The role of carbonic anhydrase in chloride uptake by larval zebrafish

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Introduction

Freshwater fish counter diffusive ion loss to their dilute environment by actively taking up ions from the surrounding water via specialised ion-transporting cells or ionocytes located in the gill. The cellular mechanism responsible for the uptake of chloride ions (Cl^-) remains poorly understood. The objective of the present study was to investigate the role of the enzyme carbonic anhydrase (CA) in Cl^- uptake via a proposed model involving Cl^-/HCO_3^- exchange. Carbonic anhydrase catalyzes the reversible hydration of CO_2 into HCO_3^- and H^+ and is therefore proposed to provide HCO_3^- for Cl^-/HCO_3^- exchange. The CA isoform of the yolk sac epithelium of larval zebrafish *(Danio rerio)*, CA II-like a, was studied; the yolk sac epithelium is the larval functional equivalent of the gill in adult fish.

Three approaches were used to evaluate the involvement of CA in Cl^- uptake. Chloride uptake was measured in zebrafish larvae experiencing functional knockdown of CA II-like a in low chloride conditions; a reduction in Cl^- uptake in larvae lacking CA II-like a was predicted. Western blotting was used to determine whether low water Cl^- levels for the first four days of development lead to increased CA II-like a protein expression to enhance Cl^- transport. Finally, an immunohistochemical approach was used to visualize and quantify CAII-like a positive ionocytes in larvae under different conditions. These CAII-like a positive cells are potentially involved in the Cl^- uptake pathway. Collectively, these experiments provided insight into the ionoregulatory mechanisms of a freshwater fish.

Materials and methods

- Zebrafish embryos were incubated post-fertilization in chloride-poor (20 μM) or control ($[Cl^-]$ = 200 μM) water for 4 days. Functional knockdown of CAII-like a was achieved using an antisense morpholino oligonucleotide.
- Rates of Cl^- uptake were measured in 4 day old larvae using ^{36}Cl.
- Western blots were carried out on proteins extracted from 4 day old larvae using a primary antibody specific for zebrafish CAII-like a and normalized against protein levels of ß-actin.
- To localize CAII-like a to specific ionocyte types, immunohistochemistry was carried out on 4 day old larvae using the CAII-like a antibody and concanavalin A as a marker for proton pump-rich (HR) ionocytes.

Results

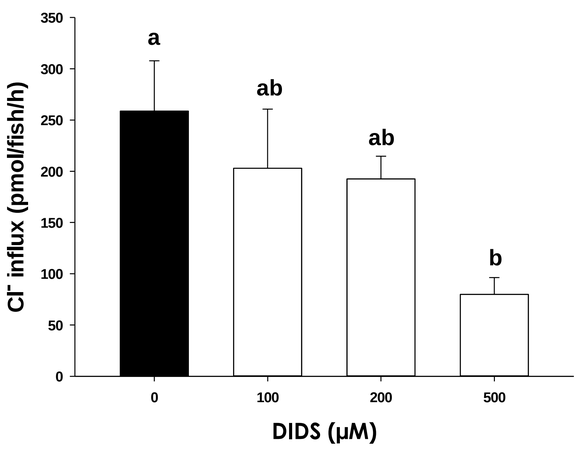


Figure 1. The rate of chloride uptake in embryos treated with increasing concentrations of DIDS, a Cl^-/HCO_3^- exchange inhibitor. Bars labeled with different letters are stastically different (One-way ANOVA, $p<0.05$). Values represent means ± SEM, $n = 6$.

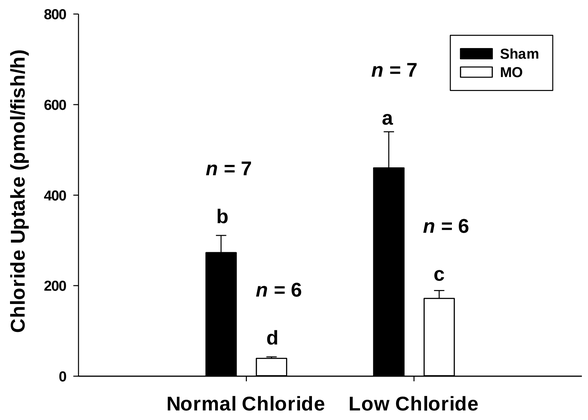


Figure 2. Zebrafish larvae experiencing functional knockdown of CAII-like a exhibited significantly lower rates of Cl^- uptake than sham-injected (control) larvae in both control water and when raised in low chloride water. Low chloride conditions significantly increased rates of Cl^- uptake. Bars labeled with different letters are statistically different (two-way ANOVA, $p<0.05$). Values represent means ± SEM. MO = CAII-like a morphant.

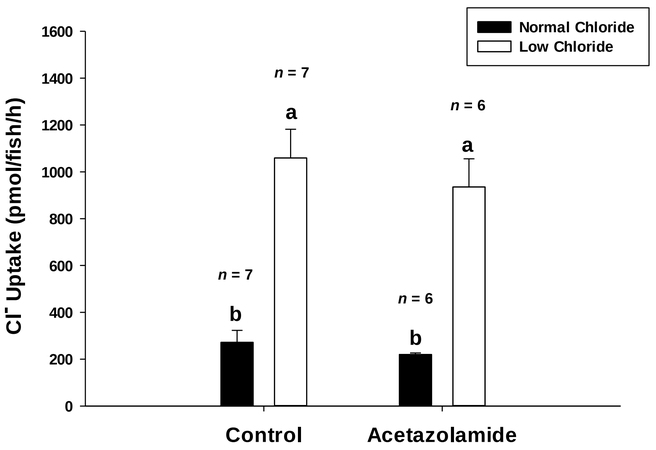


Figure 3. Pharmacological inhibition of CA (all CA isoforms) using the inhibitor acetazolamide (100 μM) did not have a significant impact on rates of Cl^- uptake in 4-day-old larvae acclimated to low chloride or control conditions. Bars labeled with different letters are staticstically different (two-way ANOVA, $p<0.05$). Values represent means ± SEM.

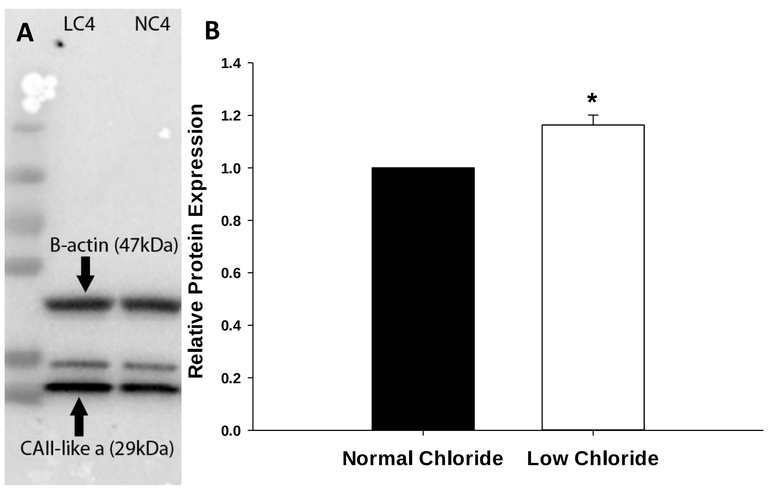


Figure 4. A representative western blot (A) depicting ß-actin at 47 kDa, CAII-like a at 29 kDa, and a nonspecific band around 35 kDa for fish raised in low chloride or control water. Quantification (B) of band densities revealed a significant elevation of CAII-like a protein levels in larvae raised in low chloride water. An asterisk indicates a statistically significant difference (Student's *t*-test, $p<0.05$). Values for low chloride water are expressed relative to the mean of control samples ± SEM. LC = low chloride. NC = normal chloride.

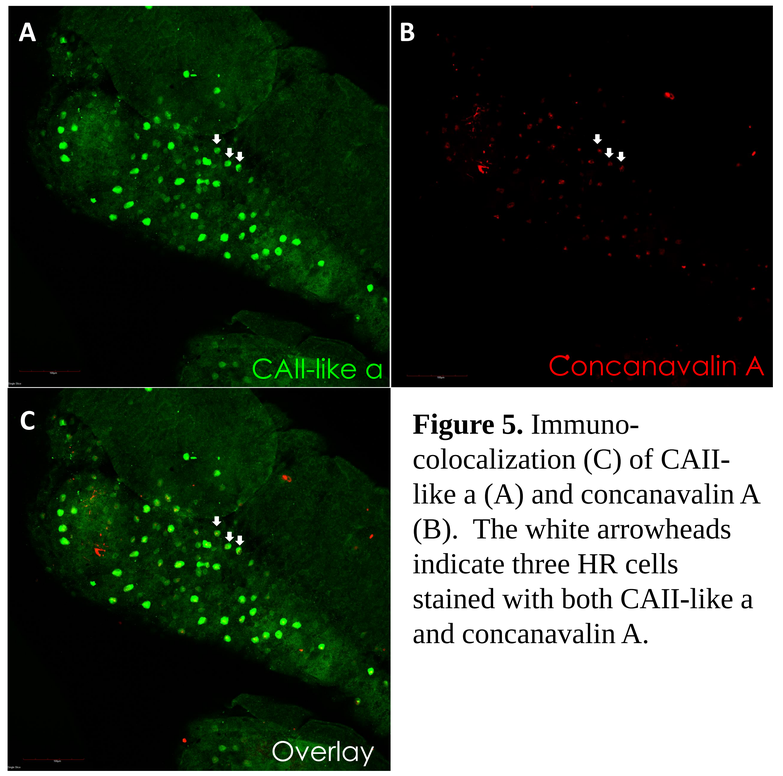


Figure 5. Immuno-colocalization (C) of CAII-like a (A) and concanavalin A (B). The white arrowheads indicate three HR cells stained with both CAII-like a and concanavalin A.

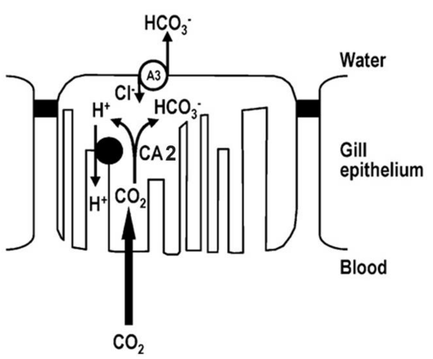
Discussion

The effectiveness of DIDS, a Cl^-/HCO_3^- exchange inhibitor, in significantly reducing chloride uptake strongly implicates a Cl^-/HCO_3^- exchange mechanism in chloride uptake in larval zebrafish.

Functional knockdown of CAII-like a caused a significant reduction in chloride uptake, implicating CAII-like a in the chloride uptake mechanism, perhaps by catalyzing the hydration of CO_2 to produce HCO_3^- ions for a Cl^-/HCO_3^- mechanism. However, treatment of fish with the CA inhibitor acetazolamide did not reduce chloride uptake. Because acetazolamide inhibits all CA isoforms, the lack of a reduction in Cl^- uptake with this treatment may have been caused by effects of other CA isoforms that masked the inhibition of CAII-like a.

Measurement of CAII-like a protein levels under low chloride conditions provided additional evidence for a role for CAII-like a in chloride uptake. The significant increase in CAII-like a protein levels in low chloride water is consistent with a need to enhance chloride uptake under these conditions.

Immunohistochemistry revealed localization of CAII-like a to HR cells, ionocytes involved in sodium uptake. Collectively, these data suggest that HR cells may also be responsible for chloride uptake through a Cl^-/HCO_3^- exchanger-mediated pathway:



Acknowledgements

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