading to lipid mixing and membrane fusion. Hence SNAREs, besides bringing opposing bilayers closer, dictate the site and size of the fusion area during cell secretion. The size of the t-/v-SNARE complex forming the channel between the secretory vesicle and the porosome, is dictated by the curvature of the opposing membranes (Fig. 2F–2H) [17^{••}]; therefore the smaller the vesicle, the smaller the channel formed.

A unique set of chemical and physical properties of the Ca^{2+} ion make it ideal for participating in the membrane fusion reaction. Calcium ion exists in its hydrated state within cells. The properties of hydrated calcium have been extensively studied using x-ray diffraction, neutron scattering, in combination with molecular dynamics simulations [38–41]. The molecular dynamic simulations include three-body corrections compared with ab initio quantum mechanics/molecular mechanics molecular dynamics simulations. First principle molecular dynamics has also been used to investigate the structural, vibrational, and energetic properties of $[\text{Ca}(\text{H}_2\text{O})_n]^{2+}$ clusters, and the hydration shell of the calcium ion [39]. These studies demonstrate that hydrated calcium $[\text{Ca}(\text{H}_2\text{O})_n]^{2+}$ has more than one shell around the Ca^{2+} , with the first hydration shell having six water molecules in an octahedral arrangement [39]. In studies using light scattering and x-ray diffraction of SNARE-reconstituted liposomes, it has been demonstrated that fusion proceeds only when Ca^{2+} ions are available between the t- and v-SNARE-apposed proteoliposomes [12,33].

Mixing of t- and v-SNARE proteoliposomes in the absence of Ca^{2+} leads to a diffuse and asymmetric diffractogram in X-ray diffraction studies, a typical characteristic of short range ordering in a liquid system [42]. In contrast, when t-SNARE and v-SNARE proteoliposomes in the presence of Ca^{2+} are mixed, it leads to a more structured diffractogram with approximately a 12% increase in X-ray scattering intensity, suggesting an increase in the number of contacts between opposing bilayers, established presumably through calcium-phosphate bridges, as previously suggested [12,33,43]. The ordering effect of Ca^{2+} on inter-bilayer contacts observed in X-ray studies [12] is in good agreement with light, AFM, and

spectroscopic studies, suggesting close apposition of PO-lipid head groups in the presence of Ca^{2+} , followed by formation of Ca^{2+} -PO bridges between the adjacent bilayers [12,33,44]. X-ray diffraction studies show that the effect of Ca^{2+} on bilayers orientation and inter-bilayer contacts is most prominent in the area of 3 Å, with additional appearance of a new peak at position 2.8 Å, both of which are within the ionic radius of Ca^{2+} [12]. These studies further suggest that the ionic radius of Ca^{2+} may make it an ideal player in the membrane fusion reaction. Hydrated calcium $[\text{Ca}(\text{H}_2\text{O})_n]^{2+}$ however, with a hydration shell having six water molecules and measuring ~6 Å would be excluded from the t-/v-SNARE apposed inter-bilayer space, hence calcium has to be present in the buffer solution when t-SNARE vesicles and v-SNARE vesicles meet. Indeed, studies demonstrate that if t- and v-SNARE vesicles are allowed to mix in a calcium-free buffer, there is no fusion following post addition of calcium [45]. How does calcium work? Calcium bridging of apposing bilayers may lead to the release of water from the hydrated Ca^{2+} ion, leading to bilayer destabilization and membrane fusion. Additionally, the binding of calcium to the phosphate head groups of the apposing bilayers may also displace the loosely coordinated water at the PO-lipid head groups, resulting in further dehydration, leading to destabilization of the lipid bilayer and membrane fusion.

We have discussed the possible mechanism of SNARE and calcium triggered membrane fusion, which brings us to the question -is there a physiological blocker of membrane fusion in cells? Recent studies demonstrate that indeed there is such a clamp or blocker in -'complexins' [46••]. Complexins are 20 kD proteins which can associate with the SNARE complex but not individual SNAREs. At the cell plasma membrane, the principal calcium sensor in the membrane fusion reaction is the calcium binding protein synaptotagmin. It is suggested that when calcium binds to the calcium sensor-synaptotagmin, the clamp is released, allowing membrane fusion to proceed [46••]. It has been demonstrated that over expression of complexin blocks membrane fusion in cells [46••].

Expulsion of intravesicular contents at the cell plasma membrane

Studies demonstrate that vesicle swelling is required for the expulsion of intravesicular content from cells during secretion [13]. It has been demonstrated [13] that the extent of swelling is directly proportional to the amount of intravesicular contents expelled, hence providing cells with the ability to precisely regulate the release of secretory products during cell secretion. Direct observation in live cells of the requirement of secretory vesicle swelling in cell secretion [13] explains the appearance of empty and partially empty vesicles following cell secretion [47,48].

Live pancreatic acinar cells in near physiological buffer, when imaged using the AFM, reveal the size of secretory vesicles called zymogen granules (ZGs) lying immediately beneath the surface of the apical plasma membrane. Within 2.5 min of exposure to a secretory stimulus, a majority of ZGs within cells swell, followed by a decrease in ZG size, and a concomitant release of secretory products [13]. These studies demonstrated for the first time in live cells that there is intracellular swelling of secretory vesicles following stimulation of secretion, and vesicle deflation following partial discharge of their contents. Measurements of intracellular ZG size further reveal that the extent of swelling differs between vesicles following a secretory stimulus. This differential swelling among ZGs within the same cell, may explain why following stimulation of secretion, some intracellular ZGs demonstrate the presence of less vesicular content than others.

The precise role of secretory vesicle swelling in the expulsion of intravesicular contents during cell secretion has been determined using an electrophysiological bilayer fusion assay employing porosome-reconstituted lipid membrane [13]. It has been demonstrated that exposure of isolated secretory vesicles to GTP results in their swelling, similar to the

observations made in live cells. Different secretory vesicles respond differently to the same swelling stimulus. This differential response to GTP has been further assessed, by measuring changes in volume of different size vesicles. Addition of isolated secretory vesicles to the *cis* compartment of the bilayer electrophysiological apparatus, demonstrates vesicle fusion at the porosome-reconstituted lipid membrane. However, even after 15 min of vesicle addition, little or no release of intravesicular content is detected in the *trans* compartment of the bilayer chamber. In contrast, addition of GTP to the *cis* compartment of the chamber induces vesicle swelling, resulting in a robust expulsion of intravesicular contents into the *trans* compartment of the bilayer chamber. This holds true for both a slow secretory cells like the acinar cells of exocrine pancreas, and in the fast secreting neuron [13]. These studies demonstrate that during cell secretion, secretory vesicle swelling is a requirement for the expulsion of intravesicular contents.

Our understanding of the molecular mechanism of secretory vesicle swelling has greatly advanced in the last decade. Isolated secretory vesicles and reconstituted swelling-competent proteoliposomes have been utilized [13,20–22,49] to determine the mechanism and regulation of vesicle swelling. As previously discussed, isolated ZGs from the exocrine pancreas swell rapidly in response to GTP [20], suggesting rapid water gating into ZGs following exposure to GTP. Results from studies demonstrate the presence of the water channel aquaporin-1 (AQP1) at the ZG membrane [21] and its participation in GTP-mediated water entry and vesicle swelling. Further, the molecular regulation of AQP1 at the ZG membrane has been studied [49], providing a general mechanism of secretory vesicle swelling. Detergent-solubilized ZGs immunoprecipitated with monoclonal AQP-1 antibody, co-isolates AQP-1, PLA2, $G_{\alpha i3}$, potassium channel IRK-8, and the chloride channel CIC-2 [49]. Exposure of ZGs to either the potassium channel blocker glyburide, or the PLA2 inhibitor ONO-RS-082, blocks GTP-induced ZG swelling. Red blood cells known to possess AQP-1 at the plasma membrane also swell on exposure to the $G_{\alpha i}$ agonist mastoparan, and responds similarly to ONO-RS-082 and glyburide, as do ZGs [48]. Artificial liposomes reconstituted with the AQP-1 immunisolated complex from solubilized ZGs also swell in response to GTP. Glyburide or ONO-RS-082 also abrogates the GTP effect in reconstituted liposomes. AQP-1 immunisolate-reconstituted planar lipid membrane demonstrate conductance, which is sensitive to glyburide and an AQP-1 specific antibody. These results demonstrate a $G_{\alpha i3}$ -PLA2 mediated pathway and potassium channel involvement in AQP-1 regulation at the ZG membrane [49], contributing to ZG swelling. Similarly, AQP-6 involvement has been demonstrated in GTP-induced and G_o -mediated synaptic vesicle swelling in neurons [22].

Conclusion

The findings outlined in this review provide our current understanding of the molecular machinery involved, and the mechanism of secretion operating at the cell plasma membrane. Porosomes are specialized plasma membrane structures present in all secretory cells, from exocrine and endocrine cells, to neuroendocrine cells and neurons. Since porosomes in exocrine and neuroendocrine cells measure 100–180 nm, and only 20–35% increase in porosome diameter is demonstrated following the docking and fusion of 0.2–1.2 μ m in diameter vesicles, it is reasonable to conclude that secretory vesicles “transiently” dock and fuse at the porosome complex to release their contents to the outside. The discovery of the porosome, an understanding of SNARE-induced membrane fusion, and the requirement of secretory vesicle swelling for expulsion of intravesicular contents, has progressed our understanding of secretion in cells. It has become clear that the secretory process in cells is co-ordinated by a highly regulated and fine-tuned orchestra.

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Figure 1. Porosomes at the plasma membrane in pancreatic acinar cells and the neuron
 (A) AFM micrograph depicting 'pits' (yellow arrow) and 'porosomes' within (blue arrow), at the apical plasma membrane in a live pancreatic acinar cell. (B) To the right is a schematic drawing depicting porosomes at the cell plasma membrane (PM), where membrane-bound secretory vesicles called zymogen granules (ZG), dock and fuse to release intravesicular contents. (C) A high resolution AFM micrograph shows a single pit with four 100–180 nm porosomes within. (D) An electron micrograph depicting a porosome (red arrowhead) close to a microvilli (MV) at the apical plasma membrane (PM) of a pancreatic acinar cell. Note association of the porosome membrane (yellow arrowhead), and the zymogen granule membrane (ZGM) (red arrow head) of a docked ZG (inset). Cross section of a circular complex

at the mouth of the porosome is seen (blue arrow head). (E) The bottom left panel shows an electron micrograph of a porosome (red arrowhead) at the nerve terminal, in association with a synaptic vesicle (SV) at the presynaptic membrane (Pre-SM). Notice a central plug at the neuronal porosome opening. (F) The bottom right panel is an AFM micrograph of a neuronal porosome in physiological buffer, also showing the central plug (red arrowhead) at its opening. It is believed that the central plug in neuronal porosomes may regulate its rapid close-open conformation during neurotransmitter release. The neuronal porosome is an order of magnitude smaller (10–15 nm) in comparison to porosome in the exocrine pancreas.

Figure 2. t-SNAREs and v-SNARE in opposing bilayers interact in a circular array resulting in the formation of conducting channels in presence of calcium

(A) Docked v-SNARE vesicles at t-SNARE-reconstituted lipid membrane. (B) Dislodgement of v-SNARE-reconstituted vesicles from the t-SNARE-reconstituted lipid membrane, exposes the pore-like (C,D) t-v-SNARE ring complexes. (C,D) Three dimension AFM micrographs of SNARE-ring complexes, at low (C) and high resolution (D). A channel at the center of the t-v-SNARE ring complex is clearly visible (Scale bar=100nm). In presence of calcium, recombinant t-SNAREs and v-SNARE in opposing bilayers drive membrane fusion. (E) When t-SNARE vesicles are exposed to v-SNARE reconstituted bilayers in the presence of calcium, vesicles fused. Similarly, vesicles containing nystatin/ergosterol and t-SNAREs when added to the *cis* compartment of a v-SNARE reconstituted bilayer, fusion of the t-SNARE containing vesicles with the v-SNARE membrane is demonstrated as current spikes due to conduction via the nystatin channels. To confirm membrane stability, the transmembrane gradient of KCl is increased to 3M, allowing gradient driven fusion of the nystatin-associated vesicles. (F–H) The size of the t-v-SNARE complex is directly proportional to the size of the SNARE-reconstituted vesicles. (F) Schematic diagram depicting the interaction of t-SNARE- and v-SNARE-reconstituted liposomes. (G) AFM images of vesicle before and after their removal using the AFM cantilever tip, exposing the t-v-SNARE ring complex at the center. (H) Note the high correlation coefficient between vesicle diameter and size of the SNARE complex.