LONG TIME ACCLIMATION TO HIGH AND LOW SALINITY OF CHLORIDE CELLS FROM THE OPERCULAR EPITHELIUM OF *FUNDULUS HETEROCLITUS*

Else Hoffmann[1], Erik Hoffmann[2], Sigfried Waldegger[3], José Zadunaisky[4] & Florian Lang[3]
[1]August Krogh Institute, 13 Universitetsparken, 2100 Copenhagen Denmark
[2]Danish Institute for Fisheries Research, 2920 Charlottenlund Denmark
[3]Universität Tübingen, Phys.Inst. Gemeinstrase 5, 72076 Tübingen, Germany
[4]University of Miami, Marine Biology and Fisheries, Miami Fl.33149.

The acclimatisation of euryhaline fish to fresh water or seawater occurs in two phases. First there is rapid signal that induces an increase (fresh to salt water) or a decrease (salt to fresh water) in the Cl^- secretion of the gill Cl^- secretary epithelium. The nature of the rapid signal has been discussed in several papers (Zadunaisky, *Kidney International* 49:1563–1567,1996). In the period following the first acclimation phase a second acclimatisation occurs which was previously shown to involve among other events a change in the number of Cl^- cells in the gill and an increase in the number of Na^+, K^+- ATPase molecules in the Cl^- cells (Karnaky et al., *J.Cell Biol.*, 70, 157–177, 1976 and Foskett et al., *J.Exp.Biol.* 106:255-281,1983). Thus far, nothing is known about the cellular signalling of long time salt-water acclimation. In the present report we look for upregulation of the Cl^- current, and we simultaneously follow the phosphorylation or expression of serine phosphorylated membrane proteins. Most recently, a serine/threonine kinase (h-sgk) has been cloned from human hepatocytes, which is transcriptionally regulated by cell volume (Waldegger et al., *Proc.Natl.Acad.Science* 94(9): 4400-4445,1997). Expression of this kinase is markedly upregulated by osmotic cell shrinkage and virtually disappears upon osmotic cell swelling. This kinase is thus considered a possible candidate for the signalling of salt acclimatisation in euryhaline fish. We therefore investigated whether a similar kinase is present in the gill epithelium of the Killifish.

Adult killifish, *Fundulus heteroclitus* were caught in the brackish, estuarine areas around Mount Desert Island from July to September 1997. The fish were kept in circulating sea-or fresh water, respectively for at least 14 days to get fully acclimated. One group was transferred back to seawater after the freshwater acclimation. Transepithelial electrophysiology has been applied to estimate transport and Western blot analysis performed to test for serine/threonine phosphorylation. Before the experiments the fish were killed and the opercular epithelium dissected out and mounted in a small Ussing chamber with teleost Ringer's on

both sides as described by Zadunaisky et al, *J. Membrane Biol.* 143, 207–217, 1995. The electrical potential difference, the short circuit current and the tissue conductance of the epithelium was measured with a voltage clamp unit. From the fresh-and salt-water acclimated fish respectively, opercular epithelia were equilibrated in teleost Ringer's and subsequently lysed in ice-cold lysis buffer for SDS–PAGE electrophoresis and Western blotting. The gills were removed, frozen and used for identification of the putative kinase.

The trans-epithelial potential difference, the steady state chloride current and the maximally obtainable chloride current after addition of mannitol plus isoproterenol were measured in salt-water acclimated, in freshwater acclimated and in fish from the same batch retransferred to salt water and measured at 5, 18, 70 and 96 hours after the transfer.

Hours after transfer to salt water	Potential Difference* mV	Short circuit Current μAcm^{-2}	Max. Current after Stimulation ** μAcm^{-2}	No. of Separate Experimt.
0 Freshwater adapt.	1.5 ± 1.1	0 ± 6	46 ± 11	3
5	2	11	43	2
18	8	34	67	2
70	7	42	145	2
96	12	119	242	2
Fully Salt-water acclim.	17.3 ± 1.3♦	148 ± 13♦	273 ± 41♦♦	♦ 29 ♦♦9

Table 1. Electrical parameters for isolated operculum epithelia of *Fundulus* acclimated to salt water or fresh water and during the first four days after transfer from fresh to salt water. Data are expressed as mean ± SD. * Open circuited: negative on the apical side, positive on the basolateral side. **Stimulation with hypertonic solution (100 mOsm mannitol) plus the β-agonist isoproterenol (20 μM)

The results are presented in Table 1. No short circuit current and no potential difference could be measured in the operculum epithelium from freshwater acclimated fish or from fish within the first 18 hours after transfer to salt water. Stimulation with hypertonic solution (100 mOsm mannitol) plus the β-agonist isoproterenol, which in salt-water accli-

mated fish causes a dramatic stimulation of the short circuit current to 273 μAcm^{-2}, did not result in any significant stimulation within the first 18 hours after transfer of the fish to salt water. Not until more than 90 hours after transfer to salt water the fish were fully acclimated as evaluated from steady state short circuit current and the maximum current after hypertonic stimulus plus stimulus with the β-agonist isoproterenol. It has previously been suggested that the primary event after increase in osmolarity is an activation of a basolateral Na^+, K^+, $2Cl^-$ co-transporter (Zadunaisky, *Kidney International,* 49, pp.1563–1567, 1996). A possible long time adaption to salt water could thus be an increase in the number of Na^+, K^+, $2Cl^-$ cotransport molecules in the membrane or alternatively an upregulation of a kinase involved in the activation of the co-transporter (Hoffmann et al., *MDIBL Bulletin*, 37, 1998)

As evidenced from Western blot analysis using antiserine-antibodies, a heavily serine phosphorylated protein of some 200 kDa is consistently observed (n=4) in the salt-water acclimated fish which is only weakly present in freshwater acclimated fish (n=4). This observation indicates that acclimatisation to salt water stimulates the expression of this 200-kDa protein and/or a serine/threonine kinase, which subsequently phosphorylates the protein. A kinase, highly homologous with the h-sgk kinase mentioned above, has been identified in the shark *Squalus acanthias* and similarly found to be transcriptionally regulated by cell volume (Waldegger et al., *MDIBL-bulletin*, 37, 1998). Using degenerated primers against highly conserved sequences of the sgk kinase we identified a similar band in the gill epithelium from salt water acclimated *Fundulus* indicating the existence of the sgk transcript in the gill. It is tempting to speculate that this kinase is upregulated in salt acclimatisation and subsequently activates the Na^+, K^+, $2Cl^-$ cotransport by phosphorylation. It is, however, also possible that hypertonicity enhances expression of functional Na^+, K^+, $2Cl^-$ cotransporters as shown in Ehrlich ascites tumor cells (Jensen & Hoffmann, *B.B.A.* 1329, 1-6, 1997). Transcriptionally regulation of a kinase and/or of the Na^+, K^+, $2Cl^-$ cotransporters will be subject of future investigations.

Acknowledgement: This research was supported by NIH Grant EY 01340.
Else K. Hoffmann was a John W. Boylan fellow of MDIBL.