

The story of cell secretion: events leading to the discovery of the 'porosome' - the universal secretory machinery in cells

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- **Introduction**
- **Earlier studies**
- **Discovery of the 'porosome' the cellular secretion machinery**
- **Regulated expulsion of vesicular contents during cell secretion**
- **Conclusion**

Abstract

Cell secretion has come of age, and a century old quest has been elegantly solved. We have come a long way since earlier observations of what appeared to be 'fibrillar regions' at the cell plasma membrane, and electrophysiological studies suggesting the presence of 'fusion pores' at the cell plasma membrane where secretion occurs. Finally, the fusion pore or 'porosome' has been discovered, and its morphology and dynamics determined at nm resolution and in real time in live secretory cells. The porosome has been isolated, its composition determined and it has been both structurally and functionally reconstituted in artificial lipid membrane. The discovery of the porosome as the universal secretory machinery in cells and the discovery of the molecular mechanism of vesicular content expulsion during cell secretion have finally enabled a clear understanding of this important cellular process. This review outlines the fascinating and exciting journey leading to the discovery of the porosome, ultimately solving one of the most difficult, significant, and fundamental cellular process -cell secretion.

Keywords: fusion pore/porosome • cell secretion

Introduction

Secretion is a universal cellular process occurring in simple organisms such as bakers yeast to complex multicellular organisms including humans. Neurotransmission, digestion, immune response, or the release of hormones, each occurs as a result of

cell secretion. Cell secretion involves the transport of vesicular products from within cells to the outside. For example, when we eat digestive enzymes stored in membranous sacs or secretory vesicles in the exocrine pancreas are released from the cell fol-

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lowing fusion of the secretory vesicle membrane at the cell plasma membrane. Similarly for neurotransmission, synaptic vesicles containing neurotransmitters are released when they fuse at the presynaptic membrane. It was therefore logical to believe that secretory vesicles fuse and completely merge and incorporate their membrane at the cell plasma membrane to enable the release of intravesicular contents from cells. In support of this mechanism of cell secretion, it was believed for nearly 50–60 years that, following stimulation of secretion, the flat cell plasma membrane (for lack of a better term at the time) invaginates toward the cytosol, eventually making contact with the secretory vesicle membrane to form the ‘fusion pore’. It was believed that the secretory vesicle then fused with the fusion pore, distending it until the vesicle membrane completely merged with the cell plasma membrane. However such a mechanism of vesicular release could not explain the accumulation of empty or partially empty vesicles following cell secretion. This conundrum was finally solved following the discovery of the ‘porosome’, the permanent cup-shaped structures at the cell plasma membrane in secretory cells where secretory vesicles transiently dock and fuse to release intravesicular contents, and with the discovery that secretory vesicle swelling is required for the regulated expulsion of secretory products during cell secretion. Today we know that following a secretory stimulus, secretory vesicles transiently fuse at the base of porosomes and release intravesicular contents dictated by the turgor pressure generated from the swelling of secretory vesicles. The amount secreted has been demonstrated to be directly proportional to the extent of vesicle swelling. The partially empty vesicle generated then dissociates from the porosome and may be available for another round of fusion, swelling, release, and dissociation at the cell plasma membrane.

Earlier studies

Thin section electron microscopic studies of stimulated secretory cells in the 60’s and the 70’s suggested that secretory vesicles often contact the cell plasma membrane at sites where the intervening cytoplasm has been expressed. It was believed that this results in the formation of the so called ‘penta-

laminae figures’, which gave rise to a state preceding membrane fusion [1–7]. In the late 70’s and the early 80’s ultrastructural freeze-fracture studies on mast cells and the frog neuromuscular junction suggested that, following stimulation of secretion, indentations appear at the plasma membrane. It was believed that indentations form at points closest to the secretory vesicle membrane and in some cases invaginate, giving rise to a tiny ‘dimple or pore’. Some of these indentations were found to have made contact or fused with the secretory vesicle membrane [8, 9]. Morphological studies, using freeze-fracture electron microscopy of stimulated mast cells, showed the presence of plasma membrane depressions ranging in size from 50–100 nm in diameter, which were thought to form following a secretory stimulus [8]. At the neck of these depressions or pores tiny protuberances called intramembrane particles were identified. In both stimulated and unstimulated mast cells small fibrils appeared to reach out to secretory vesicle membrane from the cytoplasm underneath the plasma membrane. The fibers appeared to be 50–70 nm in length and contact secretory vesicles found in close proximity to the plasma membrane (up to 100 nm), although the two membranes were separated by cytosol. Although firmly believed at the time, the total incorporation of the secretory vesicle membrane at the cell plasma membrane could not be demonstrated [8]. Even after fusion of the vesicle membrane with the so-called ‘plasma membrane pore’ and full pore distension, the remainder of the granule membrane was always found well separated from the plasma membrane [8]. Freeze-fracture studies [8, 11, 12] supported the view that fusion pores originated from specific sites at the plasma membrane, perhaps at the fibrillar region. Analogous to the presumed role of clathrin in vesiculation, it was hypothesized that the fibers may regulate pore structure and dynamics [10].

Then during the late 80’s and early 90’s, electrophysiological measurements in mast cells [13–18] and in adrenal chromaffin cells [19] suggested the presence of fusion pores at the cell plasma membrane as a ‘dynamic entity’ [10]. The results from 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 plasma membrane, with conductance similar to a large ion channel

[10]. The fusion pore either irreversibly expands or closes [10, 14, 20, 21]. The latter process, where the fusion pore opens allowing secretory vesicles to fuse momentarily and subsequently closes, was referred to as transient fusion [14, 15, 17, 20, 21]. Patch-clamp measurements of rat mast cells were the first to suggest the existence of such transient fusions [18, 21]. Experimental data from these and other studies, and from theoretical considerations, gave rise to a working model of the fusion pore [10, 22). According to this 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. The resultant structure causes the plasma membrane and secretory vesicle membranes to form a 'hemifusion' intermediate and completion of fusion by formation of an aqueous pore following rupture of the shared bilayer [10].

One needs to be critically aware that these earlier studies, performed primarily using electron microscopy and electrophysiological approaches, were based on the assumption that following stimulation of secretion, the secretory vesicle membrane fully merged with the cell plasma membrane, consequently releasing the intravesicular contents to the outside by diffusion. The incorporated vesicle membrane at the cell plasma membrane is later retrieved by endocytosis. It is precisely due to this hypothetical concept of the secretory process in cells that electron micrographs of various stages of plasma membrane invaginations were often chosen and arranged in sequential order, in support of and giving the impression that the fusion pore is formed as a consequence of dimpling of the cell plasma membrane, which eventually makes contact and fuses with the secretory vesicle membrane. The vesicle membrane then completely merges (flattens out, for lack of a better term, becoming one with the plasma membrane) with the cell plasma membrane and the excess membrane is retrieved by compensatory endocytosis at a later time. Since at the time there was no way of determining membrane structure and dynamics at nm resolution and in real time in live cells, these EM studies and their supporting electrophysiological measurements enforced the dogma and became widely accepted. However, with this model of cell secretion, numerous observations such as the appearance of empty and partially empty vesicles following cell secretion, or no loss in vesicle number following

cell secretion, could not be explained. The complete merger of synaptic vesicle membrane at the presynaptic membrane, and the compensatory retrieval of excess membrane by endocytosis, would be too tardy of a process at the nerve terminal. Moreover, the presence of neurotransmitter transporters at the synaptic vesicle membrane for refilling of spent vesicles would be of little use if vesicles completely fused at the membrane [23, 24]. In the past decade, the discovery of the new cellular structure – *the 'Porosome'*, the universal molecular machinery for cell secretion, has resolved these conundrums.

Discovery of the 'porosome' the cellular secretion machinery

Studies in the early 90's demonstrated for the first time the physical existence of fusion pores or porosomes as permanent structures at the plasma membrane in live secretory cells [25]. Discovery of the porosome, and its function as the universal secretory machinery, is *the* major discovery in cell biology since discovery of the ribosome and determination of its involvement in protein synthesis. The atomic force microscope (AFM) allowed discovery of porosomes at the plasma membrane in live cells and their structure and dynamics uncovered for the first time at nm resolution and in real time in live pancreatic acinar cells undergoing secretion [25]. The AFM images of porosomes in live cells strikingly resemble the proposed hypothetical model of the fusion pore published earlier [10], which was built on the concept of a lipid-enriched proteinaceous fusion pore having defined dimensions [26, 27].

Pancreatic acinar cells are polarized cells which secrete digestive enzymes following a meal. Unlike neurons or neuroendocrine cells, the pancreatic acinar cell is a slow secretory cell, secreting in minutes rather in seconds or milliseconds. The pancreatic acinar cell is a medium sized cell measuring 8–14 μm and is one of the well-characterized cells. Historically, the pancreatic acinar cell has been used in studies on cell secretion. These attributes may have been the primary reason for choosing the pancreatic acinar cell for cell secretion studies using the AFM leading to the discovery of the porosome [25, 28–31]. AFM studies carried out on isolated live pancreatic acinar cells in physiological

buffer demonstrate at the apical plasma membrane a group of circular 'pits' measuring 0.4–1.2 μm in diameter. Within each 'pit' typically 3–4 'depressions', each measuring 100–150 nm in diameter, are present [25]. The 'depressions' turned out to be the fusion pore or porosome, where secretory vesicles transiently dock and fuse to release intravesicular contents to outside of the cell [28–30]. Studies demonstrate that porosomes are present only at the apical plasma membrane in pancreatic acinar cells, where secretion is known to occur. The basolateral membranes of acinar cells are devoid of 'pits' or the porosome structures [25]. When pancreatic acinar cells are exposed to secretagogues (secretory stimulants) such as carbamylcholine or mastoparan, there is a time-dependent increase (20–35%) in the diameter of porosomes followed by a return to their resting size following completion of secretion [25]. The enlargement of porosome diameter and an increase in its relative depth is demonstrated and correlates with increased secretion [25]. When cells are exposed to cytochalasin B, a fungal toxin that inhibits actin polymerization, it results in a 15–20% decrease in porosome size and a consequent 50–60% loss in secretion [25]. These results suggested porosomes to be the long sought-after and elusive fusion pores. The involvement of actin in regulating both the structure and function of porosomes was further demonstrated from these studies. The membrane-bound secretory vesicles in exocrine pancreas, called zymogen granules (ZGs), contain the starch digesting enzyme amylase. Subsequent immuno-AFM studies determined the selective localization of gold-conjugated amylase antibody to porosomes, thereby conclusively demonstrating secretion to occur through these structures [28]. These findings confirmed porosomes to be permanent fusion pore structures at the plasma membrane of live pancreatic acinar cells.

Up to this point, only the topology of porosomes at the live cell surface was available. To determine the morphology of the porosome at the cytosolic compartment, pancreatic plasma membrane preparations suspended in physiological buffer solutions were used [29]. When inside-out membrane preparations in buffer and adhering to a mica surface were imaged using the AFM, scattered circular disks measuring 0.5–1 μm in diameter (the pits) containing inverted cup-shaped structures -the porosomes, were demonstrated [29]. Studies also

demonstrated the association of secretory vesicles and the presence of t-SNAREs at the base of these cup-shaped structures, further confirming them to be porosomes, the cell's secretory machinery [29]. Subsequently, the structure of the porosome was further examined and confirmed using transmission electron microscopy (TEM) [30]. TEM studies also demonstrated porosomes to possess a cup-shaped structure, with similar dimensions as determined by AFM. Using high-resolution TEM, it was demonstrated that porosomes possess a basket-like morphology, with three lateral rings and a number of vertically arranged ridges [29, 30].

Subsequently, the porosome was isolated and its composition determined. Studies demonstrated that SNAP-23, syntaxin 2, actin, α -fodrin, vimentin, the calcium channels $\beta 3$ and $\alpha 1c$, together with the SNARE regulatory protein NSF, and the chloride ion channels *ClC2* and *ClC3*, constitute the porosome complex [29]. Using yeast 2-hybrid analysis, the presence and interaction of some of these proteins within the porosome complex has further been confirmed [32]. In a recent study, the critical role of cholesterol on the structural integrity of the porosome complex has also been reported [33]. The morphology and size of the immunisolated porosome complex (protein backbone of the porosome) has also been determined, using both negative staining electron microscopy and AFM [30]. The immunisolated porosome complex has also been reconstituted into artificial liposomes, and the liposome-reconstituted complex examined using TEM [30]. Transmission electron micrographs of exocrine pancreatic tissue, and liposome-reconstituted porosome complex, demonstrate a 150–200 nm cup-shaped basket-like structure as observed when the porosome is co-isolated with secretory vesicles. Reconstituted porosomes in lipid membranes of an electrophysiological bilayer apparatus demonstrated them to be functional [30]. Addition of isolated ZGs to the *cis* compartment of the bilayer apparatus demonstrated a time-dependent increase in capacitance and conductance and the transfer of the ZG enzyme amylase from *cis* to the *trans* compartment of the bilayer chamber [30]. Furthermore, the chloride channel inhibitor DIDS was found to inhibit current activity in the porosome-reconstituted bilayer. In summary, these studies demonstrated that the porosome in the exocrine pancreas is a 100–150 nm in diameter supramolecular cup-shaped lipoprotein

structure at the cell plasma membrane, where secretory vesicles dock and fuse to release intravesicular contents to the cell's exterior [30].

Similar to the acinar cells of the exocrine pancreas, growth hormone (GH) secreting cells of the pituitary, chromaffin cells, β cells of the endocrine pancreas, mast cells, and neurons all possess porosomes at their plasma membrane, demonstrating porosomes to be the universal secretory machinery in cells. High resolution AFM examination of live GH cells [34], chromaffin cells [35], β -cells [36], mast cells [36], and neurons [37] demonstrated the presence of porosomes [36]. Similar to pancreatic acinar cells, porosomes in GH cells, when stimulated by a secretagogue, results in a 40% increase in diameter, which is cytocholasin-sensitive, demonstrating the involvement of actin in its morphology and function [34]. Analogous to the detection of amylase at the porosome opening in pancreatic acinar cells, gold-tagged growth hormone-specific antibody selectively localizes at porosomes following stimulation of GH secretion [34].

Among the porosomes identified and studied in various secretory cells, the discovery of the neuronal porosome was truly outstanding and fascinating, due primarily to its small size. High resolution transmission electron microscopy and AFM examinations at 5–8 Å resolution revealed the morphology and distribution of neuronal porosomes at the presynaptic membrane [37]. The dynamics of synaptic vesicles docked at the base of neuronal porosomes have also been successfully studied using AFM on isolated inside-out synaptosomal membrane preparations [37]. Neuronal porosomes have further been isolated and their composition determined immunochemically [37]. As in the acinar cells of the exocrine pancreas, isolated neuronal porosomes have been successfully reconstituted into lipid bilayers for both structural and functional analysis using AFM and electrophysiology. Increased capacitance of neuronal porosome-reconstituted membrane following exposure to synaptic vesicles demonstrated the functional reconstitution of isolated neuronal porosomes.

Careful examination of the pre-synaptic membrane in boutons revealed the presence of 8–10 nm cup-shaped porosomes with 40–50 nm synaptic vesicles docked at its base [37]. The porosomes appeared to have a plug-like structure at the center. Each synaptic vesicle was found docked to more than one

porosome. Ultrahigh resolution AFM imaging revealed in 3D, the 8–10 nm in diameter porosomes having a distinct plug at the center, which was also confirmed by high resolution electron micrographs [37]. Similar to the isolation of porosomes from the exocrine pancreas, neuronal porosomes were immunisolated from detergent-solubilized synaptosome preparations. Electrophoretic resolution of the immunisolates, and immunoblot analysis demonstrated the presence of SNAP-25, the P/Q-type calcium channel, actin, syntaxin-1, synaptotagmin-1, vimentin, the *N-ethylmaleimide*-sensitive factor (NSF), the chloride channel CLC-3 and the alpha subunit of the heterotrimeric GTP-binding G_o [38]. To determine if the complete porosome complex was immunisolated, the immunisolate has been reconstituted into lipid membrane prepared using brain dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylserine (DOPS) in a ratio of 7:3 [37]. At low resolution, the AFM showed the immunisolates to arrange in L- or V-shaped structures, which at higher resolution demonstrated the presence of porosomes in patches, similar to what was observed at the presynaptic membrane in intact synaptosomes [37]. Similar to the observations made in electron and AFM micrographs of the presynaptic membrane in synaptosomes, the immunisolated and lipid-reconstituted neuronal porosome demonstrated the presence of a 2 nm in diameter central plug [37]. These observations confirmed the isolated neuronal porosome to be complete.

New technological developments resulting in improved instrumentation and refined experimental procedures, have therefore resulted in the discovery of the 8–10 nm porosomes in neurons, at 5–8 Å resolution [37]. The neuronal porosome although an order of magnitude smaller than those in the exocrine pancreas or in neuroendocrine cells, possess many similarities both in structure and composition [37]. Nature has designed the porosomes as the general secretory machinery, and has fine tuned it to suit various secretory processes in different cell types [37].

Regulated expulsion of vesicular contents during cell secretion

Studies for the first time demonstrated that following the transient fusion of secretory vesicles at

the base of porosomes, vesicular contents are expelled following vesicle swelling, which generates the intravesicular pressure required for expulsion of vesicular contents [38]. These studies [38] further demonstrated that the extent of vesicle swelling was directly proportional to the amount of intravesicular contents expelled, thereby providing cells with the ability to precisely regulate the amount of secretory products to be released. These direct observations of the requirement of vesicle swelling in cell secretion provided an understanding of the appearance of empty and partially empty vesicles following completion of secretion in cells [39–41].

When live pancreatic acinar cells are imaged using AFM at greater force, the profiles of the secretory vesicles lying immediately below the plasma membrane are visible [39]. Within 2.5 min of exposure to a physiological secretory stimulus, secretory vesicles within cells swell followed by a decrease in vesicle size which correlates with intravesicular product release [39]. It has been further demonstrated that different vesicles swell differently following a secretory stimulus, which further allows cells to modulate their secretion. Differential swelling would enable different vesicles to discharge different amounts of their contents, thus explaining why following stimulation of secretion, some secretory vesicles demonstrate the presence of less content than others within the same cell [39]. Further confirmation on the role of vesicle swelling on content expulsion came from studies [39] using lipid-bilayer electrophysiological setup fusion assay. In the electrophysiological bilayer fusion assay, immunoisolated porosomes from the exocrine pancreas were reconstituted into the bilayer membrane of the apparatus and membrane conductance and capacitance continually monitored. When isolated secretory vesicles were added to the *cis* compartment of the bilayer chamber, the secretory vesicles transiently fused at the porosome base, registered as a step increase in membrane capacitance. Even after 15 min of vesicle addition to the *cis* compartment of the bilayer apparatus, little or no release of vesicular content is detected in the *trans* compartment of the bilayer chamber. However, immediately following exposure of secretory vesicles to a swelling stimulus, there is a robust expulsion of vesicular contents into the *trans* compartment of the bilayer chamber [39].

Conclusion

These studies and observations conclusively demonstrated that during cell secretion secretory vesicle swelling is required for the efficient and precise expulsion of intravesicular contents. This mechanism holds true for both slow (pancreatic acinar cell) and a fast (neurons) secretory cell [39]. The discovery of the porosome and an understanding of the molecular mechanism of secretory vesicle swelling [42–44] and its requirement for the expulsion of intravesicular contents have finally provided an understanding of cell secretion at the molecular level.

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