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RESEARCH ARTICLE

Defensive slime formation in Pacific hagfish requires Ca^{2+}- and aquaporin-mediated swelling of released mucin vesicles

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ABSTRACT

Hagfishes defend themselves from fish predators via the rapid deployment of a fibrous slime that adheres to and clogs gills. The slime transforms from a thick glandular exudate to a fully hydrated product in a fraction of a second through a process that involves the swelling and rupture of numerous mucin vesicles. Here we demonstrate that the vesicle membrane plays an important role in regulating the swelling of mucin granules, and provide evidence that the membrane contains proteins that facilitate the movement of ions and water molecules. By exposing isolated mucin vesicles to varying combinations of inorganic ions, organic compounds and membrane channel inhibitors, we found that the majority of hagfish mucin vesicles require Ca^{2+} to rupture. We also show that Ca^{2+}-dependent rupture can be pharmacologically inhibited, which suggests a role for Ca^{2+}-activated membrane transporters. We demonstrate that the aquaporin inhibitor mercuric chloride reduces the rate of vesicle swelling by an order of magnitude, which suggests that aquaporins facilitate the influx of water during vesicle deployment. Molecular evidence of two aquaporin homologues expressed in the slime glands further supports this idea. We propose a model of hagfish slime mucin vesicle rupture that involves Ca^{2+}-activated transporters and aquaporins, and suggest that the presence of these proteins is an adaptation for increasing the speed of vesicle rupture and, consequently, the speed of the sliming response of hagfishes.

KEY WORDS: Mucin, Transporter, Holocrine secretion, Mucus, Slime gland

INTRODUCTION

Hagfishes (Myxinidae) are benthic, marine craniates that are notable for their unique defense mechanism of rapidly releasing large amounts of slime exudate when threatened (Janvier, 1996; Spitzer and Koch, 1998). The exudate combines with seawater to form slime, an effective defense mechanism because of its ability to lodge on gills and impair respiration (Lim et al., 2006; Zintzen et al., 2011). The exudate consists of two principal components, long (~up to 15 cm), thin (~1–3 μm wide) protein threads and mucin-like glycoproteins (Luchtel et al., 1991; Salo et al., 1983). Recent findings have demonstrated that during mixing of slime exudate with seawater, the mucins elongate into strands and assist in the unravelling of condensed slime threads (Winegard and Fudge, 2010). Mucins also likely contribute to the slime's high hydrodynamic resistance (Winegard and Fudge, 2010).

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The slime glands are located in pairs along the entire ventro-lateral length of the animal (Fig. 1) and contain both gland thread cells (GTCs) and gland mucous cells (GMCs) (Spitzer and Koch, 1998). Holocrine secretion of mucins occurs when the gland is stimulated to expel its contents, and both mature GTCs and GMCs are forced through the narrow gland duct, causing the plasma membrane to be stripped from each cell that passes through it (Spitzer and Koch, 1998). In contrast to exocytotic release, in which mucin vesicles fuse with the apical plasma membrane of a mucous cell, holocrine release from hagfish slime glands results in the rapid and mass release of numerous mucin vesicles from many GMCs (Deyrup-Olsen and Luchtel, 1998). When the vesicles come into contact with seawater, they rapidly burst and the mucins interact with seawater and threads to produce slime (Luchtel et al., 1991).

Hagfish mucin vesicles are disc-shaped structures that are ~7 μm along the major axis, and are encased by a lipid bilayer (Luchtel et al., 1991). Vesicles can be stabilized in concentrated (1 mol l^{-1} or higher) solutions containing polyvalent anions such as citrate, sulfate and phosphate, but appear to rupture in solutions containing monovalent anions, regardless of the valency of the associated cation (Luchtel et al., 1991; Salo et al., 1983). Based on these observations, Luchtel et al. (Luchtel et al., 1991) hypothesized that ions from seawater enter the vesicle down their concentration gradients, resulting in an influx of water and the swelling and rupture of the vesicle.

In contrast to mucin granules released by exocytosis (Verdugo, 1991), hagfish slime mucins are released with intact membranes. Given the rapidity with which the slime can be deployed (~100 ms) (Lim et al., 2006), we hypothesized that the movement of ions and water molecules across the vesicle membrane may be accelerated by the presence of ion transporters and aquaporins (AQPs), the latter of which can increase the rate of water permeation by 10- to 100-fold (Agre et al., 2002).

The present study aimed to elucidate the mechanisms involved in Pacific hagfish (*Eptatretus stoutii*) slime mucin vesicle rupture in seawater. Our data demonstrate that the vesicle membrane plays an important role in regulating vesicle swelling, and suggest that the movement of ions and water molecules is facilitated by at least two kinds of membrane transporters, at least of one of which is Ca^{2+} activated.

RESULTS

Effects of Triton X-100 on mucin vesicle swelling rate

In order to determine whether mucin vesicle swelling is dependent on the vesicle membrane, we examined the effects of the membrane-disrupting detergent Triton X-100. Vesicles exposed to the simplified seawater (SSW; 10 mmol l^{-1} $CaCl_2$ + 545 mmol l^{-1} NaCl) control exhibited substantial variability in both the rate and onset of swelling, with an apparent bimodal distribution of some vesicles swelling relatively slowly and early, and others swelling rapidly after an initial delay (Fig. 2A,C). Exposure to SSW containing 0.1%

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List of abbreviations

AQP	aquaporin
ASW	artificial seawater
CaCC	calcium-activated chloride channel
DIDS	4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate
DMSO	dimethyl sulfoxide
EGTA	ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid
ETC	epidermal thread cell
GMC	gland mucous cell
GTC	gland thread cell
HMM	hidden Markov model
LMC	large mucous cell
NMDG-Cl	*N*-methyl-D-glucamine chloride
PGG	penta-*O*-galloyl-β-D-glucose hydrate
SB	stabilization buffer
SMC	small mucous cell
SSW	simplified seawater

Triton-X 100 caused a striking reduction in the variability of swelling behaviour (Fig. 2B,D; supplementary material Movie 1). The mean swelling rate for vesicles in SSW was 2.0±0.4% increase in area per millisecond, and for detergent-treated vesicles it was substantially slower (0.20±0.03% area ms^{-1}).

Ion dependence – roles of Na^+, Cl^- and Ca^{2+}

We exposed vesicles to an array of solutions that differed in the presence or absence of three abundant ions in natural seawater: Na^+, Cl^- or Ca^{2+}. Exposure to artificial seawater [ASW; following a recipe in Bidwell and Spotte (Bidwell and Spotte, 1985)] resulted in the rupture of 99.1±0.5% of vesicles (Fig. 3A) within 120 s (supplementary material Movie 2). All solutions containing 10 mmol l^{-1} Ca^{2+} or higher (SSW, 10 mmol l^{-1} Ca^{2+} gluconate + 980 mmol l^{-1} sucrose, and 333 mmol l^{-1} $CaCl_2$) also caused rupture in >98% of vesicles. This was not significantly different from ASW, indicating that the effect of Ca^{2+} was not dependent on the presence of either Na^+ or Cl^-. In the Ca^{2+}-free solution containing only 545 mmol l^{-1} NaCl, significantly fewer vesicles ruptured (39.7±2.2%) than in Ca^{2+}-containing controls. Other Ca^{2+}-free solutions also resulted in less rupture compared with the ASW control. However, 545 mmol l^{-1} NMDG-Cl (*N*-methyl-D-glucamine chloride) and ASW + 15 mmol l^{-1} EGTA [ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] both caused a higher percentage of vesicles to rupture than 545 mmol l^{-1} NaCl, but these were still both significantly lower than the ASW control.

To test the effects of mono- and divalent cations and anions (Na^+, Ca^{2+}, Cl^- and SO_4^{2-}), mucin vesicles were exposed to a salt-free solution of 1 mol l^{-1} betaine as an osmotic control, and four betaine solutions with 10 mmol l^{-1} $CaCl_2$, $CaSO_4$, NaCl or Na_2SO_4. The two solutions containing Ca^{2+} caused >98% of vesicles to rupture, regardless of the valency of the anion present in solution (Fig. 3B), while the Ca^{2+}-free solutions caused no more rupture than the betaine control.

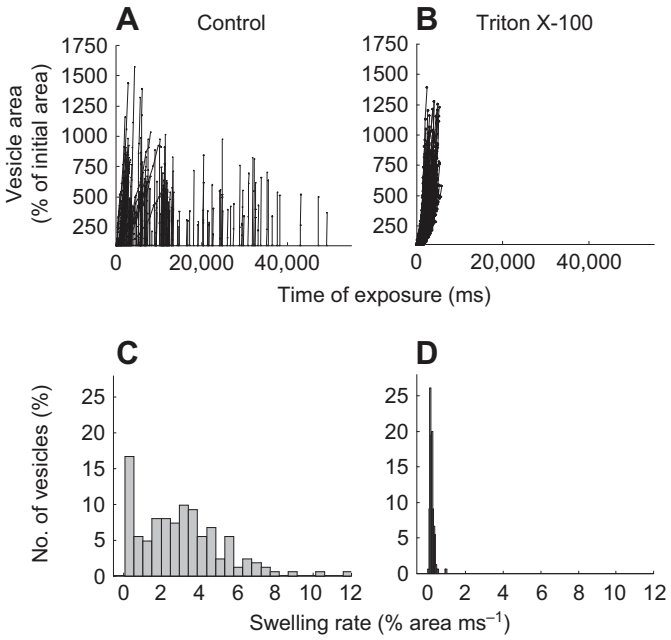

Fig. 2. The effect of a membrane-disrupting detergent on mucin vesicle swelling rates. Vesicles were exposed to simplified seawater (SSW; 10 mmol l^{-1} $CaCl_2$ + 535 mmol l^{-1} NaCl) after (A,C) control and (B,D) 0.1% Triton X-100 treatment. Triton X-100 eliminated the vesicles that swelled late and fast in SSW, and drastically reduced the variability in swelling rate.

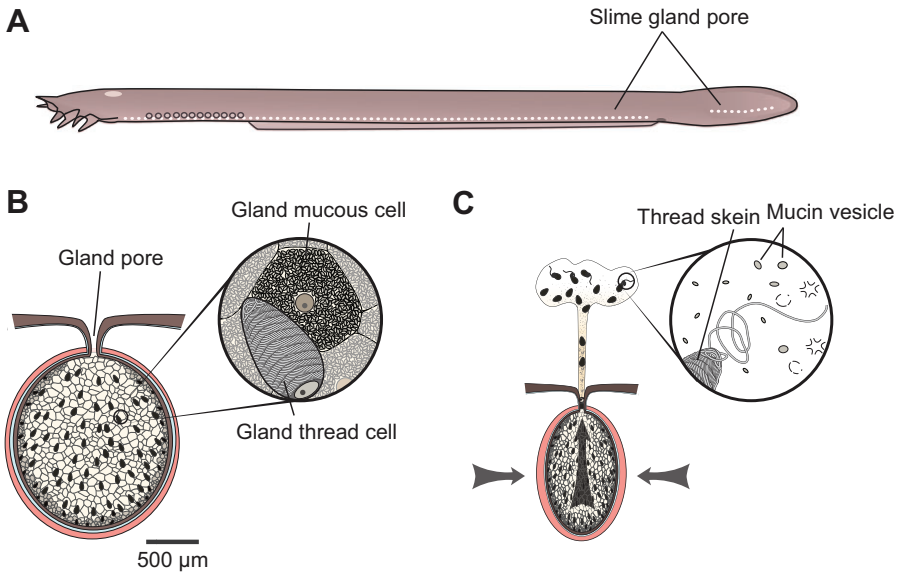

Fig. 1. The anatomy of hagfish slime glands. (A) Hagfish contain multiple paired slime glands along the ventro-lateral length of their body, and in their tail. (B) Each slime gland contains gland mucous cells (GMCs), which produce many membrane-bound mucin vesicles, and gland thread cells (GTCs), each of which produces a single thread skein. (C) When the gland is induced to slime, GMCs and GTCs are expelled through the narrow gland duct, and release their contents – mucin vesicles and thread skeins, respectively – into the seawater. Vesicles rupture and thread skeins unravel, and together they mix with seawater to form mature slime.

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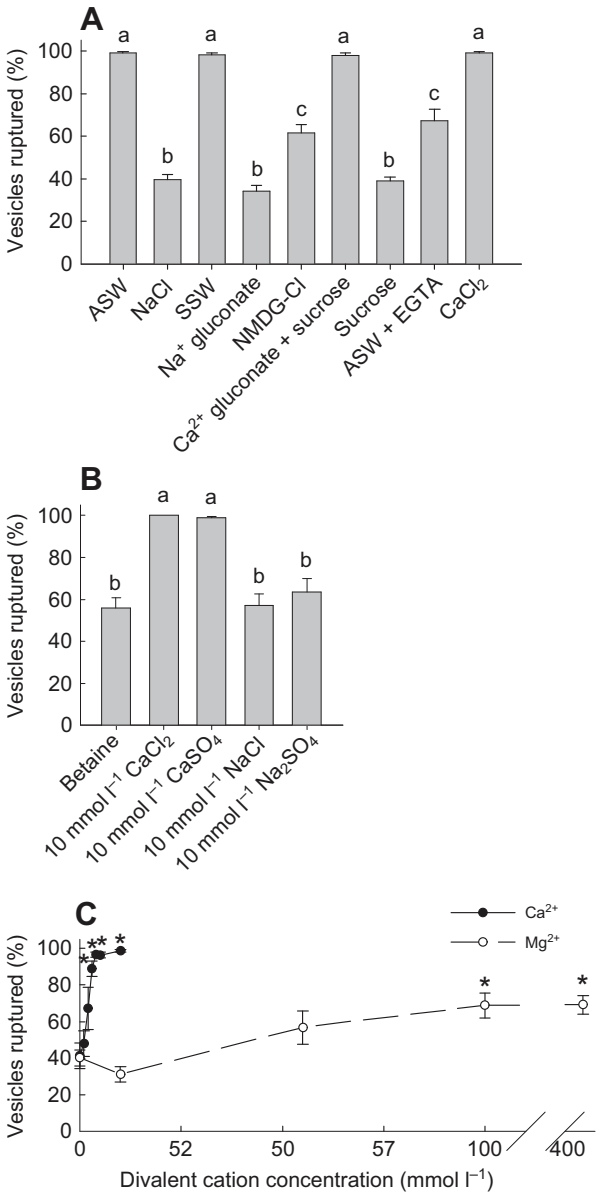

Fig. 3. The effect of solution ion content on mucin vesicle rupture. (A) Rupture of vesicles exposed to artificial seawater (ASW) and other solutions containing combinations of three ions in seawater (Na^+, Cl^- and Ca^{2+}) and impermeable analogues (NMDG, gluconate). (B) Ca^{2+} is necessary and sufficient for the rupture of 100% of vesicles, regardless of the valency of the accompanying anion. Betaine was added to each solution to bring the total osmolarity to 1 osmol l^{-1}. (C) The dependence of vesicle rupture on Ca^{2+} is not a divalent cation effect. Vesicles were exposed to serial concentrations of $CaCl_2$ and $MgCl_2$ with NaCl added to achieve seawater-strength osmolarity. In all trials, vesicle rupture was quantified for 2 min after exposure to the test solution. Error bars are s.e.m., lowercase letters indicate significant differences between treatments (P<0.05), and asterisks represent significant difference from divalent-free solutions (*P<0.05). N=6 in each experiment.

We exposed vesicles to a series of concentrations of $CaCl_2$ in NaCl to determine the threshold at which Ca^{2+} induces rupture. We also tested whether another divalent cation (Mg^{2+}) could substitute for Ca^{2+}. Concentrations of Ca^{2+} >3 mmol l^{-1} resulted in significantly more rupture than NaCl only (Fig. 3C). Mg^{2+}, the other prevalent divalent cation in seawater, only increased rupture above NaCl levels at concentrations higher than 100 mmol l^{-1} Mg^{2+}. Even 415 mmol l^{-1} $MgCl_2$ only caused 69±5% rupture, which was significantly less than the effect of Ca^{2+} concentrations two orders of magnitude lower.

Pharmacological inhibition of Ca^{2+}-dependent vesicle rupture

Hagfish slime mucin vesicles were treated with three ion channel inhibitors to test whether Ca^{2+}-induced rupture could be inhibited. Vesicles were treated with 500 µmol l^{-1}, 0.1% DMSO (dimethyl sulfoxide) or no vehicle prior to exposure to ASW, SSW, 545 mmol l^{-1} NaCl, or 10 mmol l^{-1} Ca^{2+} gluconate + 980 mmol l^{-1} sucrose. Only 37±10% of vesicles ruptured when exposed to Ca^{2+}-free NaCl as previously demonstrated. In addition, there was no significant effect of either the DMSO vehicle control or DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate) on vesicle rupture in the absence of Ca^{2+} (Fig. 4A). However, the three Ca^{2+}-containing solutions (ASW, SSW and Ca^{2+} gluconate + sucrose) showed >97% rupture, which was reduced to <27% by the presence of DIDS.

The inhibitory effect of DIDS did not depend on the presence of Cl^-, as vesicles exposed to 1 mol l^{-1} betaine or 10 mmol l^{-1} $CaSO_4$ + 980 mmol l^{-1} betaine had a similar response, with DIDS exposure resulting in significantly less rupture in the presence of Ca^{2+} than the DMSO control. However, DIDS also caused a small but significant (P=0.032) reduction in rupture in betaine alone (Fig. 4B). Similarly, treatment of vesicles with 20 µmol l^{-1} tannic acid (Fig. 4C) or 20 µmol l^{-1} PGG (penta-*O*-galloyl-β-D-glucose hydrate; Fig. 4D) resulted in significantly less rupture in solutions containing Ca^{2+} compared with controls. Tannic acid also caused a decrease in rupture in 545 mmol l^{-1} NaCl but PGG did not.

Effects of $HgCl_2$ on mucin vesicle swelling rates

We tested for the presence of AQP by exposing vesicles to four solutions (ASW, SSW, 545 mmol l^{-1} NaCl and 5 mmol l^{-1} Tris) with and without pre-treatment with 1 mmol l^{-1} $HgCl_2$, a known AQP inhibitor. $HgCl_2$ pre-treatment and solution type both had significant effects on swelling rate, and the interactive effect was also significant (Fig. 5A). Under control conditions, swelling was significantly faster in solutions containing Ca^{2+} (ASW 1.95±0.22% area ms^{-1} and SSW 2.03±0.40% area ms^{-1}) compared with Ca^{2+}-free 545 mmol l^{-1} NaCl (0.947±0.193% area ms^{-1}). Vesicles treated with ASW under control conditions exhibited both fast and slow swelling (Fig. 5B), but very few vesicles treated with $HgCl_2$ demonstrated fast swelling (Fig. 5C; supplementary material Movie 3). With $HgCl_2$, there was no significant effect of solution on swelling rate. The overall effect of $HgCl_2$ treatment was an approximate 10-fold reduction in swelling rate (mean <0.21% area ms^{-1}).

Identification of AQP-like genes in the slime gland

In order to determine whether AQPs facilitate water movement across the vesicle membrane during vesicle deployment in seawater, we searched for evidence of AQP homologues in the hagfish slime gland. A partial and a full-length sequence for two putative hagfish AQP homologues were identified following searches of two hagfish Illumina transcriptomes obtained from a combined gill/slime gland or kidney preparation. A full-length sequence for the first homologue was obtained by homologous cloning using the *Eptatretus burgeri* AQP4 (AB258403.1) as a template. Maximum likelihood phylogenetic analysis demonstrated that one sequence was a member of the aquaglyceroporin subfamily that shares a common ancestor with mammalian AQP3

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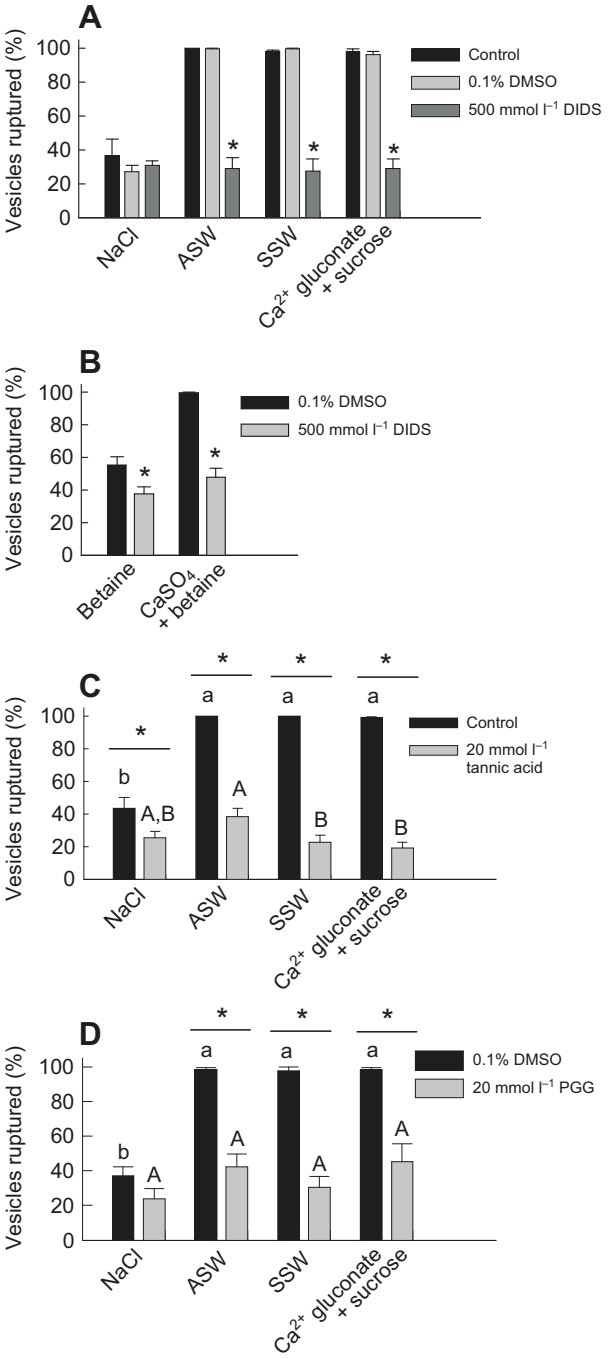

Fig. 4. Pharmacological inhibition of rupture in hagfish slime mucin vesicles. (A) Vesicles were exposed to ASW, SSW, 545 mmol l^{-1} NaCl and 10 mmol l^{-1} Ca^{2+} gluconate + 980 mmol l^{-1} sucrose after treatment with 500 µmol l^{-1} DIDS, 0.1% DMSO (vehicle) or control conditions (no vehicle). (B) To test whether the valency of anions in solution affected the efficacy of DIDS, vesicles were exposed to 1 mol l^{-1} betaine or 10 mmol l^{-1} $CaSO_4$ + 980 mmol l^{-1} betaine, containing DIDS or DMSO. Treatment of vesicles with 20 µmol l^{-1} of (C) tannic acid or (D) PGG significantly reduced vesicle rupture in solutions containing Ca^{2+} compared with controls. Error bars are s.e.m. Asterisks denote significant differences from controls (*P<0.05). Lowercase letters indicate significant differences (P<0.05) among treatments lacking the drug, and uppercase letters indicate significant differences (P<0.05) among solutions with the drug. N=6 in each experiment.

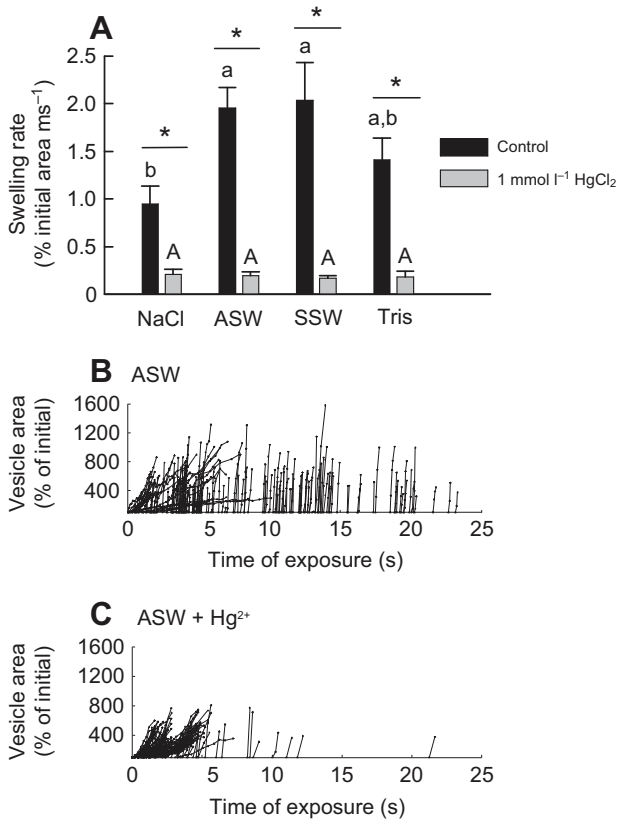

Fig. 5. The effect of $HgCl_2$ on mucin vesicle swelling rate. (A) Vesicles treated with 1 mmol l^{-1} $HgCl_2$ had significantly lower swelling rates than control vesicles exposed to three seawater-like solutions and one lightly buffered hypotonic solution (5 mmol l^{-1} Tris). (B,C) Vesicles treated with 1 mmol l^{-1} $HgCl_2$ rarely exhibited the fast swelling kinetics common in ASW. Error bars are s.e.m. Asterisks indicate significant differences between control and $HgCl_2$ treatments within each solution treatment (*P<0.05). Lowercase letters indicate significant differences (P<0.05) among control treatments, and uppercase letters indicate significant differences (P<0.05) among solutions containing mercury. NaCl, 545 mmol l^{-1} NaCl; Tris, 5 mmol l^{-1} Tris base. N=6.

(Fig. 6A). The other identified sequence grouped most closely with *E. burgeri* AQP4 and shares a common ancestor with other members identified as AQP4 (Fig. 6B). Full-length sequences can be found in the NCBI database for *EsAQP3* (KF895385) and *EsAQP4* (KF895386).

RT-PCR amplification of both *EsAQP3* and *EsAQP4* from slime gland tissue of two hagfish revealed that these two aquaporin homologues are both expressed in the slime gland (see supplementary material Fig. S1). The PCR products were sequenced and confirmed to be segments of the *EsAQP3* and *EsAQP4* genes, respectively.

DISCUSSION

We found that Pacific hagfish vesicles exposed to SSW exhibited two distinct categories of swelling behaviour, a pattern also found in Atlantic hagfish *Myxine glutinosa* (Herr et al., 2010). When the vesicle membrane was disrupted by Triton X-100, swelling behaviour was homogenized. This suggests that the two kinds of swelling can be traced to differences in the vesicle membranes. Luchtel et al. (Luchtel et al., 1991) also proposed that vesicle rupture was mediated by the vesicle membrane, and suggested that it is permeable to monovalent anions and cations of all valencies, and that the movement of these ions into the vesicle causes osmotic influx of water. Given the high concentrations of Na^+ and Cl^- in seawater, it is indeed likely that their concentration gradients would

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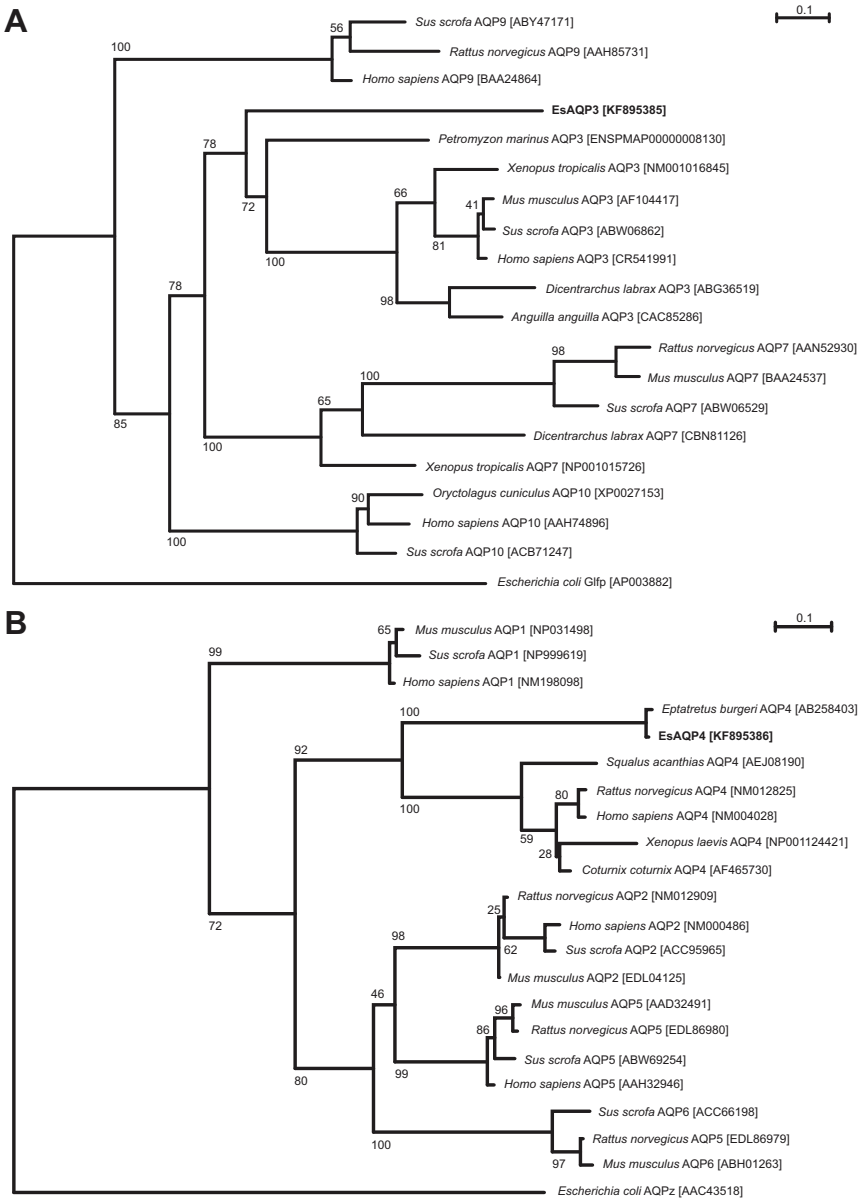

Fig. 6. Phylogenetic analysis of derived EsAQP protein sequences showed phylogenetic relationships of (A) EsAQP3 and (B) EsAQP4. This analysis used the maximum-likelihood method (RAx-ML) with 1000 bootstrap replicates. Numbers in square brackets are the GenBank and Ensemble accession numbers of the sequences. Outgroups were set as (A) *Escherichia coli* Glfp and (B) *E. coli* AQPZ.

favour net influx. Our results do not support this notion, however, as neither Na^+ nor Cl^- was required for vesicle rupture. Ca^{2+} is required for rupture in approximately 60% of vesicles. The remaining 40% of vesicles could only be stabilized in concentrated solutions of salts with polyvalent anions, and ruptured in nearly all aqueous solutions, including hyperosmotic sugar solutions. These data raise the possibility that the two classes of vesicle swelling kinetics correspond with differences in Ca^{2+} dependence. Luchtel et al. (Luchtel et al., 1991) failed to detect Ca^{2+} sensitivity in their study, but this is not surprising given that they assessed vesicle rupture by whole slime formation and were not able to examine the behaviour of individual vesicles. Ca^{2+}-independent vesicles ruptured in the absence of Ca^{2+}, Na^+ and Cl^-, and even in high concentrations of sucrose or betaine. This non-specific rupture may be a result of damaged membranes, which would let these typically impermeant molecules diffuse into the mucin network and cause swelling. Alternatively, the membranes of these vesicles may contain non-selective transporters that allow the passage of both ions and molecules as large as sucrose.

Mg^{2+}, the most abundant divalent cation in seawater, was not able to substitute for Ca^{2+} in its ability to trigger rupture, which suggests a Ca^{2+}-specific mechanism. Furthermore, Ca^{2+}-dependent rupture occurred in the absence of any monovalent cations or anions. Interestingly, Ca^{2+}-dependent rupture was inhibited by the anion channel inhibitors DIDS, tannic acid and PGG. DIDS is an effective Cl^- channel inhibitor (Jentsch et al., 2002; Schultz et al., 1999), but also has effects on a variety of integral membrane proteins, including Ca^{2+}-activated Cl^- channels (CaCCs) (Duran et al., 2010; Ferrera et al., 2010; Huang et al., 2012), bicarbonate transporters (Bevensee et al., 2000; Romero et al., 2000) and pannexins (Ma et al., 2009). Tannic acid and PGG target CaCCs but not other common Cl^- channels (Namkung et al., 2010); however, extensive pharmacological profiles of these compounds are lacking. Despite these effects, it seems unlikely that the mechanism of rupture relies on a chloride channel, as rupture occurred in the presence of impermeant monovalent anions such as gluconate, and even in the absence of any monovalent anion.

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The ability of vesicles to rupture in most test solutions suggests that the vesicle membrane contains transporters, and that these transporters are Ca^{2+}-activated in 60% of vesicles, and constitutively open in the other 40%. While we do not know the identity of these putative transporters, one possibility is that they are similar to those that form gap junctions or gap junction hemi-channels. Connexins and innexins form gap junctions in vertebrates and invertebrates, respectively, while vertebrate pannexins, which share homology with innexins, are more often found as hemi-channels without connections to other cells (reviewed in Scemes et al., 2009) and allow the passage of ions and metabolites up to 1.5 kDa (Wang et al., 2007). It is possible that a pannexin-like channel, or a similar non-specific transporter, is responsible for the movement of materials across the mucin vesicle membrane. Furthermore, some pannexins are activated by Ca^{2+} (Locovei et al., 2006). A pannexin-like transporter in the mucin vesicle membrane would allow the rapid influx of ions in seawater such as Na^+ and Cl^- down their concentration gradients. Even if the vesicle is iso-osmotic to seawater, it is likely that the most osmotically active molecules are the mucins, which are too large to exit the intact vesicle. An influx of ions would thus tip the osmotic balance in favour of net water influx. The only particles that are likely to leave the vesicle are the cationic counterions, which have not yet been identified, but must be present for electrostatic neutrality. Positive counterions also likely play a charge-shielding role and allow charged mucins to remain condensed within developing mucus cells. If these putative positive counterions are more effective at charge shielding than the cations that enter the vesicle during vesicle deployment (such as Na^+), efflux of counterions will further drive swelling via increased electrostatic repulsion among mucins.

We identified two AQP-like genes expressed in hagfish slime gland tissue that our phylogenetic analysis suggests are closely related to AQP3 and AQP4. Furthermore, when vesicles were treated with the AQP inhibitor Hg^{2+} (Agre et al., 2002; Cerdà and Finn, 2010), the mean swelling rates decreased almost 10-fold. The effect of Hg^{2+} on swelling rate was consistent regardless of whether Ca^{2+} was present, and was even observed in the ion-poor 5 mmol l^{-1} Tris treatment. These data support the hypothesis that AQPs in the vesicle membrane facilitate the rapid influx of water by greatly increasing the permeability of the vesicle membrane to water. However, it should be noted that Hg^{2+} can have inhibitory effects on a variety of other transporters including K^+ channels (Gallagher et al., 1995; Leonhardt et al., 1996; Liang et al., 2003), Na^+ channels (Kurata et al., 1998; Leonhardt et al., 1996), Ca^{2+} channels (Leonhardt et al., 1996; Pekel et al., 1993) and Na-K-2Cl cotransporters (Kinne-Saffran and Kinne, 2001). However, given that Hg^{2+} caused a reduction in swelling rate, and not the inhibition of rupture, as was seen with other chemical inhibitors (DIDS, tannic acid and PGG), ion flux was likely not affected. Indeed, the application of Hg^{2+} to vesicles resulted in a small increase in the proportion of vesicles that ruptured in the absence of Ca^{2+} (supplementary material Fig. S2), which is the opposite of what one would expect if Hg^{2+} inhibited ion channels crucial to vesicle rupture. Mercurial inhibition of AQPs is known to be reversible by reducing agents such as β-mercaptoethanol (reviewed in Cerdà and Finn, 2010); however, such agents also cleave the disulfide bonds that link mucin molecules, and have strong effects on the cohesive properties of hagfish slime (Fudge et al., 2005; Koch et al., 1991; Winegard and Fudge, 2010). For these reasons, we did not test the reversibility of Hg^{2+} inhibition, as it would be impossible to determine whether an increase in swelling rate was due to the reversal of AQP inhibition, or to changes to the mucin network (Verdugo, 1990).

AQPs have been found in secretory vesicles, and are thought to assist vesicle swelling and product release in many cases such as secretory vesicles in human small intestine (Li et al., 2005), rat parotid gland secretory vesicles (Matsuki et al., 2005) and zymogen granules of pancreatic acinar cells (Cho et al., 2002; Itoh et al., 2005). While several AQPs have been localized to intracellular vesicles, including AQP1 (Cho et al., 2002), AQP5 (Matsuki et al., 2005), AQP6 (Yasui et al., 1999), AQP10 (Li et al., 2005) and AQP12 (Itoh et al., 2005), there is no evidence of either AQP3 or AQP4 in vesicle membranes. The detection of these two AQPs in the slime gland may indicate their role in vesicle rupture, but we acknowledge that the slime gland contains several cell types, including an epithelial cell layer. We thus cannot yet conclude that these AQPs are expressed in mucous cells, and further work is required to determine whether one or both of these AQPs are found specifically in the vesicle membranes.

AQP4 is considered to be a mercury-insensitive AQP (Agre et al., 2002; Cerdà and Finn, 2010; Hasegawa et al., 1994; Yang et al., 1997), and this appears to hold true for *E. burgeri* AQP4 (Nishimoto et al., 2007). However, a more recent study of AQP4 suggests that the Hg^{2+}-sensitive domain of AQP4 is located intracellularly and is inaccessible to impermeable Hg^{2+} (Yukutake et al., 2008). While the amino and carboxy termini of AQPs typically face the cytoplasm (Verkman and Mitra, 2000), the orientation of these domains in intracellular vesicle membranes can be intravesicular (Cho et al., 2002) or extravesicular (Matsuki et al., 2005). Thus it is possible that AQP4 is Hg^{2+}-sensitive in hagfish slime mucin vesicles, as we do not know the orientation of AQPs in hagfish mucin vesicle membranes. Likewise, although western blot analysis of *E. burgeri* tissues with a *EbAQP4*-specific antibody does not reveal *EbAQP4* expression in the slime gland (Nishimoto et al., 2007), our evidence of RNA expression of *EsAQP4* in the slime gland suggests that AQPs may be more widespread in hagfish tissues than was previously assumed.

Our experiments and analysis lead us to the following model of hagfish slime mucin vesicle rupture. Ca^{2+}-sensitive vesicles are released from the slime gland into seawater, exposing them to Ca^{2+} ions that bind to transporters in the membrane, prompting their opening. A net influx of Na^+, Cl^- and other ions ensues, and water follows via osmosis, with influx facilitated by AQPs, leading to swelling and rupture of the vesicle (Fig. 7). In the case of Ca^{2+}-insensitive vesicles, the simplest model is the same without the requirement of Ca^{2+} to open the transporter.

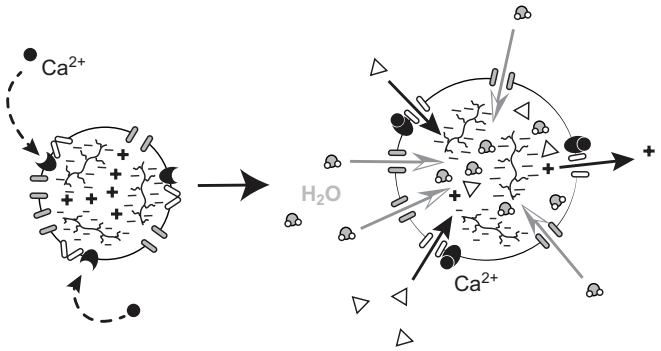

Fig. 7. A model for Ca^{2+}-dependent mucin vesicle rupture. Ca^{2+}-dependent rupture occurs when Ca^{2+} from seawater causes transporters in the mucin vesicle membrane to open and allow the passage of a variety of ions that shift the osmotic balance to favour the influx of water molecules through aquaporins, resulting in the hydration of mucins and vesicle swelling. Triangles represent seawater ions, and plus symbols represent putative positive counterions responsible for mucin condensation in the gland.

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The hagfish slime gland is unique from other mucus delivery systems in that it allows the rapid and directed release of mucus from many mucous cells. The evolution of GMCs and GTCs most likely occurred via the invagination and specialization of epidermal cells, which include epidermal thread cells (ETCs), small mucous cells (SMCs) and large mucous cells (LMCs) (Blackstad, 1968; Spitzer and Koch, 1998). SMCs are the most abundant cells in the epidermis (~92%) and are involved in merocrine secretion of epidermal mucus (Spitzer and Koch, 1998). In contrast, LMCs are fewer in number, and little is known about their function; however, Blackstad (Blackstad, 1968) indicated that mature LMCs, as well as ETCs, migrate to the surface of the epidermis and are expelled. We propose that selection for rapid and copious mucus secretion resulted in the development of larger and larger mucous cells whose contents could be ejected via the holocrine mode.

Evolutionarily speaking, holocrine secretion was surely more effective than the much slower process of exocytotic release, which requires fusion of intracellular vesicles with the apical membrane. Explosive holocrine release created its own challenges, however, as mucus granules were expelled into seawater surrounded by a lipid bilayer, which hindered the movement of ions and water into the mucin gel. The addition of transporters and AQP into the vesicle membranes helped alleviate this problem. Making the opening of the transporters dependent on Ca^{2+}, an ion that is abundant in seawater, but scarce within cells, may have reduced the risk of premature swelling of the vesicles within the gland.

Conclusions

The evolution of defensive sliming in hagfishes involved a switch from exocytotic to holocrine release of mucin vesicles, which increased the speed at which mucus could be expelled, but limited the rate at which it could be hydrated because the granules were enclosed in a lipid bilayer membrane. Our data suggest that this problem was overcome by incorporating proteins in the vesicle membrane, which increased its permeability to ions and water molecules.

MATERIALS AND METHODS

Animal care and sample collection

Pacific hagfish [*Eptatretus stoutii* (Lockington 1878)] were obtained from the Bamfield Marine Sciences Centre, Bamfield, BC, Canada, and were maintained at the University of Guelph Aqualab according to a protocol approved by the University of Guelph Animal Care Committee (protocol 09R128).

Slime exudate samples were collected from anaesthetized hagfish as described in Herr et al. (Herr et al., 2010), and were stored in a stabilization buffer {SB; 0.9 mol l^{-1} sodium citrate + 0.1 mol l^{-1} PIPES buffer [piperazine-*N*,*N*′-bis(ethanesulfonic acid)], pH 6.7} (Downing et al., 1981; Fudge et al., 2003). Stabilized slime was filtered to separate mucin vesicles from thread skeins, and samples of isolated vesicles were quantified and standardized using the linear relationship between vesicle number and absorbance (350 nm) (Downing et al., 1981).

Slime gland tissue samples were collected following euthanasia by decapitation. Slime glands were frozen in liquid nitrogen for storage at −80°C until they were used for RNA extraction.

Mucin vesicle rupture and swelling assays

Mucin vesicle rupture assays followed the methods described in Herr et al. (Herr et al., 2010). Drug/solution effects on mucin vesicles were quantified by calculating the percentage of mucin vesicles that ruptured when exposed to each solution for 120 s. Vesicle swelling rates were measured by recording videos of vesicle rupture at ~14 frames s^{-1}, and analyzing the rate at which vesicle area increased, frame-by-frame as a percentage of the initial area. The swelling rate for each vesicle was calculated as the slope of a line of best fit through the swelling data.

Triton X-100 and $HgCl_2$ exposure

To test the effect of membrane disruption, mucin vesicles from six hagfish were washed with SB containing 0.1% Triton X-100 prior to exposure to a simplified seawater (SSW) solution (10 mmol l^{-1} $CaCl_2$ + 535 mmol l^{-1} NaCl). To test the effect of $HgCl_2$ on swelling rate, vesicles were exposed to artificial seawater (ASW; 545 mmol l^{-1} NaCl), SSW and distilled water buffered with 5.0 mmol l^{-1} Tris under control conditions and after treatment with 1 mmol l^{-1} $HgCl_2$ (MacIver et al., 2009).

Ionic effects

To test whether mucin vesicle rupture is dependent on specific ions in seawater, vesicles were exposed to solutions that mimicked the osmotic strength and pH of seawater, but excluded one or more of the most abundant ions found in natural seawater (Na^+, Cl^- and Ca^{2+}). These solutions included: ASW, SSW (includes Na^+, Cl^- and Ca^{2+}), 545 mmol l^{-1} NaCl (Ca^{2+}-free), 545 mmol l^{-1} NMDG-Cl^- (Na^+- and Ca^{2+}-free), 545 mmol l^{-1} Na^+ gluconate (Ca^{2+}- and Cl^--free), 10 mmol l^{-1} Ca^{2+} gluconate + 980 mmol l^{-1} sucrose (Na^+- and Cl^--free), 1 mol l^{-1} sucrose (osmotic comparison, ion-free), ASW + 15 mmol l^{-1} EGTA (Ca^{2+}-free seawater), and 333 mmol l^{-1} $CaCl_2$ (high Ca^{2+}, Na^+-free). To test the effectiveness of a divalent anion versus a monovalent anion in the presence or absence of Ca^{2+}, vesicles were exposed to a control solution of 1 mol l^{-1} betaine, and four betaine solutions containing 10 mmol l^{-1} of $CaCl_2$, $CaSO_4$, NaCl or Na_2SO_4. The concentration of Ca^{2+} required to cause 100% rupture was measured by exposing vesicles to a series of $CaCl_2$ concentrations, and was compared with a range of concentrations of $MgCl_2$ to determine the interchangeability of divalent cations. The osmolarities of the varying concentrations of $CaCl_2$ or $MgCl_2$ were maintained by adjusting the NaCl concentration for a total osmolarity of 1 osmol l^{-1}. Solution osmolarity was measured with a Vapro Vapor Pressure Osmometer (model 5520, Wescor, Inc., Logan, UT, USA).

Pharmacological inhibition

Vesicles were exposed to the channel inhibitor DIDS at a concentration of 500 µmol l^{-1} (0.1% DMSO) in SB prior to exposure to the following three solutions: SSW, 545 mmol l^{-1} NaCl and 10 mmol l^{-1} Ca^{2+} gluconate + 980 mmol l^{-1} sucrose. Rupture of DIDS-treated vesicles was compared with control solutions with 0.1% DMSO (vehicle control), as well as solutions without a vehicle control.

Tannic acid effects were tested by exposing vesicles to the following solutions with and without 20 µmol l^{-1} tannic acid: ASW, SSW, 545 mmol l^{-1} NaCl and 10 mmol l^{-1} Ca^{2+} gluconate + 980 mmol l^{-1} sucrose. Similarly, mucin vesicles were exposed to 20 µmol l^{-1} PGG in 0.1% DMSO in SB prior to exposure to the four solutions above as well as a DMSO control.

AQP identification

Protein sequences of known AQP3 genes from a variety of species were obtained from NCBI using the standard BLASTp algorithms (Altschul et al., 1990) using *Xenopus laevis* aquaporin 3 (NP_001081876) as a query sequence. Eighteen AQP homologues were obtained and aligned with MUSCLE (www.ebi.ac.uk/Tools/msa/muscle/), and using HMMER3 (v3.0; Janelia Farm; hmmer.janelia.org), a hidden Markov model (HMM) profile was calculated from the resulting alignment. We conducted two separate HMMER searches through both a translated hagfish gill/slime gland transcriptome database and a translated hagfish kidney transcriptome (BGI, Beijing, China) using the compiled AQP HMM profile as a query. The sequences that were returned by the HMMER search were BLAST analyzed on NCBI to verify that they belonged to the AQP family of genes. From these searches, we were able to obtain a full-length sequence of *EsAQP3* and a partial sequence of *EsAQP4*. In order to obtain a full-length coding sequence for *EsAQP4*, interspecies primers (see supplementary material Table S1) were developed using published AQP4 from *E. burgeri* (NCBI accession BAE93686).

Determination of molecular sequences of *E. stoutii* AQP3 and AQP4

Total RNA was obtained from the hagfish gill (~100 mg) using a TRIzol extraction. The RNA sample was then cleaned of genomic contents using

DNase I (Ambion/Life Technologies, Carlsbad, CA, USA) and first-strand gill cDNA was synthesized using RevertAid H-minus M-MuLV reverse transcriptase (Fermentas/Thermo Scientific, Pittsburgh, PA, USA). PCR reactions were conducted using Phusion DNA polymerase (Thermo Scientific) and interspecies or species-specific primers using the manufacturer's specifications for the reaction for 30 cycles. DNA products were sequenced using BigDYE Terminator 3.1 according to the manufacturer's specifications.

Phylogenetic analysis of *EsAQP3* and *EsAQP4*

Identified *EsAQP3* and *EsAQP4* protein sequences were aligned separately against other known AQPs belonging to the AQP and aquaglyceroporin subfamilies, respectively (Tingaud-Sequeira et al., 2010), using MUSCLE (Edgar, 2004) in Seaview (Galtier et al., 1996; Gouy et al., 2010) for MacOS. The two aligned sequence files were then refined using GBlocks (Castresana, 2000) to subtract gaps and residues of low/noisy homology with parameters selected to allow for more relaxed stringency (Talavera and Castresana, 2007). Phylogenetic analysis was conducted using RAxML-HPC BlackBox v7.6.3 (Stamatakis, 2006; Stamatakis et al., 2008) hosted on the CIPRES science gateway (Miller et al., 2010) using the LG evolutionary model (Le and Gascuel, 2008). For phylogenetic analysis, base frequencies were determined by the model and the proportion of invariable sites was determined using the GTRGAMMA model. Bootstrap analysis was conducted with 1000 replications. Nineteen (*EsAQP3*) and 21 (*EsAQP4*) sequences from a range of aquaglyceroporin and aquaglyceroporin subfamilies, respectively, were used in the analysis (Fig. 6), with *GlfP* (AP003882) and *AQPZ* (AAC43518) from *Escherichia coli* selected as an outgroup for the aquaglyceroporin and AQP trees, respectively.

RT-PCR

RT-PCR was used to test whether the identified AQP sequences were expressed in slime gland tissue. Total RNA was extracted from the gland tissue from two hagfish using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), and reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA), following treatment with Amplification Grade DNase 1 (Sigma-Aldrich, St Louis, MO, USA). Detection of *EsAQP3* and *EsAQP4* sequences in the sample via PCR was through a TopTaq DNA Polymerase Kit (Qiagen Sciences, MD, USA) using species-specific primers (see supplementary material Table S2). Sequencing was performed at the University of Guelph Advanced Analysis Centre Genomics Facility to confirm identity. All kits were used following the manufacturer's protocol.

Statistical analysis

Statistical analyses on Triton X-100 and $HgCl_2$ experiments were conducted using SPSS Statistics v19 (IBM, Armonk, NY, USA). Vesicle swelling rates were calculated as the slope of 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. Because the hagfish used for the control solution experiments were different animals 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.

Statistical analysis of ion dependence and chemical inhibition experiment data was conducted using SigmaStat for Windows (v3.5). All percent rupture data were transformed by taking the arcsine of the square root of each value. The ion sensitivity experiments, as well as concentration threshold experiments for Ca^{2+} and Mg^{2+}, were analyzed by repeated-measures 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. Repeated measures two-way ANOVAs were used to test for the effects and interactions of solution and drug treatments for the experiments involving DIDS, tannic acid and PGG. All ANOVA results that indicated significant differences ($P<0.05$) were followed by Holm–Sidak *post hoc* analysis.

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Competing interests

The authors declare no competing financial interests.

Author contributions

All authors contributed to designing the experiments. J.E.H. and A.M.C. conducted experiments, analyzed the data and wrote the paper. All authors contributed to final manuscript editing. D.S.F. and G.G.G. provided materials and animals.

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Supplementary material

Supplementary material available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.101584/-/DC1

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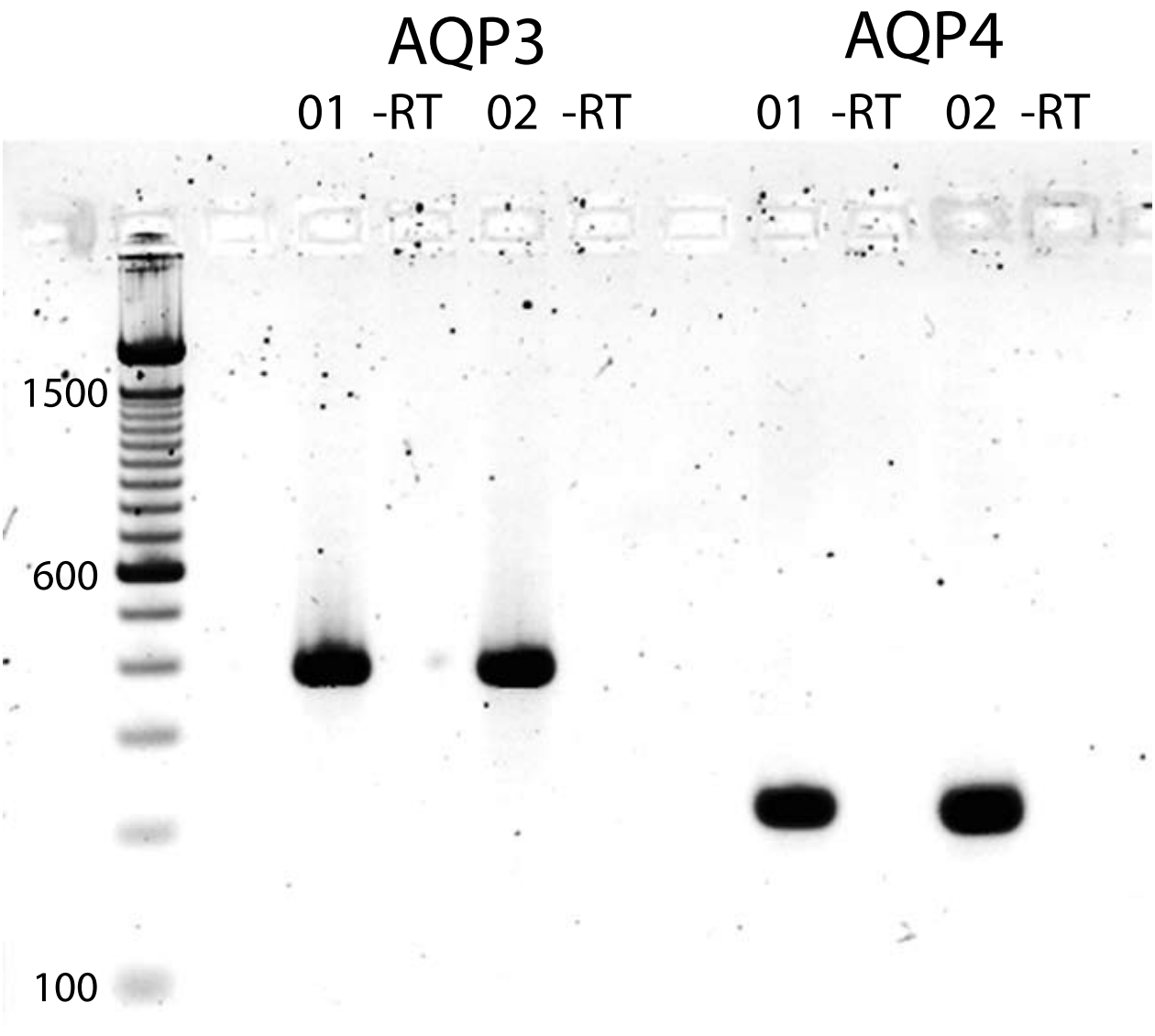

Fig. S1. Expression of EsAQP3 and EsAQP4 in hagfish slime gland tissue. Total RNA was extracted from the slime glands of two hagfish (01 and 02), and was reverse transcribed to cDNA. Target sequences 344 bp long for *EsAQP3* and 181 bp long for *EsAQP4* were amplified using reverse transcription (RT) PCR, and both homologs were found to be expressed 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.

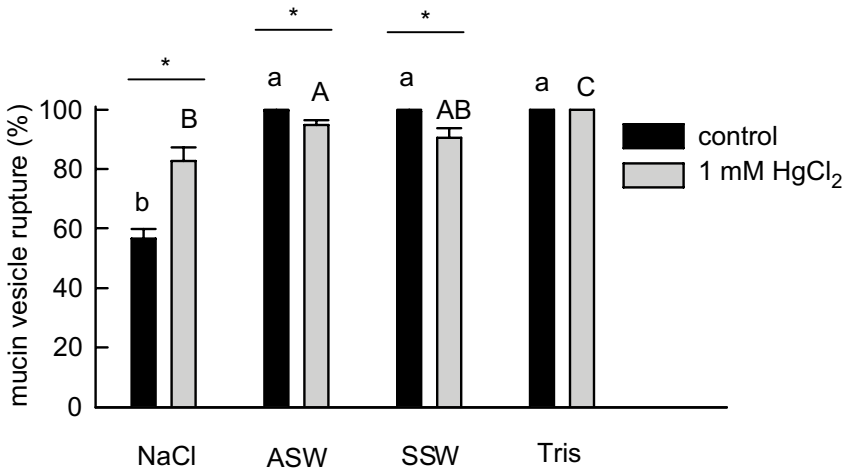

Fig. S2. The effect of $HgCl_2$ on mucin vesicle rupture. Vesicles treated with 1 mM $HgCl_2$ exhibited significantly higher percent rupture in 545 mM NaCl (Ca^{2+}-free) than in control NaCl, but in artificial seawater (ASW) and simplified seawater (SSW, 10 mM $CaCl_2$ + 535 mM NaCl), significantly fewer vesicles ruptured over the exposure period (2 min). $HgCl_2$ did not affect vesicle rupture in a very hypotonic solution (5 mM Tris). Error bars are s.e.m.. Asterisks (*) indicate significant differences between control and $HgCl_2$ treatments within each solution treatment. Lowercase letters indicate significant difference ($p < 0.05$) among control treatments, and uppercase letters indicate significant differences ($p < 0.05$) among solutions containing mercury. N = 6.

Movie 1. Hagfish slime mucin vesicles treated with 0.1% Triton X-100 prior to exposure to simplified seawater (SSW, 10 mM $CaCl_2$ + 535 mM NaCl). Videos were recorded at ~14 frames per second using a monochrome digital camera (Q-imaging Retiga Exi Fast1394) connected to a Nikon Eclipse 90i microscope (Nikon Instruments, Inc., Melville, NY). Videos were processed using NIS-Elements A.R. 3.0 software (Nikon Instruments, Inc.).

Movie 2. Hagfish slime mucin vesicles exposed to artificial seawater (ASW). Videos were recorded at ~14 frames per second using a monochrome digital camera (Q-imaging Retiga Exi Fast1394) connected to a Nikon Eclipse 90i microscope (Nikon Instruments, Inc., Melville, NY). Videos were processed using NIS-Elements A.R. 3.0 software (Nikon Instruments, Inc.).

Movie 3. Hagfish slime mucin vesicles treated with 1 mM $HgCl_2$ prior to exposure to artificial seawater (ASW). Videos were recorded at ~14 frames per second using a monochrome digital camera (Q-imaging Retiga Exi Fast1394) connected to a Nikon Eclipse 90i microscope (Nikon Instruments, Inc., Melville, NY). Videos were processed using NIS-Elements A.R. 3.0 software (Nikon Instruments, Inc.).

Table S1. Primers used to obtain full length coding sequence for *EsAQP3* and *EsAQP4*. Interspecies primers were developed using published AQP4 from *Eptatretus burgeri* (NCBI accession BAE93686.1).

Interspecies primers for *AQP4*	
5'-GTTCGCAAAGCCACTCC-3',	F1_E.stouti
5'-CCCTGTCTGAACAAATGAGC-3',	R1_E.burgeri
5'-TCTTCTGAGAAAGGACAGTCG-3',	F2_E.burgeri
5'-CCATTCTCACTCTGGATTTGC-3'.	R2_E.burgeri
AQP3 full length primers	
F: 5'-CTGAGTACCTGATACCTCCTGA-3'	
R: 5'-GATGATAGATGCAGGACGAAGG-3'	
AQP4 full length primers	
F: 5'-CATGCCGCAAATAAGCAGAC-3'	
R: 5'-TCT TCTCCCTGTATGAACAAATGA-3'	

Table S2. *EsAQP3* and *EsAQP4* primers used in the detection of expression of these genes in the hagfish slime gland via RT-PCR.

RT-PCR primers	
5'-GCGGGTCCTCGCACAGCGAATGCGC-3'	F_EsAQP3
5'-CCGCGGTCTCCATGCTCCAGGGT-3'	R_EsAQP3
5'-CGACCTACACATCGCACTTG-3'	F_EsAQP4
5'-ACGAGGTTGTGAAGGGTGAC-3'	R_EsAQP4