

**IMMUNOCYTOCHEMICAL TRACKING OF CFTR AND NKCC
TRANSPORTERS IN CHLORIDE CELLS AND INTESTINE OF
KILLIFISH (*FUNDULUS HETEROCLITUS*):
CHANGES WITH SALINITY ADAPTATION**

W.S. Marshall, Biology Department, St. Francis Xavier University,
Antigonish, NS, Canada (bmarshall@stfx.ca),

E.M. Lynch, J.A. Howard and R.R.F. Cozzi

EXTENDED ABSTRACT ONLY - PLEASE DO NOT CITE

The gill epithelia of teleost fish secrete NaCl in seawater (SW) and absorb NaCl in freshwater (FW). Na⁺,K⁺-ATPase pump in both salinities provides the driving force, but it is the placement of passive transporters that provides the direction. In SW a basolaterally located Na⁺,K⁺,2Cl⁻ cotransporter (NKCC) transports Cl⁻ into the ion secreting cell. At the apical membrane is an anion channel that is a homolog for Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) that allows Cl⁻ exit (review: Marshall 2002). Killifish CFTR (kfCFTR) has been cloned from the euryhaline teleost fish *Fundulus heteroclitus* and this protein is expressed in gill, opercular epithelium and intestine, as detected by Northern blot analysis (Singer et al. 1998). CFTR, detected immunocytochemically, is restricted to the membrane in apical crypts of chloride cells of seawater adapted mudskippers (Wilson et al. 2000). In salinity adaptation from FW to SW, ion transport direction reverses from uptake to secretion, but it is not clear how this reversal occurs. In linear time courses of salinity adaptation in tilapia (*Oreochromis mossambicus*) larvae (Hiroi et al. 1999), more than 70% of the same chloride cells are still present 96h after transfer to sea water, so the redistribution of transporters in extant cells is an important component of adaptation. This study traces, using immunocytochemistry, NKCC and CFTR in chloride cells during SW adaptation.

Methods

Opercular membranes containing mitochondria rich chloride cells were dissected and stained with Mitotracker Red (Molecular Probes), then fixed (80% MeOH/20% DMSO) and incubated with primary antibodies, mouse monoclonal anti hCFTR carboxy terminus (R&D systems) and mouse monoclonal anti hNKCC (T4, Iowa Hybridoma Bank). The secondary antibody was goat anti mouse Oregon Green 488 (Molecular Probes). Whole opercular membranes were viewed with a confocal microscope (Olympus FV300).

Opercular Membrane CFTR Immunofluorescence

Cellular distribution of CFTR immunofluorescence was observed in chloride cells of FW adapted fish and animals transferred to SW for 24h, 48h and 14+ days. Confocal microscopy allowed localization within mitochondria rich (MR) cells to be determined as superficial (in the membrane of the apical crypt) or in the basolateral membrane of the cells. In FW, 90 percent of MR cells had diffuse kfCFTR immunofluorescence in the central part of the cell (Figure 1B), with only 8.1 percent having apical kfCFTR that was 6.6 ± 0.54 (mean \pm SEM) μm below the microridges of surrounding pavement cells. Curiously, pavement cells from FW (but not SW) killifish had positive immunofluorescence for kfCFTR, suggesting that pavement cells contribute to ion uptake in FW. After 24h in SW, a time when kfCFTR expression is elevated (Singer et al. 1998), there appeared among 18.8 percent of MR cells, a

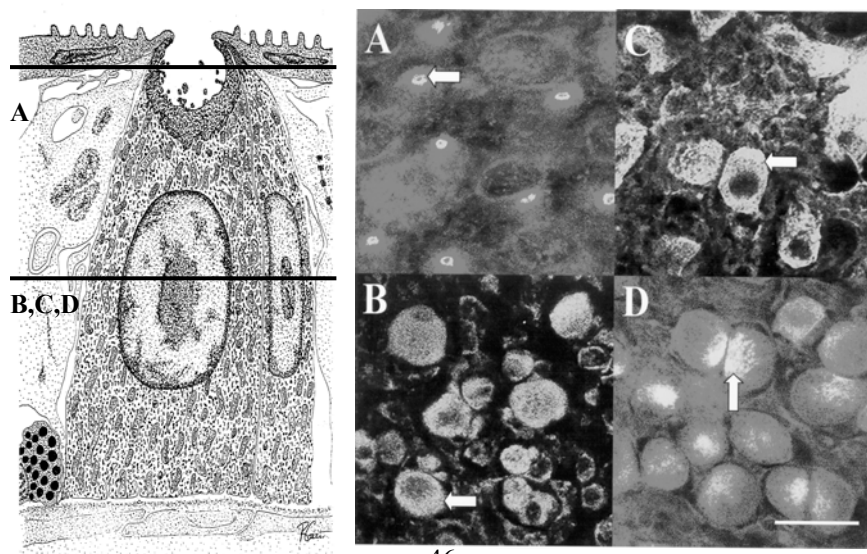


Figure 1. LEFT Drawing of chloride cell showing planes of optical sections. RIGHT Immunocytochemistry of opercular membranes (white; arrows) and mitotracker stain (Grey). A) CFTR apical distribution in SW. B) CFTR diffuse basolateral distribution in FW. C) NKCC diffuse basolateral distribution in SW. D) Eccentric NKCC distribution in FW. Bar is 20 μm . (fm. Marshall et al 2002)

condensed punctate immunofluorescence that was $13.4 \pm 0.66 \mu\text{m}$ below the surface of the cells. By 48h a majority (76.3%) of MR cells had punctate kfCFTR distribution and the distance from the surface was less ($7.8 \pm 0.2 \mu\text{m}$), a distribution approaching the SW acclimated condition. Often the apical membrane CFTR immunofluorescence was identifiable as the characteristic ring at the apex of the cell (Figure 1A). In SW acclimated fish all MR cells had the ring shape kfCFTR immunofluorescence that was $6.1 \pm 0.04 \mu\text{m}$ below the surface. Thus CFTR, the anion channel responsible for Cl^- secretion in marine teleosts, redistributes in MR cells during SW acclimation by condensation of a diffuse distribution below the apical crypt followed by translocation and insertion in the apical membrane.

Opercular membrane NKCC Immunofluorescence

Unlike the CFTR distribution in FW, NKCC immunofluorescence was condensed and localized in lateral parts of MR cell complexes in FW animals (Figure 1D). Where chloride cells were paired, NKCC immunofluorescence appeared where the two cells abutted. NKCC redistributed to the whole basal cytoplasm after acclimation to SW (Figure 1C). Because SW ion secretion is blocked by basolateral furosemide and bumetanide, the NKCC fluorescence is very likely in the tubular system, not the cytosol. NKCC, the cotransporter that translocates Cl^- across the basolateral membrane, moves from an eccentric cytosolic location in fresh water to a diffuse basolateral localization in SW chloride cells.

Intestine NKCC and CFTR immunofluorescence

CFTR was immunocytochemically localized in intestine frozen sections and found to be present in basolateral membranes of all enterocytes and in the brush border membrane of some (approx. 25%) cells. In contrast, NKCC immunofluorescence was in the basolateral and brush border membranes of most enterocytes and in the basolateral membrane only in a minority (approx. 25%) of cells. Sections of killifish posterior intestine were induced to secrete

NaCl and fluid by the calcium ionophore ionomycin (1.0 μ M) in combination with agents to elevate intracellular cyclic AMP, dibutyryl-cAMP (db-cAMP) 0.5 mM with 0.1 mM 3-isobutyl-1-methylxanthine (IBMX). Mucosal application of the anion channel blocker 1.0 mM diphenylamine-2-carboxylate (DPC) after ionomycin + db-cAMP + IBMX significantly reduced serosal to mucosal unidirectional Cl⁻ flux ($P < 0.001$), net Cl⁻ flux ($P < 0.05$), short circuit current (I_{sc} , $P < 0.001$) and tissue conductance (G_t , $P < 0.001$), while 0.1 mM of the disulfonic stilbene DIDS was without effect. Teleost intestine is capable of salt and fluid secretion if intracellular Ca²⁺ and cAMP pathways are stimulated together. The ion and fluid secretion appears to involve activation of CFTR ion channels in the apical membrane of a subpopulation of enterocytes.

Conclusions

- 1) The anion channel CFTR redistributes from the basolateral membrane of chloride cells in FW to the apical crypt membrane during SW adaptation.
- 2) The cotransporter NKCC moves from an punctate locus eccentric to the nucleus to a diffuse basolateral location during SW adaptation.
- 3) The teleost chloride cell may be an ideal model to study trafficking of ion transport proteins in epithelial cells.

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Acknowledgements

This work was funded by NSERC research grant to W.S.M. and USRA scholarship to J.A.H. and confocal microscope was purchased by CFI and ACOA.

