NKCC-EXPRESSION AND ACTIVATION IN YOLK SAC CHLORIDE CELLS OF JAPANESE MEDAKA (*ORYZIA LAPIDES*) EMBRYOS

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Euryhaline fish frequently migrate to brackish water for breeding, and eggs hatch in this water of variable salinity. Consequently mechanisms for salt adaptation have to be established early during the development. Adult euryhaline fish adapt to increased environmental salinity by increasing secretory activity of mitochondria rich chloride cells (MR-cells) in fish gills. Major transport molecules for salt secretion in these cells are Na-K-ATPase and Na-K-2Cl cotransporter (NKCC) (Flemmer, A. et al., Bull MDIBL 38, 1999). In killifishembryos, MR-cells can be detected in the yolk sac, and chloride secretion is likely to proceed across yolk sac epithelium of these cells (Guggino, W.B. et al. Am. J. Physiol. 238: R42-49, 1980). During salt adaptation of euryhaline tilapia (*Oreochromis mossambicus*), yolk sac MR-cells undergo morphological and functional changes comparable to MR cells in gills of adult fish. In salt water adapted fish, cell surface area increases, and the expression of Na-K-ATPase increases as compared to fresh water adapted fish (Shiraishi K. et al., Cell Tissue Res. 288: 583-590, 1997). However, NKCC expression and activation in embryonic yolk sac MR-cells have not been elucidated. The purpose of this study was to investigate NKCC expression and activation in yolk sac chloride cells of Japanese medaka *(Oryzia lapides)* embryos.

Adult medaka were kept in fresh water on a 12 hour light cycle to induce dayly spawning. Fertilized eggs were harvested every morning, dissected free from chorion, and transferred to rearing solutions of two different salinities (low salt: 15 mM NaCl, 0.4 mM KCl, 0.27 $CaCl_2$, 1.33 mM $MgSO_4$; high salt: 150 mM NaCl, 0.4 mM KCl, 0.27 mM $CaCl_2$, 1.33 mM $MgSO_4$). Embryos were studied 2 and 6 days after fertilization and salt exposition (hatching at 9-10 days). NKCC- and Na-K-ATPase-expression were investigated with specific antibodies (Anti-T84 for NKCC; C. Lytle, UC, Riverside, CA; and a5 for Na-K-ATPase, Iowa Hybridoma, University of Iowa) in fixed cryosections of whole fish on a laser scanning microscope. NKCC-activation was determined with a phospho-specific antibody (R5), determining phosphorylated NKCC (P-NKCC) as previously described. This antibody is detecting two phophorylated threonin residues (T_{184}/T_{189} corresponding to human T_{212}/T_{217}) of a highly conserved region of the NKCC protein (Flemmer, A. et al., FASEB J. 13: A399, 1999). Embryos from the same rearing solution were dissected free from the egg, incubated with or without forskolin (10 μM) and the phosphatase inhibitor caliculin A (0.5 μM) for 60 min (+f). Subsequently, an equal number of embryos were homogenized in 1 M H_3PO_4 / 1% SDS, and expression of P-NKCC in embryo homogenates was examined by immunoblotanalysis. A single band was detected at 180 kD using the R5 antibody (Fig. 1C); thus, subsequent analysies were performed on dotblots. NKCC activation was determined densitometrically. R5 signal after 60 minutes of forskolin + caliculin A incubation was referred to as total NKCC. Densitometric results were analyzed by ANOVA and subsequent LSD-testing, when appropriate.

Immunofluorescence staining showed NKCC expression in single, cone shaped cells in yolk sac of both 2 and 6 day old fry. NKCC was expressed in parallel with Na-K-ATPase, identifying these cells as chloride cells (Fig. 1A, 1B). The cytosolic expression pattern of both proteins was comparable to that previously described for adult killifish gill MR-cells (Flemmer, A. et al. Bull

MDIBL 38, 1999). NKCC and Na-K-ATPase expression levels in fish incubated in low salt solutions were no different than those in fish incubated in high salt solutions.

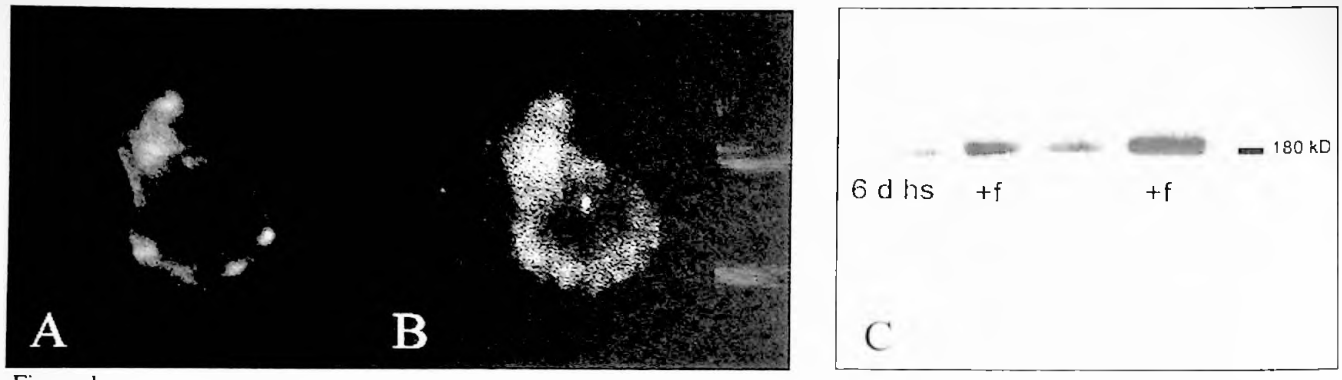

Figure 1
Fluorescence micrograph of yolk sac cell of 6 day old embryos. Cryosection was probed with both, anti-T84 (NKCC: A; secondary antibody: FITC) and a5 (Na-KA-TPase: B; secondary antibody: TRITC); 2000x. C: Immunoblot, probed with R5, detecting NKCC activation; fish were maintained in high salinity for 6 days (6d hs). +f indicates forskolin-incubation.

Immunoblots showed that forskolin significantly increased activation of NKCC in embryos maintained in high salt conditions (after 2 and 6 days) (Figure 1C, 2A, B: $p < 0.05$), indicating a cAMP-mediated pathway for cotransporter activation in yolk-sac MR-cells. Total NKCC expression (after forskolin + CalA) tended to be highest when eggs were maintained in high salt conditions for 6 days as compared to low salt conditions ($p = 0.14$, 2d hs+f vs. 6d hs+f). Baseline NKCC activity was not different in fish of both salinities (Figure 2A, B).

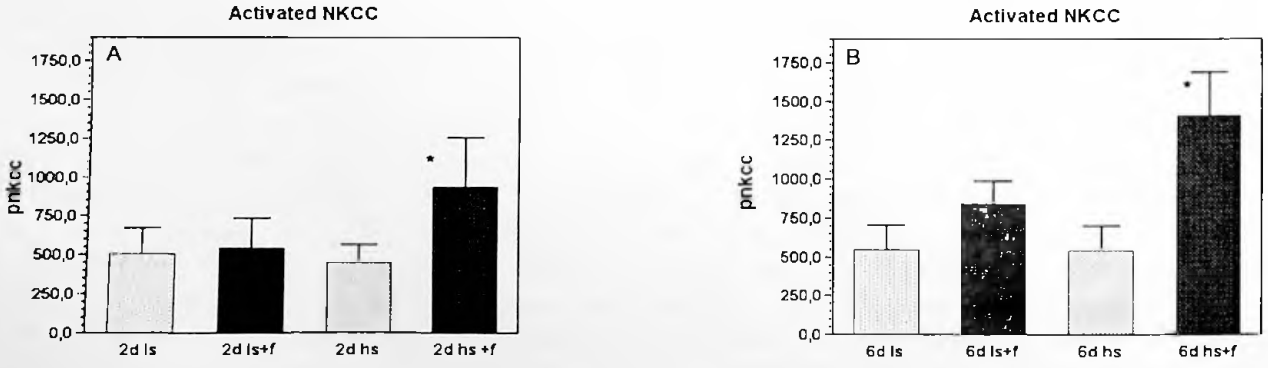

Figure 2:
Activation of NKCC in medaka embryos 2 days (A) and 6 days (B) after fertilization. Fish were maintained in low salt (ls) or high salt (hs) conditions after harvesting and incubated without and with forskolin (10 μM) + caliculin A (0.5 μM) (+f) for 60 minutes prior to testing. MEAN ± SEM, * $p<0.05$ vs. hs and ls;

Our results indicate that embryonic medaka yolk sac chloride cells, identified by Na-K-ATPase expression, express NKCC. In addition, embryonic NKCC is regulated by a cAMP mediated pathway and high salt conditions might result in adaptive changes in these cells.

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