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Abstract:

The cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel widely expressed in many tissues and many organisms. Mutations of CFTR cause the disease cystic fibrosis in humans. In the intestine of fish CFTR may be a regulator of further downstream processes. CFTR in both fish and mouse intestines has been shown to regulate the secretion of HCO_3^- into the lumen by the Slc26 class of transporters. In fish it is possible for CFTR to have an osmoregulatory role due to the ability of CFTR to transport chloride ions. Previous experiments conducted in our lab have shown that relative CFTR protein levels do not change upon acclimation of a fish salinities of 0.1 ppt, 5 ppt, and 32 ppt water. In this experiment I will test the hypothesis that CFTR activated through a cGMP-dependent pathway may only have a minimal role in directly regulating osmoregulation. *Fundulus grandis* were acclimated to 0.1, 5, and 32 ppt for at least 1 week each. The short circuit current (I_{sc}) and resistance changes across the anterior intestine epithelium were measured before and after the addition of the cGMP analog 8-Br-cGMP and the irreversible CFTR inhibitor s-methyl methanethiofulfonate (s-methyl). My findings showed that cGMP stimulation and s-methyl inhibition of CFTR showed no differences in changes to epithelium I_{sc} and resistance, leading to the conclusion that CFTR does not have a major role in osmoregulation. Instead, intestinal CFTR in fish may be a regulatory mechanism of downstream effectors such as Slc26 transporters that control functions such as HCO_3^- secretion into the intestinal lumen.

Introduction:

In humans cystic fibrosis is a hereditary disease in which the affected individuals possess mutations of cystic fibrosis transmembrane conductance regulator (CFTR) proteins that are defective (Kerem, 2005; Kitson *et al*, 2013). CFTR is a chloride channel that allows the secretion of chloride ions out of the cell. The loss of chloride transport across epithelial cells due to defective CFTR transporters leads to mucous accumulation on the epithelial surfaces (Hanrahan *et al*, 2013). It is the mucous accumulation on the surfaces of lung tissue that causes most of the mortalities due to cystic fibrosis in humans (Hanrahan *et al*, 2013).

CFTR is found in the fish gill where it is a crucial component of chloride excretion (Singer *et al*, 1998, Scott *et al*, 2005). CFTR is also present within the fish intestine however work needs to be done to fully understand its role in maintaining ionic balance (Scott *et al*, 2006). The intestine of teleost fish contains many membrane-bound ion transporters such as $Na^+/K^+/2Cl^-$ cotransporters (NKCC) and Na^+/Cl^- cotransporters powered by the electrochemical gradient generated by the Na^+/K^+-ATPase (Scott *et al*, 2006). Regulation of ion transporters within the intestine can thus lead to the regulation of water absorption across the intestinal membrane in both freshwater and saltwater acclimated fish. The ability of CFTR to operate as a chloride channel means that it potentially has a role in chloride secretion and thus osmoregulation.

A possible mechanism of activation of CFTR is by through its phosphorylation by the cGMP-dependent kinase, cGMP protein kinase II (cGKII) (Figure 1) (Hofmann *et al*; Lohmann *et al*, 1997). A cell permeable analog of cGMP, 8-Bromoguanosine 3', 5'-cyclic monophosphate (8-Br-cGMP), has been shown to mimic the effect of cGMP and selectively activate cGKII in the

rat intestine (Markert *et al*, 1995). Additionally, Tuo *et al* (2009) found that 8-Br-cGMP induced CFTR phosphorylation in duodenal epithelial cells in mice. CFTR in the intestine is suggested to facilitate ion absorption by replenishing the depleted luminal chloride concentrations caused by NKCC activity (Grosell *et al*, 2011). CFTR has also recently been shown to have an influence on the secretory activity of the Slc26 anion transporters in mouse intestines, influencing HCO_3^- secretion (Singh *et al*, 2010).

The electrical properties of a membrane such as short circuit current (I_{sc}) and membrane resistance depend on factors that determine the movement of ions across a membrane. An example of a factor that determines a membrane's electrical properties is the availability of transport mechanisms for the ion to cross the membrane such as ion transporters and channels. I_{sc} is a measure of the movement of ions across a membrane and resistance is a measure of the ease of travel for ions. Thus, following the changes in these two values in a membrane can provide insight into the changes in activity of into ion transport mechanisms such as channel openings. Trischetta *et al* (1996) found that the application of 8-Br-cGMP to the basal intestinal epithelium in the European eel (*Anguilla anguilla*) resulted in a decrease of I_{sc} from its control value.

Fundulus grandis is a euryhaline fish species that lives in brackish habitats within the Gulf of Mexico (Scott, 2005). *F. grandis* possesses the ability to thrive in a wide range of salinity tolerances and is a popular model for studying osmoregulatory plasticity in fish (Scott *et* al, 2004; Scott *et al*, 2006; Laverty and Skadhauge, 2012). Fish acclimated to seawater are exposed to an environment hyperosmotic to their internal fluids. Due to the hyperosmotic stress, seawater acclimated fish constantly lose water to the environment through osmosis (Evans, 2005; Evans, 2008). Thus, seawater-acclimated fish must constantly drink water in order to replenish the water lost to the environment and actively excrete ions into their surroundings (Evans *et al*, 2005;

Evans, 2008, Marshall *et al*, 2002). Conversely, fish acclimated to freshwater face a hypotonic environment and undergo a net loss of ions to the surroundings through the gills and the urine. Freshwater fish must uptake ions through their gills in order to maintain osmotic balance and also have a slower drinking rate (Evans *et al*, 2005; Evans, 2008). The changing excretion and absorption mechanisms that *F. grandis* incorporate serve to maintain a constant salt balance within the organism.

This set of experiments aims to characterize the role of CFTR in the anterior intestine of *F. grandis* by specifically targeting the phosphorylation of CFTR by cGK II. This will be achieved by the application of 8-Br-cGMP to the anterior intestine of *F. grandis* acclimated to a range of salinities and measuring the resulting I_{sc}. I will also apply the CFTR inhibitor s-methyl methanethiosulfonate (s-methyl) and compare its effects in fish acclimated to each salinity. Previous dot blot analyses have shown that the relative protein levels of CFTR did not change with salinity acclimation. If my results are consistent with our lab's previous results then the effect of activating CFTR by cGKII, and inactivation by s-methyl, will produce similar effects in fish acclimated to any salinity, due to consistent CFTR protein levels.

Materials and Methods:

Tissue preparation

Fundulus grandis were caught from Cocodrie, La and acclimated in the lab to 10 ppt (Instant Ocean®) water for at least three weeks (24 ±1.8°C, 12 h light and 12 h dark cycle). *F. grandis* were transferred and acclimated separately to 5 ppt and 0.1 ppt for at least one week. After one week some 5 ppt acclimated *F. grandis* were then transferred and acclimated to 32 ppt for at least one additional week prior to use. The salinities were selected to allow fish

acclimations to a range of salinities from freshwater to saltwater. Between 15 and 18 fish were acclimated to each salinity.

The fish were euthanized by decapitation preceding tissue preparation. The anterior intestine was immediately cut out of each fish. The intestines were cut open to lie flat. Spatulas were used to both stretch the intestinal membranes and to try to scrape away the muscular layer as best as possible. The intestines were then mounted onto pair of sliders with a diameter of 0.1 cm^2. The sliders were used to hold the intestinal membrane between the chambers and expose the membrane to the bathing solutions.

Ussing Chamber preparation

An Ussing Chamber was used to measure the I_{sc} across the intestinal epithelia mounted on the sliders. The chamber was set up so that I_{sc} and membrane resistance were measured relative to the apical side of the membrane. 2 mL of modified Cortland's Saline Solution (151 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.88 mM $MgSO_4 \cdot 7H_2O$, 0.46 mM Na_2HPO_4, 0.48 mM K_2HPO_4, 11 mM HEPES-free acid, 4.5 mM urea, pH 7.8) was used to bathe both the apical and basal sides of the membrane within the Ussing Chamber. The solution was constantly bubbled with 97% O_2 and 3% CO_2 throughout each experiment in order to keep the membrane functional for as long as possible. Additionally, the apical chamber solution contained 3 mM mannitol and the basal chamber solution contained 3 mM glucose. The glucose was added to the basal side to provide and energy substrate and the mannitol was added to provide osmotic balance. Agar was dissolved in 3 M KCl to form a 3% wt agar solution that was used to make the agar bridges. 8-Br-cGMP was dissolved into Milli-Q® water and stored at -20 °C. S-methyl methanethiofulfonate (s-methyl) was dissolved in DMSO and also stored at -20 °C.

Data collection

A dose response test was conducted to determine the concentration of 8-Br-cGMP to sufficiently stimulate the membrane epithelium CFTR. This was done by letting membrane sections establish a control I_{sc} before bringing the concentration of 8-Br-cGMP in the bathing solution to 0.05, 0.1, and 0.2 mM 8-Br-cGMP. The dose response test led to the conclusion that 0.2 mM 8-Br-cGMP stimulated the membrane significantly more 0.05 mM 8-br-cGMP and 0.1 mM 8-Br-cGMP.

The I_{sc} was measured constantly throughout each replicate and each replicate was allowed to establish a constant baseline I_{sc} before applying either 0.2 mM 8-Br-cGMP basally or 0.1 mM s-methyl apically to the solution of either chamber. A replicate constituted a single experimental test that involved the establishment of a control I_{sc} and the I_{sc} measured after the addition of a single drug for a fish membrane acclimated to a single salinity. The membranes with stabilized I_{sc} values after basal 8-Br-cGMP addition were then administered apical s-methyl. Each of these additions was also considered one replicate for a single salinity. No single fish intestine was used for two of the same kinds of replicates. It usually took 45 – 60 minutes to establish this baseline I_{sc}.

Data points were selected 11, 10, and 9 minutes prior to the addition of the each drug of each experiment to serve as the control data points for that drug and replicate. These points were selected to provide consistent and equal data values of I_{sc} and membrane resistance after every drug addition. After each addition a period of 30 minutes was given to allow the I_{sc} to stabilize before any further additions of drugs to the chambers. Data points were also selected 9, 10, and

11 minutes after any addition of drugs to the chambers. A 2 mV pulse was constantly applied at a frequency of one pulse/minute to every replicate.

The I_{sc} values were measured at each of the previously mentioned data points, giving three I_{sc} values before and after each treatment. The set of I_{sc} values before each treatment were averaged, as well as the set of I_{sc} values collected after each treatment. With these two sets of average I_{sc} values I measured the percent change of I_{sc} by diving the calculated I_{sc} average for the three points after the treatment by the I_{sc} average for the points before the treatment.

At each data point the ΔI_{sc} could be measured. The applied 2 mV pulse divided by the ΔI_{sc} was equal to the membrane resistance for that data point. The membrane resistance was also averaged for the set of three data points before each treatment and the set of data points after each treatment. The percent changes in membrane resistance were calculated from the resulting calculated average membrane resistances before and after each treatment. Within each replicate the same data points were used to collect both the I_{sc} and membrane resistance values.

Three different possible drug combinations were applied to the intestinal membrane. The first was the only the application of the CFTR inhibitor s-methyl to the apical membrane and the second was only the application of the CFTR activator 8-Br-cGMP to the basal membrane. I measured both the changes in I_{sc} and membrane resistance from the control value for these two combinations. The third combination was the application of apical s-methyl to inactivate the CFTR on an intestinal membrane that had already been stimulated by basal 8-Br-cGMP.

Statistical analysis

ANOVA (alpha = 0.05) was performed with SAS 9.3 (SAS Institute®) to compare the I_{sc} percent changes due to the addition of 0.05, 0.1, and 0.2 mM basal 8-Br-cGMP for both 5 ppt and 32 ppt acclimated fish, as part of the dose response test. ANOVA was also used to compare the resulting changes in I_{sc} and membrane resistance resulting from each drug combination and salinity acclimation. Additionally, a two-way ANOVA was performed for all the drug and salinity treatment combinations.

Results:

Dose response test

5 ppt acclimated fish showed significantly different percent I_{sc} changes between application of 0.05, 0.1, and 0.2 mM basal 8-Br-cGMP (F (3, 50) = 12.64, $p < 0.0001$). Basal application of 0.2 mM 8-Br-cGMP showed the greatest percent I_{sc} change and 0.05 mM 8-Br-cGMP showed the least. 32 ppt acclimated fish also showed significantly different percent I_{sc} changes (F (3, 44) = 4.69, $p = 0.01$). The I_{sc} percent change again increased with increasing concentration of 8-Br-cGMP, with the maximum change occurring after the basal concentration of 8-Br-cGMP had been brought to 0.2 mM. These results are illustrated in Figure 2.

Comparisons of I_{sc} and membrane resistance changes

ANOVA revealed that were similar effects of just s-methyl on I_{sc} for fish acclimated to each salinity (F (2, 17) = 0.65, $p = 0.5$). The effect of 8-Br-cGMP on I_{sc} also did not change with salinity acclimation variation (F (2, 12) = 2.71, $p = 0.1$). The addition of s-methyl to a membrane already stimulated by 8-Br-cGMP did not produce significantly different effects on I_{sc} between

fish acclimated to each of the salinities (F (2, 12) = 0.90, p = 0.4). Figure 3 shows the average effects of each treatment on I_{sc} for each of the salinities.

The effects of each treatment on membrane resistance produced results similar to those of the I_{sc}. 0.1, 5, and 32 ppt acclimated fish all showed insignificantly different responses after the addition of just s-methyl (F (2, 14) = 2.39, p = 0.1). The effects from 8-Br-cGMP on membrane resistance were similar in all fish despite the salinity differences (F (2, 12) = 1.95, p = 0.2). S-methyl resistance change responses in membranes already treated with 8-Br-cGMP also showed no significant difference among salinities (F (2, 12,) = 3.23, p = 0.08). These resistance changes are illustrated in Figure 4. A comparison between all of the drug combinations and salinities also showed no significant difference between all of the groups (F (4, 41) = 0.35, p = 0.8).

Discussion:

Our results show that there is a similar activation effect from 8-Br-cGMP for fish acclimated to each tested salinity. The inactivation effect from s-methyl is also similar among each of the salinities. This suggests that CFTR expression was similar in all fish, despite the acclimated salinity. This conclusion agrees with results previously found in our lab that showed that the intestinal CFTR protein levels were not significantly different between 0.1 ppt, 5 ppt, and 32 ppt acclimated fish (Meng, unpublished).

I propose that intestinal CFTR activation is necessary for purposes other than maintaining chloride ion balance. Activation of CFTR and the subsequent chloride flux possibly leads to further activation of more highly conserved downstream elements, perhaps mechanisms of HCO_3^- exchange. As referenced before, Singh *et al* (2010) demonstrated that CFTR activity in the rat jejunum stimulates HCO_3^- secretion by the Slc26 family of transporters. HCO_3^- exchange

between the organism and the environment is a mechanism of pH regulation in the fish intestine (Carvalho *et al,* 2012). My experiments supplied the intestinal membrane with consistent bubbling of 3% CO_2. Carbonic anhydrase in blood facilitates the conversion of CO_2 to carbonic acid which rapidly dissociates into HCO_3^-. Because HCO_3^- is a weakly acidic ion it can play a buffering role in an organism's pH regulation mechanisms. This is a possible explanation for the consistent CFTR activity in my experiments where pH was not a variable.

Slc26 transporters have also been found in sea bream where they play a role in removing luminal calcium by precipitating calcium carbonate (Gregório *et al*, 2013). This mechanism would suggest that the role of CFTR in fish intestines is to not directly maintain ionic balance through chloride transport, but through the indirect downstream effects for calcium removal. The fish used in my experiments were not acclimated to varying concentrations of calcium ions. Thus if an important role of CFTR is to remove luminal calcium then that could possibly explain the lack of CFTR activity differences among the fish acclimated to the range of salinities tested. However, this mechanism may be not be well supported by my experiments as a calcium removal mechanism would not be expected to be significant in freshwater acclimated fish due to the low calcium concentrations.

I acknowledge that a limiting aspect of this study was the conservative use of ion channel blockers. S-methyl was used to monitor the activity of CFTR; however there is the possibility of variation in the activity of other ion channels and transporters, possibly due to addition of the cGMP analog. The application of specific blockers such as oubain for the Na^+/K^+-ATPase could help more accurately isolate and describe the effect of CFTR in the fish intestine in response to activators such as cGMP. This would help isolate the response in membrane I_{sc} and resistance to

just CFTR. Furthermore, methanthiosulfonates have been shown to interact with cysteine residues other than those of CFTR through covalent binding (Angelow and Yu, 2009).

The evidence in this paper supports the hypothesis that CFTR protein levels in the anterior intestine of *F. grandis* are similar among fish acclimated to different salinities. This is likely because the chloride excreted by CFTR plays a role in regulating further downstream effectors such as the Slc26 transporters. Further experiments should target possible mechanisms that are regulated by CFTR activity. If the purpose of CFTR is to regulate HCO_3^- secretion, calcium removal, and pH homeostasis (Singh *et al*, 2010, Carvalho *et al*, 2012, Gregório *et al*, 2013), then possibly monitoring CFTR expression in fish acclimated to varying pH or calcium concentration would yield more conclusive results.

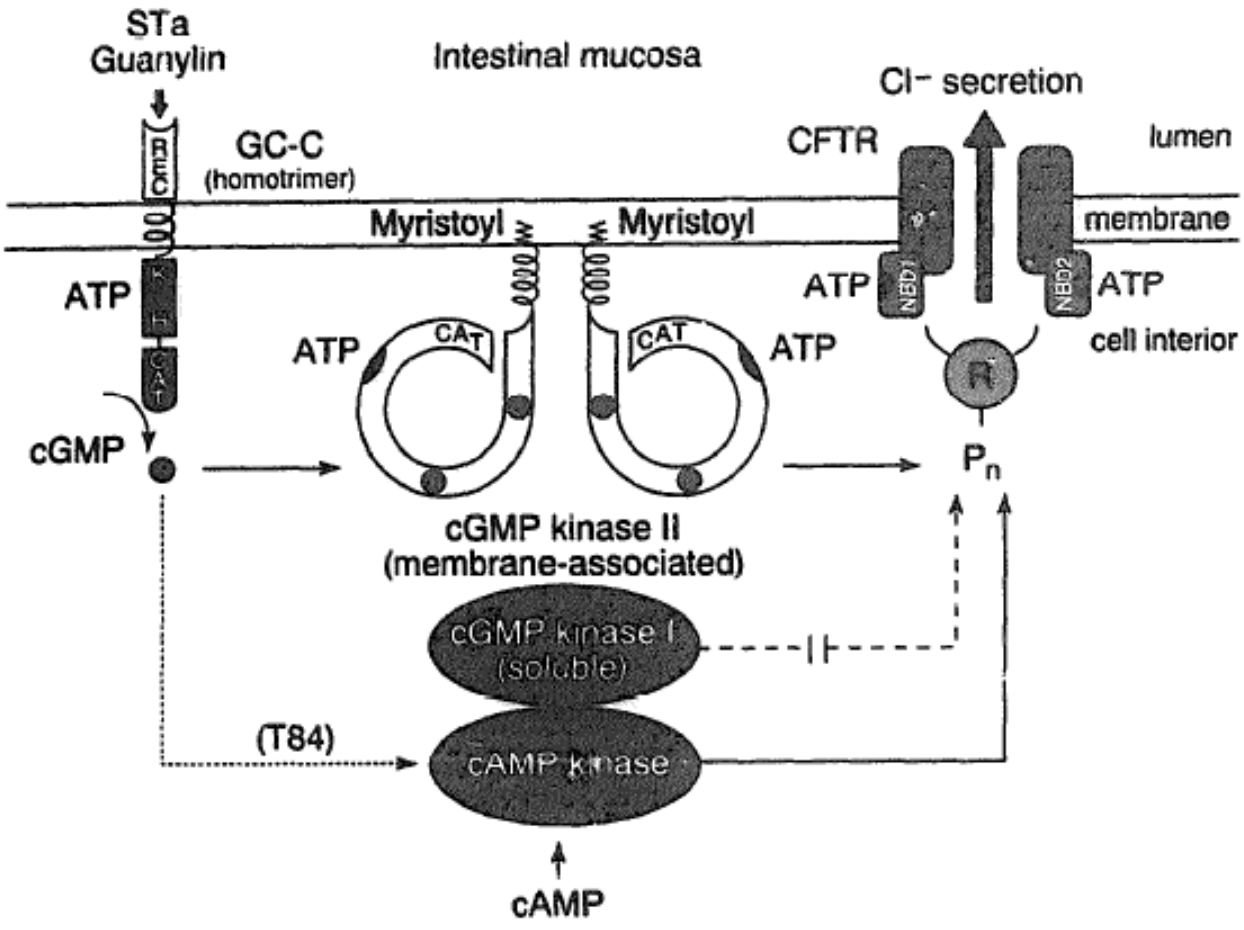

Figure 1. Model of chloride excretion via CFTR, induced by a cGMP-dependent mechanism (Lohmann *et al*, 1997)

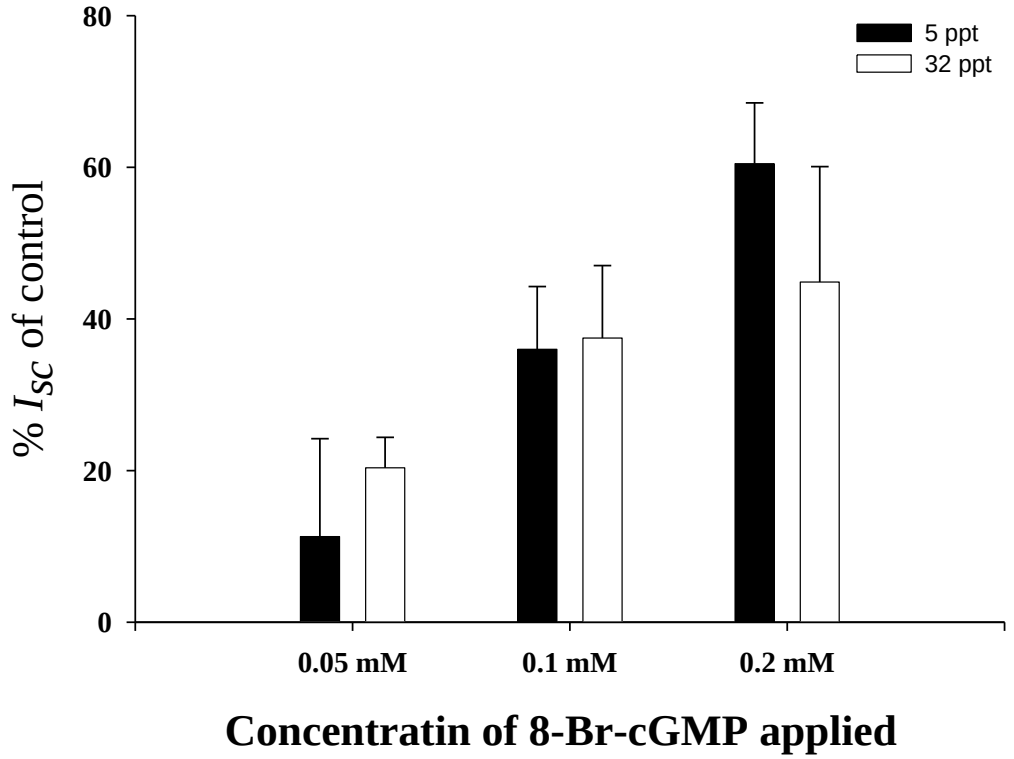

Figure 2. The percent change of membrane I_{sc} from the control value showed significant increases correlated to increasing concentration of applied 8-Br-cGMP for both 5 ppt and 32 ppt acclimated fish (N = 12). The bars shown represent the standard error of each mean.

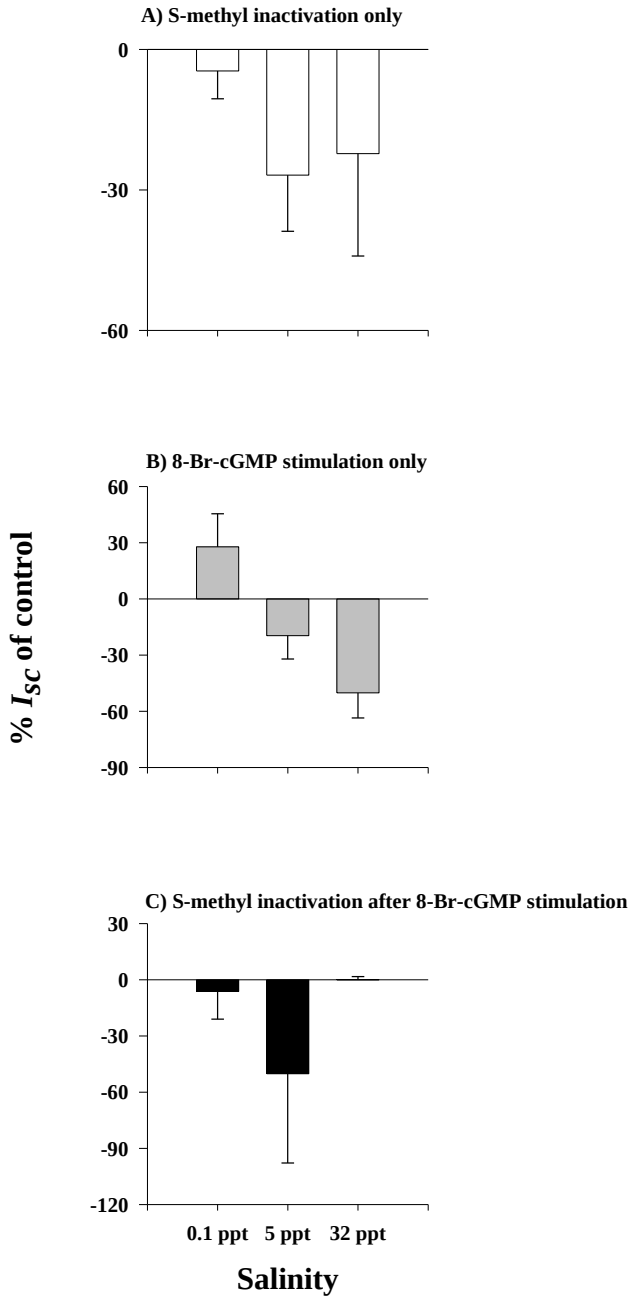

Figure 3. The percent changes in membrane I_{sc} for each combination of treatment and acclimation (N = 5 - 8). A) The percent changes in membrane I_{sc} from the control value to the resulting I_{sc} from s-methyl addition. B) The control value to the I_{sc} resulting from 8-Br-cGMP addition. C) The percent change in I_{sc} resulting from s-methyl addition to an already stimulated membrane by 8-Br-cGMP. The bars represent the standard error of each treatment.

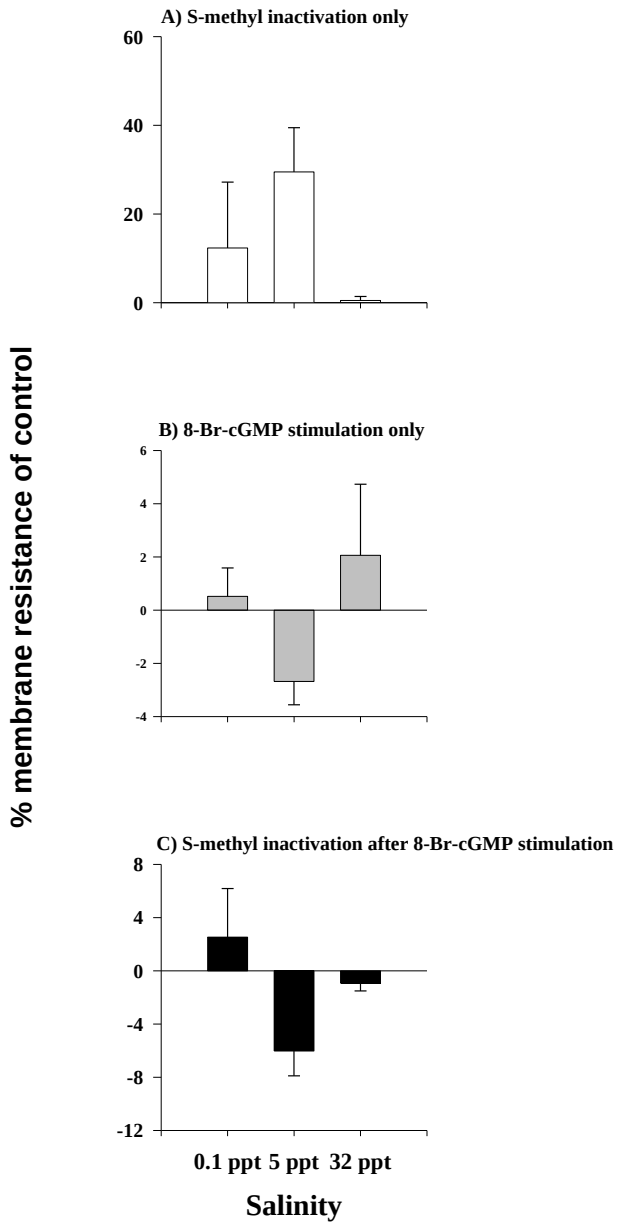

Figure 4. The percent changes in membrane resistance for each combination of treatment and acclimation (N = 5 - 8). A) The percent changes in membrane resistance from the control value to the resulting I_{sc} from s-methyl addition. B) The control value to the resistance resulting from 8-Br-cGMP addition. C) The percent change in resistance resulting from s-methyl addition to an already stimulated membrane by 8-Br-cGMP. The bars represent the standard error of each treatment.

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