Mechanisms of Rupture of Mucin Vesicles from the Slime of Pacific Hagfish (*Eptatretus stoutii*): Roles of Inorganic Ions and Aquaporin Water Channels

by

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ABSTRACT

MECHANISMS OF RUPTURE OF MUCIN VESICLES FROM THE SLIME OF PACIFIC HAGFISH (EPTATRETUS STOUTII): ROLES OF INORGANIC IONS AND AQUAPORIN WATER CHANNELS

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Pacific hagfish (Eptatretus stoutii) slime mucin vesicles are released by holocrine secretion with membranes that remain intact until the vesicle contacts seawater and ruptures. This thesis is an investigation of the mechanisms that drive mucin vesicle rupture for mucin release. Using isolated mucin vesicles collected from the slime glands of the hagfish, I tested the effects of a variety of solutions and drugs on vesicle rupture. I found that there are two categories of mucin vesicle that differ in their sensitivity to calcium ions, and that calcium-dependent vesicle rupture was inhibited with anion channel inhibitors. I also found that vesicle swelling rate was reduced by the aquaporin inhibitor mercuric chloride. Together, these data suggest that mucin vesicle rupture is partially dependent on the movement of chloride ions from seawater through calcium-activated anion channels and the rapid influx of water through aquaporin-like proteins in the vesicle membrane.
Dedicated with love to my nágyapa,

Dr. Ferenc Herr (1922 – 2011)
ACKNOWLEDGEMENTS

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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AQP</td>
<td>Aquaporin</td>
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<td>bp</td>
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<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
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<td>Calcium-activated chloride channel</td>
</tr>
<tr>
<td>CaSR</td>
<td>Calcium-sensing receptor</td>
</tr>
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<td>DIDS</td>
<td>4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate</td>
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<td>Dimethyl sulfoxide</td>
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<td>EbAQP4</td>
<td><em>Eptatretus burgeri</em> aquaporin 4</td>
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<tr>
<td>fps</td>
<td>Frames per second</td>
</tr>
<tr>
<td>GMC</td>
<td>Gland mucous cell</td>
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<tr>
<td>GTC</td>
<td>Gland thread cell</td>
</tr>
<tr>
<td>HMM</td>
<td>Hidden Markov models</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>NPA</td>
<td>Asparagine, proline, alanine motif</td>
</tr>
<tr>
<td>PGG</td>
<td>Penta-O-galloyl-β-D-glucose hydrate</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
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<tr>
<td>SB</td>
<td>Stabilization buffer</td>
</tr>
<tr>
<td>SSW</td>
<td>Simplified seawater (10 mM CaCl(_2) + 535 mM NaCl)</td>
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<tr>
<td>TMAO</td>
<td>Trimethylamine oxide</td>
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CHAPTER 1: GENERAL INTRODUCTION
1.1 Mucus: biochemical and physical properties

Mucus is a mixture of high-molecular weight glycoproteins, ions, water, and an assortment of proteins that together form a hydrophilic, viscoelastic polymer gel (Aitken and Verdugo, 1989; Thornton and Sheehan, 2004; Verdugo, 1990). Mucus has a variety of functions including respiration, ionic and osmotic regulation, reproduction, pathogen resistance, locomotion, and protection (Shephard, 1994). Mucins are the glycoproteins that give mucus its characteristic properties, and are composed of a peptide core rich in serine and threonine residues that provide attachment sites for short polysaccharide chains with sulphate and sialic acid terminals that give them a polyanionic character at neutral or near-neutral pH (Thornton and Sheehan, 2004; Verdugo, 1990; Verdugo, 1991). In typical mucins, the peptide core represents approximately 20% of the total weight of the molecule, with the carbohydrate side chains representing the remaining 80% (Thornton and Sheehan, 2004). The physical and chemical properties of mucins cause them to swell and interact with one another through entanglement or through covalent disulfide bonds to form a complex gel-like network when exposed to water (Shephard, 1994; Verdugo, 1990). It was long assumed that since disulfide bond-cleaving agents cause mucins to disperse, mucin networks were held together by cross-links, however the manner in which mucin chains diffuse suggests they are not restricted by the presence of disulfide bonds. This entangled-network model suggests that mucin networks are held together by inter-chain tangles, and that disulfide bonds exist in the protein backbone of the molecule. Since diffusion times of polymer chains are proportional to the square of the chain length, in this model, cleaving of disulfide bonds would result in shorter mucin chains, and therefore much faster dispersion of mucus networks (Verdugo, 1990).

Mucus is produced by goblet cells and mucous cells, and is packaged into membrane-
enclosed granules or vesicles (these terms are used in the literature interchangeably, but for this thesis, I will use the term “vesicle” unless referring to specific studies) that are produced by the Golgi system. In vertebrates, these cells are typically found in the epithelium of respiratory, digestive, and reproductive tracts, as well as in the skin of animals found in aquatic or semi-aquatic environments such as fish and amphibians (Deyrup-Olsen and Luchtel, 1998). The typical method of mucus secretion is by exocytosis, a process in which the granule or vesicle containing the mucous substances docks with the apical plasma membrane of the secretory cell, and forms a fusion pore that expands and allows the free exchange of water and electrolytes between the interior of the vesicle and the extracellular space. This movement of water and ions results in a phase transition of the intravesicular contents, and the release of the secretory cell products (Deyrup-Olsen and Luchtel, 1998; Verdugo, 1991). Although it is unclear whether swelling of intracellular vesicles occurs before or after the fusion pore is formed, in the case of mucus exocytosis, the decondensation and release of the contents of a mucin vesicle is triggered by the movement of water and ions through the pore, rather than the pore being formed by the swelling vesicle (Verdugo, 1990; Verdugo, 1991).

The decondensation of the polyanionic mucin matrix is a process that is governed by a Donnan equilibrium, and follows first-order kinetics (Aitken and Verdugo, 1989; Tam and Verdugo, 1981). A Donnan equilibrium-controlled process is one in which a semi-permeable membrane limits the movement of some charged particles but not others, so that the movement of unconstrained particles along their concentration gradients may result in differences in electrical potential across the membrane (Donnan, 1924). In the case of a polymer gel, or a mucus gel, the polyanionic macromolecules – mucins – that form the gel act like a semi-permeable membrane that limits the movement of certain particles, but simultaneously act as the
constrained polyanion that induces the osmotic drive (Tam and Verdugo, 1981). The hydration of the mucus gel is therefore determined by the charge density of the mucins, and the movement of water and ions into the mucin network (Aitken and Verdugo, 1989).

1.2 The “Jack-in-the-Box” hypothesis of mucin exocytosis

The highly negative charge that the polysaccharide side chains confer to a mucin molecule gives a mucin network its gel-like properties and causes it to swell in a more rapid manner than simple diffusion would allow, since mucin molecules naturally repel one another. However, this property also poses problems for mucin condensation within the mucous cell vesicles, prior to product release. Verdugo et al. (1987a) hypothesized that mucin condensation within the mucin vesicle was made possible by the presence of cations such as Ca\(^{2+}\), which would act as charge-shielding ions and prevent negatively-charged mucin molecules from repelling one another, and allow them to be packed more densely. In this model, the rapid expansion of the mucin network following exocytosis results from the removal or replacement of Ca\(^{2+}\) with a less effective charge-shielding ion, which then leaves the mucins free to electrostatically repel one another (Verdugo et al., 1987b; Verdugo, 1991).

Named the “Jack-in-the-box” mechanism of mucin exocytosis, this hypothesis is supported by the observation that the release of mucins from the giant mucous granules of the terrestrial land slug *Ariolimax columbianus* is preceded by a release of Ca\(^{2+}\), suggesting that the removal of Ca\(^{2+}\) from within the granule triggers mucin network expansion (Verdugo et al., 1987b). Verdugo (1991) originally suggested that exchange of Ca\(^{2+}\) for Na\(^{+}\) through the secretory pore of a goblet cell drives the swelling of mucous granules. However, Nguyen et al. (1998) demonstrated that Ca\(^{2+}\) oscillations in mucin granules from rabbit goblet cells are dependent on
ion exchange through an inositol-1,4,5-trisphosphate sensitive Ca\(^{2+}\) channel and an apamin-sensitive Ca\(^{2+}\)-activated K\(^+\) channel. It is therefore possible that K\(^+\) exchange for Ca\(^{2+}\) may also play a role in mucin decondensation during exocytosis. The “Jack-in-the-Box” mechanism of exocytosis is not, however, restricted to mucus secretion through exocytosis. In fact, the giant mucous granules produced by *A. columbianus* that were used to support the “Jack-in-the-Box” hypothesis are extruded from the cell intact by apocrine secretion, and are disrupted after the granule has left the cell (Deyrup-Olsen et al., 1983). Although exocytotic secretion of mucus is the most commonly employed strategy, there are some systems in which mucus secretion is achieved by other means, including apocrine and holocrine secretion.

1.3 Roles of ions and water flux in the secretion of mucus and other membrane-bound structures

The secretion of mucous and other exocytotic vesicles has been shown to be associated with ion movement through a variety of transmembrane channels. For example, Cl\(^-\) movement is well known to be associated with mucus production due to symptoms of airway diseases caused by Cl\(^-\) channel dysfunction. Cystic fibrosis is linked to the dysfunction or absence of the cystic fibrosis transmembrane regulator (CFTR), a cAMP-dependent Cl\(^-\) channel. While dysfunction of CFTR is associated with airway surface liquid depletion causing impaired ciliary function and the impaired ability of the mucous layer to adhere to the epithelium (Williams et al., 2006), its presence in mucous vesicle membranes suggests that it also plays a role in intravesicular matrix organization, including the regulation of intravesicular ion concentrations (Bacconnais et al., 2005; Perez-Vilar, 2007). Similarly, in asthmatic patients, an increase in Ca\(^{2+}\)-activated Cl\(^-\) channel (CaCC) expression has been linked to mucus over-production (Hoshino et al., 2002), and CaCC expression has been found in a variety of exocrine secretory epithelia in mouse models.
Ion channels have been localized to the membranes of a variety of other intracellular secretory vesicles, and ion movement, together with water movement, has been linked to swelling and exocytosis. Jena et al. (1997) found evidence for GTP-mediated opening of Cl\(^-\) and K\(^+\) channels in the zymogen granule membrane, and hypothesized that it was associated with a net influx of ions and water into the granule lumen, causing the granule to swell and fuse with the plasma membrane prior to exocytosis. In rat synaptic vesicles, GTP-induced swelling has been linked to facilitated water transport through an aquaporin (AQP) water channel, AQP6, due to vH\(^+\)-ATPases in the vesicle membrane, which cause intravesicular acidification (Jeremic et al., 2005; Shin et al., 2010). Aquaporins, as water-selective membrane pores, are of particular interest to understanding mucus secretion because they provide a 10- to 100-fold higher capacity for water permeation than a membrane lacking them (Agre et al., 2002). Since vesicle swelling may be central to plasma membrane fusion and subsequent exocytosis (Kelly et al., 2004), it follows that facilitated water influx through water-selective channels would be an asset to such a strategy. In addition to synaptic vesicles, AQPs have been found in the membranes of secretory vesicles from the human small intestine (Li et al., 2005), in rat parotid gland secretory vesicles (Matsuki et al., 2005), and in zymogen granules of pancreatic acinar cells (Cho et al., 2002; Itoh et al., 2005), where they are thought to be important for the swelling of the vesicle and the release of their contents.

1.4 Hagfish slime, a unique mucous substance, a unique mode of secretion

Hagfish (Craniata, Hyperotreti) are jawless, marine chordates that are characterized by having a large notochord without any bony or cartilaginous vertebrae, a cartilaginous skull, a
single nostril through which water is brought into the internal gill pouches, and large 
ventrolateral slime glands (Fernholm, 1998; Janvier, 1996). They are well known for their unique 
defense mechanism of producing alarming amounts of slime when disturbed, and it is this slime 
that is of particular interest for the unique strategy of mucus release that is employed for its 
production. The slime is produced by many slime glands that are located ventrolaterally along 
both sides of the hagfish’s body, in each body segment (Newby, 1946; Spitzer and Koch, 1998). 
The slime is a combination of mucin-like glycoproteins and intermediate filament-based protein 
threads that mix together to form a sieve-like arrangement of fibres and mucus that entraps and 
slow down seawater, forming a slippery, mucous mass (Fudge et al., 2005). It is thought that 
this slime is primarily a defense mechanism against gill-breathing predators, as its fibrous 
threads allow it to become caught on filamentous structures such as gills (Lim et al., 2006). 
Recently, Zintzen et al. (2011) directly observed multiple instances of hagfish repelling large, 
gill-breathing predators such as sharks with their slime, and provided some evidence that the 
slime may also be used for prey capture when hagfish exhibit predatory behaviour. Hagfish slime 
may also provide some defence against microbial pathogens, as higher levels of innate immune 
substances such as alkaline phosphatase, lysozyme, and cathepsin B are found in the hagfish’s 
extruded slime than in its epidermal mucus (Subramanian et al., 2008).

The protein threads and mucins that make up hagfish slime are produced by gland thread 
cells (GTCs) and gland mucous cells (GMCs) respectively, both of which are found in the slime 
glands (Figure 1.1). GTCs are prolate ellipsoidal cells that have a mean size of 64 x 123 µm 
along the minor and major axes (Spitzer and Koch, 1998). Each GTC produces a single, large, 
tapered thread that is assembled from multiple intermediate filaments, and is tightly coiled into a 
thread skein bundled within the confines of the GTC plasma membrane (Downing et al., 1981a;
The hagfish slime gland (a) contains two unique cell types: (b) irregularly shaped gland mucous cells (GMCs) and ellipsoidal gland thread cells (GTCs). Each GMC is packed with many membrane-bound, disc-shaped mucin vesicles that are approximately 7 µm along the major axis, while each GTC produces a single, large, tapered thread that is tightly coiled into a thread skein. When stimulated, the muscle surrounding the gland contracts (c), and the products of these two cell types are expelled forcefully through the gland duct. As the cells pass through the narrow duct (e), their plasma membranes are stripped away, and the cell contents – mucin vesicles and thread skeins – are released into the external environment intact. When the slime gland exudate contacts seawater (d), the thread skeins unravel, and mucin vesicles swell and rupture, releasing the mucin molecules they contain. Together, mucins and unravelled threads entrap water to form the hagfish’s defensive slime.
Koch et al., 1991; Spitzer and Koch, 1998). GMCs are irregularly shaped cells that are approximately 72-93 µm in diameter, and each one is packed with a multitude of membrane-bound, disc-shaped mucin vesicles that are approximately 7 µm along the major axis (Luchtel et al., 1991b; Spitzer and Koch, 1998). Mature slime is 99.996% seawater, 0.002% threads, and 0.0015% mucin by weight, and is produced when these two cell products – threads and mucins – are released from the gland and contact convectively mixing seawater (Fudge et al., 2005). The interaction of both components is crucial for slime formation as it has been shown that whole slime will not form from mucins or threads alone (Koch et al., 1991). Furthermore, the disruption of the mucin network by disulfide bond-cleaving agents such as dithiothreitol will delay the onset of whole slime formation, reduce the degree of thread skein unravelling, and reduce the volume of whole slime formed by disrupting the mucin network (Fudge et al., 2005; Koch et al., 1991; Winegard and Fudge, 2010). In Atlantic hagfish (Myxine glutinosa), it has been suggested that thread skeins uncoil only when the mucins released from the vesicles form elongated strands that attach to the coiled thread skeins and allow the transmission of hydrodynamic forces to the threads (Winegard and Fudge, 2010).

While the mucus produced by small epidermal mucous cells to coat the surface of the hagfish’s skin is released via merocrine secretion through small channels connecting the mucous cells to the surface of the epidermis, the mucus produced by GMCs in the slime gland is released from the gland by holocrine secretion (Patzner et al., 1982; Spitzer and Koch, 1998). When the muscle fibres surrounding the slime gland contract, GTCs and GMCs are expelled toward the gland duct, which, having a width of approximately 100 µm, is just wide enough to allow the passage of one cell at a time (Downing et al., 1981b; Spitzer and Koch, 1998). This restriction causes the cell membrane to be stripped from the cells, and the cell contents – thread skeins and
mucin vesicles – are released into the external environment by the holocrine mode (Koch et al., 1991; Spitzer and Koch, 1998). In contrast to exocytotic mucin release, when hagfish slime mucins are expelled from the slime gland, they are still encapsulated in their vesicle membrane. It is only after they come into contact with seawater that the vesicles begin to swell, and the mucins are released to interact with the thread skeins, causing them to unravel to lengths of up to 10-17 cm (Fudge et al., 2005; Luchtel et al., 1991b). The slime mucins themselves differ from typical vertebrate mucins in that they contain only 12% carbohydrate by dry mass in contrast with the typical 80% carbohydrate content of other mucins, and have a high sulfate composition relative to carbohydrate, as well as high proline and valine content, and a low serine content (Salo et al., 1983; Thornton and Sheehan, 2004).

Luchtel et al. (1991b) demonstrated that hagfish slime mucin vesicles are composed of a single lipid bilayer that encapsulates the mucin matrix, and tested the permeability characteristics of the vesicle membrane by adding slime exudate to a variety of solutions in order to determine which solutions caused slime formation, and which solutions were able to stabilize the exudate. They found that slime will form when exudate is placed in 1 M solutions containing univalent anions (e.g. chloride, nitrate, bicarbonate), regardless of the valency of the cations present, but will not rupture when placed in 1 M solutions containing polyvalent anions (e.g. sulphate, citrate, phosphate) (Luchtel et al., 1991b). Likewise, Salo et al. (1983) found that slime exudate was best stabilized by sodium citrate at concentrations between 0.5-1.0 M, but divalent tartrate, and monovalent acetate were also able to stabilize exudate at a concentration of 3 M. Given these characteristics, Luchtel et al. (1991b) speculated that the hagfish mucin vesicle membranes were permeable to univalent anions and to cations of all valencies. They hypothesized that rupture occurs when ions from seawater enter the vesicle along their diffusion gradients, and cause a
secondary influx of water into the vesicle that hydrates the mucin gel and results in the swelling and rupture of the vesicle (Luchtel et al., 1991b). However, whole slime formation is dependent upon high ionic strength and the presence of Ca$^{2+}$ such that a much smaller mass of slime will form in NaCl solutions that lack Ca$^{2+}$ than in solutions that contain it (Fudge, 2002). Whether this is due to a requirement of the mucin component or the thread component is not clear.

Hagfish are osmoconformers with little capacity to regulate Na$^+$ and Cl$^-$ concentrations, and have a plasma osmolarity and ionic composition similar to that of seawater (Currie and Edwards, 2010). However, there is evidence that the osmolyte composition in the slime gland is, in fact, regulated (Herr et al., 2010). In the slime gland of *M. glutinosa*, the fluid component of slime exudate, which consists of intra- and extracellular fluid from GTCs and GMCs, is high in methylamines such as betaine and trimethylamine oxide (TMAO), and low in Ca$^{2+}$ (Herr et al., 2010). Interestingly, approximately half of mucin vesicles exposed to betaine and TMAO can be stabilized with solutions composed of these methylamines, suggesting that their presence helps counteract protein destabilization within the slime gland (Herr et al., 2010). It is unclear why some vesicles rupture in these solutions, and other do not, but in the same study, Herr et al. (2010) demonstrated that in a sample of isolated vesicles exposed to seawater, there appears to be a dichotomy of swellings rates, with some vesicles swelling very slowly, and other swelling quite rapidly. Whether these findings together suggest that there is more than one type of mucin vesicle released from the slime gland has yet to be determined.

The mechanisms behind hagfish slime mucin deployment have still only been partially described, but it appears as though mucin vesicle rupture is the result of the interaction of the vesicle with some ions in seawater, as well as mucin hydration and expansion by water influx. It is tempting to compare hagfish slime mucin vesicle deployment to the deployment of the giant
mucous granules of *A. columbianus* as the two strategies are, in some ways, similar, but there are many ways in which they differ. For example, the giant mucous granules of *A. columbianus* are bound by two lipid bilayers, one originating from the Golgi system, and one from the plasma membrane (Luchtel et al., 1991a). In terms of vesicle or granule rupture, hagfish mucin vesicles show greater ion sensitivity, while slug mucous granules display sensitivity to rupture-inducing agents such as adenosine triphosphate (ATP) (Luchtel et al., 1991a). However, even among related species of pulmonates, the mechanism of mucin vesicle rupture varies, such that in some species of slug, ATP-dependent rupture may be related to the activation of \( \text{Ca}^{2+} \)-channels, or to the maintenance of a transmembrane proton gradient (Deyrup-Olsen, 1996). The mechanism of hagfish slime mucin vesicle rupture may simply represent yet another unique strategy of mucin deployment.

1.5 **Thesis objectives**

In this thesis, I asked the question: what mechanisms govern hagfish slime mucin vesicle rupture? Using stabilized and isolated mucin vesicles from the Pacific hagfish (*Eptatretus stoutii*), I approached this question by looking at how characteristics of the mucin vesicle membrane regulate swelling and rupture behaviour of mucin vesicles.

In Chapter 2, I tested the hypothesis that mucin vesicle rupture is dependent on the movement of specific ions from seawater into the mucin vesicles via ion channels in the membrane. This hypothesis predicts that mucin vesicle rupture should be prevented in the absence of these required ions, or if the ion channels upon which rupture is dependent are blocked, and was tested by exposing stabilized vesicles to a variety of seawater-strength solutions, and treating vesicles with the anion channel inhibitor DIDS (4,4′-
Diisothiocyanatostilbene-2,2’-disulfonic acid disodium salt hydrate). Finding that mucin vesicle rupture was in part dependent upon Ca\(^{2+}\), I hypothesized that Ca\(^{2+}\)-dependent rupture was due to either (a) Ca\(^{2+}\) channels in the vesicle membrane, or (b) Ca\(^{2+}\)-activated channels for other ions. These alternative hypotheses predicted (a) that vesicle rupture should be inhibited by Ca\(^{2+}\) channel inhibitors, and (b) rupture should be prevented by chemically inhibiting Ca\(^{2+}\) receptors when Ca\(^{2+}\) is present, or initiated by chemically activating the Ca\(^{2+}\) receptors when Ca\(^{2+}\) is absent.

In Chapter 3, I tested the hypothesis that the rate at which mucin vesicles swell is primarily dependent upon characteristics of the mucin vesicle membrane, and not on biophysical properties of the mucin gel. This hypothesis predicts that vesicles with disrupted membranes should swell in an atypical fashion when exposed to artificial seawater. Finding that swelling rate was regulated by the vesicle membrane, I hypothesized that the rate at which water enters the mucin vesicle is dependent upon aquaporins in the vesicle membrane. This hypothesis predicts that aquaporin proteins would be expressed in the slime gland, and that inhibition of aquaporins will decrease the rate at which the mucin vesicles swell. To explain the differences in swelling rate of vesicles exposed to seawater, my second hypothesis proposes that differences in the rate at which vesicles swell is related to Ca\(^{2+}\) sensitivity of different vesicles. This hypothesis predicts that vesicles exposed to Ca\(^{2+}\)-free solutions will have a different swelling rate than vesicles exposed to solutions with Ca\(^{2+}\).

The studies presented in this thesis provide evidence for two categories of vesicle: one which ruptures in Ca\(^{2+}\)-free solutions, and one that requires Ca\(^{2+}\) in order for rupture to occur. Chapter 2 demonstrates that Ca\(^{2+}\)-dependent rupture of hagfish slime mucin vesicles can be inhibited by anion channel inhibitors that are known to block CaCCs, suggesting that Ca\(^{2+}\)
triggers anion influx to cause the vesicle to rupture. Chapter 3 provides evidence that treatment with the AQP inhibitor Hg\(^{2+}\) causes a significant decrease in mucin vesicle swelling rate, and demonstrates the expression of two AQP-like genes in the slime gland, suggesting that rapid water influx is facilitated by AQPs in the vesicle membrane. These two studies provide novel insight into the mechanisms of hagfish slime mucin vesicle rupture.
CHAPTER 2: CA$^{2+}$-DEPENDENT RUPTURE OF MUCIN VESICLES FROM THE SLIME OF

THE PACIFIC HAGFISH (*Eptatretus stoutii*)
The defensive slime produced by hagfish is composed of mucins and intermediate filament-based thread fibres. The mucins are packaged into relatively large vesicles that rupture when they come into contact with seawater. I hypothesized that mucin vesicle rupture is due to the influx of specific ions in seawater through ion channels in the vesicle membrane. I exposed isolated vesicles to a variety of seawater-strength solutions and ion channel inhibitors to determine the mechanism of rupture of mucin vesicles. I found that mucin vesicles fall into two categories: Ca\textsuperscript{2+}-independent and Ca\textsuperscript{2+}-dependent. Ca\textsuperscript{2+}-dependent vesicles were sensitive to three compounds known to inhibit Ca\textsuperscript{2+}-activated chloride channels (CaCCs). I propose that Ca\textsuperscript{2+}-dependent rupture is caused by the influx of Cl\textsuperscript{-} from seawater through CaCCs in the vesicle membrane, resulting in an osmotic drive for the secondary influx of water into the vesicle that results in mucin hydration and vesicle swelling.
2.1 Introduction

Pacific hagfish (*Eptatretus stoutii*) are well known for the defensive slime they produce when physically disturbed. The slime is produced in multiple slime glands that are located in a line along both sides of the animal, next to the ventral fin, and is a result of the combined products of two unique cell types, the gland thread cells (GTCs) and the gland mucous cells (GMCs) (Blackstad, 1968; Spitzer and Koch, 1998). The slime is thought to be primarily a defence mechanism against gill-breathing predators as it effectively reduces flow over fish gills and successfully deters a variety of predators, and may also play a role in immobilizing prey (Lim et al., 2006; Zintzen et al., 2011). It may also provide some defence against microbial pathogens, as higher levels of innate immune substances such as alkaline phosphatase, lysozyme, and cathepsin B are found in the hagfish’s extruded slime than in its epidermal mucus (Subramanian et al., 2008).

Within the slime gland, each GMC produces many vesicles that contain mucin-like glycoproteins (Luchtel et al., 1991b). The mucins found in hagfish slime are unique in that they contain only 12% carbohydrate by dry mass, in contrast to conventional teleost mucins, which contain approximately 85% carbohydrate (Salo et al., 1983; Spitzer and Koch, 1998). When the hagfish gland is stimulated to release slime, the GMCs – as well as GTCs – are pushed through the gland duct, the cell membranes are stripped from the cells so that the contents of the cell – mucin vesicles, in the case of GMCs – are released into the external environment via holocrine secretion (Koch et al., 1991; Spitzer and Koch, 1998). When the intact vesicles come into contact with seawater, they rupture and release the mucins they contain (Luchtel et al., 1991b). The interaction of the free mucins with the slime threads that are produced by GTCs is necessary for producing the fully formed slime, and it has been shown that disruption of the mucin network by
disulfide bond-cleaving agents such as dithiothreitol will delay the onset of whole slime formation, reduce the volume of whole slime formed, and reduce the degree of thread skein unravelling (Fudge et al., 2005; Koch et al., 1991; Winegard and Fudge, 2010).

The nature of the mucin vesicle’s sensitivity to seawater has still only been partially described. Luchtel et al. (1991b) demonstrated that hagfish slime mucin vesicles have a single lipid bilayer that encapsulates the mucin component, and are produced by the Golgi apparatus in the gland mucous cell. They found that whole slime will form when slime gland secretions are placed in 1 M solutions containing univalent anions (e.g. chloride, nitrate, bicarbonate), regardless of the valency of the cations present, but slime will not form when gland secretions are placed in 1 M solutions containing polyvalent anions (e.g. sulphate, citrate, phosphate). Given these observations, Luchtel et al. (1991b) speculated that the hagfish mucin vesicles are permeable to univalent anions and cations of all valencies. According to this model, rupture occurs when ions from seawater enter the vesicle along their diffusion gradient, causing a secondary influx of water that results in mucin hydration and swelling, and vesicle rupture.

Luchtel et al. (1991b) did not find evidence for specific ion channels in the vesicle membrane, but observed that rupture was inhibited by high concentration of zinc ions, which have been shown to inhibit a variety of different membrane channels, including Ca\(^{2+}\) ATPases (Hogstrand et al., 1996), voltage-activated Ca\(^{2+}\) channels (Büsselberg et al., 1994), and aquaporins (Yukutake et al., 2009). Herr et al. (2010) found that the fluid component of unruptured slime exudate contains relatively low concentrations of divalent cations Mg\(^{2+}\) and Ca\(^{2+}\), and relatively high concentrations of the methylamines betaine and trimethylamine oxide (TMAO), but attempts to stabilize isolated mucin vesicles with high concentrations of these organic osmolytes yield mixed results, since almost half of mucin vesicles exposed to betaine
and TMAO rupture, suggesting that there may be differences among mucin vesicles. In addition to these studies, there is strong evidence that the formation of whole slime is dependent upon both the high ionic strength of seawater, and the presence of calcium, but it is unknown whether Ca\(^{2+}\)-dependent slime formation is due to an effect on the mucin vesicles or the thread skein, or both (Fudge, 2002).

The relationship between mucus deployment and Ca\(^{2+}\) is an important one in other systems of mucus secretion. Verdugo et al. (1987b) suggested that the explosive nature of mucus release from mucous granules or goblet cells was due to the electrostatic repulsion among negatively charged mucin carbohydrate side chains. This “Jack-in-the-box” hypothesis of mucus release posits that within mucous granules, Ca\(^{2+}\) plays the role of a charge-shielding ion and allows negatively charged mucins to be packed more tightly than they would be if no cation were present. When Ca\(^{2+}\) is removed, or exchanged with a monovalent cation like Na\(^{+}\) that has weaker charge shielding properties, the mucins are once again in the position to repel one another, causing explosive expansion of the mucin network (Verdugo et al., 1987b; Verdugo, 1991). Ca\(^{2+}\) may also play an important role in cross-linking salivary mucin molecules to form larger aggregates, providing another level of macromolecular organization in addition to the covalent disulfide bonds that lend mucus networks their characteristic integrity (Raynal et al., 2003).

While it is well-known that disulfide bonds are important for the cohesiveness of hagfish slime (Fudge et al., 2005; Koch et al., 1991; Winegard and Fudge, 2010), it is possible that Ca\(^{2+}\) cross-links are also important for the cohesiveness of the network of mucin strands upon which whole slime formation depends.

Based on this information, it is easy to see why Ca\(^{2+}\) might be important to mucus deployment, but it does not answer the question of whether or not Ca\(^{2+}\) plays a role in causing
mucin vesicles to rupture, and if it does, how this is accomplished. Ca$^{2+}$ is known to play a number of important physiological roles in cells, often as a signalling molecule (see for example reviews by Berridge, 1997; Berridge et al., 2000; Bootman et al., 2001; Clapham, 2007). In mucus secretion, because of the extensive research done on Ca$^{2+}$ release prior to mucin network expansion, there has been a great deal of focus on ion channels that would allow an exchange of Ca$^{2+}$ for another ion. Verdugo (1991) suggested that exchange of Ca$^{2+}$ for Na$^+$ through the secretory pore of a goblet cell drives the swelling of mucous granules. It was later shown that Ca$^{2+}$ oscillations in mucin granules from rabbit goblet cells were dependent on ion exchange through an inositol-1,4,5-trisphosphate sensitive Ca$^{2+}$ channel and an apamin-sensitive Ca$^{2+}$-activated K$^+$ channel, suggesting that K$^+$ exchange for Ca$^{2+}$ may be responsible for mucin decondensation (Nguyen et al., 1998).

In this study, I ask the questions: what causes hagfish slime mucin vesicles to rupture when they come into contact with seawater, what characteristics of the mucin vesicle membrane might regulate this phenomenon, and what, if any, is the role of Ca$^{2+}$ in mucin vesicle rupture?

The first set of experiments in this study were designed to test the hypothesis that mucin vesicle rupture is dependent on the movement of specific ions from seawater into the mucin vesicles via ion channels in the membrane. This hypothesis, which I will refer to as the ion channel hypothesis, predicts that mucin vesicle rupture should be prevented in the absence of these required ions, or if the ion channels upon which rupture is dependent are blocked. These predictions were tested by first exposing hagfish slime mucin vesicles to seawater-strength solutions in which three major ions present in seawater were systematically eliminated, and second, by employing the use of the anion channel inhibitor DIDS (4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate) to attempt to stop mucin
vesicles from rupturing.

The second set of experiments in this study expands on the results of these first ion sensitivity experiments, which revealed that mucin vesicle rupture is highly dependent upon the presence of Ca$^{2+}$ ions. I tested two hypotheses that could explain how Ca$^{2+}$ causes mucin vesicles to rupture. The Ca$^{2+}$ channel hypothesis proposes that mucin vesicle rupture is caused by Ca$^{2+}$ entering the mucin vesicle through Ca$^{2+}$ channels in the membrane, and directly causing vesicle swelling from within. The Ca$^{2+}$ trigger hypothesis proposes that vesicle rupture is due to an indirect effect of Ca$^{2+}$, which acts as a signalling molecule that triggers the opening of ion channels in the vesicle membrane and allows the passage of ions across the membrane. If mucin vesicle rupture can be explained by the Ca$^{2+}$ channel hypothesis, then rupture should be prevented by Ca$^{2+}$ channel inhibitors. If mucin vesicle rupture can be explained by the Ca$^{2+}$ trigger hypothesis, then rupture should be prevented by chemically inhibiting extravesicular Ca$^{2+}$ receptors when Ca$^{2+}$ is present, or initiated by chemically activating the Ca$^{2+}$ receptors when Ca$^{2+}$ is absent. Although there are many channels and mechanisms through which Ca$^{2+}$ can act, I focused on the effects of a common calcium channel inhibitor (nicardipine), a calcium-sensing receptor agonist (spermine), and two related calcium-activated chloride channel inhibitors (tannic acid and penta-O-galloyl-β-D-glucose hydrate) on isolated hagfish slime mucin vesicles.

2.2 Methods and materials

2.2.1 Chemicals

Sodium citrate, Tris base, NaCl, MgSO$_4$, KCl, NaHCO$_3$, sucrose, citric acid, dimethyl sulfoxide (DMSO), and HCl were obtained from Fisher Scientific (Fair Lawn, NJ). Clove oil,
PIPES [piperazine-N,N'-bis(ethanesulfonic acid)], CaCl$_2$, MgCl$_2$, N-Methyl-D-glucamine (NMDG), sodium D-gluconate, nicardipine, spermine, tannic acid, and penta-O-galloyl-β-D-glucose hydrate (PGG) were obtained from Sigma-Aldrich (St. Louis, MO). Calcium gluconate and DIDS (4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate) were produced by Acros Organics and obtained through Fisher Scientific. Anhydrous betaine was produced by Fluka, and obtained through Sigma-Aldrich.

2.2.2 Animals, anaesthesia, and slime collection

Pacific hagfish (*Eptatretus stoutii*) were obtained from the Bamfield Marine Sciences Centre, Bamfield, BC, and were maintained at the University of Guelph, ON in ECARS (Environmentally Controlled Aquatic Recirculating System) tanks in the Hagen Aqualab at 10°C in 2000 L of artificial seawater with a salinity of 34‰. Prior to sample collection, hagfish were anaesthetized with a 1:9 dilution of clove oil in ethanol, added to artificial seawater for a final concentration of clove oil of 50 µl/l (Herr et al., 2010). The skin of the anaesthetized hagfish was washed clean with distilled water and dried, and the slime glands were stimulated to express exudate with a mild electric impulse delivered by a GRASS Instruments SD9 stimulator (6 V, 80 Hz; Quincy, MA). The exudate was collected into a stabilization buffer (SB) composed of 0.9 M sodium citrate and 0.1 M PIPES buffer, pH 6.7 (modified from Downing et al., 1981b; Fudge et al., 2003). The hagfish were transferred to clove oil-free seawater until they recovered from the anaesthetic, and were returned to their ECARS. All procedures used were approved by the University of Guelph Animal Care Committee (Protocol 09R128).

The stabilized slime was filtered through nylon mesh with a pore diameter of 53 µm in order to separate mucin vesicles, which have an average diameter of approximately 7 µm, from
thread skeins, which have an average length of approximately 150 µm (Downing et al., 1984; Luchtel et al., 1991b). After filtration, the thread skeins were discarded and isolated mucin vesicles were quantified using spectrophotometry. Absorbance measurements were taken at a wavelength of 350 nm with a Biochrom Novaspec II Visible Spectrophotometer (Cambridge, UK), and were plotted linearly with corresponding counts of mucin vesicles on a haemocytometer, similar to the method of Downing et al. (1981b) who found the relationship between optical density at 350 nm and dry weight. A linear relationship between absorbance at 350 nm and vesicle number was found, and had an R-squared value of 0.94. The concentrations of all subsequent samples were estimated using the equation, absorbance = (0.0096)(number of vesicles). All samples were then diluted to a concentration of approximately 75 vesicles per µl. Samples were stored in SB at 4°C for up to one week before use in rupture assays.

2.2.3 Mucin vesicle rupture assay

In order to analyze the swelling and rupture behaviour of stabilized mucin vesicles when various treatments were applied, open-ended rupture chambers were constructed using glass slides and coverslips, separated by two lines of 0.1 mm diameter glass beads embedded in Vaseline petroleum jelly spaced 6 mm apart (Herr et al., 2010). The chambers held a volume of approximately 20 µl. Stabilized mucin vesicles were added to each chamber and allowed 2 min to settle to the surface of the glass slide to which they adhered. After the settlement period, the chamber was washed with 60 µl of SB to remove any loose vesicles. The settled mucin vesicles within the chamber were then exposed to 30 µl of a given experimental solution by adding a drop of the solution at one end of the chamber, and drawing it through with a strip of filter paper applied to the other end of the chamber. During this event, the vesicles were observed using the 40X DIC objective of a Nikon Eclipse 90i microscope (Nikon Instruments, Inc., Melville, NY),
and time lapse videos were captured using a monochrome digital camera (Q-imaging Retiga Exi Fast1394), at a set capture timeline of 5 frames per second (fps) for 20 seconds, followed by 2 fps for 40 sec, followed by 1 fps for 1 min using NIS-Elements A.R. 3.0 software (Nikon Instruments, Inc.). Videos were later analyzed using the same software.

2.2.4 Ionic requirement

To test the hypothesis that mucin vesicle rupture is dependent on specific ions in seawater, the mucin vesicles were exposed to a number of solutions that mimicked the osmotic strength and pH of seawater, but excluded one or more of the most abundant ions found in natural seawater. All solutions were buffered with 5 mM Tris base to have a final pH of 8, and had an osmolarity of 1000 mOsmol, verified with a Vapro Vapor Pressure Osmometer (model 5520, Wescor, Inc., Logan, UT). Artificial seawater (ASW) was used as a control, made according to a recipe in Bidwell and Spotte (1985), and was compared to the effects of a variety of simplified seawater solutions. Mucin vesicles were exposed to a solution of 545 mM NaCl to test the effect of the two most abundant ions found in seawater, Na\(^+\) and Cl\(^-\), on mucin vesicle rupture. To test how mucin vesicle rupture changes in the presence of Ca\(^{2+}\), isolated vesicles were exposed to a solution that was composed of 10 mM CaCl\(_2\) + 535 mM NaCl. To test the effect the absence of Na\(^+\) has on mucin vesicle rupture, Na\(^+\) was replaced with the impermeable Na\(^+\) analog NMDG, and vesicles were exposed to a 545 mM NMDG-Cl solution. To test the effect of the absence of Cl\(^-\), Cl\(^-\) was replaced with the impermeable Cl\(^-\) analog gluconate, and vesicles were exposed to a solution composed of 545 mM Na\(^+\) gluconate. To test the effect of the absence of both Na\(^+\) and Cl\(^-\), vesicles were exposed to a solution containing 10 mM Ca\(^{2+}\) gluconate + 980 mM sucrose, and were compared to a control treatment of 1000 mM sucrose. NMDG-Cl was prepared by adding an equimolar concentration of HCl to NMDG, adjusting for
pH. The pH of Na\(^+\) gluconate was adjusted with citric acid to avoid use of HCl. In order to determine the sensitivity threshold of mucin vesicles to Ca\(^{2+}\) and Mg\(^{2+}\), mucin vesicles were exposed to serial concentrations of CaCl\(_2\) or MgCl\(_2\), with osmolarity remaining constant by adjusting NaCl concentration for a total osmolarity of 1000 mOsm. The effect of each treatment was determined as the percentage of mucin vesicles that rupture when exposed to each solution, and was calculated by comparing the number of intact vesicles prior to exposure to the number of intact vesicles after 2 minutes of exposure. The start time for exposure to a particular solution was set as the frame when solution flow within the chamber was first observed.

### 2.2.5 Anion channel inhibition

The effect of the anion channel inhibitor DIDS was determined by exposing stabilized mucin vesicles to the inhibitor prior to the addition of a rupture solution. DIDS has been shown to be an effective Cl\(^-\) channel inhibitor at concentrations as low as 500 µM (Goss et al., 2001), therefore, in this experiment, DIDS was dissolved at a stock concentration of 250 mM in DMSO, and diluted to a final concentration of 500 µM (0.2% DMSO) in SB and the following three rupture solutions: 10 mM CaCl\(_2\) + 535 mM NaCl, 545 mM NaCl, and 10 mM Ca\(^{2+}\) gluconate + 980 mM sucrose. Mucin vesicles were added to rupture chambers and allowed to settle for 2 minutes before they were washed with SB + DIDS. After a two minute incubation period in the dark to avoid any deterioration of DIDS due to light exposure, DIDS-treated vesicles were exposed to rupture solutions that also contained 500 µM DIDS, and were compared to control solutions with 0.2% DMSO, as well as solutions without a vehicle control. Time lapse videos were analyzed, and the percentage of vesicles that ruptured within a two minute exposure was calculated.
2.2.6 *Calcium channel inhibition*

Because the Ca$^{2+}$ channel inhibitor nicardipine was found to be poorly soluble in both seawater solutions and SB, it was dissolved at a concentration of 1 M in DMSO, and was added to betaine-based solutions with the same osmolarity as seawater. Betaine (1 M) was deemed an acceptable, inert salt substitute as it is found at relatively high concentrations in the slime gland itself, and has been shown to cause rupture in only ~35% of Atlantic hagfish mucin vesicles (Herr et al., 2010). In the betaine-based solutions, nicardipine was less likely to come out of solution. After preliminary experiments, a concentration of 1 mM nicardipine was chosen as the best concentration for these experiments. However, because betaine alone only stabilizes a portion of mucin vesicles, the mucin vesicles were sequentially exposed to 1 M betaine with 1 mM nicardipine, followed by 10 mM CaCl$_2$ + 980 mM betaine with 1 mM nicardipine, so that nicardipine would have the opportunity to act on Ca$^{2+}$ channels before Ca$^{2+}$ was introduced into the system. Mucin vesicle samples were also exposed to control solutions with and without 0.1% DMSO. The effect of each treatment was calculated as the total percentage of mucin vesicles that ruptured.

2.2.7 *Calcium-sensing receptor activation*

To test for the presence of an extravesicular calcium sensing receptors (CaSR), isolated mucin vesicles were exposed to spermine, a CaSR agonist. Although spermine is a strong CaSR agonist alone, it has been shown to have a greater effect on CaSRs in the presence of low concentrations of Ca$^{2+}$ (Quinn et al., 1997). For this reason, the mucin vesicles were exposed to a Ca$^{2+}$-free solution and a solution with a concentration of Ca$^{2+}$ below that which causes 100% rupture in mucin vesicles (2 mM). Stabilized vesicles were added to rupture chambers as
described earlier, and were exposed to the following two solutions with or without 4 mM spermine added: 545 mM NaCl, and 2 mM CaCl₂ + 545 mM NaCl. This concentration of spermine was used to mimic the CaCl₂ concentration requirement for 100% rupture of mucin vesicles. Time lapse videos were analyzed, and the percentage of vesicles that ruptured was calculated.

2.2.8 Calcium-activated chloride channel inhibition

To test for the presence of calcium-activated chloride channels (CaCCs), stabilized mucin vesicles were exposed to tannic acid and PGG, which have both been shown to inhibit CaCCs (Namkung et al., 2010). To determine the concentration threshold of tannic acid, it was first dissolved in dH₂O at a stock concentration of 100 mM, and then added to ASW in a concentration series that ranged from 2 µM to 50 µM. To compare the effectiveness of tannic acid in different rupture solutions, mucin vesicles were exposed to the following solutions with and without 20 µM tannic acid added: ASW, 10 mM CaCl₂ + 535 mM NaCl, 545 mM NaCl, and 10 mM Ca²⁺ gluconate + 980 mM sucrose. A similar approach was taken to determine the effect of PGG. PGG was dissolved at a stock concentration of 20 mM in DMSO (final ≤ 0.2%) before it was added to the stabilization buffer. Mucin vesicles were exposed to serial concentrations of PGG in SB ranging from 2 µM to 40 µM by washing the solution through the rupture chamber, after which they were exposed to ASW. Like tannic acid, 20 µM PGG was chosen as an effective inhibitory concentration to compare the effects of the four above-mentioned rupture solutions. To control for any effect of DMSO, mucin vesicles were also exposed to a vehicle control of 0.1% DMSO in SB prior to the rupture assay.
2.2.9 Statistical analysis

Statistical analyses were conducted using SigmaStat for Windows (v. 3.5). All percent rupture data were transformed by taking the arc sine of the square root of each value. The ion sensitivity experiments, as well as concentration threshold experiments for Ca\(^{2+}\), Mg\(^{2+}\), tannic acid, and PGG were analyzed using one-way ANOVA. A t-test was used to compare the highest concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) to which the vesicles were exposed. Two-way ANOVAs were used to look at the effects and interactions of solution and drug treatments for the experiments involving DIDS, nicardipine, spermine, tannic acid, and PGG. All ANOVA results that indicated significant differences (p < 0.05) were followed by Holm-Sidak post-hoc analysis.

2.3 Results

2.3.1 Ionic requirement

Isolated hagfish slime mucin vesicles were exposed to an array of seawater-like solutions that differed in the presence or absence of three of the most ubiquitous free ions in natural seawater: calcium, sodium, and chloride ions. Analysis by one-way ANOVA revealed a significant difference in the percentage of mucin vesicles that ruptured depending on the solution to which they were exposed (p < 0.001), and Holm-Sidak post-hoc analysis revealed that the difference was dependent on the presence or absence of Ca\(^{2+}\) in the solutions. I found that in solutions that contained 10 mM Ca\(^{2+}\), almost all the mucin vesicles ruptured. When exposed to ASW, 99.14 ± 0.28 % (mean ± s.e.m., n = 8) of mucin vesicles ruptured. The percentage of vesicles that ruptured with exposure to ASW did not differ significantly from the percentage of
vesicles that ruptured in samples that were exposed to a solution composed of 10 mM CaCl$_2$ + 535 mM NaCl, or to a sodium- and chloride-free solution composed of 10 mM calcium gluconate + 980 mM sucrose (Figure 2.1). However, exposure to 545 mM NaCl resulted in significantly less (p<0.001) rupture (41.4 ± 7.0% (n = 6)) than Ca$^{2+}$ containing controls. Among all Ca$^{2+}$-free solutions (545 mM NaCl, 545 mM sodium gluconate, 545 mM NMDG-Cl, and 1 M sucrose), post-hoc analysis revealed no significant difference in the percentage of mucin vesicles that ruptured.

One-way ANOVA of the effects of exposing mucin vesicles to NaCl solutions with serial concentrations of CaCl$_2$ showed that there was a significant trend of an increase in the percentage of mucin vesicles that ruptured as CaCl$_2$ concentration was increased (p < 0.001, Figure 2.2). Concentrations of CaCl$_2$ that were 3 mM and above cause significantly more vesicles to rupture than the solution that contained 0 mM CaCl$_2$, and therefore, 3 mM Ca$^{2+}$ can be considered the lowest concentration of Ca$^{2+}$ required for complete rupture of stabilized mucin vesicles, with little variation in the number of vesicles that rupture in higher concentrations (Figure 2.2a). The ability to cause complete rupture of stabilized mucin vesicles appears to be exclusive to Ca$^{2+}$, as the other most prevalent divalent cation in seawater, Mg$^{2+}$, did not have the same effect as did the solutions containing Ca$^{2+}$ (Figure 2.2b). At 55 mM, a concentration equivalent to that found in seawater, Mg$^{2+}$ does not cause significantly more vesicles to rupture than a Mg$^{2+}$-free NaCl solution. Furthermore, even the high concentration of 415 mM MgCl$_2$ only caused 85.35 ± 3.7% of mucin vesicles to rupture, significantly fewer vesicles than when samples were exposed to 10 mM Ca$^{2+}$ (p < 0.001).
2.3.2 Anion channel inhibition

Stabilized mucin vesicles were significantly affected by both the solution to which they were exposed (10 mM CaCl$_2$ + 535 mM NaCl, 545 mM NaCl, and 10 mM Ca$^{2+}$ gluconate + 980 mM sucrose) (p < 0.001), and the 500 µM DIDS treatment (p < 0.001). Two-way ANOVA also revealed a significant interactive effect of the two factors (p < 0.001). For both Ca$^{2+}$-containing solutions, treatment of stabilized vesicles with 500 µM DIDS resulted in significantly fewer vesicles rupturing than was observed for vesicles treated with the 0.2% DMSO vehicle control, or the no-vehicle control. In the Ca$^{2+}$-free solution, there were no significant differences in the percentage of mucin vesicles that ruptured if they were treated with DIDS or not, but treatment with 0.2% DMSO caused significantly more mucin vesicles to rupture (Figure 2.3). However, the effect of the DMSO vehicle alone was in the opposite direction of the effect of the drug it carried, indicating that significant decreases in the percentage of vesicles ruptured are due to DIDS, and not its vehicle.

2.3.3 Calcium channel inhibition

In mucin vesicles that were treated with nicardipine, and then exposed to betaine solutions with and without 10 mM CaCl$_2$, two-way ANOVA showed a significant difference in the effect of solution, with 1 M betaine causing significantly fewer vesicles to rupture than did 10 mM CaCl$_2$ + 980 mM betaine (p < 0.001). However, there was no significant effect of nicardipine treatment (p = 0.156) (Figure 2.4).

2.3.4 Calcium-sensing receptor activation

Stabilized mucin vesicle samples that were exposed to Ca$^{2+}$-free NaCl, or to NaCl with 2
mM Ca\(^{2+}\) did not differ in the percentage of vesicles that ruptured (p = 0.245) nor did the presence or absence of the CaSR agonist spermine at a concentration of 4 mM have an effect on the percentage of vesicles that ruptured (p = 0.655) (Figure 2.5).

### 2.3.5 Calcium-activated chloride channel inhibition

Stabilized vesicles that were exposed to ASW containing concentrations of tannic acid that were 6 µM and higher exhibited a significantly lower percentage of rupture than control samples (p < 0.001). There was also a significant difference between the percentage of vesicles that ruptured when exposed to 6 µM tannic acid versus 8-50 µM tannic acid (p < 0.001), with higher concentrations exhibiting an ability to stabilize a larger proportion of the vesicles in a sample. Vesicles treated with SB containing concentrations of PGG that were 5 µM and higher, prior to ASW exposure, also had a significantly lower percentage of mucin vesicles rupturing than control samples (p < 0.001), with no significant differences among concentrations higher than 5 µM (Figure 2.6).

Vesicles that were exposed to different rupture solutions containing 10 mM Ca\(^{2+}\) exhibited a significantly lower rupture percentage in solutions with 20 µM tannic acid, compared to the same rupture solutions that did not contain tannic acid (p < 0.001). However, the stabilizing effect of tannic acid was not as strong in 10 mM calcium gluconate in 980 mM sucrose as it was in ASW and 10 mM CaCl\(_2\) + 535 mM NaCl (p < 0.001), but this may be due to tannic acid being less soluble in sucrose than in salt solutions. When samples were exposed to Ca\(^{2+}\)-free NaCl, a lower proportion of mucin vesicles ruptured than in ASW and 10 mM CaCl\(_2\) + 545 mM NaCl, but this effect was not dependent on the presence of tannic acid (Figure 2.7a). Similarly, in mucin vesicle samples that were treated with 20 µM PGG prior to exposure to
solutions that contained 10 mM Ca\textsuperscript{2+} there was a significant stabilizing effect of PGG in comparison to the 0.1% DMSO vehicle control (p < 0.001). There was no significant effect of PGG in samples exposed to Ca\textsuperscript{2+}-free NaCl (Figure 2.7b).
**Figure 2.1.** Effect of seawater-like solutions on isolated Pacific hagfish (*Eptatretus stoutii*) slime mucin vesicles. Solutions that contained 10 mM Ca$^{2+}$ cause significantly more vesicles to rupture than solutions that were Ca$^{2+}$-free. Error bars are s.e.m., and asterisks (*) indicate significant differences from artificial seawater (ASW).
Figure 2.2. Effects of various concentrations of CaCl₂ (a and b), and MgCl₂ (b) on isolated Pacific hagfish (*Eptatretus stoutii*) slime mucin vesicles. Solutions contained adjusted concentrations of NaCl so that all solutions had the same osmolarity of 1000 mOsmol/L. Error bars indicate s.e.m. Significant differences from the respective Ca²⁺- or Mg²⁺-free control are denoted by asterisks (*).
Figure 2.3. Effect of the anion channel inhibitor DIDS on mucin vesicle rupture. Isolated Pacific hagfish (*Eptatretus stoutii*) slime mucin vesicles were exposed to three rupture solutions after being treated with 500 µM DIDS, 0.2% DMSO (the vehicle for the DIDS), or no vehicle. Error bars indicate s.e.m. Within each solution treatment, asterisks (*) denote significant differences from the no vehicle control samples, and daggers (†) denote significant differences from the DMSO control.
Figure 2.4. Effect of the calcium channel inhibitor nicardipine on mucin vesicle rupture. Pacific hagfish (*Eptatretus stoutii*) slime mucin vesicles were exposed to 1 M betaine followed by betaine with 10 mM CaCl$_2$ containing 1 mM nicardipine, 0.1% DMSO (the vehicle for the nicardipine), or no vehicle. Error bars indicate s.e.m. There was a significant difference in the percentage of vesicles ruptured among solution treatments (lower case letters), but not among drug treatment. Nicardipine did not inhibit rupture of isolated mucin vesicles.
Figure 2.5. Effect of the calcium-sensing receptor agonist spermine on mucin vesicle rupture. Pacific hagfish (*Eptatretus stoutii*) slime mucin vesicles that were exposed to solutions with CaCl$_2$ concentrations that were below the 4 mM rupture-inducing threshold (0 mM and 2 mM) and contained 4 mM spermine did not cause significantly more vesicles to rupture than solutions not containing spermine (control). Error bars indicate s.e.m.
Figure 2.6. Rupture inhibition curve for various concentrations of Ca$^{2+}$-activated Cl$^{-}$ channel inhibitors tannic acid and PGG. Isolated Pacific hagfish (*Eptatretus stoutii*) slime mucin vesicles were exposed to a series of concentrations of tannic acid and PGG (penta-O-galloyl-β-D-glucose hydrate) in the presence of artificial seawater. Concentrations that had significantly fewer vesicles rupture than samples treated with inhibitor-free controls are indicated by asterisks (*) or daggers (†) for tannic acid and PGG, respectively.
Figure 2.7. Rupture inhibition by tannic acid and PGG in a variety of seawater-like solutions. Pacific hagfish (*Eptatretus stoutii*) slime mucin vesicles that were treated with (a) 20 µM tannic acid or (b) 20 µM PGG (penta-O-galloyl-β-D-glucose hydrate) were less susceptible to rupturing when exposed to solutions containing Ca\(^{2+}\). Mucin vesicles were not affected by the vehicle DMSO (0.1%). Error bars indicate s.e.m. Asterisks (*) denote significant differences from control treatments within solution groups. Lower case letters denote significant differences among solutions within control treatments, and upper case letters denote significant differences among solutions within drug treatments.
2.4 Discussion

Hagfish slime mucin vesicle rupture has been described as an event that is governed by the movement of ions from seawater across the mucin vesicle membrane (Luchtel et al., 1991b). The *ion channel hypothesis* states that mucin vesicle rupture is dependent on ion influx through channels in the vesicle’s membrane. It predicts that there are ion-specific channels in the membrane, and therefore either inhibition of the channels or the elimination of the ions that pass through them would inhibit vesicle rupture altogether. The data presented here provide evidence for this hypothesis. My experiments revealed that there are two categories of mucin vesicles released from the slime gland that exhibit different ion sensitivity profiles. I found that approximately 30-40% of mucin vesicles ruptured regardless of the composition of the solution to which they were exposed, with the exception of multivalent anion solutions like sodium citrate, but the remaining 60% of vesicles only ruptured in solutions that contained Ca$^{2+}$. For the purposes of this discussion, I will classify the former category of vesicles as Ca$^{2+}$-independent vesicles, and the latter as Ca$^{2+}$-dependent vesicles. The possibility that two types of mucin vesicles exist was first suggested based on differences in the swelling rates of individual vesicles exposed to seawater, where a proportion of vesicles in a given sample had a relatively slow swelling rate compared to the remaining vesicles, which ruptured quite rapidly (Herr et al., 2010). Although I cannot yet say that the differences in ion sensitivity correspond to differences in swelling rate, it is possible that the differences in Ca$^{2+}$ dependence revealed by this study reflect the differences in swelling rate observed by Herr et al. (2010). It is not, however, obvious what the functional advantage of having two types of mucin vesicles might be, or how they might be separately produced within the gland mucous cells.

The existence of the Ca$^{2+}$-independent vesicles undermines the *ion channel hypothesis* as
these vesicles appear to rupture even in the complete absence of the ions found in seawater. These vesicles ruptured in Ca\(^{2+}\), Na\(^{+}\), and Cl\(^{-}\)-free solutions, including a solution composed solely of sucrose. There are two possible explanations for this phenomenon. The first is that the membrane of these vesicles is damaged, whether by the process of passing through the slime gland duct, or by the filtration process required to separate the mucin vesicles from the thread skeins. When Salo et al. (1983) used transmission electron microscopy to image isolated mucin vesicles, they found that the membranes of some of the vesicles were not intact, but it is unclear whether this was an artefact of sample preparation or if the vesicles are indeed damaged when released from the gland. The second possible explanation for Ca\(^{2+}\)-independent rupture is that these vesicles contain non-selective pores in their membranes that allow ions and molecules at least as large as sucrose to pass. The characterization of this group of vesicles will require further study, as I am not currently able to explain their sensitivity. Their presence is interesting, however, as it may explain the results seen by Luchtel et al. (1991b), who hypothesized that vesicle rupture was due to the movement of monovalent anions and all cations across the vesicle membrane. This model was based on the observation that slime would form in solutions composed of any combination of these ions, including those that lack Ca\(^{2+}\), however, Luchtel et al. (1991b) assessed mucin vesicle rupture by whole slime formation, and then examined samples for unruptured vesicles. My method allows me to examine the effect of solutions on isolated mucin vesicles in a more controlled manner, and without the interference of other slime components. The formation of slime in Ca\(^{2+}\)-free solutions observed by Luchtel et al. (1991b) can be explained if the rupture of 40\% of the mucin vesicles released from the slime gland is sufficient to form mature slime. This explanation is consistent with evidence that slime can form in NaCl, but with a smaller mass than is produced when Ca\(^{2+}\) is present (Fudge et al., 2005).
The other group of mucin vesicles consists of Ca\(^{2+}\)-dependent vesicles that only rupture when Ca\(^{2+}\) is present. I found that close to 100% of vesicles rupture when the solutions to which they were exposed contained Ca\(^{2+}\) at concentrations greater than or equal to 3 mM. I caution against making too much of this particular threshold value, however, as the Ca\(^{2+}\)-chelating effect of any residual citrate stabilization buffer in the rupture chamber would require higher amounts of Ca\(^{2+}\) to overcome. However, even if my results overestimate the requirement for Ca\(^{2+}\) concentration, they indicate that the threshold for total rupture is well below the concentration of Ca\(^{2+}\) found in seawater. I found that Ca\(^{2+}\)-dependent rupture was not dependent on any other specific ion found in seawater, as the vesicles ruptured in Na\(^+\) - and Cl\(^-\)-free solutions. However, I cannot rule out a role for anions in seawater, as I found that DIDS-treated vesicle samples that were exposed to solutions that contained 10 mM Ca\(^{2+}\) exhibited significantly less rupture than untreated samples. As an anion channel inhibitor, DIDS acts non-specifically on a variety of different channels (Wulff, 2008), and its effect on mucin vesicle rupture would suggest that there is also a role for anion movement across the vesicle membrane in the process of mucin release. The ion sensitivity experiments included exposing vesicles to a solution that was composed of 10 mM Ca\(^{2+}\) gluconate + 980 mM sucrose, designed with the intention of testing how the vesicles would react to a Cl\(^-\) -free solution. Gluconate is a large monovalent anion that is usually considered incapable crossing biological membranes, and it was used as a Cl\(^-\) analog for this reason. However, it may be more accurate to say that gluconate simply has very low permeability, with a permeability profile for a typical Cl\(^-\) channel listing gluconate as the least permeable of monovalent anions (Wölfl et al., 1996). It is possible that the anion channel affected by DIDS has enough gluconate permeability to allow the initiation of vesicle rupture in Cl\(^-\)-free solutions. In seawater, allowing a sudden influx of Cl\(^-\) ions into the hagfish slime mucin vesicle
could initiate vesicle rupture by two methods. First, a Cl\(^-\) influx could cause a secondary influx of water molecules into the vesicle, allowing the hydration of mucin molecules, much as Luchtel et al. (1991b) suggested; or second, Cl\(^-\) ions could initiate Jack-in-the-box-like rupture by increasing the charge of an already negatively charged environment, and increasing electrostatic repulsion among mucins, as Verdugo et al. (1987b) suggested.

While it is clear that a large fraction of hagfish slime mucin vesicles require Ca\(^{2+}\) for rupture, the mechanism by which Ca\(^{2+}\) exerts its effects is less clear. The discovery of the Ca\(^{2+}\)-dependent vesicles is of particular interest because it seems counter-intuitive to well-established strategies of mucus exocytosis in vertebrates and invertebrates alike (Verdugo et al., 1987a; Verdugo et al., 1987b). If we consider the process of mucus secretion in the context of the Jack-in-the-box hypothesis, then mucin vesicle rupture should be preceded by Ca\(^{2+}\) release, as is seen in the giant mucin vesicles of the terrestrial slug *Ariolimax columbianus* (Verdugo et al., 1987b). Based on my results, however, the opposite appears to occur, where external Ca\(^{2+}\) is required to initiate mucin vesicle rupture. However, this does not eliminate the possibility that Ca\(^{2+}\) also provides the charge-shielding that allows the mucin molecules to condense more tightly within the vesicle. The concentrations of divalent cations Ca\(^{2+}\) and Mg\(^{2+}\) from all slime constituents as determined by Munz and MacFarland (1964) are much greater than the concentrations of these same ions in the fluid released from the slime gland prior to mucin vesicle rupture and thread skein unravelling (Herr et al., 2010). This suggests that it is possible that some of the additional Ca\(^{2+}\) is released from the vesicle when rupture occurs.

I hypothesized that Ca\(^{2+}\) acts either by entering the vesicle through Ca\(^{2+}\) channels in the membrane, or by a Ca\(^{2+}\)-triggered mechanism by acting as a signalling molecule that initiates the process of vesicle rupture. I found no evidence of a nicardipine-sensitive Ca\(^{2+}\) channel, nor of a
CaSR. However, due to difficulties with getting most common Ca$^{2+}$ channel inhibitors to dissolve in most seawater-strength solutions, I was unable to test many more inhibitors, and therefore I cannot eliminate the possibility that a Ca$^{2+}$ channel exists in the vesicle membrane. The CaSR experiments were more convincing, as spermine was readily soluble in seawater, and even high concentrations of spermine acting in the presence of below-threshold Ca$^{2+}$ did not cause more vesicles to rupture than solutions lacking spermine.

I also looked into the possibility that mucin vesicle rupture is dependent upon Ca$^{2+}$-activated chloride channels. Recently, the TMEM16A channel protein (also called anoctamin-1, part of the anoctamin family) was identified as a CaCC as it demonstrates characteristics of typical CaCCs such as its permeability sequence (NO$_3^– > I^- > Br^- > Cl^- > F^–$) and its sensitivity to inhibitors such as niflumic acid, NPPB, and DIDS (Duran et al., 2010; Ferrera et al., 2010). In addition to these common Cl$^- $ channel inhibitors, Namkung et al. (2010) found that tannic acid and related gallotannins such as PGG in green tea and red wine effectively inhibit TMEM16A and other unidentified CaCCs at micromolar concentrations. Furthermore, these tannic acid derivatives do not inhibit CFTR Cl$^- $ channels, suggesting that these compounds have a more specific inhibitor effect on CaCCs than the common Cl$^- $ channel inhibitors (Namkung et al., 2010). The fact that Ca$^{2+}$-dependent mucin vesicle rupture can be inhibited with DIDS is consistent with the presence of a CaCC-like protein in the mucin vesicle membrane. I tested this hypothesis further by treating vesicles with the CaCC inhibitors tannic acid and PGG, and found that both compounds were capable of inhibiting rupture in Ca$^{2+}$-dependent mucin vesicles at micromolar concentrations. This supports the hypothesis that rupture is dependent on an anion channel similar to the tannic acid and gallotannin-sensitive CaCCs.

The behaviour of tannic acid- and PGG-treated vesicles in the Cl-free Ca$^{2+}$ gluconate +
sucrose solution is consistent with my observations of DIDS-sensitive vesicles, and can also be explained by gluconate permeability. There is evidence for some gluconate permeability in TMEM16A, although it is very small (Manoury et al., 2010). The observation that mucin vesicles rupture in a Cl\(^{-}\)-free solution containing Ca\(^{2+}\) gluconate, does not, therefore, eliminate the possibility that rupture is dependent on a CaCC-like ion channel. The permeability to all monovalent anions, and the reliance upon their presence may help explain why it is necessary to use polyvalent anions such as citrate, sulphate, phosphate, and tartrate to stabilize mucin vesicles (Luchtel et al., 1991b; Salo et al., 1983). It is possible that if the role of this anion channel is to allow an influx of anions that initiates water influx as well, then even the passage of a small amount of gluconate into the vesicle may be sufficient to induce the swelling and rupture of the vesicle. Until the Ca\(^{2+}\)-activated anion channel in question can be isolated and its conductance can be tested, I cannot eliminate this possibility. Given the current evidence, I proposed that Ca\(^{2+}\)-dependent mucin vesicle rupture relies on environmental Ca\(^{2+}\) triggering a sudden influx of Cl\(^{-}\) into the vesicle through this Ca\(^{2+}\)-activated anion channel. The mucin vesicle, being packed with negatively charged mucins, is likely relatively Cl\(^{-}\)-free and thus Cl\(^{-}\) at seawater concentration would quickly diffuse into the vesicle down its concentration gradient, causing a secondary influx of water, much like Luchtel et al. (1991b) hypothesized (Figure 2.8). Having a requirement of Ca\(^{2+}\) for mucin vesicle rupture may be advantageous for stabilization purposes within the hagfish’s slime gland. With an osmoregulatory strategy that appears more similar to that of marine invertebrates than marine teleosts, the inorganic salt content of hagfish plasma is similar to that of seawater, with little capacity to regulate Na\(^{+}\) and Cl\(^{-}\) concentrations (Currie and Edwards, 2010; Sardella et al., 2009). However, there is evidence that hagfish are able to regulate divalent cation concentration, as the concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) in the plasma are
Figure 2.8. A new hypothesis for Ca$^{2+}$-dependent hagfish slime mucin vesicle rupture. Ca$^{2+}$ in seawater triggers Ca$^{2+}$-activated anion channels in the vesicle membrane to open, allowing the sudden influx of Cl$^-$. Cl$^-$ enters the vesicle and contributes to the negatively charged internal environment by displacing charge-shielding cations. The movement of Cl$^-$ is accompanied by the secondary influx of water molecules, which hydrate the mucins. Mucin vesicle rupture is a combination of the electrostatic repulsion of negatively charged particles, and the swelling of the mucin network through gel hydration.
roughly half of those in seawater, and in muscle and liver tissues, intracellular Ca\(^{2+}\) concentrations are as low as 2.6 and 1.3 mM/kg H\(_2\)O respectively (Bellamy and Jones, 1961; Sardella et al., 2009). Furthermore, in the slime gland of Atlantic hagfish, the fluid component of slime exudate, which consists of intra- and extracellular fluid from gland thread cells and gland mucous cells, contains negligible concentrations of Ca\(^{2+}\) at nanomolar ranges, and relatively high concentrations of methylamines (Herr et al., 2010). Herr et al. (2010) speculated that the high concentration of methylamines helps counteract protein destabilization by the relatively high concentrations of Cl\(^-\) and Na\(^+\) concentrations within the slime gland, but it is possible that it is the lack of Ca\(^{2+}\) that is most critical for mucin vesicle stabilization. The absence of Ca\(^{2+}\) may be enough to provide stability to keep mucin vesicles intact until they are released into a high Ca\(^{2+}\) concentration environment such as seawater.

I have described two distinct groups of mucin vesicles released from the slime gland with different ion-sensitivity profiles: Ca\(^{2+}\)-independent and Ca\(^{2+}\)-dependent vesicles. While I am currently unable to describe the mechanism of rupture of Ca\(^{2+}\)-independent vesicles, I proposed that Ca\(^{2+}\)-dependent vesicles contain Ca\(^{2+}\)-activated anion channels in the mucin vesicles membrane that allow Cl\(^-\) influx in the presence of Ca\(^{2+}\). This hypothesis is supported by the ion sensitivity and pharmacological properties of the hagfish slime mucin vesicle, and the evidence I have presented here provides a clear direction for future studies of hagfish slime mucin deployment. The characterization of a hagfish Ca\(^{2+}\)-activated anion channel through molecular studies and electrophysiology will add to our understanding of hagfish slime mucin deployment, and contribute to our knowledge of the growing family of proteins classified as CaCCs.
CHAPTER 3: Ca^{2+} and Hg^{2+}-SENSITIVE SWELLING RATES OF MUCIN VESICLES
FROM THE SLIME OF PACIFIC HAGFISH (Eptatretus stoutii): EVIDENCE FOR
AQUAPORIN-MEDIATED WATER PERMEABILITY
Abstract

Pacific hagfish slime mucin vesicles are released by holocrine secretion so that their membranes remain intact until the vesicle contacts seawater. The vesicle ruptures under the influence of ions in seawater, and the freed mucins interact with slime threads to form the hagfish’s defensive slime. I hypothesized that mucin vesicle swelling rate is regulated by the vesicle membrane through the presence of Ca\(^{2+}\)-sensitive proteins and aquaporins (AQP) that permit a more rapid influx of water for the hydration of mucins. I found that vesicles that rupture in the absence of Ca\(^{2+}\) have a lower mean swelling rate than those that require Ca\(^{2+}\), and that treating mucin vesicles with AQP-inhibiting HgCl\(_2\) caused a decrease in the swelling rate of vesicles. This evidence that aquaporins play a role in facilitating water movement across the mucin vesicle membrane is supported by molecular evidence of AQP-like protein mRNA expression in the slime gland.
3.1 Introduction

The defensive slime generated by Pacific hagfish (*Eptatretus stoutii*) when they are physically disturbed is produced in many slime glands that are located in a line along both sides of the animal, and is composed of the products of two unique cell types, the gland thread cells (GTCs) and the gland mucous cells (GMCs) (Blackstad, 1968; Spitzer and Koch, 1998). It is thought to be primarily a defence mechanism against gill-breathing predators as it reduces flow over fish gills and deters a variety of predators (Lim et al., 2006; Zintzen et al., 2011). Within the slime gland, each GMC produces many vesicles that contain mucin-like glycoproteins (Luchtel et al., 1991b; Salo et al., 1983). When the hagfish gland is stimulated to release slime, the cell membrane is stripped from the GMCs as they are pushed through the gland duct, and the mucin vesicles are released intact into the external environment (Koch et al., 1991; Spitzer and Koch, 1998). When the vesicles come into contact with seawater, they rupture and release the mucins they contain (Luchtel et al., 1991b). The interaction of the free mucins with the slime threads that are produced by GTCs is necessary for producing the fully formed slime (Fudge et al., 2005; Koch et al., 1991; Winegard and Fudge, 2010).

Luchtel et al. (1991b) demonstrated that hagfish slime mucin vesicles have a single lipid bilayer that encapsulates the mucin component, and are produced by the Golgi apparatus in GMCs. They speculated that vesicles are permeable to univalent anions and cations of all valencies, as they found that whole slime will form when slime gland secretions are placed in 1 M solutions containing univalent anions (e.g. chloride, nitrate, bicarbonate), regardless of the valency of the cations present. In the previous study, I demonstrated that approximately 60% of isolated vesicles require Ca\textsuperscript{2+} for rupture to occur, while the remaining vesicles will rupture upon exposure to any solution, barring solutions with high concentrations of polyvalent anions. These
results suggest that there are two distinct categories of mucin vesicles that can be described by their dependence on Ca$^{2+}$ in seawater. Evidence for two types of mucin vesicles is also presented by Herr et al. (2010), who observed that vesicles could be categorized by the rate at which they swell when exposed to seawater.

Hagfish slime mucin vesicle swelling is thought to be regulated by the influx of ions from seawater into the vesicle, which then causes a secondary influx of water that results in mucin hydration and swelling, and causes vesicle rupture (Luchtel et al., 1991b). The hydration of a mucous gel has been described as a Donnan equilibrium-controlled process in which a semi-permeable membrane limits the movement of certain charged particles but not others, and the movement of unconstrained particles along their concentration gradients can result in differences in electrical potential across the membrane (Donnan, 1924). In the case of a mucous gel, the network of glycoproteins that form the gel act as both the semi-permeable membrane that limits the movement of polyions, and as the limited polyanion that induces the osmotic drive (Tam and Verdugo, 1981). According to this model, the hydration of the mucous gel is determined by the charge density of mucins, and the movement of water and electrolytes into the mucin network (Aitken and Verdugo, 1989). The characteristics of the mucin molecule itself can also contribute to the hydration dynamics of a mucin network, as the time it takes for mucin chains to diffuse away from one another increases proportional to the square of the chain length (Verdugo, 1994).

In typical modes of mucin exocytosis, the swelling of the secretory vesicle occurs after the formation of the fusion pore, and the mucous gel is formed in the extracellular space following release from goblet cells (Verdugo, 1991). Hagfish slime mucin vesicles are unique in that they are intact when they are released from the gland into the environment, and the swelling of the mucin network must be triggered by environmental components moving across the vesicle.
membrane to initiate mucin release. Luchtel et al. (1991b) did not specify the mechanism of water influx when they conceived of their vesicle swelling hypothesis, but is implied that it occurs through passive diffusion across the vesicle membrane (Luchtel et al., 1991b). However, the rapidity with which slime is deployed by a disturbed hagfish (Lim et al., 2006) may suggest that water movement across the vesicle membrane is somehow facilitated so that influx is quicker than simple diffusion through the lipid bilayer would allow.

Aquaporins (AQP) are intrinsic membrane water channels that provide a 10- to 100-fold higher capacity for water permeation than a membrane lacking them (Agre et al., 2002). First characterized in human erythrocytes twenty years ago (Preston et al., 1992), the aquaporin superfamily has since grown to include 13 subfamilies (AQP0-12) that fall roughly into two categories: classical aquaporins (AQP0, -1, -2, -4, -5, -6) that are water-selective, and aquaglyceroporins (AQP3, -7, -9, -10) that are permeable to small solutes such as glycerol and urea as well as to water (Cerda and Finn, 2010). AQPs exhibit high water selectivity such that they can exclude even protons, a characteristic that is thought to be due to the presence of two highly conserved asparagine, proline, alanine (NPA) motifs (Agre et al., 2002; Engel and Stahlberg, 2002). Reversible mercury inhibition has been observed in the majority of described AQPs, and is thought to be caused by the covalent attachment of Hg\(^{2+}\) to the sulphhydryl group of a cysteine (Cys) residue in close proximity to one of these NPA motifs in the water pore (Cerda and Finn, 2010). While highly involved in both mammalian and piscine osmoregulation, AQPs have also been found in the membranes of secretory vesicles such as in those found in the human small intestine (Li et al., 2005) and the zymogen granules of pancreatic acinar cells (Cho et al., 2002; Itoh et al., 2005), where they are thought to be important for the swelling of the vesicle and the release of its contents. These characteristics make AQPs potential candidates for the
rapid water influx necessary for hagfish slime mucin gel hydration.

In this study, I ask the question: what regulates the swelling rate of hagfish slime mucin vesicles? My first hypothesis, which I will refer to as the *membrane-regulated swelling hypothesis*, is that mucin vesicle swelling rate is regulated primarily by characteristics of the mucin vesicle membrane, and not by biophysical properties of the mucin gel. It predicts that the swelling behaviour of mucin vesicles with a disrupted membrane will differ from the swelling behaviour of vesicles with intact membranes. This prediction was tested by exposing stabilized mucin vesicles to the membrane-disrupting detergent Triton X-100 prior to exposing them to a variety of solutions.

My second two hypotheses stem from results that supported the *membrane-regulated swelling hypothesis*. I ask the questions, what characteristics of the mucin vesicle membrane regulate swelling rate, and what causes natural differences in the swelling rates of vesicles? The *aquaporin-dependent swelling hypothesis* states that the rapid rate at which mucins within the vesicle are hydrated is facilitated by aquaporin water channels in the vesicle membrane that increase the water permeability, and that any differences in vesicle swelling rates are due to differences in the number of aquaporins in the membrane. It predicts the expression of aquaporin proteins in the slime gland, and that treatment of vesicles with an aquaporin inhibitor will reduce the rate at which the vesicles swell when they are exposed to seawater and similar solutions. This prediction was tested by treating vesicles with the aquaporin inhibitor HgCl$_2$, and by looking for the expression of aquaporins in the slime gland using reverse transcription (RT) PCR. The *Ca$^{2+}$-sensitivity swelling hypothesis* states that natural differences in swelling rates of individual vesicles are due to a vesicle’s sensitivity to Ca$^{2+}$. It predicts that the vesicles that swell when exposed to a Ca$^{2+}$-free solution will have swelling rates that differ from vesicles that require Ca$^{2+}$
to swell. This prediction was tested by measuring the mean swelling rate of vesicles exposed to a variety of solutions that differed in their inclusion of Ca\(^{2+}\).

3.2 Methods and materials

3.2.1 Animals, anaesthesia, and slime collection

Pacific hagfish (*Eptatretus stoutii*) were obtained from the Bamfield Marine Sciences Centre, Bamfield, BC, and were maintained at the University of Guelph, ON in ECARS (Environmentally Controlled Aquatic Recirculating System) tanks in the Hagen Aqualab at 10°C in 2000 litres of artificial seawater with a salinity of 34‰. Prior to sample collection, hagfish were anaesthetized with a 1:9 dilution of clove oil (Sigma-Aldrich, St. Louis, MO) in ethanol, added to artificial seawater for a final concentration of clove oil of 50 µl/l (Herr et al., 2010). The skin of the anaesthetized hagfish was washed clean with distilled water and dried, and the slime glands were stimulated to express exudate with a mild electric impulse delivered by a GRASS Instruments SD9 stimulator (6 V, 80 Hz; Quincy, MA). The exudate was collected into a stabilization buffer (SB) composed of 0.9 M sodium citrate and 0.1 M PIPES [piperazine-N,N'-bis(ethanesulfonic acid)] buffer (Sigma-Aldrich, St. Louis, MO), pH 6.7 (modified from Downing et al., 1981b; Fudge et al., 2003). The hagfish were transferred clove oil-free seawater until they recovered from the anaesthetic, and were returned to their ECARS. All procedures used were approved by the University of Guelph Animal Care Committee (Protocol 09R128).

The stabilized slime was filtered through nylon mesh with a pore diameter of 53 µm in order to separate mucin vesicles, which have an average diameter of approximately 7 µm, from
thread skeins, which have an average length of approximately 150 µm (Downing et al., 1984; Luchtel et al., 1991b). After filtration, the thread skeins were discarded and isolated mucin vesicles were quantified using spectrophotometry. Absorbance measurements were taken at a wavelength of 350 nm with a Biochrom Novaspec II Visible Spectrophotometer (Cambridge, UK), and were plotted linearly with corresponding counts of mucin vesicles on a haemocytometer, similar to the method of Downing et al. (1981b) who found the relationship between optical density at 350 nm and dry weight. A linear relationship between absorbance at 350 nm and vesicle number was found, and the concentrations of all subsequent samples were estimated using the equation, absorbance = \((0.0096)(\text{number of vesicles})\). All samples were then diluted to a concentration of approximately 75 vesicles per µl. Samples were stored in SB at 4°C for up to one week before use in rupture assays.

### 3.2.2 Mucin vesicle swelling assay

Glass slide rupture chambers were constructed as in Herr et al. (2010), and held a volume of approximately 20 µl. Stabilized mucin vesicles were added to each chamber and allowed 2 minutes to settle to the surface of the glass slide to which they adhered. After the settlement period, the chamber was washed with 60 µl of SB to remove any loose vesicles.

Mucin vesicles from 6 hagfish were exposed to artificial seawater (ASW) made according to a recipe in Bidwell and Spotte (1985) to determine the swelling rates of vesicles under control conditions. They were also exposed to the Ca\(^{2+}\)-free solution of 545 mM NaCl, a simplified seawater (SSW) solution composed of 10 mM CaCl\(_2\) + 535 mM NaCl, and to distilled water lightly buffered with 5.0 mM Tris. After the settlement period, vesicles within the chamber were exposed to 30 µl of one of the above four experimental solutions by adding a drop of the
solution at one end of the chamber, and drawing it through with a strip of filter paper applied to the other end of the chamber. During this event, the vesicles were observed using the 40X DIC objective of a Nikon Eclipse 90i microscope (Nikon Instruments, Inc., Melville, NY), and time lapse videos were captured using a monochrome digital camera (Q-imaging Retiga Exi Fast1394, Surrey, BC), at the highest frame rate possible (approximately 14 fps). Sodium citrate, Tris base, NaCl, MgSO$_4$, KCl, and NaHCO$_3$ and were obtained from Fisher Scientific (Fair Lawn, NJ). CaCl$_2$ and MgCl$_2$, were obtained from Sigma-Aldrich (St. Louis, MO).

To test the effect of mucin vesicle membrane disruption, mucin vesicles collected from 6 hagfish were washed with SB containing 0.1% Triton X-100 (Sigma-Aldrich) prior to exposure to rupture solutions. Triton X-100-treated vesicles were exposed to SSW, and time lapse videos were recorded.

To test the effect of the aquaporin inhibitor HgCl$_2$ on mucin vesicle swelling rate, HgCl$_2$ (Sigma-Aldrich) was dissolved in distilled water at a stock concentration of 100 mM, and was then diluted in stabilization buffer for a final concentration of 1 mM in SB. This final concentration of HgCl$_2$ was used by MacIver et al. (2009) to inhibit eel AQPs, and was found to be the an effect concentration for use on mucin vesicles in preliminary studies. Mucin vesicles from 6 hagfish were washed with 1 mM HgCl$_2$ in SB, and were left to incubate at 4°C in the dark for 30 minutes, as it has been previously found that a 30 minute incubation period is ideal for aquaporin inhibition (MacIver et al., 2009). Following this period, the HgCl$_2$-treated vesicles were exposed to ASW, 545 mM NaCl, SSW, and 5 mM Tris, and time lapse videos were recorded. Videos were analyzed for both the swelling rates of individual vesicles, and the total percentage of vesicles that ruptured under HgCl$_2$ and control conditions in all four solutions.
3.2.3 Swelling video analysis

Each video of mucin vesicle swelling was assigned a random four digit number prior to analysis to avoid bias during analysis. Analysis was done using NIS-Elements A.R. 3.0 software (Nikon Instruments, Inc.). In each video, individual vesicles were numbered sequentially from top left to right bottom, and the area (in $\mu m^2$) that each vesicle occupied as it swelled was measured over the period of time it took for the vesicle to swell completely. A vesicle was considered completely ruptured when the vesicle was no longer visually discernible. This was done for a maximum of 30 vesicles per video, with each video representing a single replicate. To accurately represent mean swelling rates of vesicles under different treatments, swelling rates were not included for vesicles that did not swell at all, but these vesicles were represented in calculations of the total percentage of vesicles that ruptured with each treatment. There were six replicates per treatment, with each replicate representing vesicles collected from one of six hagfish.

Vesicle area measurements were normalized as a percentage of the initial area, prior to the commencement of swelling so that the original area a vesicle occupied was considered 100%. Time zero of solution exposure was normalized as the frame immediately before the first vesicle began to swell. The swelling rate for each vesicle in relative area over time was calculated as the linear slope of a line of best fit through the swelling curve.

3.2.4 Statistical analysis

Statistical analyses were conducted using SPSS Statistics v.19 (IBM, Armonk, NY). Percent rupture data were transformed by taking the arcsine of the square root of each value. Vesicle swelling rates were calculated in Excel 2007 (Microsoft, Redmond, WA) using the linear
equation function, and were log$_{10}$ transformed. The mean of the log transformed swelling rates was calculated for each trial to obtain a single value for each true replicate (hagfish) under each treatment and solution condition. Since the hagfish used for the control solution experiments were different hagfish from the six used for the HgCl$_2$ treatments, the comparison of control vesicles to HgCl$_2$-treated vesicles was analyzed using a split plot design. Both percent rupture data and mean swelling rate data were analyzed with a two-way ANOVA for solution and HgCl$_2$ treatment effects, with HgCl$_2$ treatment nested within hagfish identity. ANOVA results that indicated significant differences were followed by Tukey HSD post-hoc pairwise comparisons based on both split plot and whole plot error terms.

3.2.5 **Euthanasia and tissue collection**

Hagfish were anaesthetized in 250 mg/l MS-222 (Ethyl 3-aminobenzoate methanesulfonate; Syndel Laboratories Ltd, Vancouver, BC) buffered with 500 mg/l NaHCO$_3$, and then euthanized by decapitation. The slime glands were removed by cutting away the skin and separating it from the connective tissue and muscle of the body. Slime glands were individually removed from the skin and frozen in liquid nitrogen for storage at -80°C until they were used for RNA extraction.

3.2.6 **Aquaporin sequences identification**

Protein sequences of known AQP genes from a variety of species were gathered from NCBI (Table 3.1). These homologs were aligned using MUSCLE (European Bioinformatics Institute, www.ebi.ac.uk/Tools/msa/muscle/), and a HMM profile was calculated from the
resulting alignment using HMMER3 v. 3.0 (Janelia Farm; http://hmmer.janelia.org). The HMM profile was compared to a translated hagfish gill and slime gland transcriptome database\(^1\) (BGI, Beijing, China). The HMMER search yielded two sequences that will be henceforth referred to \(E.\ stoutii\) aquaporin-like protein 1 (AQP-ES1) and \(E.\ stoutii\) aquaporin-like protein 2 (AQP-ES2). Both sequences were BLAST analyzed on NCBI to verify that they belonged to the AQP family of genes, and were aligned with the closest matched sequences using ClustalW2 v. 2.1 (European Bioinformatics Institute, http://www.ebi.ac.uk/Tools/msa/clustalw2/). Amino acid sequences were determined by BLAST searching the three amino acid sequences translated from the three reading frames using EMBOSS Transeq (European Bioinformatics Institute, http://www.ebi.ac.uk/Tools/st/emboss_transeq/). The nucleotide sequences for these homologs were used to develop species-specific primers for reverse transcription (RT) PCR analysis. Forward (F) and reverse (R) primers for the two AQPs were as follows: AQP-ES2_F: GCGGGTCCTCGCACAGCGAA TGCGC, AQP-ES2_R: CCGCGGTCTCCATGCTCCAG GGT, AQP-ES1_F: CGACCTACACATCGCACTTG, and AQP-ES1_R: ACGAGGGTTGTAAGGGTGAC.

3.2.7 RNA extraction, cDNA synthesis, and RT-PCR

Total RNA was extracted from the gland tissue from two hagfish using TRIzol Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol, and the RNA pellet was reconstituted in RNase-free water. RNA samples were quantified using a NanoDrop 8000 spectrophotometer (software version 2.2.0, Thermo Scientific, Wilmington, DE). Prior to reverse transcription, 1-2 µg of total RNA was treated with Amplification Grade DNase 1 (Sigma, St. Louis, MO) according to the protocol provided by the manufacturer. The samples were then

\(^1\) Provided in collaboration with Dr. Greg Goss, University of Alberta, Edmonton, AB
reverse transcribed to cDNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA), following the manufacturer’s protocol. To control for genomic DNA contamination, non-reverse transcribed (-RT) controls were included during cDNA synthesis. The cDNA reaction took place using a Techne thermal cycler (FTGENE2D, St. Louis, MO). The AQP-ES1 and AQP-ES2 sequences were amplified from the samples by PCR using a TopTaq DNA Polymerase kit (Qiagen Sciences, Maryland, USA), following the manufacturer’s protocol, including the addition of the CoralLoad gel loading reagent and tracking dyes. The 50 µl reactions contained approximately 2 µg of template DNA or -RT control samples, and 1 µl each of the AQP-ES1_F and AQP-ES1_R, or AQP-ES2_F and AQP-ES2_R primers. The PCR amplification was performed following the manufacturer’s cycling protocol: initial denaturation at 94°C for 3 minutes; 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min; final extension at 72°C for 10 min. The products were loaded on a 1% agarose gel containing GelRed Nucleic Acid Gel Stain (Biotium, Hayward, CA), and was run at 59 V for 1-2 h. Products were located using an ultraviolet (UV) light box, and product sizes were compared to a 100 bp DNA ladder (Invitrogen). The product bands were excised from the gel, and the DNA was extracted using the QIAquik Gel Extraction kit (Qiagen Sciences) according to the manufacturer’s protocol. In the final step, the DNA was eluted from the QIAquik membrane with water, and the extracted product samples were sent to the University of Guelph Advanced Analysis Centre Genomics Facility for sequencing. The sequences were compared to the target transcriptome sequences using the ClustalW2 Multiple Sequence Alignment tool (European Bioinformatics Institute (EBI), http://www.ebi.ac.uk/Tools/msa/clustalw2/), and BLAST searched to confirm similarity to known aquaporin sequences.
Table 3.1. The list of species and accession numbers for the AQP gene sequences used to identify AQP-like sequences in a translated hagfish gill and slime gland transcriptome database.

<table>
<thead>
<tr>
<th>Species</th>
<th>Aquaporin type</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>African clawed frog (<em>Xenopus laevis</em>)</td>
<td>AQP3 (Gill blood group)</td>
<td>NP_001081876.1</td>
</tr>
<tr>
<td>Chicken (<em>Gallus gallus</em>)</td>
<td>Predicted: hypothetical protein</td>
<td>XP_424500.2</td>
</tr>
<tr>
<td>Common marmoset (<em>Callithrix jacchus</em>)</td>
<td>Predicted: AQP3-like</td>
<td>XP_002743063.1</td>
</tr>
<tr>
<td>Common quail (<em>Coturnix coturnix</em>)</td>
<td>AQP water channel protein</td>
<td>ACF19804.1</td>
</tr>
<tr>
<td>Cow (<em>Bos taurus</em>)</td>
<td>AQP3</td>
<td>NP_001073262.1</td>
</tr>
<tr>
<td>Dog (<em>Canis familiaris</em>)</td>
<td>Predicted: similar to AQP3</td>
<td>XP_854596.1</td>
</tr>
<tr>
<td>European seabass (<em>Dicentrarchus labrax</em>)</td>
<td>AQP3</td>
<td>ABG36519.1</td>
</tr>
<tr>
<td>Gray short-tailed opossum (<em>Monodelphis domestica</em>)</td>
<td>Predicted: AQP3-like</td>
<td>XP_001362292.1</td>
</tr>
<tr>
<td>Horse (<em>Equus caballus</em>)</td>
<td>Predicted: AQP3 (Gill blood group)</td>
<td>XP_001917822.1</td>
</tr>
<tr>
<td>Human (<em>Homo sapiens</em>)</td>
<td>AQP3</td>
<td>NP_004916.1</td>
</tr>
<tr>
<td>Japanese eel (<em>Anguilla japonica</em>)</td>
<td>AQP3</td>
<td>BAH89253.1</td>
</tr>
<tr>
<td>Japanese tree frog (<em>Hyla japonica</em>)</td>
<td>AQP</td>
<td>BAF63030.1</td>
</tr>
<tr>
<td>Mouse (<em>Mus musculus</em>)</td>
<td>AQP3</td>
<td>NP_057898.2</td>
</tr>
<tr>
<td>Platypus (<em>Ornithorhynchus anatinus</em>)</td>
<td>Predicted: similar to AQP3</td>
<td>XP_001512694.1</td>
</tr>
<tr>
<td>Rat (<em>Rattus norvegicus</em>)</td>
<td>AQP3</td>
<td>NP_113891.1</td>
</tr>
<tr>
<td>Rhesus macaque (<em>Macaca mulatta</em>)</td>
<td>Predicted: AQP3</td>
<td>XP_001099318.1</td>
</tr>
<tr>
<td>Wild boar (<em>Sus scrofa</em>)</td>
<td>AQP3</td>
<td>NP_001103642.1</td>
</tr>
<tr>
<td>Zebrafish (<em>Danio rerio</em>)</td>
<td>AQP3a</td>
<td>NP_998633.1</td>
</tr>
</tbody>
</table>
3.3 Results

3.3.1 Effects of Triton X-100 on mucin vesicle swelling rate

Stabilized mucin vesicles that were exposed to a simplified seawater solution (SSW) composed of 10 mM CaCl$_2$ and 535 mM NaCl exhibited a range of swelling rates (Figure 3.1a). The mean swelling rate of all vesicles exposed to SSW was $2032 \pm 396\%$ (mean ± s.e.m.) increase in area per second. While the majority of vesicles appeared to swell rapidly, a small portion of vesicles appeared to swell more slowly (Figure 3.1b). The time at which vesicles ruptured following the beginning of exposure to SSW varied from less than 1 s to 50 s. All of the slowly swelling vesicles began swelling within the first 3 s of exposure.

Stabilized vesicles that were treated with 0.1% Triton X-100 before being exposed to SSW exhibited a mean swelling rate of $197.6 \pm 32.9\%$ increase in area per second (Figure 3.2), a mean swelling rate that was significantly slower than untreated vesicles ($p < 0.001$). All the vesicles began swelling within 2 s of exposure to SSW, and exhibited relatively similar swelling patterns.

3.3.2 Ca$^{2+}$ and HgCl$_2$-sensitive mucin vesicles

Isolated mucin vesicles were exposed to four solutions (ASW, 545 mM NaCl, SSW, and 5 mM Tris) under control conditions, or after being treated with 1 mM HgCl$_2$. A two-way ANOVA of the overall effect of the treatments on the percentage of vesicles that ruptured when exposed to them revealed a significant main effect of solution ($p < 0.001$), and a significant interactive effect of solution with HgCl$_2$ treatment ($p < 0.001$) (Figure 3.3). Pairwise Tukey’s HSD post-hoc analysis revealed that under the control treatment, 545 mM NaCl caused
significantly fewer vesicles to rupture than in all other solutions. Under the HgCl₂ treatment, exposure to 545 mM NaCl resulted in the lowest percentage of vesicles rupturing, and exposure to 5 mM Tris resulted in the highest. Within ASW and SSW exposures, treatment with HgCl₂ caused significantly fewer vesicles to rupture than control treatments, and within 545 mM NaCl exposures, treatment with HgCl₂ caused significantly more vesicles to rupture (Figure 3.3).

Two-way ANOVA of the mean swelling rates of vesicles revealed a significant main effect of HgCl₂ treatment (p < 0.001), and a significant interactive effect of solution by HgCl₂ treatment (p < 0.05) (Figure 3.4). Pairwise Tukey’s HSD post-hoc analysis revealed that under control conditions, there was a significant effect of solution, with 545 mM NaCl causing slower swelling rates (947 ± 193% area/s) in rupturing vesicles than ASW (1952 ± 217% area/s) and SSW (2032 ± 396% area/s). Under HgCl₂ conditions, there was no significant effect of solution on swelling rate. The mean swelling rate of vesicles exposed to HgCl₂ was approximately 10 times slower than that of vesicles exposed to control conditions under all solution treatments.

3.3.3 Identification of AQP-like proteins in the slime gland

A HMMER search of a translated E. stoutii gill and slime gland transcriptome database yielded two sequences that fit an HMM profile based on 18 aquaporin homologs gathered from NCBI. Both sequences were BLAST searched and found to belong to the aquaporin family. The two sequences were designated AQP-ES1 and AQP-ES2.

A BLAST search of AQP-ES1 revealed that it was most similar to Eptatretus burgeri aquaporin 4 (AQP4; accession number AB258403.1). The 375 bp long nucleotide sequence of AQP-ES1 found in the hagfish transcriptome was not a complete sequence, but was highly homologous to a section of E. burgeri AQP4 that contains one of two highly conserved NPA
motifs in the most likely open reading frame (Figure 3.5). Nucleotide alignment analysis of the AQP_ES1 nucleotide sequence with the *E. burgeri* AQP4 nucleotide sequence using ClustalW2 revealed an alignment score of 98% (Figure 3.6). Amino acid alignment analysis using ClustalW2 gave an alignment score of 96% (Figure 3.7).

A BLAST search of AQP-ES2 revealed that it was most similar to *Hyla chrysoscelis* aquaporin 3-like (HC-3) mRNA (AQP3; accession number DQ364245.1) and to *Takifugu obscurus* aquaporin 10a (AQP10a; accession number AB610920.1). In contrast to AQP-ES1, the AQP-ES2 sequence found in the hagfish transcriptome was 1284 bp long, and may represent the entire protein as it includes start and stop codons, as well as two NPA motifs in the most likely open reading frame (Figure 3.8). Nucleotide alignment analysis of the AQP_ES2 with *H. chrysoscelis* AQP3 and *T. obscurus* AQP10a using ClustalW2 revealed alignment scores of 57% and 64% respectively (Figure 3.9). Amino acid alignment using ClustalW2 gave alignment scores of 47% and 26% for *H. chrysoscelis* and *T. obscurus* respectively (Figure 3.10).

RT-PCR amplification of both AQP-ES1 and AQP-ES2 from the slime gland tissue of two hagfish revealed that these two aquaporin homologs are expressed in the slime gland (Figure 3.11). The PCR products were sequenced and confirmed to be segments of the AQP-ES1 and AQP-ES2 genes.
Figure 3.1. Pacific hagfish (*Eptatretus stoutii*) slime mucin vesicles exposed to SSW (10 mM CaCl$_2$ and 535 mM NaCl) as they swell over time. Each line represents a single vesicle. Individual vesicles exhibit different swelling behaviours when exposed to SSW. (a) Stabilized mucin vesicles take < 1 to 50 s to begin swelling after exposure. (b) Detail: slow-swelling vesicles begin swelling within the first few seconds of exposure.
Figure 3.2. Mucin vesicles treated with the membrane-disrupting detergent Triton X-100 as they swell over time. Pacific hagfish (*Eptatretus stoutii*) slime mucin vesicles treated with 0.1% Triton X-100 prior to exposure to 10 mM CaCl$_2$ + 535 mM NaCl exhibit slow, more uniform swelling rates. Each line represents a single vesicle swelling over time. Vesicles begin swelling within 2 s of exposure.
Figure 3.3. The percentage of mucin vesicles that rupture when exposed to a variety of solutions under control and HgCl$_2$ treatment. Pacific hagfish (*Eptatretus stoutii*) slime mucin vesicles were exposed to three seawater-like solutions and one lightly buffered hypotonic solution (5 mM Tris) under control conditions, or after being treated with 1 mM HgCl$_2$. Asterisks (*) indicate significant differences between control and HgCl$_2$ treatments within each solution treatment. Uppercase letters denote significant differences among solutions within control treatments, and lowercase letters denote significant differences among solutions within HgCl$_2$ treatments.
Figure 3.4. The mean swelling rates of Pacific hagfish (*Eptatretus stoutii*) slime mucin vesicles that rupture when exposed to a variety of solutions under control and HgCl$_2$ treatment. Mean swelling rates were calculated for mucin vesicles exposed to three seawater-like solutions and one lightly buffered hypotonic solution (5 mM Tris), with and without exposure to 1 mM HgCl$_2$. There were significant differences in mean swelling rate for control vs. HgCl$_2$ treatments among all solutions (*). Vesicles treated with 545 mM NaCl under control conditions had a significant lower mean swelling rate than the two other seawater-like solutions (uppercase letters). There were no significant differences in mean swelling rate among solutions under the HgCl$_2$ treatment (lowercase letters).
Figure 3.5. Nucleotide and translated amino acid sequences for the aquaporin-like protein AQP-ES1. The nucleotide sequence for AQP-ES1 was obtained from a search of an *Eptatretus stoutii* gill and slime gland transcriptome database, and the mostly likely amino acid sequence was translated from the first reading frame [F1]. The characteristic aquaporin NPA motif is boxed. Translation done using EMBOSS Sixpack (European Bioinformatics Institute, http://www.ebi.ac.uk/Tools/st/emboss_sixpack/).
Figure 3.6. Nucleotide sequence comparison of *Eptatretus stoutii* AQP-like protein 1 (AQP-ES1) with *Eptatretus burgeri* aquaporin 4 (AQP4). AQP-ES1 was obtained from a translated gill and slime gland transcriptome database, *E. burgeri* AQP4 obtained from NCBI (accession number AB258403). Analysis with ClustalW2 gave an alignment score of 98%. Shown here is the region of the 1271 bp long *E. burgeri* AQP4 sequence that overlaps with the 375 bp long AQP-ES1 sequence retrieved from the transcriptome database. Alignment graphic created with CLC Sequence Viewer v. 6.6.1 (CLC bio, Aarhus, Denmark).
Figure 3.7. Amino acid sequence comparison of Eptatretus stoutii AQP-like protein 1 (AQP-ES1) Eptatretus burgeri aquaporin 4 (AQP4). AQP-ES1 was obtained from a translated gill and slime gland transcriptome database, E. burgeri AQP4 obtained from NCBI (accession number AB258403.1). Asterisks (*) indicate the location of NPA motifs. Analysis with ClustalW2 gave an alignment score of 96%. Alignment graphic created with CLC Sequence Viewer v. 6.6.1 (CLC bio, Aarhus, Denmark).
Figure 3.8. Nucleotide and translated amino acid sequences for the aquaporin-like proteins AQP-ES2. AQP-ES2 translated nucleotide sequence from an Eptatretus stoutii gill and slime gland transcriptome database, and the mostly likely amino acid sequence was translated from the second reading frame [F2]. Asterisks (*) indicate stop codons, mostly likely start codon (ATG) and stop codon (TAG) are underlined and bolded. The characteristic aquaporin NPA motifs are boxed. Translation done using EMBOSS Sixpack (European Bioinformatics Institute, http://www.ebi.ac.uk/Tools/st/emboss_sixpack/).
Figure 3.9. Nucleotide sequence comparison of *Eptatretus stoutii* AQP-like protein 2 (AQP-ES2) with *Hyla chrysoscelis* aquaporin 3-like protein (AQP3) and *Takifugu obscurus* aquaporin 10a (AQP10a). AQP-ES2 was obtained from a translated gill and slime gland transcriptome database, *H. chrysoscelis* AQP3 (accession number DQ364245) and *T. obscurus* AQP10 (accession number AB610920) were obtained from NCBI. Analysis with ClustalW2 gave alignment scores of 57% and 64% for *H. chrysoscelis* and *T. obscurus* respectively. Alignment graphic created with CLC Sequence Viewer v. 6.6.1 (CLC bio, Aarhus, Denmark).
Figure 3.10. Amino acid sequence comparison of *Eptatretus stoutii* AQP-like protein 2 (AQP-ES2) with *Hyla chrysoscelis* aquaporin 3-like protein (AQP3), and *Takifugu obscurus* aquaporin 10a (AQP10a). AQP-ES2 was obtained from a translated gill and slime gland transcriptome database, *H. chrysoscelis* AQP3 (accession number ABC98210) and *T. obscurus* AQP10 (accession number BAL44697) were obtained from NCBI. Asterisks (*) indicate the location of NPA motifs. Analysis with ClustalW2 gave alignment scores of 47% and 26% for *H. chrysoscelis* and *T. obscurus* respectively. Alignment graphic created with CLC Sequence Viewer v. 6.6.1 (CLC bio, Aarhus, Denmark).
Figure 3.11. Expression of two aquaporin-like proteins in hagfish slime gland tissue. Primers were designed for two aquaporin homologs (AQP_ES1 and AQP-ES2) found in a translated Pacific hagfish (*Eptatretus stoutii*) gill and slime gland transcriptome database. Total RNA was extracted from the slime glands of two hagfish (01 and 02), and was reverse transcribed to cDNA. Target sequences 344 bp long for AQP-ES2 and 181 bp long for AQP-ES1 were amplified using reverse transcription (RT) PCR, and both of these homologs were found to be produced in the slime gland. The products were run on a 1% agarose gel, and were sequenced to confirm their identity. First lane is a 100 bp DNA ladder, -RT lanes are non-reverse transcribed controls.
3.4 Discussion

Hagfish slime deployment is a rapid defense mechanism that depends in part upon the fast release of mucins from vesicles that are intact when they are expelled from the slime gland. The membrane-regulated swelling hypothesis states that swelling rate is regulated primarily by characteristics of the mucin vesicle membrane, and less so by the biophysical properties of the mucin gel. This hypothesis is supported by my observation that treatment with the membrane-disrupting detergent Triton X-100 reduced the mean swelling rate of vesicles exposed to SSW, and homogenized the swelling behaviour so that all vesicles began swelling at roughly the same time and at the same slow rate. This was in contrast to control conditions, in which stabilized vesicles treated with SSW exhibited a dichotomy of swelling behaviours: one very rapid, and one much slower, similar to the observation of Herr et al. (2010) of Atlantic hagfish mucin vesicles exposed to ASW. Because the swelling rate of the Triton X-100-treated vesicles likely represents the swelling rate of the mucin gel unconstrained by a membrane, this method may be a valuable technique for future studies characterizing the biophysical properties of hagfish slime mucins. The membrane disruption results also raise the possibility that vesicles that swell slowly under control conditions may have damaged membranes, since, superficially, the slowly swelling vesicles and the Triton X-100-treated vesicles appear to have similar swelling rates. An alternative possibility is that whatever mechanism causes vesicles to rupture rapidly is lacking in the slow swelling vesicles. In either scenario, it is still apparent that the timing of rupture, and the rapid rate at which the majority of vesicles swell are highly dependent upon specific characteristics of the intact vesicle membrane.

In SSW, the time after exposure at which swelling began varied from vesicle to vesicle, with an apparent delay before the majority of rapidly rupturing vesicles began to swell. The fact
that this behaviour was eliminated when vesicles were treated with Triton X-100 suggests that the vesicle membrane is responsible for delayed, rapid rupture. This slight delay in the timing of rupture may be adaptive for a sliming hagfish if it would allow released vesicles to disperse farther away from the body of the hagfish before the vesicles are deployed and release their mucins. To test this idea more thoroughly would require the measurement of swelling rates of native vesicles in seawater without first stabilizing them in sodium citrate, since the stabilization step may introduce vesicle behaviours that are not physiologically relevant. For example, it is possible that the delay in rupture observed in this study was due to the Ca\(^{2+}\)-chelating effects of citrate, which is present in high concentrations in SB. If the vesicles require a certain threshold concentration of Ca\(^{2+}\) to rupture, and residual citrate in the rupture chamber makes Ca\(^{2+}\) inaccessible, then it follows that the rupture of Ca\(^{2+}\)-dependent vesicles will be delayed until the citrate is sufficiently diluted in the chamber.

Similarly, the decrease in swelling rate caused by Triton X-100 treatment may be an artefact of the experimental setup. In the presence of a detergent, swelling of the mucus network should be uninhibited by a membrane, and it is puzzling that these vesicles swell more slowly than vesicles with an intact membrane. The fact that the vesicles remain intact in SB even after treatment with Triton X-100 suggests that high concentrations of citrate can keep the mucin network condensed even in the absence of the vesicle membrane. The swelling rate of vesicles with damaged membranes may therefore be governed simply by the rate at which SB is replaced by SSW during the experiment. In contrast, vesicles with intact membranes are delayed in their swelling by the presence of the membrane, but when the membrane is eventually breached, swelling is rapid because most of the citrate in the chamber has been flushed away and diluted.

Ca\(^{2+}\) had significant effects on both vesicle rupture and swelling rate. Only a portion of
vesicles ruptured when exposed to Ca\(^{2+}\)-free NaCl, and the mean swelling rate of the vesicles that did rupture was significantly slower than the mean swelling rate of vesicles that ruptured in solutions containing Ca\(^{2+}\). This suggests that the differences in the two apparent swelling rates of vesicles exposed to SSW in this study, and to ASW in Herr et al. (2010) are due to Ca\(^{2+}\) sensitivity, and supports the Ca\(^{2+}\)-sensitivity swelling hypothesis. However, not all Ca\(^{2+}\)-independent vesicles were slowly swelling vesicles. Of the vesicles that ruptured with exposure to NaCl, a larger portion of them appeared to be slowly swelling vesicles, but rapidly rupturing vesicles were not eliminated. Since the mechanism of rupture of Ca\(^{2+}\)-independent vesicles is still unknown, it is difficult to explain at this point why they exhibit both slow and fast swelling rates. It is likely, however, that if the Ca\(^{2+}\)-dependent vesicles make up the majority of the rapidly swelling vesicles, then rapidly swelling vesicles are underrepresented in Ca\(^{2+}\)-free solutions because they do not rupture, and it is for this reason that the mean swelling rate of NaCl-exposed vesicles is much lower than ASW- and SSW-exposed vesicles.

HgCl\(_2\) treatment caused an almost tenfold decrease in the mean swelling rates of stabilized vesicles, regardless of the composition of the solution to which they were exposed. However, there were also significant effects of Hg\(^{2+}\) on the total percentage of vesicles that ruptured in all solutions except 5 mM Tris, such that fewer vesicles ruptured in ASW and SSW, and more vesicles ruptured in NaCl than under control conditions. The former observation can be explained by the time constraint of these experiments. The vesicles were exposed to a given solution for 2 min, which, under control conditions, is more than sufficient to observe 100\% rupture in Ca\(^{2+}\)-containing solutions. However, if rapid swelling is dependent on aquaporins in the vesicle membrane, then the inhibition of these aquaporins could result in incomplete vesicle swelling in the 2 min timeframe. The observation that a greater number of vesicles ruptured in
NaCl when exposed to HgCl$_2$ is more difficult to explain, however, it is possible that vesicles that require Ca$^{2+}$ to rupture are affected by other divalent cations such as Hg$^{2+}$, and will rupture in the absence of Ca$^{2+}$ after treatment with Hg$^{2+}$. While Hg$^{2+}$ has been shown to adversely affect the conductance of many Ca$^{2+}$ channels (Büsselberg et al., 1994; Büsselberg, 1995; Tarabová et al., 2006), there is evidence for Hg$^{2+}$-induced Ca$^{2+}$ release from scallop sarcoplasmic reticulum through the interaction of Hg$^{2+}$ with sulfhydryl groups linked to channel components (Burlando et al., 1997). The effects of Hg$^{2+}$ on CaCCs have yet to be determined.

The nearly tenfold decrease in swelling rate with treatment by HgCl$_2$ suggests the presence of aquaporins in the vesicle membrane, as HgCl$_2$ is a known inhibitor of most aquaporins (Agre et al., 2002; Cerda and Finn, 2010), and has been shown to reduce the water permeability of AQP-reconstituted proteoliposomes (Zeidel et al., 1992) and *Xenopus laevis* oocytes expressing AQPs (MacIver et al., 2009). It also inhibits lysis of treefrog erythrocytes via AQP-mediated water influx (Goldstein et al., 2010). Given the effect on swelling rate that HgCl$_2$ treatment had on hagfish mucin vesicles, it is likely that aquaporins play an important role in water influx-driven rupture of the vesicles. The deployment of slime by an aggravated hagfish is a rapid defensive response that makes use of mucin strands to conduct mixing forces to thread skeins in order to form slime in less than 100 ms (Lim et al., 2006; Winegard and Fudge, 2010). Although mixing is necessary for full slime formation, mucin vesicles have no such requirement for rupture to occur, and vesicle rupture appears to be osmotically driven (Herr et al., 2010; Luchtel et al., 1991b). If mucin release must occur prior to thread unraveling and slime formation, and mucin vesicle rupture is driven by water influx, then the presence of aquaporins in the vesicle membrane could facilitate a more rapid response. However, given the confounding effects that Hg$^{2+}$ has on the Ca$^{2+}$-dependent aspect of mucin vesicle rupture, I am currently
unable to separate the Ca$^{2+}$-dependence of swelling rate from the water influx-dependence. Since Hg$^{2+}$ may mimic Ca$^{2+}$ with respect to Ca$^{2+}$-dependent rupture, then it may also mimic Ca$^{2+}$ with respect to Ca$^{2+}$-dependent swelling rates. I cannot, therefore, definitively say that differences in vesicle membrane aquaporins are or are not a possible source of natural differences in mucin vesicle swelling rates in seawater. I can, however, infer that water influx in general is facilitated by the presence of aquaporins in the vesicle membrane.

Both aquaporin-like RNA sequences that were identified in an E. stoutii gill and slime gland transcriptome database were found to be expressed in slime gland tissue. Although this study was done on whole gland tissue, and it is not specific to gland mucous cells, the identification of the AQP-ES1 and AQP-ES2 sequences provide a starting point for future studies. Both sequences have close homologies to aquaporins in other species, and both contain channel-forming NPA motifs that are highly conserved (>98%) among aquaporins (Engel and Stahlberg, 2002; Zeuthen and MacAulay, 2002). While the AQP-ES1 sequence appears to represent only a portion of the entire protein, including only one of two predicted NPA motifs, it was highly homologous (98%) to E. burgeri AQP4 (EbAQP4) identified by Nishimoto et al. (2007). The AQP-ES2 sequence, in contrast, may represent the entire protein, as it appears to includes both stop and start codons, and contains two NPA motifs. Further investigation into this protein is required to properly classify it, however, it most closely resembles H. chrysoscelis AQP3 (57%) and T. obscurus AQP10a (64%).

Known characteristics of AQP4 in other species may make the AQP-ES1 a controversial candidate for AQP-mediated swelling in hagfish slime mucin vesicles. Yang et al. (1997) demonstrated that AQP4 has a low activation energy, and has a single channel water permeability that is 3-fold greater than that of AQP1, which makes it a compelling option for the
basis of rapid slime deployment. However, AQP4 has long been considered to be a mercury-insensitive aquaporin (Agre et al., 2002; Cerda and Finn, 2010; Hasegawa et al., 1994; Yang et al., 1997), and this characteristic appears to hold true for *E. burgeri* AQP4 (Nishimoto et al., 2007). Inhibition of AQPs by Hg$^{2+}$ is thought to be due to the covalent binding of Hg$^{2+}$ to the sulfhydryl group of a cysteine residue found extracellularly near the pore of the channel, in proximity to one of the NPA motifs, and Hg$^{2+}$-insensitivity of AQP4 is attributed to the lack of this Cys residue (Cerda and Finn, 2010). However, when rat AQP4 channels are reconstituted into proteoliposomes, osmotic permeability decreases significantly with HgCl$_2$ treatment (Yukutake et al., 2008). Yukutake et al. (2008) speculated that this effect was seen in AQP4-reconstituted proteoliposomes and not in oocytes because of the orientation of the channel. They found that the reconstituted AQP4 is incorporated randomly into the proteoliposome membrane in both orientations, and identified a Cys residue on a cytoplasmic loop of AQP4 as the target of mercuric inhibition (Yukutake et al., 2008). While aquaporins are typically inserted into cellular membranes with the amino and carboxy termini facing the cytoplasm (Verkman and Mitra, 2000), the orientation of aquaporins in intracellular vesicles is not so predictable. Cho et al. (2002) found that AQP1 in rat pancreatic zymogen granules was oriented such that the carboxyl domain was located intravesicularly, whereas Matsuki et al. (2005) found that AQP5 was oriented in rat parotid gland secretory vesicles with the carboxyl domain located extravesicularly. This evidence is reason enough to consider AQP-ES2 as a possible candidate for AQP-facilitated water influx into *E. stoutii* mucin vesicles, as the orientation of the AQP channel in this intracellular vesicle is unknown. Although Western blot analysis of *E. burgeri* tissues with a EbAQP4-specific antibody did not reveal EbAQP4 expression in the slime gland (Nishimoto et al., 2007), my evidence of RNA expression of AQP-ES1 in the slime gland
suggests that AQPs may be more widespread in hagfish tissues than was previously assumed.

The other candidate AQP, AQP-ES2, was most similar to AQP3 and AQP10, which both fall into the category of aquaglyceroporin as they allow the passage of small solutes such as glycerol and urea in addition to water (Cerda and Finn, 2010). AQP3 and AQP10 are functionally similar, and both are known to be inhibited by HgCl$_2$ (Ishibashi et al., 2002; Ishibashi, 2009). Furthermore, Li et al. (2005) found human AQP10 in the cytoplasm of gastro-entero-pancreatic endocrine cells in the duodenum, closely associated with secretory vesicles. They concluded that AQP10 was present in the vesicle membrane, and speculated that it played a role in vesicle swelling and membrane fusions, similar to the way AQP1 participates in zymogen granule swelling and exocytosis in mast cells (Cho et al., 2002; Li et al., 2005). An AQP10-like channel such as AQP-ES2 could have a similar function in the membrane of the hagfish slime mucin vesicle.

In conclusion, the swelling rates of hagfish slime mucin vesicles can be characterized both by the ion sensitivity of the vesicle, and by HgCl$_2$-sensitivity. It appears as though the differences in the swelling rates of stabilized vesicles exposed to seawater are due to the presence or absence of Ca$^{2+}$ in solution, where Ca$^{2+}$-free solutions cause an overall slower mean swelling rate. This difference is likely due to the fact that a significant proportion of mucin vesicles are highly dependent upon Ca$^{2+}$ for rupture to occur at all, but the mechanism causing these differences requires further investigation. The HgCl$_2$-sensitivity of mucin vesicles suggests that aquaporins play an important role in mucus deployment driven by rapid water influx, and this is further supported by evidence for the RNA expression of two AQP-like proteins in the slime gland. Future work will require the characterization of these AQPs, and the localization of one or both of these proteins to the mucin vesicle membrane within the slime gland. However,
this study provides the first indication that AQP water channels may be involved in the rapid mucus swelling necessary for hagfish slime deployment.
CHAPTER 4: GENERAL DISCUSSION
4.1 Major findings

In this thesis, I investigated the mechanism of mucin release from the mucin vesicles that are expelled from the slime glands of Pacific hagfish (*Eptatretus stoutii*) during the formation of their defensive slime. In Chapter 2, I demonstrated that there are two categories of mucin vesicles released from the slime gland, Ca\(^{2+}\)-independent vesicles and Ca\(^{2+}\)-dependent vesicles. The former represented approximately 40% of the vesicles observed, and consisted of vesicles that ruptured in solutions with a similar osmotic concentration to that of seawater, including a sucrose solution that lacked any inorganics ions, but were equally stable as all other vesicles in high concentrations of sodium citrate. In contrast, the Ca\(^{2+}\)-dependent vesicles, which represented the remaining 60% of vesicles, required at least 3 mM Ca\(^{2+}\) in order to rupture from a stabilized state. Furthermore, rupture of these vesicles was inhibited by the anion channel inhibitor DIDS (4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate), as well as by tannic acid and PGG (penta-O-galloyl-β-D-glucose hydrate), all three of which have been shown to inhibit calcium-activated chloride channels (CaCCs) (Ferrera et al., 2010; Namkung et al., 2011). These results suggest that Ca\(^{2+}\)-dependent vesicle rupture is due to a Ca\(^{2+}\)-triggered influx of anions – most likely Cl\(^-\) – across the mucin vesicle membrane.

In Chapter 3, I demonstrated that the way in which mucin vesicles swell is mediated at least in part by the vesicle membrane, and not only by the properties of the mucin gel. I found that the mucin vesicle swelling rate was drastically reduced when the vesicles were treated with the aquaporin (AQP) inhibitor HgCl\(_2\), which suggests the presence of AQPs in the mucin vesicle membrane. The presence of aquaporins was further supported by RT-PCR revealing that two AQP-like proteins are expressed in the slime gland. Mean mucin vesicle swelling rate was also reduced when vesicles were exposed to Ca\(^{2+}\)-free solutions, which suggests that differences in
vesicle swelling rate may be attributable to Ca\textsuperscript{2+}-dependence, but these results were not separable from the effect of HgCl\textsubscript{2} because Hg\textsuperscript{2+} caused a greater percentage of vesicles to rupture in Ca\textsuperscript{2+}-free solutions, and may have activated CaCCs. It is therefore unclear whether the slow rate at which some vesicles swelled was a result of Ca\textsuperscript{2+} sensitivity or a limitation on water influx due to a reduced number of AQPs in the vesicle membrane.

4.2 Two vesicle types

Together, these results draw a clearer picture of the mechanism of hagfish slime mucin deployment, and clarify earlier studies that were conducted on this system. Prior to the studies presented in this thesis, the most extensive characterization of hagfish slime mucin vesicles was done by Luchtel et al. (1991b), who found that slime will form in solutions with monovalent anions (Cl\textsuperscript{-}, NO\textsubscript{3}\textsuperscript{-}, HCO\textsubscript{3}\textsuperscript{-}) associated with any valency of cation, but not in solutions with high concentrations of polyvalent anions, such as citrate. Because the experimental setup I used in these studies allowed the direct observation of isolated, stationary mucin vesicles as they were exposed to a given solution, I was able to more directly quantify the percentage of vesicles that ruptured in a particular solution. Luchtel et al. (1991b) found no specific requirement for Ca\textsuperscript{2+}, but it may be the case that the 40% of vesicles that rupture in the absence of Ca\textsuperscript{2+} are sufficient to form slime. Whole slime will form in a Ca\textsuperscript{2+}-free NaCl solution, but with a smaller mass than when slime exudate is exposed to solutions that contain an additional 10 mM CaCl\textsubscript{2} (Fudge, 2002). This is consistent with a system in which less than half of the available mucus is released in the absence of a key ion trigger.

The Ca\textsuperscript{2+}-independent vesicles present a puzzling finding as their swelling behaviour appears to be entirely non-specific. The fact that they rupture even when exposed to a
concentrated sucrose solution that lacks any inorganic ions suggests that they may have pores in the vesicle membrane that allow the passage of large solutes, or that their membranes are damaged. However, if all vesicles that ruptured in the absence of Ca\(^{2+}\) were damaged, then one would expect to see Ca\(^{2+}\)-independent vesicles exhibit the same swelling rates as vesicles whose membranes were intentionally damaged with a detergent. This is not the case, as Triton X-100-treated vesicles and NaCl-treated vesicles appear to have much different patterns of swelling.

Vesicle samples that were exposed to Ca\(^{2+}\)-containing and Ca\(^{2+}\)-free solutions included vesicles that exhibited both fast and slow swelling behaviours, similar to those that were observed by Herr et al. (2010). The slow swelling rate observed under these control conditions does, however, appear to be superficially similar to that of Triton X-100-treated vesicles. It is not clear if this implies that there are not two, but three vesicle “types”, but it is possible that slowly swelling vesicles have damaged membranes, and rapidly swelling vesicles can be categorized as Ca\(^{2+}\)-independent and Ca\(^{2+}\)-dependent. While this adds another level of complexity to the characterization of hagfish slime mucin vesicles, this possibility cannot be eliminated until Ca\(^{2+}\)-independent vesicles are better described, and slow swelling rates are better understood. In Chapter 3, I speculated that the slow swelling rate of Triton X-100-treated vesicles was due to the experimental setup, implying that in natural conditions – i.e. vesicles directly released from the gland into seawater – such a slow swelling rate would not be seen. It is only by testing the swelling rate of vesicles directly from the slime gland in seawater without first stabilizing them that the true nature of slowly swelling vesicles can be described.

If Ca\(^{2+}\)-independent vesicles do represent a real vesicle sub-category, then it raises the question of what the functional advantage might be of having two vesicle types, and how they are produced. Two possibilities include differences in mucin content playing different roles in
slime formation, and differences in the timing of mucin release caused by differences in membrane sensitivity. The former seems unlikely as, in the absence of a functional vesicle membrane, there appeared to be no differences in the rates at which the mucin gels swelled, at least from a swelling kinetics perspective. However, the possibility that the mucins contained by the two vesicles types are different could be tested if the Ca$^{2+}$-independent vesicles could be isolated from the Ca$^{2+}$-dependent vesicles. If slime could be made by separating these components and recombining them in different ways, then comparisons of whole slime produced by Ca$^{2+}$-independent vs. Ca$^{2+}$-dependent vesicles could give clues as to how these vesicles differed. A hypothesis of differences in mucin deployment timing may also have some functional merit. In Chapter 3, I speculated that a delay in rupture could be adaptive in allowing the dispersal of vesicles before the slime is formed. At the same time, however, slime formation occurs incredibly quickly, and appears to require the formation of mucin strands before complete thread unraveling can take place (Winegard and Fudge, 2010). If Ca$^{2+}$-independent vesicles are sensitive to a greater variety of solutes, this may allow them to rupture sooner than Ca$^{2+}$-dependent vesicles if the ion channels responsible for this permeability are not gated. Perhaps having two types of vesicles that each are optimal for either fast or slow mucus release provides a balance between quick slime formation and mucus dispersal. If this is true, then the machinery for producing two distinct vesicle types must exist in the slime gland. This raises the question of whether or not a single gland mucous cell can produce multiple types of mucin vesicles, and if it can, how it might do this. If the two mucin vesicle types are produced by two different types of gland mucous cell, then we might ask how the cells differ and if they can be identified.

4.3 The role of Ca$^{2+}$ in slime formation

The role of Ca$^{2+}$ in hagfish slime mucin vesicle rupture is unique when considered in the
context of what is known about how Ca\textsuperscript{2+} acts in mucus deployment in other organisms. Typically, Ca\textsuperscript{2+} is thought to play an important role as an intravesicular charge-shielding cation that allows the condensation of many negatively charged mucins within a secretory vesicle. It is the removal of Ca\textsuperscript{2+} from this system that triggers the mucin network to expand through the “Jack-in-the-box” mechanism of mucin exocytosis (Verdugo, 1991). Furthermore, the presence of high concentrations of Ca\textsuperscript{2+} in an external medium will reduce the rate at which the exocytosed mucin network will expand (Verdugo et al., 1987a), and may provide an additional level of organization to the tangled mucin network by providing Ca\textsuperscript{2+}-mediated protein cross-links between mucin molecules (Raynal et al., 2003). In both cases, Ca\textsuperscript{2+} limits mucin network swelling or changes its rheological properties. It is interesting, therefore, that 60% of hagfish slime mucin vesicles require that Ca\textsuperscript{2+} be present in the external medium before mucin release occurs.

In Chapter 2, I hypothesized that the Ca\textsuperscript{2+} requirement is due to CaCCs in the mucin vesicle membrane, and that it is the movement of other ions (Cl\textsuperscript{-}, Na\textsuperscript{+}) in seawater that cause rupture to occur. It is highly likely, however, that intravesicular Ca\textsuperscript{2+} plays the same role in hagfish mucin vesicles as it does in the mucous vesicles of other organisms, and that there are two roles of Ca\textsuperscript{2+} at play in this system: one of condensing mucins, and one of triggering rupture. The former role has yet to be tested in hagfish slime mucin vesicles, but a greater understanding of slime formation would be achieved through evidence of high intravesicular Ca\textsuperscript{2+} concentrations. Furthermore, although the experiments from Chapter 2 suggest that the low mass of slime that forms in Ca\textsuperscript{2+}-free seawater (Fudge, 2002) is due to fewer vesicles rupturing, if Ca\textsuperscript{2+} promotes the aggregation of mucin molecules and contributes to the organization of the mucin network structure by providing cross-links (Raynal et al., 2003), then perhaps Ca\textsuperscript{2+} in seawater
also plays a role in slime formation after rupture occurs. This could be tested by inducing vesicle rupture with Triton X-100 in Ca\(^{2+}\)-free solutions in order to see how fully deployed slime forms in the absence of Ca\(^{2+}\). Similarly, it could be tested by examining how different ratios of mucin to threads affect slime mass, in order to determine if Ca\(^{2+}\) plays a greater role in the mass of slime formed than does the amount of available mucin. The experiments presented in this thesis provide insight into one role of Ca\(^{2+}\) in slime formation in seawater, but it is likely that it has other roles as well.

4.4 A new model of Ca\(^{2+}\)-dependent mucin vesicle rupture

From the data presented in this thesis, I propose a new model for Ca\(^{2+}\)-dependent hagfish slime mucin vesicle rupture (Figure 4.1). We must first assume that the intravesicular environment is composed of tightly packed mucins whose charges are shielded from one another by a cation such as Ca\(^{2+}\), as is proposed for many other mucous vesicles (Verdugo et al., 1987b). The net charge of particles within the vesicle is likely balanced, such that the mucins within are tightly condensed. Vesicle rupture is therefore induced when this charge balance is disrupted so that the mucins electrostatically repel one another. Given the evidence for both CaCC- and AQP-like proteins in the mucin vesicle membrane, hagfish slime mucin vesicle rupture is likely initiated by the binding of Ca\(^{2+}\) from seawater to Ca\(^{2+}\)-activated anion channels in the vesicle membrane. When these channels open, Cl\(^{-}\) from seawater can move freely across the vesicle membrane, and given that the intravesicular concentration of Cl\(^{-}\) is likely considerably less than the Cl\(^{-}\) concentration in seawater, Cl\(^{-}\) moves into the vesicle down its concentration gradient. The influx of Cl\(^{-}\) disrupts the charge balance within the vesicle, and initiates the electrostatic repulsion of adjacent mucins, while simultaneously creating the osmotic drive for water influx through AQPs. Together, these events result in the swelling of the mucin network and the
Figure 4.1. A new hypothesis of Ca$^{2+}$-dependent hagfish slime mucin vesicle rupture. The mucins within the vesicle are surrounded by a charge-shielding cation (Ca$^{2+}$), which allows them to condense. The vesicle membrane contains aquaporin (AQP) water channels and calcium-activated chloride channels (CaCCs). Rupture is initiated when Ca$^{2+}$ binds to CaCCs, and causes them to open. Cl$^{-}$ enters the vesicle along its concentration gradient, and causes the secondary influx of water through AQPs down the osmotic gradient. Cl$^{-}$ disrupts the intravesicular cation/mucin charge balance, and causes mucins to repel one another. Hydration of the mucins causes the mucin matrix to expand, resulting in vesicle swelling and rupture.
subsequent rupturing of the vesicle membrane as it is stressed beyond its limits. Any damage to the membrane caused by swelling would then allow the free movement of all ions and water molecules into the mucin network, and result in its subsequent expansion.

Although the vesicles did not appear to be dependent upon the presence of Na\(^+\) for rupture to occur, it is possible that Cl\(^-\) influx would be accompanied by an influx of Na\(^+\) from seawater. The influx of negatively charged Cl\(^-\) without a counterion such as Na\(^+\) would result in a buildup of negative charge within the vesicle, which would contribute to the electrostatic swelling of the mucin network, but would also limit the amount of Cl\(^-\) influx. It is possible that even a small, limited amount of Cl\(^-\) influx can trigger the events that lead to mucin vesicle rupture, but the possibility that concurrent Na\(^+\) influx takes place should be considered in future studies. A concomitant influx of Na\(^+\) ions along with Cl\(^-\) would pull in more water than Cl\(^-\) alone would, and has the further benefit of displacing divalent cations like Ca\(^{2+}\) or Mg\(^{2+}\), which likely play a role in the condensation of mucin molecules via shielding negative charges between adjacent mucins. The loss of charge shielding would lead to an increase in electrostatic repulsion among mucins, which would further drive vesicle swelling and the uptake of water through AQPs in the vesicle membrane.

The decondensation of mucins in other organisms is associated with the loss or replacement of Ca\(^{2+}\) from the mucin network. In the giant mucous granules of *Ariolimax columbianus*, for example, rupture is preceded by Ca\(^{2+}\) release (Verdugo et al., 1987b), while in the mucin vesicles of rabbit goblet cells, an exchange of Ca\(^{2+}\) for K\(^+\) may be responsible for mucin network swelling (Nguyen et al., 1998). Given that a rupturing hagfish slime mucin vesicle is surrounded by seawater with high concentrations of Na\(^+\) (~463 mM) and Cl\(^-\) (~543 mM) (Bidwell and Spotte, 1985), it is likely that the exchange of Na\(^+\) with Ca\(^{2+}\) could potentially
disrupt the charge-shielding actions of Ca\textsuperscript{2+}. In Chapter 2, I found no evidence of a requirement for Na\textsuperscript{+} in order for rupture to occur, as rupture occurred in Na\textsuperscript{+}-free solutions if a sufficient concentration of Ca\textsuperscript{2+} was available. Although no pharmacological experiments were done to test the effects of the inhibition of Na\textsuperscript{+} channels in Chapter 2, Luchtel et al. (1991b) tested the effects of the Na\textsuperscript{+}-K\textsuperscript{+}-Cl\textsuperscript{-} cotransport inhibitor furosemide on hagfish slime mucin vesicles, and found no effect of that particular drug on vesicle rupture, despite its effects on \textit{A. columbianus} (Deyrup-Olsen et al., 1992). It is also unclear if rupture is preceded by an efflux of Ca\textsuperscript{2+} from the vesicles, as the presence of Ca\textsuperscript{2+} channels in the vesicle membrane remains unconfirmed. In Chapter 2, I found no evidence for a nicardipine-sensitive Ca\textsuperscript{2+} channel, but given the difficulty of dissolving many Ca\textsuperscript{2+} channel inhibiting drugs in seawater-strength solutions, I was unable to test any other Ca\textsuperscript{2+} channel inhibitors. However, Deyrup-Olsen et al. (1992) demonstrated that ATP-induced rupture of the giant mucous granules of \textit{A. columbianus} can be inhibited by verapamil, suggesting that ATP-induced rupture is due to the actions of an ATP-dependent Ca\textsuperscript{2+} channel. Since rupture of hagfish mucin vesicles cannot be induced with ATP, it is unlikely that the hagfish mucin vesicle membrane contains an ATP-dependent Ca\textsuperscript{2+} channel (Luchtel et al., 1991b).

4.5 Future Directions

The evidence presented in these two studies strongly suggests the presence of both CaCC- and AQP-like proteins in the membrane of hagfish slime mucin vesicles, which will require detailed characterization in the future. The evidence for the CaCC-like channel is based on pharmacological and ion sensitivity experiments, and further investigation of this channel will benefit from the identification of the gene and the characterization of the protein. With access to a transcriptome database for \textit{E. stoutii} gill and slime gland tissue, and the abundance of recent
studies describing TMEM16A and related CaCCs in a variety of organisms and tissues (Huang et al., 2012), the information is available to begin the search for candidates for this protein. At the same time, the functional characteristics of this channel will require more detailed description in order to explain why vesicles ruptured in solutions that lacked Cl− but contained the typically impermeant anion gluconate. It would be highly beneficial to establish the ion selectivity and conductance of the channel by patch-clamp electrophysiology.

As we have two candidate AQP genes that both show expression in the slime gland, the characterization of the specific AQP involved with mucin vesicle rupture has advanced slightly more than the characterization of the CaCC-like channel. However, both sequences should be completed and compared extensively to other known aquaporins in order to accurately place them in the correct AQP subfamily. Since expression of both AQPs was found in the slime gland as a whole, it will also be necessary to determine if one or both of these proteins are localized to the vesicle membrane within the gland mucous cells through, for example, immunostaining methods. Additionally, the ability to clone and express these AQPs in oocytes or proteoliposomes would allow us to quantify their water permeability and solute selectivity. The analysis done in Chapter 3 suggested that AQP-ES2 can be characterized as either an AQP3 or an AQP10, both of which are considered aquaglyceroporins, while AQP-ES1 was most similar to a water-selective AQP4. Given the differences in solute selectivity of the members of these two AQP subfamilies, one method of gaining evidence of one AQP over the other would be to test if hagfish slime mucin vesicles can also be induced to rupture in aquaglyceroporin permeating ions such as urea and glycerol.

The full characterization of the mechanism of rupture of hagfish slime mucin vesicles is still far from complete, and while I have presented a model based on the findings of this thesis,
there are still many questions to be answered. The role of Na\(^+\) in vesicle rupture is one area that requires further investigation, as it is still unclear whether or not Na\(^+\) accompanies Cl\(^-\) influx when rupture is triggered. The role of Ca\(^{2+}\) is similarly speculative, and evidence for the simultaneous release of Ca\(^{2+}\) with mucin vesicle rupture would support a mechanism of mucin deployment as it is described by the “Jack-in-the-box” hypothesis (Verdugo, 1991). Finally, determining how Ca\(^{2+}\)-independent mucin vesicles rupture will greatly advance our understanding of hagfish slime mucin vesicles and slime formation as a whole.

### 4.6 Conclusions

In summary, this thesis presents the first evidence of Ca\(^{2+}\)-dependent rupture of the mucin vesicles from the slime of Pacific hagfish. The data presented here strongly suggest that rupture is dependent upon a CaCC-like channel, and is facilitated by the rapid influx of water through Hg\(^{2+}\)-sensitive AQP-like water channels. These results add significantly to our understanding of how mucus deployment is achieved in the defensive sliming event, and provide the basis for a new hypothesis of hagfish slime mucin vesicle rupture. Furthermore, the pharmacological and molecular evidence of these channels opens the door for future characterization of two membrane proteins in a “primitive” chordate, which will add to our understanding of an ion channel and a water channel whose structures and functions have only been elucidated in the last twenty years.
REFERENCES


### Table A.1. Artificial seawater composition. From Bidwell and Spotte (1985).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc. (mmol/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>468</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>25</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>28</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>10</td>
</tr>
<tr>
<td>KCl</td>
<td>10</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>2.4</td>
</tr>
<tr>
<td>Tris Buffer</td>
<td>5</td>
</tr>
</tbody>
</table>
Figure A. 1. Mucin vesicle samples were quantified by calculating vesicle densities based on the relationship between absorbance (Abs) of stabilized samples at 350 nm and number of vesicles as counted on a haemocytometer. The relationship between Abs and vesicle number is described by the linear equation: $y = 0.0096x$ ($R^2 = 0.94$).
Figure A. 2. Individual trials of hagfish slime mucin vesicles treated with 0.1% Triton X-100 and exposed to SSW (10 mM CaCl$_2$ and 535 mM NaCl).
Figure A.3. Hagfish slime mucin vesicles exposed to artificial seawater (ASW) as they swell over time. Each line represents a single vesicle. Individual vesicles exhibit different swelling behaviours when exposed to ASW. (a) Stabilized mucin vesicles take < 1 to 26 s to begin swelling after exposure. (b) Detail: slow-swelling vesicles begin swelling within the first ten seconds of exposure.
Figure A. 4. Hagfish slime mucin vesicles exposed to 545 mM NaCl as they swell over time. Each line represents a single vesicle. (a) Stabilized mucin vesicles take < 1 to 40 s to begin swelling after exposure. (b) Detail: slow-swelling vesicles begin swelling within the first ten seconds of exposure.
Figure A. 5. Hagfish slime mucin vesicles exposed to 5 mM Tris as they swell over time. Each line represents a single vesicle. (a) Stabilized mucin vesicles take < 1 to 17 s to begin swelling after exposure. (b) Detail: slow-swelling vesicles begin swelling within the first ten seconds of exposure.
Figure A.6. Hagfish slime mucin vesicles treated with 1 mM HgCl₂ and exposed to artificial seawater (ASW) as they swell over time. Each line represents a single vesicle. (a) Stabilized mucin vesicles take < 1 to >120 s to begin swelling after exposure. (b) Detail: slow-swelling vesicles begin swelling within the first ten seconds of exposure.
Figure A.7. Hagfish slime mucin vesicles treated with 1 mM HgCl$_2$ and exposed to 545 mM NaCl as they swell over time. Each line represents a single vesicle. (a) Stabilized mucin vesicles take $< 1$ to $> 16$ s to begin swelling after exposure. (b) Detail: slow-swelling vesicles begin swelling within the first ten seconds of exposure.
Figure A.8. Hagfish slime mucin vesicles treated with 1 mM HgCl$_2$ and exposed to simplified seawater (SSW) as they swell over time. SSW is composed of 10 mM CaCl$_2$ and 535 mM NaCl. Each line represents a single vesicle. (a) Stabilized mucin vesicles take < 1 to >120 s to begin swelling after exposure. (b) Detail: slow-swelling vesicles begin swelling within the first ten seconds of exposure.
Figure A.9. Hagfish slime mucin vesicles treated with 1 mM HgCl$_2$ and exposed to 5 mM Tris as they swell over time. Each line represents a single vesicle. (a) Stabilized mucin vesicles take < 1 to 30 s to begin swelling after exposure. (b) Detail: slow-swelling vesicles begin swelling within the first ten seconds of exposure.