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Pharmacological evidence that DAPI inhibits NHE2 in *Fundulus heteroclitus* acclimated to freshwater

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ABSTRACT

Ionoregulation in the euryhaline killifish *Fundulus heteroclitus* has been intensively studied over the last two decades using a variety of techniques. However, there has been limited use of pharmacological inhibitors to identify proteins involved in ion transport for this species. In this study, we used a range of pharmacological inhibitors (EIPA, DAPI, ethoxzolamide, bumetanide, bafilomycin, phenamil, hydrochlorothiazide) to investigate the proteins involved in Na^+ transport in freshwater (1 mM Na^+) acclimated *F. heteroclitus*. Our results indicate that Na^+ uptake under these conditions is sensitive to both EIPA (NHE-specific inhibitor) and DAPI (putative ASIC-specific inhibitor), but not to any of the other inhibitors. Results for EIPA are consistent with previous studies indicating *F. heteroclitus* relies solely on NHE2 for Na^+ transport across the apical membrane of ionocytes. In contrast, results for DAPI are surprising given previous studies that have indicated the H^+-ATPase is basolaterally located in *F. heteroclitus* and so cannot contribute to Na^+ uptake via ASIC. The lack of bafilomycin sensitivity in the current study is consistent with a basolaterally located H^+-ATPase. This suggests that DAPI is not an ASIC-specific inhibitor as has been previously assumed, and that it may also inhibit NHE2. Finally, we did not observe Na^+ uptake to be sensitive to ethoxzolamide, suggesting that carbonic anhydrase may not be involved in generating the H^+ needed to maintain NHE activity in freshwater as has been previously proposed.

1. Introduction

In freshwater, fish compensate for diffusive salt loss by actively taking up ions against their concentration gradients at the gill primarily by specialized ionocytes. Sodium uptake is thought to be primarily driven by Na^+/K^+-ATPase located on the basolateral side of the ionocytes. Uptake of Na^+ across the apical membrane of ionocytes may occur by several different transport proteins. The specific apical Na^+ transport proteins involved varies by species and as a function of environmental conditions (e.g., Na^+ concentration, pH, temperature) (Brix et al., 2015; Hwang et al., 2011).

The most ubiquitous mechanism of Na^+ uptake at the apical membrane of ionocytes is via a Na^+/H^+ exchanger (NHE; SLC9), with two isoforms, NHE2 and NHE3, commonly found in freshwater and euryhaline fish (Hwang et al., 2011). Many fish species also utilize a H^+-ATPase/Na channel system (H/NaC) for Na^+ uptake (Hwang et al., 2011). A few species have also been shown to utilize a Na-Cl co-transporter (NCC; SLC12) or a Na^+-K^+-$2Cl^-$ co-transporter (NKCC; SLC12) to facilitate apical Na^+ uptake (Brix and Grosell, 2012; Wang et al., 2009; Yang et al., 2011).

Confirmation of the involvement of these proteins has been accomplished by a combination of techniques including the use of pharmacological inhibitors, manipulation of Na^+, H^+, and Cl^- gradients, immunohistochemistry to visualize protein localization, gene expression, and gene knock down/out studies. However, for the H/NaC system, the presence of a Na^+ channel in particular has historically only been indicated by inhibitor studies, especially with phenamil (Bury and Wood, 1999; Preest et al., 2005; Reid et al., 2003), which is thought to be a specific blocker of the epithelial Na^+ channel (ENaC) (Garvin et al., 1985). However, genomic studies have failed to identify ENaC in any Actinopterygii species, though it is present in multiple Sarcopterygii species (Uchiyama et al., 2015), suggesting phenamil is not specific to ENaC and can block other Na^+ transport proteins as well. Recently, several studies have tentatively identified the Na^+ channel in Actinopterygii to be an acid sensitive ion channel (ASIC). Specifically, ASIC4 has been localized to the apical membrane of the gill of both rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*) (Dymowska et al., 2015; Dymowska et al., 2014). In both species, DAPI (4′,6-diamidino-2-phenylindole), a putative ASIC-specific inhibitor (Chen et al., 2010), significantly inhibits Na^+ uptake, providing

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functional evidence that ASIC may be the Na^+ channel in fish gills involved in maintaining Na^+ homeostasis (Dymowska et al., 2015; Dymowska et al., 2014).

Pharmacological inhibition of Na^+ transport proteins has been a useful tool in elucidating the various mechanisms by which aquatic organisms maintain Na^+ homeostasis. Interestingly, the model euryhaline fish *Fundulus heteroclitus*, which has been intensively studied with respect to Na^+ ionoregulation, has not been subjected to the standard array of pharmacological inhibitors. Patrick and Wood (1999) conducted the only study of this type on *F. heteroclitus*, demonstrating that Na^+ uptake in freshwater acclimated fish is inhibited by 1×10^{-4} M amiloride, a concentration typically thought to inhibit both NHE and Na^+ channels such as ENaC and ASICs (Brix and Grosell, 2012; Kleyman and Cragoe, 1988). Instead the proteins involved in ion homeostasis in this species have primarily been identified using immunohistochemistry, gene expression, and western blot techniques (Edwards et al., 2005; Edwards et al., 2010; Scott et al., 2005).

Like all fish, Na^+ homeostasis by *F. heteroclitus* is driven by a basolaterally located Na^+-K^+-ATPase. However, *F. heteroclitus* is somewhat unusual in that apical entry of Na^+ appears to occur exclusively via a Na^+/H^+ exchanger (NHE-2) (Scott et al., 2005). While the H^+-ATPase is also found in ionocytes of *F. heteroclitus*, immunohistochemistry studies indicate it is located on the basolateral, rather than apical membrane (Katoh et al., 2003). The lack of an apical H/NaC system in *F. heteroclitus* may be characteristic of euryhaline fishes in the order Cyprinodontiformes, as several other species in this order also appear to lack an H/NaC system (Brix and Grosell, 2012, 2013).

In this study, we sought to characterize proteins involved in Na^+ uptake in freshwater acclimated *F. heteroclitus* using a range of pharmacological inhibitors (EIPA, DAPI, ethoxzolamide, bumetanide, bafilomycin, phenamil, hydrochlorothiazide). The objective of these experiments was to further characterize the relative contribution of various Na^+ transport proteins to Na^+ uptake across the apical membrane of this species. Further, because only NHE is thought to be involved in Na^+ uptake, it allowed us to test the specificity of other inhibitors that have undergone limited testing in fish (e.g., DAPI, phenamil).

2. Methods and materials

2.1. Animal collection and holding

Adult *F. heteroclitus* (2–5 g ww) were shipped from Aquatic Research Organisms (Hampton, New Hampshire) to the University of British Columbia (Vancouver, BC) and held for ~2 weeks at a salinity of $15\,g\,l^{-1}$ [dechlorinated City of Vancouver tapwater with Instant Ocean™ (Spectrum Brands, Blacksburg, VA, USA)] in a closed 700 l recirculating system with biological filtration maintained at 20–22 °C. After this initial holding period, fish were acclimated over the course of 7 d to freshwater (dechlorinated City of Vancouver tapwater with reagent grade salts added to reach the following ionic composition: ~1.0 mM Na^+, 1.0 mM Cl^-, 0.5 mM Ca^{2+}, 0.2 mM Mg^{2+}, 0.5 mM SO_4^{2-}, 0.8 mM HCO_3^-, pH 7.9) in the same 700 l recirculating system. Fish were held under these conditions for an additional 4 weeks prior to experiments. During holding, fish were fed flake food (Tetramin™ Tropical Flakes, Spectrum Brands, Blacksburg, VA, USA) once daily to satiation and 70% water changes were performed on the system weekly to maintain water quality.

2.2. Experiments with pharmacological inhibitors

In our initial screening experiments with pharmacological inhibitors, we exposed fish separately to 7 different inhibitors: DAPI (4′,6-diamidino-2-phenylindole, 1×10^{-6} M) a putative ASIC-specific inhibitor (Chen et al., 2010), phenamil (2×10^{-5} M) a Na^+ channel (ENaC) blocker (Garvin et al., 1985), EIPA (5-(*N*-ethyl-*N*-isopropyl)-amiloride) (5×10^{-5} M) a NHE-specific inhibitor (Kleyman and Cragoe, 1988), ethoxzolamide (6-ethoxy-1,3-benzothiazole-2-sulfonamide) (1×10^{-4} M) a carbonic anhydrase inhibitor (Gordon, 1958), bafilomycin A1 (1×10^{-6} M) a H^+-ATPase inhibitor (Werner et al., 1984), bumetanide (1×10^{-4} M) a NKCC inhibitor (Gimenez, 2006), and hydrochlorothiazide (1×10^{-4} M) a NCC inhibitor (Stokes et al., 1984). All inhibitors except DAPI were dissolved in dimethyl sulfoxide (DMSO) with a final DMSO concentration of 0.1% in each treatment. A separate carrier control (0.1% DMSO only) was tested to evaluate the effect of DMSO on Na^+ uptake.

We used a relatively standard approach in which for each treatment, 8 fish (0.8–4.2 g) were placed individually in containers with 250 ml of freshwater of the same composition to which fish were acclimated. Fish were not fed for 48 h prior to the experiment. Test solutions were continuously aerated to maintain dissolved oxygen levels during the flux period. Fish were allowed to settle in their containers for 10 min after which 1–2 μCi of ^{22}Na was added to the solution. The flux solution was sampled after 1 min for measurements of $[Na^+]$ and ^{22}Na activity. The total flux exposure period was 1–2 h for all inhibitors except bafilomycin which was limited to 15 min due to its relatively high toxicity. In all cases, the internal specific activity was < 1% of the external specific activity such that correction for backflux was unnecessary (Maetz, 1956). At the end of the exposure period, water samples for $[Na^+]$ and ^{22}Na activity were again collected, fish were removed from the exposure media, double rinsed in a 100 mM NaCl solution to displace any loosely bound ^{22}Na, euthanized with an overdose of MS-222, blotted dry, weighed to nearest 0.1 mg and then assayed individually for radioactivity.

2.3. Dose-response experiments for DAPI and EIPA

Based on results from the initial inhibitor experiments, we conducted additional experiments to fully characterize the dose-response of Na^+ uptake to DAPI and EIPA and to allow estimation of an IC50 for each inhibitor. For each inhibitor, five concentrations on a logarithmic scale were tested along with a carrier control, with six replicates per concentration using the same methods previously described.

2.4. Analytical methods, calculations and statistical analysis

Total Na^+ in water samples was measured by atomic absorption spectrophotometry (Varian SpectraAA220, Mulgrave, Australia). Water and fish samples were measured for ^{22}Na activity using a gamma counter with a window of 15–2000 keV (Wallac 1470 Wizard, Perkin Elmer, Waltham, MA, USA). Rates of Na^+ uptake as measured by the appearance of radioactivity in the fish (in $nmol\,g^{-1}\,h^{-1}$) were calculated using previously described methods (Boisen et al., 2003).

All values are expressed as means ± SEM throughout. Results for DAPI were compared to a water only control while all other inhibitors were compared to the carrier control. Data were compared by Welch's *t*-test (DAPI) or by ANOVA when multiple treatments were evaluated (all other inhibitors). For ANOVA, a Dunnett's test was used for post-hoc multiple comparisons. Statistical tests were performed in R v3.4.3 (Core Team, 2017). Dose response data for DAPI and EIPA were fitted to a 3-parameter non-linear regression model using log-transformed data for dose (Hall and Langmead, 2010) using GraphPad Prism™ (Version 7) (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. Pharmacological inhibitors

We observed no significant difference in Na^+ uptake rate between the control and carrier control ($t = 0.67$, d.f. = 14, $p = 0.53$), suggesting DMSO has no effect on Na^+ uptake (Fig. 1). Of the inhibitors

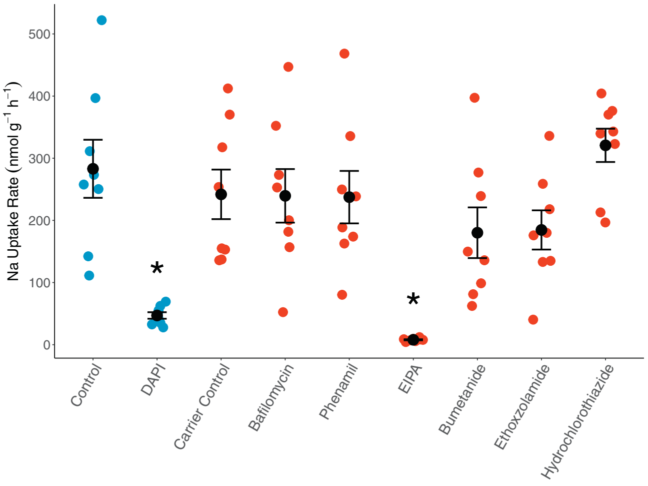

Fig. 1. Effect of pharmacological inhibitors on Na^+ uptake in *F. heteroclitus*. Raw data plotted in blue are comparisons between control and inhibitors with no carrier. Raw data plotted in red are comparisons between carrier control and inhibitors with carrier. Black data are mean ± SEM for each treatment. * = statistically significant difference compared to the appropriate control ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tested, only two had a significant effect on Na^+ uptake rate. DAPI elicited an 83% reduction in Na^+ uptake rate compared to the control ($t = 5.02$, d.f. = 7.2, $p = 0.001$) while EIPA caused a 97% reduction in Na^+ uptake rate (F = 7.71, d.f. = 6, 49, $p < 0.001$) (Fig. 1).

3.2. DAPI and EIPA dose response experiments

Dose response data for DAPI and EIPA were fitted to 3-parameter logistic models (Hall and Langmead, 2010) (Fig. 2). For DAPI, the first significant inhibition occurred at 10^{-6} M, whereas for EIPA, it occurred at 10^{-5} M. For DAPI, we estimated an IC50 of 5.45×10^{-7} M (95% CI: 7.08×10^{-8}–4.12×10^{-6} M) while for EIPA the estimated IC50 was 3.59×10^{-6} M (95% CI: 1.16×10^{-6}–1.12×10^{-5} M).

4. Discussion

The current working model for apical Na^+ uptake in freshwater *F. heteroclitus* was developed by Scott et al. (2005). They proposed that apical Na^+ uptake is accomplished by NHE2 and that this is driven, at least in part, by the generation of intracellular H^+; with intracelluar H^+ generated by hydration of CO_2 mediated by cytosolic carbonic anhydrase (CA_c). The generation of intracellular H^+ provides a motive force for the otherwise unfavorable thermodynamic gradient for NHE function in freshwater (Parks et al., 2008). The model developed by Scott et al. (2005) is based on their own gene expression studies which demonstrated upregulation of NHE2 and CA_c in the gill upon transfer of *F. heteroclitus* from brackish to fresh water, as well as previous studies characterizing the expression and localization of proteins potentially involved in Na^+ uptake (Edwards et al., 2005; Katoh et al., 2003; Lacy, 1983; Patrick et al., 1997; Patrick and Wood, 1999).

We note that this model, and in particular the constraints on NHE function in freshwater requiring the generation of intracellular H^+ as a motive force, is based on the assumption that intracellular Na^+ concentrations are ≥3 mM. This assumption has similar implications for ASIC function, where intracellular Na^+ concentrations ≥3 mM will necessitate the presence of an apical H^+-ATPase to provide the necessary electrochemical motive for Na^+ uptake. For purposes of our data interpretion, we have assumed this to be the case. However, if intracellular Na^+ is ≤1 mM either generally or in micro-environments in the ionocyte, then in the freshwater used in our experiments, NHE will function without the generation of excess H^+ and ASIC may function without a H^+-ATPase. This is a continuing area of uncertainty for all models of Na^+ transport in freshwater fish ionocytes.

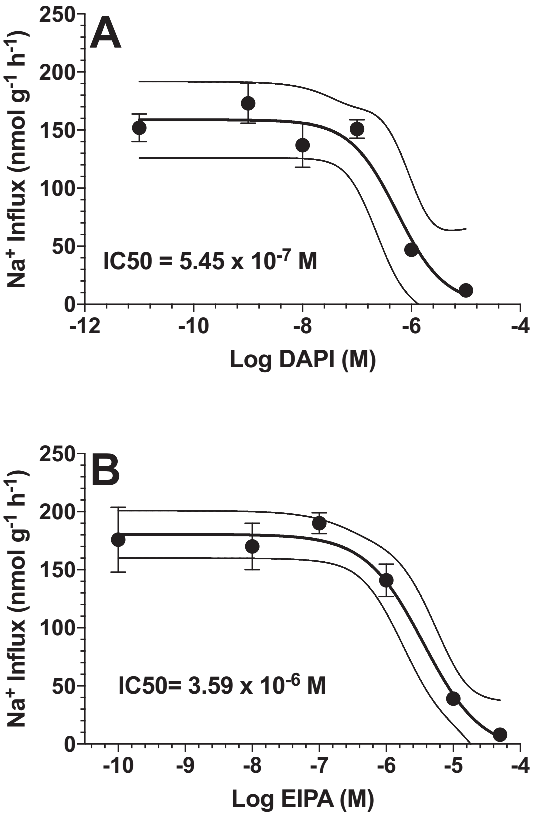

Fig. 2. Dose response relationship for A) DAPI and B) EIPA. Data fit to 3-parameter nonlinear model to estimate the IC50 for each inhibitor. Outer bands represent 95% confidence intervals for the mean regression.

In this study, we sought to evaluate this model by pharmacologically identifying proteins involved in Na^+ transport at the apical membrane of ionocytes in freshwater acclimated *F. heteroclitus*. Results from our study provided several insights into the proteins used by *F. heteroclitus* in freshwater Na^+ ionoregulation, and additionally raised a potential issue regarding the assumed specificity of one of the inhibitors.

Consistent with our current understanding that *F. heteroclitus* relies solely on NHE2 for apical Na^+ uptake in relatively low Na^+ (< 2 mM) fresh waters, EIPA proved an effective inhibitor nearly abolishing (97% inhibition) Na^+ uptake at a concentration of 5×10^{-5} M in our water which contained 1 mM Na^+. We further estimated an IC50 for EIPA on *F. heteroclitus* of 3.59×10^{-6} M, which to the best of our knowledge is the first such estimate for a fish. However, using the data presented in Boyle et al. (2016) we were able to estimate IC50s for rainbow trout larvae (ion uptake across the skin) of 8.5 and 9.3×10^{-6} M for larvae exposed to low (100 μM) and high (2200 μM) Na^+ waters, respectively. Similarly, IC50s of 1.1 and 2.7×10^{-5} M EIPA were estimated for 20-d old juvenile (ion uptake across the gill) rainbow trout exposed to low and high Na^+ waters.

Previously, studies on the pupfish *Cyprinodon variegatus variegatus* and *C. v. hubbsi* using the same EIPA concentration as in the current study, observed comparable inhibition of Na^+ uptake in *C. v. hubbsi* (91%) but more modest inhibition in *C. v. variegatus* (51%) (Brix and Grosell, 2012). Interestingly, *C. v. variegatus* utilizes NHE2 for Na^+ uptake while *C. v. hubbsi* utilizes NHE3 (Brix et al., 2015), suggesting that relative sensitivity to EIPA does not correlate with NHE isoform. Indeed, the effect of EIPA across fish species is quite variable, but this

Table 1
Summary of the effects of EIPA on Na^+ uptake rate in fish. Summary of effects of EIPA on freshwater fish species. Fish classified as juvenile or adult accomplish Na^+ uptake primarily across the gill while larval fish predominantly take up Na^+ across the skin and/or yolk sac. Negative % inhibition are studies where Na^+ uptake was stimulated by EIPA.

Species	Lifestage	Water [Na] (μM)	Water pH	[EIPA] (μM)	% Inhibition	Reference
Acipenser transmontanus	Juvenile	100	7.0	40	88*	Shartau et al. (2016)
Carassius auratus	Adult	300	6–7	50	46*	Preest et al. (2005)
Cyprinodon v. hubbsi	Adult	100	7.0	50	95*	Brix and Grosell (2012)
Cyprinodon v. hubbsi	Adult	2000	7.0	50	91*	Brix and Grosell (2012)
Cyprinodon v. hubbsi	Adult	7000	7.0	50	29*	Brix and Grosell (2012)
Cyprinodon macularius	Adult	2000	7.0	50	12*	Brix and Grosell (2013)
Cyprinodon macularius	Adult	7000	7.0	50	10	Brix and Grosell (2013)
Cyprinodon v. variegatus	Adult	2000	7.0	50	51*	Brix and Grosell (2012)
Cyprinodon v. variegatus	Adult	7000	7.0	50	21*	Brix and Grosell (2012)
Danio rerio	Adult	35	6.0	50	−51*	Boisen et al. (2003)
Danio rerio	Adult	1480	8.2	50	−44	Boisen et al. (2003)
Danio rerio	Adult	50	6.0	100	−38	Dymowska et al. (2015)
Danio rerio	Adult	500	8.5	100	−68	Dymowska et al. (2015)
Danio rerio	Larval	686	6.7–7.4	10	18*	Esaki et al. (2007)
Danio rerio	Larval	686	6.7–7.4	100	50*	Esaki et al. (2007)
Danio rerio	Larval	780	7.4	100	−2	Kumai and Perry (2011)
Danio rerio	Larval	780	4.0	100	53*	Kumai and Perry (2011)
Fundulus heteroclitus	Adult	1000	7.9	50	97*	This study
Oncorhynchus mykiss	Larval	100	5.9	10	51*	Boyle et al. (2016)
Oncorhynchus mykiss	Larval	100	5.9	100	85*	Boyle et al. (2016)
Oncorhynchus mykiss	Larval	2200	8.1	10	44*	Boyle et al. (2016)
Oncorhynchus mykiss	Larval	2200	8.1	100	81*	Boyle et al. (2016)
Oncorhynchus mykiss	Juvenile	100	5.9	10	48*	Boyle et al. (2016)
Oncorhynchus mykiss	Juvenile	100	5.9	100	86*	Boyle et al. (2016)
Oncorhynchus mykiss	Juvenile	2200	8.1	10	36*	Boyle et al. (2016)
Oncorhynchus mykiss	Juvenile	2200	8.1	100	78*	Boyle et al. (2016)
Oncorhynchus mykiss	Larval	600	8.0	100	50*	Zimmer et al. (2017)
Oncorhynchus mykiss	Juvenile	600	8.0	100	85*	Zimmer et al. (2017)
Oryzias latipes	Larval	< 100	6.8	100	54*	Wu et al. (2010)
Paracheirodon innesi	Adult	50	6–7	50	−8	Preest et al. (2005)

* Inhibition of Na^+ uptake that was significantly different from the control ($p < 0.05$).

drug has been shown to inhibit Na^+ uptake in the majority of species tested to date (Table 1). The notable exceptions are the neon tetra (*Paracheirodon innesi*) (Preest et al., 2005) and the adult life stage of zebrafish (*Danio rerio*) (Boisen et al., 2003; Dymowska et al., 2015). It is unclear why EIPA inhibits Na^+ uptake in *D. rerio* larvae but not adults, given that both life stages appear to utilize NHE-3b for Na^+ uptake (Hwang, 2009).

The assumed unfavorable Na^+ gradient for NHE to function in the freshwater gill must be counteracted by a (locally) high concentration of intracellular H^+ (Parks et al., 2008). The high H^+ concentration is provided by either CA_c facilitated hydration of CO_2 or by the deprotonation of intracellular ammonium (NH_4^+) as ammonia (NH_3) exits across the apical membrane of the gill cell via Rh proteins (Kumai et al., 2011; Wright and Wood, 2012; Wu et al., 2010). Scott et al. (2005) proposed that the former process (CA_c) is involved in Na^+ uptake in *F. heteroclitus* based on an observed ~3-fold upregulation of CA_c gene expression after transfer from brackish to freshwater. Contrary to this, we did not observe a significant reduction in Na^+ uptake rate when *F. heteroclitus* were exposed to 1×10^{-4} M ethoxzolamide, a potent CA inhibitor (Fig. 1).

Previously, Boisen et al. (2003) demonstrated ethoxzolamide did not inhibit Na^+ uptake rate in zebrafish (*Danio rerio*) acclimated to low Na^+ water. Subsequent studies have demonstrated that zebrafish use the NHE-Rh metabolon for Na^+ uptake in ion poor water (Kumai and Perry, 2011). In comparison, Brix et al. (2015) showed a significant reduction in Na^+ uptake rate in *C. variegatus variegatus* (58%) and *C. v. hubbsi* (32%) exposed to the same concentration of ethoxzolamide, while Shartau et al. (2016) observed an ~85% reduction in Na^+ uptake rate in the sturgeon, *Acipenser transmontanus*. For each of these species, additional experiments demonstrated NHE function in freshwater was not facilitated by the NHE-Rh metabolon and that CA_c derived H^+ were likely the primary motive force in freshwater NHE function. Hence, there is typically consistency across experimental observations with respect to H^+ generation for NHE function in freshwater, in that fish with ethoxzolamide-sensitive Na^+ uptake lack an NHE-Rh metabolon.

Our experimental results with ethoxzolamide suggest CA_c generated H^+ may not be an important factor in freshwater NHE function for *F. heteroclitus*. However, unlike studies on *D. rerio*, Patrick et al. (1997); Patrick and Wood (1999) demonstrated that Na^+ uptake rate is not sensitive to high environmental ammonia, that inhibition of Na^+ uptake by amiloride, a broad spectrum Na^+ transport inhibitor, has no effect on ammonia excretion rate, and that Na^+ uptake was not altered by metabolic acidosis. All of this suggests *F. heteroclitus* is not using an NHE-Rh metabolon for Na^+ uptake in 1 mM Na^+ freshwater and that additional experiments are needed to further explore how NHE functions in freshwater acclimated *F. heteroclitus*. For example, it is possible that previously observed upregulation of CA_c during freshwater acclimation results in it being sufficiently over abundant in *F. heteroclitus* ionocytes such that the ethoxzolamide concentration used in our experiment was insufficient to inhibit Na^+ uptake.

DAPI also proved an effective inhibitor of Na^+ uptake in the current study, with 1×10^{-6} M DAPI reducing the uptake rate by 83% (Fig. 1). A subsequent dose-response experiment resulted in an IC50 of 5.45×10^{-7} M (Fig. 2). As previously discussed, freshwater Na^+ uptake in *F. heteroclitus* is thought to be accomplished exclusively via NHE2. Unlike many freshwater teleosts, involvement of a Na^+ channel (e.g., ASICs) is not considered likely given the basolateral location of the H^+-ATPase which would need to be located on the apical membrane to provide the necessary electrochemical gradient for Na^+ channel function. The fact that the H^+-ATPase inhibitor bafilomycin did not inhibit Na^+ uptake rate (Fig. 1) further supports this model.

Consequently, the inhibition of Na^+ uptake rate by DAPI is quite surprising given it has been hypothesized to be an ASICs-specific inhibitor in studies with rainbow trout and zebrafish (Dymowska et al., 2015; Dymowska et al., 2014). It is also noteworthy that phenamil, a drug thought to be specific for ENaC yet effective in blocking Na^+ uptake in some freshwater fish (Brix and Grosell, 2012; Bury and Wood, 1999; Preest et al., 2005; Reid et al., 2003) was completely without effect on Na^+ influx rate in *F. heteroclitus* suggesting that the phenamil-sensitive Na^+ channel, whatever its nature, is not present in the killifish.

Results from this study raise questions regarding the specificity of DAPI for ASICs and suggest it may also be an effective inhibitor of NHE2. Interestingly, recent studies with larval rainbow trout have similarly found that both EIPA and DAPI cause approximately equal and marked inhibition of Na^+ uptake rate whereas phenamil has no significant effect (A.M. Zimmer, P.A. Wright, and C.M. Wood, unpublished results). Thus, the specificity of DAPI, as well as phenamil, in freshwater fish is now in question. Importantly, this does not mean that the previous studies by Dymowska et al. were incorrect in identifying the role of ASICs in Na^+ uptake in *O. mykiss* and *D. rerio*. Dymowska et al. (2014) demonstrated that EIPA, but not DAPI, inhibited pH*i* recovery in isolated *O. mykiss* ionocytes, providing convincing evidence that DAPI does not inhibit NHE (presumably NHE3b) in this species. Further, Na^+ uptake rate in both of these species is also sensitive to bafilomycin indicating the function of an apical H^+-ATPase that is presumably associated with ASICs (Horng et al., 2007; Lin and Randall, 1993; Lin et al., 2006). The only other species that we are aware of that has been evaluated with DAPI is the white sturgeon, *Acipenser transmontanus*. In this species, Na^+ uptake is EIPA-sensitive but not DAPI-sensitive with the NHE isoform involved in Na^+ uptake currently unknown (Shartau et al., 2016). Collectively, these data indicate that inhibition of NHE by DAPI may be isoform and/or species-specific with current data suggesting NHE2, but not NHE3, is DAPI-sensitive. Studies on a wider range of species will help to resolve this uncertainty.

5. Conclusions

A variety of tools (pharmacological inhibitors, immunohistochemistry, gene/protein expression, morpholinos) can contribute to our understanding of ion transport processes in aquatic organisms. The use of pharmacological inhibitors provides a relatively easy method for quickly identifying the types of proteins that may be involved in ion transport, but it is critical that the efficacy and specificity of the inhibitors be understood. In this study, we demonstrated that DAPI was an effective inhibitor of Na^+ uptake rate in a freshwater acclimated *F. heteroclitus*. This suggests that, at least in *F. heteroclitus*, DAPI can also inhibit NHE2 and the possibility that this effect extends to other species should be investigated.

Further, experiments with ethoxzolamide suggest that CA may not be actively involved in providing protons for NHE function in freshwater, as has been previously proposed for this species. Given that previous studies also indicate *F. heteroclitus* does not use an NHE-Rh metabolon, further studies into how NHE function is accomplished in freshwater acclimated *F. heteroclitus* are needed.

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