Summary of our work in the past 20 years (1994-2014)

“discovery of the ‘porosome’ -the universal secretory portal in cells”

Porosome-mediated secretion in cells
The contribution made by our laboratory in the past 20 years is the fundamental discovery of a new cellular structure the ‘porosome’ and how this structure is involved in the regulation of fractional release of intravesicular contents from cells during secretion. Porosomes are cup-shaped supramolecular lipoprotein structures at the cell plasma membrane ranging in size from 15 nm in neurons and astrocytes, to 100-180 nm in endocrine and exocrine cells. Neuronal porosomes are composed of nearly 40 proteins, including a number of integral membrane proteins. Elucidation of the porosome structure, its chemical composition, and functional reconstitution into artificial lipid membrane, and the molecular assembly of membrane-associated t-SNARE and v-SNARE proteins in a ring complex resulting in the establishment of membrane continuity to form a fusion pore at the porosome base, has also been demonstrated in great detail by our group. Furthermore, the molecular mechanism of secretory vesicle swelling, and its requirement for intravesicular content release during cell secretion has been determined. Collectively, these studies provide a molecular understanding of fractional release of intravesicular contents from cells during secretion, resulting in a paradigm-shift in our understanding of the secretory process.

Using the atomic force microscope (AFM), our laboratory was the first to break the 5 nm barrier in imaging live cells and to study their structure-function in real time (1997 PNAS 94:316-321; 2003 Science 302:1002-1005), and achieved >1 nm resolution in imaging the neuronal porosome complex in isolated synaptosome preparations (2004 CBI 28:699-708; 2012 J. Proteomics 75:3952-3962). Similarly, the assembly and disassembly of membrane-associated SNARE proteins in buffer at >1 nm resolution was determined in our laboratory over 12 years ago using the AFM (2002 Biophys J. 83:2522-2527; 2005 JACS 127:10156-10157; 2006 JACS 128:26-27).

Introduction
Secretion is a fundamental cellular process in living organisms, from yeast to cells in humans. Secretion is both responsible and required for a variety of physiological activities, such as neurotransmission and the release of hormones and digestive enzymes. Correspondingly, secretory defects in cells are responsible for a host of debilitating diseases. Since the mid 1950’s, it was believed that secretory vesicles completely merged with the cell plasma membrane during secretion, resulting in release of the entire vesicular contents. While this provides one mechanism for secretion, the observation of partially empty vesicles in cells following secretion [Fig. 1] is incompatible with complete vesicle merger, suggesting the presence of an additional mechanism that allows partial discharge of intra-vesicular contents during secretion. This proposed mechanism would require the involvement of a plasma membrane structure, which would serve to prevent the complete collapse of secretory vesicles at the cell plasma membrane. This structure would enable the vesicle to transiently establish continuity with the plasma membrane, expel a portion of its contents, and disengage while remaining partially filled [Fig. 1a,b, (√)]

Fig. 1a. Electron micrographs of rat peritoneal mast cells, in resting (A, extreme left) and following secretion (B, extreme right). Note the fractional release of intravesicular contents following secretion
(B) *J. Exp. Med.* 1975, 142:391-401). This fractional release of intravesicular contents could only be possible via the porosome (P)-mediated transient fusion mechanism shown (√).

The late Prof. Bruno Ceccarelli was a pioneer in the field of ‘transient’ or ‘kiss-and-run’ mechanism of secretory vesicle fusion at the cell plasma membrane enabling fractional release of intravesicular contents. Ceccarelli proposed in 1973 the existence of such a process in cells (1). In 1990 Prof. Wolfhard Almers and F.W. Tse hypothesized that the fusion pore, a continuity established between the vesicle membrane and the cell plasma membrane, results from a “preassembled ion channel-like structure that could open and close” (2). A later 1992 review (3) by Jonathan Monck and Prof. Julio Fernandez, opined that the principal difficulty in observing these structures and fusion pore formation at these structures, was the lack of ultrahigh resolution imaging tools to directly monitor their presence and study their activity in live cells.

**Fig. 1b: Schematic depiction of porosome-mediated cell secretion.**

Porosomes are cup-shaped lipoprotein structures at the cell plasma membrane. Secretory vesicles dock at the porosome base via t-SNAREs present at the porosome base and v-SNAREs present at the secretory vesicle membrane to form a t-/v-SNARE ring complex, establishing continuity between the opposing membranes [fusion pore] through which pressurized intravesicular contents [intravesicular pressure established via active transport of water through aquaporin or water channels (AQP) at the secretory vesicle membrane] are expelled to the outside during cell secretion. Following secretion, the SNARE ring complex is disassembled by NSF-ATP and the fused lipid membrane is cleaved by dynamin-GTP. The resultant partially empty vesicle then dissociates from the cell membrane.

**Discovery of the ‘porosome’**

In the mid 1990’s, motivated by the goal of identifying cellular structures at the plasma membrane involved in the regulated fractional release of intravesicular contents from cells, our laboratory employed the then newly developed technique of atomic force microscopy (AFM) to image the morphology and dynamics of the live pancreatic acinar cell surface at the nanometer scale during secretion. The major breakthrough came in 1996, when circular pit-like structures containing 100-180 nm depressions or pores were observed at the apical plasma membrane of pancreatic acinar cells, where secretion is known to occur. During secretion, the depressions or pores grew larger, returning to their resting size following completion of cell secretion. We first reported these results on January 1, 1997 in the Proceedings of the National Academy of Sciences of the USA (4).

After another five years of study, our next breakthrough was communicated via a number of papers by our research team, with the results establishing that the observed depressions are secretory portals at the plasma membrane in cells. In January 2002 and February 2003, we reported in two studies, one in Cell Biology International in collaboration with Prof. Douglas J. Taatjes, and the other in Biophysical Journal, that following stimulation of cell secretion, gold-conjugated amylase antibodies (amylase being one of the major intravesicular enzymes secreted by pancreatic acinar cells) accumulate at depressions, establishing that depressions are the long sought-after secretory portals in cells (5,6). The study reported in the Biophysical Journal (6), further demonstrated the presence of t-SNAREs at the porosome base facing the cytosol, firmly establishing depression
structures to be secretory portals where vesicles transiently dock and fuse for intravesicular content release during secretion (6). In March 2002, our laboratory in collaboration with Prof. Lloyd L. Anderson reported in the journal Endocrinology (7) on the depressions and their dynamics at the cell plasma membrane in growth hormone (GH) secreting cells of the pig pituitary gland, and on the accumulation of GH-immuno-gold at depressions following secretion. In the same year in a separate study, in collaboration with Prof. Arun Wakade and Prof. George Pappas, we reported the depression structure and their dynamics in chromaffin cells (8). In September 2003 (9) following immunoisolation of these structures from acinar cells of the exocrine pancreas, our research team finally determined their composition, and we managed to functionally reconstitute the isolated porosomes into artificial lipid membranes (9). In the same study (9), morphological details of the porosome complex associated with docked secretory vesicle were revealed using electron microscopy (9) [Fig. 2A].

![Fig. 2. Porosomes in the exocrine pancreas (A,B) and neurons (C,D).](image)

(A) An electron micrograph of a single porosome at the apical plasma membrane (PM) of a pancreatic acinar cell is shown. Note the porosome membrane (POM, yellow arrowhead) associated with the membrane of a secretory vesicle (ZGM). A circular ring structure (blue arrowhead) forms the neck of the porosome complex. (B) An atomic force micrograph of the apical surface of a live pancreatic acinar cell, demonstrating the presence of four openings or porosomes (one indicated by the yellow arrowhead). Porosomes in the exocrine pancreas range in size from 100-180 nm in diameter. (C) An electron micrograph of a neuronal porosome (red arrowheads) in association with a synaptic vesicle (SV) at the presynaptic membrane (Pre-SM) of the nerve terminal is demonstrated. Note a central plug in the porosome complex. (D) An atomic force micrograph of a neuronal porosome at the presynaptic membrane in a live cell. Note the central plug (red arrowhead). The neuronal porosome is an order of magnitude smaller (10-17 nm in diameter) in comparison to the porosome in the exocrine pancreas. [Images obtained from: Proc Natl Acad Sci 94:316-321 (1997); Biophys J 85:2035-2043 (2003); Cell Biol Int 28:699-708 (2004); J Microscopy 232:106-111 (2008).]

![Fig. 3. Docked synaptic vesicles at neuronal porosome complex in the presynaptic membrane of the nerve terminal, observed using atomic force microscopy (AFM), electron microscopy (EM), and small angle X-ray solution scattering (SAXS).](image)

(a) AFM micrograph obtained in fluid of a synaptic vesicle (SV) docked at the cup-
shaped porosome complex (P) at the cytosolic compartment of the presynaptic membrane. Note the 35 nm SV docked to a 15 nm porosome complex. (b) An EM micrograph of a 35 nm SV docked to a 15 nm P at the presynaptic membrane [Cell Biol. Int. 28:699-708 (2004)]. Note the central plug of the porosome complex in the electron micrograph. (c) The averaged SAXS 3-D structure of synaptic vesicle (purple) docked at the cup-shaped neuronal porosome complex (pink) at the presynaptic membrane in isolated synaptosomes, is presented [Micron (published on-line ahead of print) http://dx.doi.org/10.1016/j.micron.2013.10.002. (2013)]. Note that AFM, EM, and SAXS, all demonstrating similarity in the docking and interaction of synaptic vesicles with the neuronal porosome complex at the presynaptic membrane.

In the past nearly 20 years following the initial observation of the porosome employing a combination of approaches such as AFM, biochemistry, electrophysiology, conventional EM, mass spectrometry, and X-ray solution scattering analysis, our laboratory discovered these structures to be present in all secretory cells examined, including, perhaps most significantly, neurons [Fig. 2C,D; Fig. 3] (10-13). Our studies have established this new supramolecular lipoprotein structure at the cell plasma membrane to be secretory portals that performs the specialized function of fractional discharge of intravesicular contents from cells during secretion. This specialized structure was named by us the ‘porosome’ (11-13). The discovery of the porosome provides new understanding on how cells secrete.

The significance of the identification of the porosome is reflected by the great number of publications on porosomes and associated transient fusion mechanism accompanied by fractional discharge of intravesicular contents from cells. A few representative publications are: In 2011 the porosome in hair cells was discovered by Prof. Dennis Drescher [Cell Biol. Int. Rep. (2011) 18:31-34.]; in 2010 porosome in RBL-2H3 and BMMC cells were reported by Prof. Gang-yu Liu [J. Phys. Chem. B. (2010) 114:5971-5982.]; porosomes in the exocrine pancreas have been further elaborated by the groups of Prof. Elshennawy in 2011[J. Am. Sci. (2011) 7:835-843.] and Prof. Constantin Craciun in 2013 [Micron (2013) 44:137-142.]

Fig. 4. Docked synaptic vesicles (SV) at the base of a cup-shaped neuronal porosome complex (P) in the presynaptic membrane (PSM) of the nerve terminal, observed using electron microscopy. Micron (2012) 43:948-953. Courtesy of Prof. M. Zhvania.


Membrane fusion and secretory vesicle volume regulation for fractional release of intravesicular contents from cells: In the past two decades, our research team has also made seminal contributions to our understanding of the fundamental molecular process involved in Ca++ and SNARE-mediated membrane fusion (14-22), and on
secretory vesicle volume regulation required for the regulated fractional release of intravesicular contents (23-29) via the porosome during cell secretion. This process enables cells to precisely regulate the discharge of a portion of their intravesicular contents during a secretory episode, while retaining full integrity of both the vesicle membrane and the cell plasma membrane.

**Role of SNAREs in membrane fusion:** In 1988 Prof. Richard Scheller discovered a secretory vesicle associated membrane protein (VAMP-1 or v-SNARE) (30), and then in 1992 he and his team discovered another important protein present in the plasma membrane called syntaxin. Syntaxin is one of the two target SNARE or t-SNARE proteins (31). Then in 1989 Prof. Michael Wilson discovered SNAP-25, the other t-SNARE protein (32). Understanding the properties of the three SNARE proteins in membrane fusion requires a molecular understanding of their interactions, with the different SNARE proteins being present in opposing membranes (VAMP-1 in secretory vesicle membrane, and syntaxin and SNAP-25 in the cell plasma membrane). Since SNAREs are membrane-associated proteins, crystals of membrane-associated SNARE complex are required for X-ray crystallography. This has not been possible. To circumvent issues associated with the solubility of membrane associated SNAREs, Prof. Axel Brunger and Prof. Reinhard Jahn in 1998 truncated the hydrophobic membrane anchoring domains of syntaxin and VAMP, to obtained crystals of a non-membrane associated t-/v-SNARE complex. Utilizing X-ray crystallography, Professors Brunger and Jahn determined the atomic structure of the soluble SNARE complex at 2.4Å, which they reported in Nature (33). It was unclear however, whether the structure of the resolved soluble SNARE complex was identical to the native membrane-associated SNARE complex.

To address this issue, we carried out high-resolution AFM studies combined with electrophysiological measurements. In a study reported in the Biophysical Journal in 2002 (14), our group demonstrated that in the absence of membrane association, SNAREs fail to appropriately bind to each other or establish continuity between the opposing bilayers in presence of calcium. We demonstrated that VAMP-1 proteins present in one membrane interact with syntaxin and SNAP-25 proteins present in an opposing membrane, and assemble in a rosette or ring configuration, establishing continuity between the opposing bilayers in the presence of calcium. While it had been hypothesized that the interaction between t-SNAREs and v-SNARE present in opposing bilayers form rosette or ring structures (34), the experimental confirmation of this was first reported by our group in the 2002 in the Biophysical Journal paper (14) [Fig. 5], and further established using high resolution EM (17-22).

**Fig. 5.** Actual t-/v-SNARE ring complexes or rosettes formed following the interaction between membrane-associated t-SNAREs and v-SNAREs. Top panel is a schematic drawing depicting the interaction between t-SNAREs and v-SNAREs in opposing vesicles. AFM micrographs of the actual SNARE complex rings or rosettes are presented in the top right panel, in the middle panel, and in the right lower panel (14,15).
This SNARE rosette arrangement between opposing bilayers during membrane fusion is now widely accepted as the fundamental structure of the t-/v-SNARE complex associated with membrane fusion and cell secretion, and is widely published (35,36).

**Role of Ca\(^{2+}\) in membrane fusion:** In the 1970’s, the late Prof. Demetrious Papahadjopoulos had proposed the involvement of inter-membrane Ca\(^{2+}\)-phospholipid complex in the fusion of opposing lipid membranes (37). To determine the involvement of Ca\(^{2+}\) in membrane fusion at the atomic level, we performed X-ray diffraction studies involving t- and v-SNARE reconstituted liposomes (15). Results from this study demonstrated that SNAREs overcome the repulsive forces between the opposing negatively charged lipid membranes to bring them within a distance of 2.8Å (15). We therefore concluded that if calcium was involved in the bridging of opposing bilayers via oxygens of the phospholipid head groups, calcium must be present at the site where the t-SNARE vesicles and v-SNARE vesicles make contact. T-SNARE vesicles and v-SNARE vesicles complexed in the absence of calcium, would therefore fail to establish continuity between the opposing bilayers since hydrated calcium (with 6 water molecules surrounding it) measuring nearly 7Å would be unable to fit within the 2.8Å spacing separating the two opposing membranes. In 2004, this hypothesis was tested and confirmed experimentally by us (16). From these results we further hypothesized that following bridging of the opposing phospholipids by hydrated Ca\(^{2+}\), the loss of coordinated water associated with the calcium ion as well as those associated with the oxygens of the phospholipid head groups must result in local dehydration, lipid mixing, and membrane fusion. We tested this hypothesis using blind molecular dynamic simulations involving dimethyl phosphate (DMP), calcium, and water molecules (13). Confirming this hypothesis, results from the study demonstrated that hydrated Ca\(^{2+}\) is capable of bridging phospholipid head groups, and that this process results in the expulsion of water from both phospholipid head groups and the calcium ion (38). The simulation further demonstrated that the distance between the anionic oxygens in DMP bridged by calcium is 2.92Å, which is in close agreement with the 2.8Å reported from X-ray diffraction measurements (15). These findings provide new insights into our understanding of the chemistry of membrane fusion.

**Secretory vesicle volume increase and regulated content expulsion during cell secretion:** In the early 1990’s it was reported that secretory vesicles undergo an increase in volume during cell secretion (39,40). However, the molecular mechanism underlying volume regulation of secretory vesicles, and the role of this volume increase on secretory vesicle function during cell secretion, was poorly understood. Our studies showed that water channels or aquaporins in conjunction with several ion channels present at the secretory vesicle membrane regulate the vesicle volume through GTP-binding G-proteins (23-29). The role of various ion channels at the secretory vesicle membrane was also demonstrated using single vesicle patch studies (41). In 2004 we reported that secretory vesicle volume increase is a requirement for the regulated release of vesicular contents from cells (25). The relative increase in vesicle volume during cell secretion is proportional to the fraction of the intravesicular contents released.

In summary, our studies in the past 20 years demonstrate the presence of a new cup-shaped lipoprotein structure at the cell plasma membrane called porosome, -the universal secretory portals in cells, and elucidated how the porosome is involved in the regulate fractional release of intravesicular contents from cells with exquisite precision involving membrane fusion and secretory vesicle volume regulation. We have isolated the porosome from a number of secretory cells including neurons, determined its composition, functionally reconstituted it in lipid membrane, and determined its dynamics and high-resolution structure using a variety of approaches including atomic force microscopy, electron microscopy, and X-ray solution scattering. Complementing the regulation of the porosome function, our studies have further contributed to our understanding of membrane fusion and secretory vesicle volume regulation, both required for the regulated fractional release of intravesicular contents during cell secretion. These results provide for the first time a molecular understanding of the regulated fractional release of intravesicular contents from cells during secretion. Recent studies by our group using mass spectrometry, demonstrate interaction between the cystic fibrosis trans-membrane conductance regulator (CFTR) and the porosome complex in human airways epithelia,
shedding light on the possible regulatory role of CFTR on the quality of mucus secretion via the porosome complex (42). Results from this study provide critical insights into the etiology of CF disease and for potential therapies. Similarly, recent studies using mass spectrometry provide some understanding of the lipidome of the neuronal porosome complex (43), and the role of Hsp90 in porosome assembly and function (44), enabling further understanding of the porosome structure-function (45). Currently, to further our understanding of the porosome, the major focus of our laboratory is to determine the assembly, structure, and conformation of the neuronal porosome using single particle electron crystallography, cryoelectron microscopy, X-ray solution and neutron scattering, and other approaches.

References:


