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RESEARCH ARTICLE

The role of acid-sensing ion channels in epithelial Na^+ uptake in adult zebrafish (*Danio rerio*)

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ABSTRACT

Acid-sensing ion channels (ASICs) are epithelial Na^+ channels gated by external H^+. Recently, it has been demonstrated that ASICs play a role in Na^+ uptake in freshwater rainbow trout. Here, we investigate the potential involvement of ASICs in Na^+ transport in another freshwater fish species, the zebrafish (*Danio rerio*). Using molecular and histological techniques we found that *asic* genes and the ASIC4.2 protein are expressed in the gill of adult zebrafish. Immunohistochemistry revealed that mitochondrion-rich cells positive for ASIC4.2 do not co-localize with Na^+/K^+-ATPase-rich cells, but co-localize with cells expressing vacuolar-type H^+-ATPase. Furthermore, pharmacological inhibitors of ASIC and Na^+/H^+-exchanger significantly reduced uptake of Na^+ in adult zebrafish exposed to low-Na^+ media, but did not cause the same response in individuals exposed to ultra-low-Na^+ water. Our results suggest that in adult zebrafish ASICs play a role in branchial Na^+ uptake in media with low Na^+ concentrations and that mechanisms used for Na^+ uptake by zebrafish may depend on the Na^+ concentration in the acclimation medium.

KEY WORDS: Gill, Acid-sensing ion channels, Zebrafish, Sodium uptake, Ionoregulation

INTRODUCTION

In fresh water, teleost fishes experience the continuous diffusive loss of ions to a more hypo-osmotic external environment. To maintain homeostasis, freshwater fishes must take up ions including Na^+, Cl^- and Ca^{2+} from the surrounding water and/or reduce the diffusive ion loss by maintaining low paracellular permeability. Uptake of ions is achieved by specialized ionocytes called mitochondrion-rich cells (MRCs) that are located on the gill epithelium (see reviews by Dymowska et al., 2012; Evans et al., 2005; Hwang et al., 2011). However, despite considerable research efforts, the mechanisms of ion acquisition and identity of the transporters involved in ion uptake in fresh water are still not fully understood.

The most favoured current model of Na^+ uptake in freshwater fish gills proposes Na^+ uptake to occur via a Na^+/H^+ exchanger (NHE) (reviewed by Hwang et al., 2011). This model is supported by empirical evidence from several freshwater fish species (e.g. Bradshaw et al., 2012; Esaki et al., 2007; Hirata et al., 2003; Yan et al., 2007); however, a limitation of this model is the questionable ability of the electroneutral NHE to function in low-Na^+ waters (Na^+<0.1 mmol l^{-1}) and/or low pH (pH<5), where gradients for Na^+ and H^+ would be reversed (Avella and Bornancin, 1989; Parks et al., 2008). Recently, the NHE model has been extended by the addition of an ammonia (NH_3)-transporting Rhesus (Rh) protein (Nakada et al., 2007; Nawata et al., 2007), whereby NHE2/3 and Rh protein form a metabolon, which locally decreases H^+ concentration in the boundary layer and facilitates NHE function in acidic environments (Wright and Wood, 2009). However, although the NHE/Rh metabolon model provides a solution for constraints at low pH, it cannot alleviate the thermodynamic constraints imposed by low-Na^+ environments (see 'rainbow trout' section in Dymowska et al., 2012).

An alternative model for Na^+ uptake, where Na^+ transport is mediated by an apical channel that works in concert with vacuolar-type H^+-ATPase (VHA), better fits gill Na^+ uptake in low-Na^+ environments, as it would theoretically not be limited by external ion concentrations (Avella and Bornancin, 1989). Support for this model comes from studies that have shown decreased Na^+ uptake in the whole organism and in *in vitro* preparations of isolated MRCs exposed to bafilomycin, a selective VHA inhibitor (Bury and Wood, 1999; Goss et al., 2011; Reid et al., 2003). Additionally, VHA was localized to the apical region of the MRCs in the rainbow trout (Sullivan et al., 1995; Wilson et al., 2000). However, until recently, the putative Na^+ channel had not been identified, and as a result, the VHA/Na^+ channel model has received less support from researchers in recent years. Recently, we have demonstrated that acid-sensing ion channels (ASICs) play a role in Na^+ acquisition in the gill epithelium of rainbow trout (Dymowska et al., 2014). ASICs are H^+-gated Na^+ channels that belong to the epithelial Na^+ channel/degenerin (ENaC/DEG) superfamily (for a review, see Holzer, 2009). In mammals, seven different ASIC subunits (ASIC1a, ASIC1b, ASIC1c, ASIC2, ASIC4a, ASIC4b and ASIC5) have been identified and are encoded by four different genes, whereas in zebrafish, a fish species with the most extensive functional characterization of ASICs, six subunits are present (ASIC1.1, ASIC1.2, ASIC1.3, ASIC2, ASIC4.1 and ASIC4.2) and each subunit is encoded by a different gene (Gründer et al., 2000; Paukert et al., 2004; Sakai et al., 1999; Waldmann and Lazdunski, 1998). In the recent study by Dymowska et al. (2014) *asic1* and *asic4* were cloned from the gill cDNA of the adult rainbow trout, and using immunohistochemistry, ASIC4 was apically localized to MRCs rich in Na^+/K^+-ATPase (NKA) (Dymowska et al., 2014). Additionally, ASIC-specific inhibitors 4′,6-diamidino-2-phenylindole (DAPI) and diminazene (Chen et al., 2010), were demonstrated to decrease Na^+ uptake in a dose-dependent manner in juvenile rainbow trout acclimated to a low-Na^+ (~50 µmol l^{-1}) environment (Dymowska et al., 2014).

In the study presented here, we investigated whether ASICs are involved in branchial Na^+ transport in another model organism for ion transport research, the zebrafish (*Danio rerio*). Zebrafish have become an increasingly popular model organism for use in studies on ionoregulation in freshwater fish (e.g. Boisen et al., 2003; Hwang, 2009; Kumai and Perry, 2011; Shih et al., 2012; Yan et al.,

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List of abbreviations

ASIC	acid-sensing sodium channel
DAPI	4′,6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
EIPA	ethyl-iso-propyl-amiloride
ENaC/DEG	epithelial Na^+ channel/degenerin
HR	VHA-rich
KS	K^+-secreting
MRC	mitochondrion-rich cell
NaR	NKA-rich
NCC	Na^+/Cl^- co-transporter
NHE	Na^+/H^+ exchanger
NKA	Na^+/K^+-ATPase
Rh	Rhesus
SIET	scanning ion-selective technique
VHA	vacuolar-type H^+-ATPase

2007) because of the availability of their genetic databases, as well as ease of husbandry, gene expression analysis and genomic manipulation (Lawrence, 2011). Unlike trout, which have two distinct MRC sub-types, zebrafish have been demonstrated to have at least four types of MRC in the gill and skin epithelia: VHA-rich cells (HR cells), NKA-rich cells (NaR cells), cells expressing Na^+/Cl^- co-transporter (NCC cells) and K^+-secreting cells (KS cells), with HR cells proposed to be the main site for Na^+ uptake (for a review, see Hwang et al., 2011). The current model for zebrafish HR cells also places an unidentified epithelial Na^+ channel on the apical side (Dymowska et al., 2012; Hwang et al., 2011). Based on the previous findings in rainbow trout (Dymowska et al., 2014), we hypothesized that the role of the epithelial Na^+ channel in adult zebrafish gill is assumed by proteins from the ASIC family.

The objective of our study was to verify that ASICs are present in the gills of the adult zebrafish and then to determine whether ASICs are involved in Na^+ uptake at the whole-animal level using flux experiments. We measured Na^+ uptake using radiotracer ($^{22}Na^+$) analysis in zebrafish exposed to ultra-low (Na^+~50 µmol l^{-1}; pH 6) and low (Na^+~500 µmol l^{-1}; pH 8.5) Na^+-containing medium in the presence and absence of common Na^+-uptake pharmacological inhibitors, including DAPI. Na^+ concentrations in both exposure media were chosen to span the theoretical limits of the NHE model (Parks et al., 2008). Expression levels of ASIC mRNA were also evaluated in the ultra-low, low and high (Na^+~1400 µmol l^{-1}; pH 7) Na^+ exposures, to examine changes in expression associated with a change in environment.

RESULTS

Pharmacological inhibition of Na^+ uptake

To determine whether DAPI, an ASIC selective inhibitor (Chen et al., 2010) displayed a similar dose-response inhibition effect on Na^+ uptake in zebrafish to that of rainbow trout, flux experiments using $^{22}Na^+$ were performed on adult zebrafish exposed to low-Na^+ medium (~500 µmol l^{-1} Na^+; Table 1). After 90 min, zebrafish showed reduced Na^+ uptake with increasing concentrations of DAPI

Table 1. Ion composition and pH of the exposure media

	Concentration (µmol l^{-1})			
	Na^+	Cl^-	Ca^{2+}	pH
Ultra-low Na^+	49.5±0.5	64±1	308±15	6
Low Na^+ (tap water)	511±25	304±32.5	1179±30	8.5
High Na^+	1420±28	1051±35.5	425±31	7

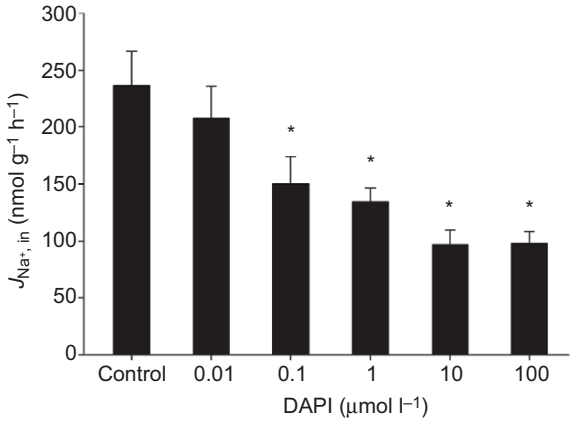

Fig. 1. The effect of DAPI on Na^+-uptake rates in adult zebrafish acclimated to low-Na^+ medium (~500 µmol l^{-1}). Values are means±s.e.m. (N=6). *P<0.05, significantly different from the control group.

(Fig. 1). The maximal (but not complete) inhibition of Na^+-uptake rate was observed at 10 µmol l^{-1} DAPI and was equal to 59% of control Na^+ uptake. Increasing DAPI concentration to 100 µmol l^{-1} did not result in further decreases in Na^+ uptake.

To determine the relative involvement of Na^+ transporters in Na^+ uptake, DAPI and two other Na^+ inhibitors – ethyl-iso-propyl-amiloride (EIPA), which has a high selectivity for NHE (Ito et al., 2014; Kleyman and Cragoe, 1988) and amiloride, which inhibits both NHE and Na^+ channels (Kleyman and Cragoe, 1988) – were tested in low-Na^+ (511±25 µmol l^{-1}) and ultra-low-Na^+ (49.5±0.5 µmol l^{-1}) conditions (Fig. 2). Zebrafish acclimated to low-Na^+ medium exhibited a 55% decrease in Na^+ uptake in the presence of DAPI (10 µmol l^{-1}) (Fig. 2A), which was in agreement with the dose-response experiment (Fig. 1). Amiloride (200 µmol l^{-1}) also reduced Na^+ uptake by 55%, whereas EIPA (100 µmol l^{-1}) had no significant effect, although a consistent trend towards increase in Na^+ uptake was noted (Fig. 2B). In contrast, when zebrafish were acclimated to ultra-low-Na^+ medium, the control rate of flux was reduced by ~50% (Fig. 2A) and the effect of the pharmacological agents on Na^+ uptake was no longer observed.

ASICs mRNA expression in the gills

The expression pattern of different ASIC subunits (*asic1.1*, *asic1.2*, *asic1.3*, *asic2*, *asic4.1* and *asic4.2*) mRNAs in the gill tissue of zebrafish acclimated to high-, low- and ultra-low-Na^+ water was examined by RT-PCR. Elongation factor 1α1 (*ef1α1* was used as an internal control; primers used are shown in Table 2). All six ASIC mRNAs were present in the zebrafish gill tissue regardless of the acclimation medium (Fig. 3).

Immunolocalization of ASIC4.2 in zebrafish gills

With the use of a custom-made anti-zebrafish ASIC4.2 antibody, we verified the expression of ASIC4.2 in the gills of zebrafish by immunoprecipitation and a single band corresponding to ~65 kDa (predicted size 62.8 kDa) was identified in the gills of multiple animals (Fig. 4). Immunohistological analysis with the anti-ASIC4.2 antibody demonstrated that ASIC4.2 was present in the gills of the adult zebrafish (Fig. 5B and Fig.6B). To determine the cell type to which ASIC4.2 localizes, gills were initially double stained with anti-ASIC4.2 and anti-NKA, a marker of the NaR MRC type in zebrafish (Hwang and Lee, 2007). Cells positive for anti-ASIC4.2 and anti-NKA were

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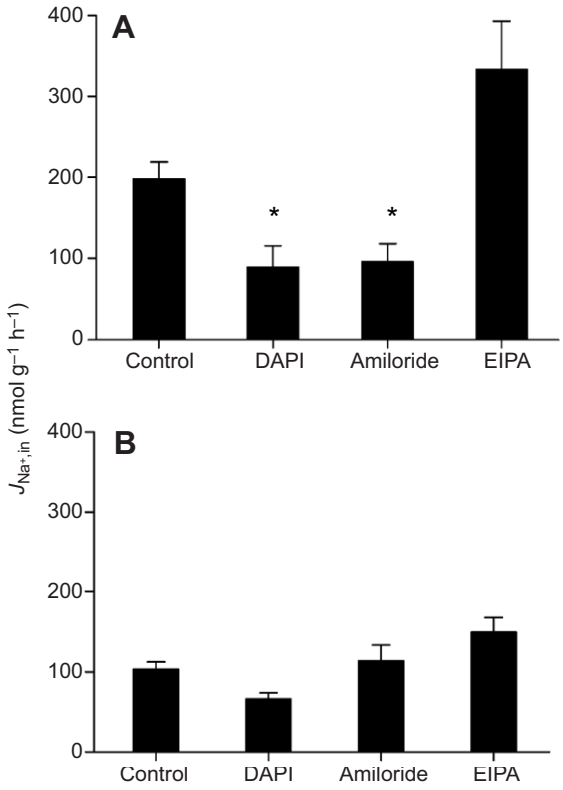

Fig. 2. The effect of DAPI, amiloride and EIPA on Na^+-uptake rates in adult zebrafish acclimated to low- and ultra-low-Na^+ media. Na^+-uptake rates were measured in (A) low-Na^+ medium (~500 μmol l^{-1}) and (B) ultra-low-Na^+ medium (~50 μmol l^{-1}) with DAPI (10 μmol l^{-1}), amiloride (200 μmol l^{-1}) and EIPA (100 μmol l^{-1}). Values are means±s.e.m. (*N*=6). *P<0.05, significantly different from the control group.

observed in the lamellae and interlamellar region of the gills (Fig. 5B,C); however, ASIC4.2 and NKA did not co-localize, suggesting that the ASIC4.2 transporter is not present in NaR MRCs (Fig. 5D). To determine if ASIC4.2 co-localizes with other transporting proteins, gills were also stained with ASIC4.2 and VHA, a marker for HR-type MRC. Since both the anti-ASIC4.2 and anti-VHA antibodies were raised in rabbit, staining was carried out on consecutive 4 μm sections, to avoid cross-reactivity. Comparison of staining patterns in both sections revealed that many cells positive for anti-ASIC4.2 were also positive for anti-VHA (Fig. 6D), suggesting that ASIC4.2 protein is localized to HR-type MRCs.

DISCUSSION

The objective of the present study was to verify whether ASICs are expressed in the gill tissue of the adult zebrafish, and if they are involved in branchial Na^+ uptake, as shown recently for rainbow trout (Dymowska et al., 2014). Previous studies on ASICs in zebrafish have demonstrated their widespread expression in the central and peripheral nervous systems, specifically in brain, retina, intestine and taste buds, where they have been suggested to be involved in nerve transduction and neural communication (Levanti et al., 2011; Paukert et al., 2004; Viña et al., 2013). However, their presence and function in the gill epithelium of zebrafish has never before been tested. The results of the present study demonstrate that multiple ASIC subunits are expressed in the gill tissue at the mRNA level, with ASIC4.2 protein being localized to MRCs. Pharmacological blockade of Na^+ uptake with ASIC-specific inhibitors revealed that ASICs are involved in Na^+ regulation at the whole-animal level. Based on the findings in the present study, we propose that ASICs play a role in Na^+ uptake in the gills of adult zebrafish.

Recently, *asic4* and *asic1* genes have been demonstrated to be expressed in the MRCs from the gill tissue of rainbow trout (Dymowska et al., 2014). Moreover, ASIC4 protein has been found to co-localize with the NKA, a classic marker for MRCs in rainbow trout, and expressed in the apical region of the cell (Dymowska et al., 2014). In the present study, we observed that similar to rainbow trout, ASIC4.2 was present in the lamellar and interlamellar region of the adult zebrafish gill. However, staining with anti-ASIC4.2, anti-VHA and anti-NKA antibodies revealed that in zebrafish gill epithelium ASIC4.2 protein co-localized to the cells expressing VHA, but not to the cells that express NKA. We infer that in zebrafish gills, ASIC4 protein is present in the HR type of ionocytes. Previous studies using *in situ* hybridization and immunohistochemistry techniques have demonstrated that HR ionocytes of zebrafish are abundant in NHE3b and VHA, but in contrast to rainbow trout, they are not characterized by a high abundance of NKA (Dymowska et al., 2012; Esaki et al., 2007; Lin et al., 2006; Yan et al., 2007; for a review on MRC sub-types in freshwater fish, see Dymowska et al., 2012). Our finding could be further corroborated by co-localization of ASIC4 with NHE3b; however, this remains to be determined.

In zebrafish, HR cells are suggested to be the primary site for Na^+ uptake and H^+ secretion. This was determined by measurement of H^+ fluxes in the vicinity of HR cells using the scanning ion-selective electrode technique (SIET) (Lin et al., 2006). Currently, the most favoured model for Na^+ uptake in freshwater fish, including zebrafish, incorporates an electroneutral NHE because a Na^+

Table 2. Gene-specific primers used for RT-PCR

Gene	Primer sequence (5′–3′)	Accession number	Amplicon (bp)
asic1.1	F: AACCCAGACGTCAAAGGAACGCTA R: AAGACAGTTTCGAGCCGTCGCTAT	AJ609615	264
asic1.2	F: TCATTGGAGCCAGTATTCTTACC R: GAGAGAGAACAACCACGAGATG	AJ609616	312
asic1.3	F: CACACCTGAGCAGTACAAAGA R: CCACCGATATCACCAAGTAACC	AJ609617	300
asic2	F: GGAAAGCAGATGCTTGTGGACCT R: AGCAGCCAATTGAGATGCGGAAAC	AJ609618	339
asic4.1	F: AACACCATCCTCCCGAATCACCAT R: AGTCCTGCGAAAGGAGTTGGGAAA	AJ609619	305
asic4.2	F: CCAGGAACAGAGGCTAACATA R: CCATAGAGAGCTCTTTCCCATAC	AJ609620	305
elf1α1	F: GGGTCTGTCCGTTCTTGGAG R: TTCTCAGGCTGACTGTGCTG	NM_131263	83

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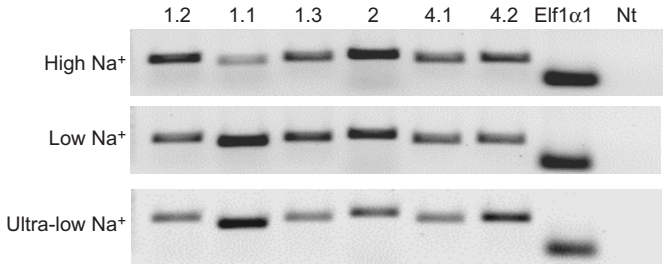

Fig. 3. RT-PCR analysis of the expression of ASICs family subunits in the gills of adult zebrafish. Fish were acclimated to high-Na^+ (~1200 μmol l^{-1}), low-Na^+ (~500 μmol l^{-1}) and ultra-low-Na^+ (50 μmol l^{-1}) media. Elf1α1, zebrafish elongation factor used as a positive control; Nt, no template. Details of primers used and amplicon sizes are shown in Table 2.

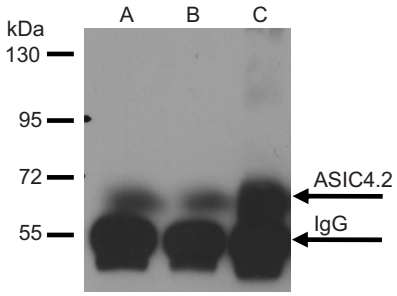

Fig. 4. Western blot analysis with anti-zASIC4.2 antibody of whole-gill homogenates from zebrafish acclimated to high-Na^+ medium (~1400 μmol l^{-1}). Lanes A and B are gill homogenates from one individual fish, lane C is pooled sample of gill homogenates from two fish. IgG, rabbit immunoglobulin G.

epithelial channel that could alternatively perform that function in the freshwater gill epithelium had never previously been identified. However, previous studies in zebrafish adults and larvae demonstrated that bafilomycin, a specific VHA inhibitor, caused a substantial reduction of Na^+-uptake rates (i.e. Boisen et al., 2003; Kumai and Perry, 2011; Kwong and Perry, 2013). Moreover, knockdown of VHA subunit A (*atp6v1a*) in zebrafish larvae caused a decrease in whole-body Na^+ content in morphants (Horng et al., 2007). Therefore, it is likely that in zebrafish, there exists a secondary, VHA-dependent mechanism for Na^+ transport. The mechanism by which VHA activity promotes Na^+ uptake is not yet known; however, two alternatives may exist: firstly, VHA charges the apical membrane potential thereby increasing the electrochemical gradient for Na^+ to enter in the cell through ASIC, and secondly, VHA provides a gating signal to ASIC by acidification of the boundary layer, since ASICs are gated by extracellular H^+ ions that activate several amino acid residues located in different extracellular domains (Bonifacio et al., 2014; Paukert et al., 2008). One complicating factor is that ASIC4.2 has been demonstrated to be insensitive to extracellular H^+ (Chen et al., 2007). However, there is evidence that ASICs assemble into homo- or heteromeric trimers (Jasti et al., 2007), therefore it is possible that ASIC4.2 forms a channel with other ASIC subunits that show extracellular H^+ gating. Given that we have demonstrated by PCR the presence of all known isoforms of ASIC in the gill of zebrafish, this may be a scenario by which ASIC4.2 could mediate Na^+ transport in conjunction with the VHA.

In the present study, use of the ASIC-specific inhibitor DAPI verified involvement of ASICs in Na^+ uptake in adult zebrafish. DAPI is a diarylamidine, a class of drugs that have been recently shown to block ASIC Na^+ currents in cultured mice hippocampal neurons, but do not block other epithelial Na^+ channels, such as ENaCs (Chen et al., 2010). Recently, DAPI has also been demonstrated to inhibit Na^+ uptake in rainbow trout exposed to ultra-low ionic strength/low pH water (Dymowska et al., 2014). Moreover, DAPI did not affect the NHE-mediated alkalinization in isolated NHE-expressing (PNA^+) MR cells from rainbow trout gill, indicating lack of inhibitory effect of DAPI on the trout NHEs (Dymowska et al., 2014). Since DAPI had not been previously employed in Na^+-transport studies in zebrafish, we demonstrated a dose-dependent inhibitory effect on Na^+ uptake rate in animals acclimated to low ionic strength water. However, unlike in rainbow trout, where DAPI almost completely (>90%) inhibited Na^+ uptake (Dymowska et al., 2014), in zebrafish the maximal inhibition of Na^+ flux was ~59% and further increases in DAPI concentration did not cause any further reduction in Na^+ uptake. This result suggests that

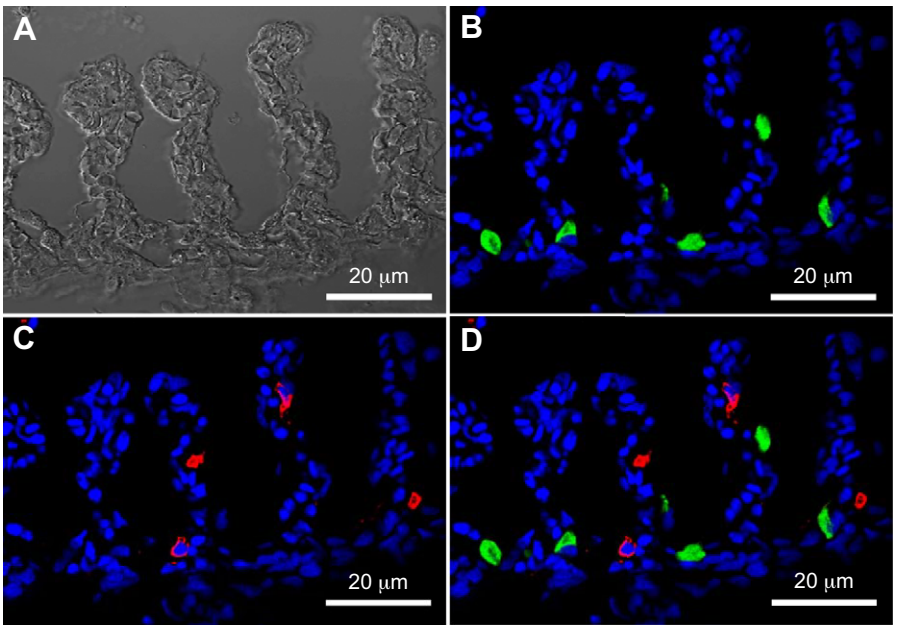

Fig. 5. Double immunostaining with anti-Na^+/K^+-ATPase (α5) antibody and anti-ASIC4.2 antibody in zebrafish gill. (A) Bright field, (B) anti-Na^+/K^+-ATPase (green), (C) anti-ASIC4.2 (red), (D) merged images from B and C.

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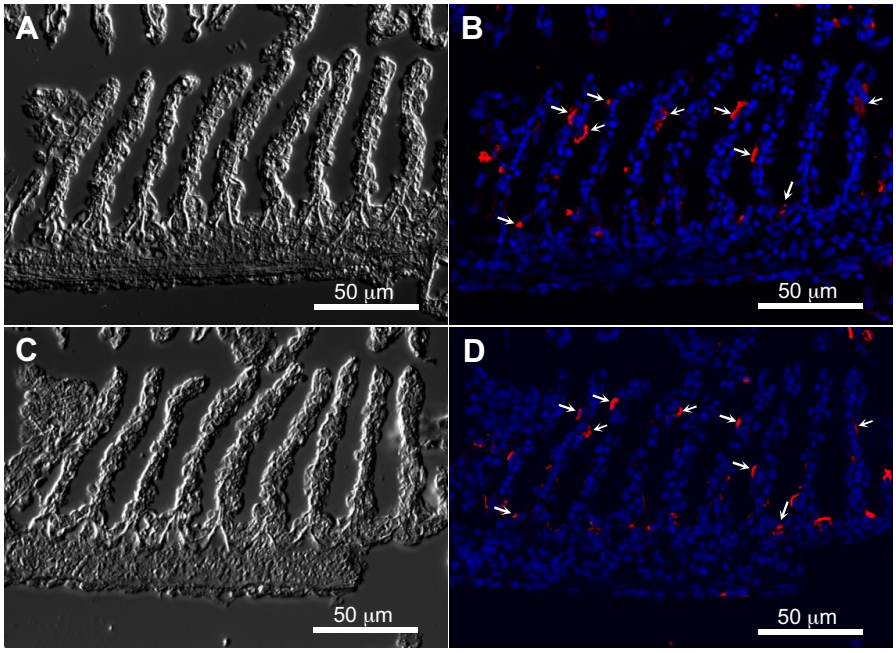

Fig. 6. Immunostaining with anti-ASIC4.2 antibody and anti-V-H$^+$-ATPase antibody in consecutive sections (4 μm) of zebrafish gill. (A,C) Bright-field images; (B) anti-ASIC4.2; (D) anti-V-H$^+$-ATPase. Arrows indicate cells displaying co-localization of ASIC4.2 and V-H$^+$-ATPase.

other DAPI-insensitive mechanism(s) for Na^+ transport exist in the zebrafish gill that could account for the remaining 41% of Na^+ transport capacity (see below).

Given the thermodynamic limitations of various transport systems at low- and ultra-low-Na^+ concentrations, we combined the use of pharmacological agents inhibiting different Na^+ transporters with acclimation media varying in Na^+ concentration. Our results revealed that the effect of the inhibitors on Na^+ uptake differed significantly between the exposure media at the different Na^+ concentrations examined. Zebrafish acclimated to low-Na^+ water showed a significant reduction in Na^+-uptake rates in the presence of DAPI and amiloride, whereas fish exposed to ultra-low-Na^+ medium were insensitive to the presence of DAPI, amiloride or EIPA. These results suggest the existence of another mechanism for Na^+ uptake in adult zebrafish that is insensitive to any of the inhibitors used in this study.

It should be noted that our experimental protocol included changes in H^+ and Ca^{2+} concentration in the exposure media. For the ultra-low-Na^+ medium, the lower pH (pH 6) was chosen specifically to impose thermodynamic constraints on the NHE function. In regard to ASICs, it has been previously shown in toadfish ASIC1 that increase of the external H^+ concentration resulted in activation and opening of more channels (Zhang et al., 2006). Similar, extracellular Ca^{2+} can modulate ASIC dependence on pH, whereby an increase in external Ca^{2+} concentration shifts the pH required for activation of mammalian ASIC1 and ASIC3 to more acidic values (Babini et al., 2002; Immke and McCleskey, 2003). Additionally, one study demonstrated that decrease in external Ca^{2+} resulted in increased number of open toadfish ASIC1 channels (Zhang et al., 2006). Based on these findings, it has been suggested that Ca^{2+} and H^+ compete for the same binding site (Kellenberger and Schild, 2015). In our study, we observed reduced Na^+ uptake together with insensitivity to ASIC inhibitor in the ultra-low-Na^+ exposure medium when compared with low-Na^+ conditions, which in our case suggests no apparent stimulatory effects of increases in external H^+ and/or decreased (~4-fold) Ca^{2+} on ASIC4-mediated Na^+ uptake.

Previous studies using amiloride and EIPA inhibitors in zebrafish have reported conflicting results. Similar to our findings, Boisen and colleagues (2003) demonstrated no effect of either amiloride or EIPA on Na^+ uptake in adult zebrafish acclimated to ultra-low ionic strength water (Na^+ ~35 μmol l^{-1}) (Boisen et al., 2003), while other studies (i.e. Kumai and Perry, 2011; Shih et al., 2012) have demonstrated an effect of EIPA on Na^+ transport in zebrafish larvae. Kumai and Perry (2011), reported a significant reduction in Na^+ uptake by EIPA in 4 dpf zebrafish larvae reared in acidic water, while Shih et al. (2012) observed supressed Na^+ gradients (as measured with a SIET electrode) in larvae acclimated to water with very low Na^+ concentration (Na^+ ~50 μmol l^{-1}). Therefore, it is possible that amiloride and EIPA sensitivity of Na^+ uptake in ultra-low-Na^+ environments is highly variable between zebrafish developmental stages (embryos to adults).

As mentioned above, the results from fluxes in low- and ultra-low-Na^+ media with DAPI, amiloride and EIPA suggested the involvement of an alternative Na^+-uptake mechanism to NHE and ASIC. One apparent candidate for this alternative Na^+-uptake mechanism is the Na^+/Cl^- co-transporter (SLC12A10.2) that has been demonstrated to be apically expressed in the NCC sub-type of MRCs in zebrafish gill and embryonic skin (Hwang and Lee, 2007; Hwang et al., 2011). Translational knockdown of either *nhe3b* or *gcm2*, the latter being a transcription factor that controls differentiation into HR cells (Chang et al., 2009) in zebrafish larvae, resulted in a substantial increase in the number of NCC type of MRCs in the morphants, which also coincided with an increased Na^+ content in the *gcm2* morphants (Chang et al., 2013). However, the function of an electroneutral NCC operating as currently described with 1:1 inward-directed co-transport to take up Na^+ from a low- or ultra-low-Na^+environment is highly questionable based on the thermodynamics principles (Hwang et al., 2011; Dymowska et al., 2012).

In conclusion, we have demonstrated the presence of ASIC4.2 in the HR cells located on the zebrafish gill epithelium. Further, using pharmacological blockade with DAPI and amiloride, we demonstrated the involvement of ASIC channels in Na^+ uptake in zebrafish acclimated to low-Na^+ medium. Additionally, we demonstrated that Na^+ transport in low- and ultra-low-Na^+ waters are controlled by a number of different transport systems and that in ultra-low-Na^+ waters,

Na^+ uptake is insensitive to NHE- and ASIC-specific inhibitors, suggesting that an alternative mechanism is working at these Na^+ levels.

MATERIALS AND METHODS

Animals

Adult zebrafish (wild-type strain A/B; *Danio rerio* Hamilton 1822) were obtained from the University of Alberta Aquatic Facility where they were maintained in 30 l flow-through tanks supplied with aerated and de-chlorinated conditioned reverse-osmosis (RO) water (high Na^+ water Table 1; temp. 28°C). Fish were fed twice daily with live brine shrimp (*Artemia salina*; INVE Aquaculture Nutrition) and trout chow (O.S.I. Marine Lab Inc.) and were kept on a 14 h:10 h light:dark photoperiod. All animals used in the experiments were males because females varied in mass depending on the presence of eggs. The experiments were conducted in compliance with University of Alberta Animal Care protocol AUP00000072.

Exposure to ultra-low- and low-Na^+ media

For acclimation experiments, zebrafish were transferred to temperature-controlled (temp. 28°C) 20 l glass tanks containing either ultra-low- or low-Na^+ medium (Table 1) for 1 week prior to $^{22}Na^+$-uptake experiments. To prepare ultra-low-Na^+ water, ions were added from stock solutions (1 mol l^{-1} NaCl, 10 mol l^{-1} $CaSO_4$, 10 mmol l^{-1} $MgSO_4$) to reverse osmosis (deionized) water, followed by addition of KOH or H_2SO_4 to obtain the desired pH level of 6. For low-Na^+ water, City of Edmonton tap water was used. For all exposures, approximately half of the water volume was changed daily to prevent accumulation of nitrogenous waste. Fish were not fed for the duration of the acclimation. Water ion concentrations were monitored daily using atomic absorption spectrophotometery (Perkin Elmer, Model 3300, CT, USA).

Pharmacological inhibition of Na^+ uptake

Sodium uptake in zebrafish acclimated to ultra-low- or low-Na^+ water was measured in 600 ml, aerated flux chambers using radiolabelled $^{22}Na^+$ as described previously (Goss and Wood, 1990). Briefly, fish (N=6 per treatment) were transferred from the acclimation tank to the flux chamber 1 h prior to the measurement to enable chamber acclimation. Radiolabelled $^{22}Na^+$ (either 8 µCi l^{-1} or 19 µCi l^{-1} as appropriate) was then added to each chamber and allowed to mix for 10 min. For experiments involving pharmacological treatment, pharmacological agents were added 10 min prior to $^{22}Na^+$. After 10 min of mixing of $^{22}Na^+$, an initial 3 ml water sample was collected while a final flux sample was taken after 90 min of exposure. Zebrafish were then killed with an overdose with MS-222, removed from the flux chambers, rinsed three times in 300 mmol l^{-1} NaCl solution, blotted dry, weighed and individually analysed for $^{22}Na^+$ activity with a gamma counter (Packard Cobra II, Auto Gamma, Model 5010, Perkin Elmer, MA, USA). Unidirectional $^{22}Na^+$ influx $J_{Na^+,in}$ (nmol g^{-1} h^{-1}) was calculated as:

$$J_{Na^+,in} = \frac{CPM}{SA\ t\ M}, \quad (1)$$

where CPM (counts per minute) is the total radioactivity of one fish (n=1), SA is the specific media activity (CPM µmol^{-1}), t is the time lapsed (h) and M is the mass of the fish (g).

The first series of fluxes investigated concentration-dependent effects of 4′,6-diamidino-2-phenylindole (DAPI) at 0, 0.01, 0.1, 1, 10 and 100 µmol l^{-1} on Na^+ uptake. Since DAPI had not been used in adult zebrafish previously, we needed to determine the effective dose required. The dose–response study was performed only in zebrafish acclimated to low-Na^+ medium, since preliminary Na^+ flux experiments showed insensitivity to DAPI in zebrafish acclimated to ultra-low-Na^+ medium. The second series of fluxes investigated the effect of amiloride (200 µmol l^{-1}), an inhibitor of NHE, ENaC and ASICs (Kleyman and Cragoe, 1988; Paukert et al., 2004), ethyl-iso-propyl-amiloride (EIPA; 100 µmol l^{-1}), an inhibitor with high affinity for NHE but not Na^+ channels (Kleyman and Cragoe, 1988) and DAPI (10 µmol l^{-1}) an inhibitor of ASICs but not ENaCs (Chen et al., 2010) on Na^+ uptake in zebrafish acclimated to either ultra-low- or low-Na^+ water. All pharmacological agents were dissolved in 0.1% dimethyl sulfoxide (DMSO) and for the control group, only 0.1% DMSO was used.

Immunoprecipitation and western blot

An anti-zebrafish ASIC4.2 antibody was generated as described previously (Dymowska et al., 2014). Immunoreactivity of anti-ASIC4.2 antibody in zebrafish was validated using immunoprecipitation (IP) technique according to the protocol used in our previous study (Dymowska et al., 2014). Briefly, whole gill baskets were dissected out of adult zebrafish, washed in ice-cold PBS, and cells lysed in 1 ml of IP buffer containing 1% Triton X-100 for 30 min. The whole lysate was incubated with 4 µl of anti-ASIC4.2 antibody at 4°C overnight with agitation. Following the incubation, 60 µl of pre-swelled and pre-blocked protein A–Sepharose CL4b beads (Sigma, St Louis, MO) were added to the lysate and incubated for 6 h on a rotator. Next, samples were briefly centrifuged, supernatant was removed and the beads were washed three times with 1 ml IP buffer. Washed beads were incubated with 35 µl Laemmli buffer for 15 min at 65°C, centrifuged and the supernatant retained for western blot analysis, as described previously (Dymowska et al., 2014). Briefly, the samples were separated on a 7.5% polyacrylamide mini-gel, transferred to a nitrocellulose membrane, blocked in 5% skimmed milk in Tris-buffered saline containing Triton X-100 (0.2%) (TBST) and incubated with anti-ASIC4.2 antibody (1:1000) on a rocker at 4°C overnight. Subsequently, the membrane was washed, blocked again with 5% skimmed milk in TBST and incubated for 1 h at room temperature with a secondary horseradish-peroxidase-conjugated goat anti-rabbit antibody (1:10,000; Santa Cruz Biotechnology, Dallas, TX). Finally, the membrane was washed and immunoreactive bands were visualized with a SuperSignal West Pico Chemiluminescence Substrate kit (Thermoscientific) according to the manufacturer's protocol.

Immunohistochemistry

Gills of zebrafish acclimatized to high Na^+ (Table 1) were examined for the presence of ASIC4.2, VHA and Na^+/K^+-ATPase. Zebrafish were killed with an overdose of MS-222 (1 g l^{-1}), and the second and third gill arches were removed from the gill basket, fixed in 4% paraformaldehyde in PBS (pH 7.4) overnight at 4°C, embedded in paraffin blocks and processed for immunochemistry as described previously (Dymowska et al., 2014). Briefly, serial sections (4 µm) were cut, rehydrated and incubated for 1 h in 10 mmol l^{-1} citrate buffer at 70°C for epitope retrieval. For co-localization of ASIC4.2 and NKA, sections were incubated with the anti-ASIC4.2 polyclonal antibody (1:250) and anti-NKA monoclonal antibody (1:250; Developmental Studies Hybridoma Bank, University of Iowa) overnight, followed by incubation for 1 h with TRITC-conjugated anti-rabbit (1:500; Invitrogen, OR, USA) and FITC-conjugated anti-mouse (1:500; Invitrogen) secondary antibodies. To analyze co-localization of ASIC4.2 with VHA, consecutive sections were incubated overnight with either anti-ASIC4.2 antibody as described above or anti-VHA antibody (1:300; kindly donated by Dr Steve Perry, University of Ottawa), since both polyclonal antibodies were raised in the same host. Subsequently, sections were incubated for 1 h with secondary TRITC-conjugated anti-rabbit antibody (1:500; Invitrogen) and sections stained with anti-ASIC4.2 were compared with consecutive sections stained with anti-VHA in order to determine co-localization. All slides were analyzed with a laser-scanning confocal microscope (Zeiss LSM 710, Germany) at the Cross Cancer Institute Cell Imaging Facility, Edmonton, Alberta. Images were processed with an LSM Image Browser (v.4.2.0.121; Carl Zeiss) and Adobe Photoshop software.

Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from zebrafish gill tissues using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. Following the isolation, RNA was treated with DNase I (Ambion, Austin, TX, USA) in order to remove genomic DNA and further purified with an on-column cleanup using RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada). The quality of the RNA was then assessed by visualization on a formaldehyde gel and the concentration was measured with a NanoDrop® ND-1000

UV-vis Spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). First-strand cDNA was synthesized from 1 µg of RNA using SuperScript III Reverse Transcriptase (Invitrogen) according to protocols provided by the manufacturer. Gene-specific primers for zebrafish *asic1.1*, *asic1.2*, *asic1.3*, *asic2*, *asic4.1* and *asic4.2* were designed with PrimerQuest (Table 2). PCR was performed with Phusion polymerase (New England Biolabs, MA, USA) under the following conditions: 98°C for 1 min of initial denaturation followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 63°C for 30 s, elongation at 72°C for 40 s and with a final elongation at 72°C for 10 min. PCR products were visualized by 1% agarose gel electrophoresis followed by ethidium bromide staining.

Statistical analysis

Data are reported as means±s.e.m. For Na^+ flux data, one-way ANOVA and *post hoc* comparison with Tukey test was performed (SigmaPlot version 11, Systat, Chicago, IL, USA).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

A.K.D. and G.G.G. are responsible for conception and design of the research; A.K.D., D.B. and A.G.S. performed the experiments and analyzed the data; A.K.D. and G.G.G. interpreted the results of the experiments and drafted the manuscript; A.K.D. prepared the figures; all authors edited and revised the manuscript and approved the final version.

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