IMMUNOLOCALIZATION OF Na^+/K^+ ATPASE IN GILL MITOCHONDRIA-RICH CELLS OF ATLANTIC HAGFISH (*MYXINE GLUTINOSA* L.)

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Although the complete physiological function of fish gill chloride cells is still being determined, it is likely that their primary roles include ion uptake in hyperosmotic regulators (Perry, S. F., *Annu. Rev. Physiol.* 59:325-47, 1997) and ion excretion in hypoosmotic regulators (Karnaky, K. J. J., *Am. Zool.* 26:209-224, 1986). Surprisingly, the gills of hagfishes have been shown to contain morphologically similar cells (Mallatt, J. and C. Paulson, *Am. J. Anat.* 177:243-269, 1986; Elger, M., *Anat. Embryol.* 175:489-504, 1987) even though they are not able to regulate extracellular NaCl concentrations (Evans, D. H., Osmotic and ionic regulation. *In The Physiology of Fishes*, 314-341, 1993). Biochemical data on the distribution and quantity of Na^+/K^+-ATPase (a chloride cell marker) in hagfish is limited to one study which used a potassium-dependent p-nitrophenylphosphase (K-NPPase) histochemical technique on the Pacific hagfish (*Eptatretus stouti*; Mallatt, J. et al., *Can. J. Zool.*, 65:1956-1965, 1987). Thus, in the present study, an antibody specific for the α-subunit of the Na^+/K^+-ATPase (Witters, H. et al., *Cell Tiss. Res.* 283:461-468, 1995) and a vital mitochondria dye were used to determine the distribution of Na^+/K^+-ATPase relative to mitochondria-rich cells within the hagfish gill filament.

Hagfish gill pouches were dissected and immersion fixed in 3% paraformaldehyde in 0.01 M PBS for three hours at room temperature. After rinsing with PBS, aldehyde fixed pouches were cryoprotected in 20% sucrose, 3% polyethylene glycol overnight at 4 °C and frozen in Tissue Tek imbedding medium by immersion in liquid nitrogen. Cryosections (8-10 µm) were cut longitudinal to the filaments with a Reichert-Jung cryostat and attached to poly-L-lysine coated slides. After drying to the slide, sections were rehydrated with PBS and blocked with fish gelatin (2% fetal calf serum, 1% BSA, 0.2% fish gelatin, and 0.05% azide in PBS) for 15 minutes. Primary antibody, α5, diluted to 5 µg/ml in fish gelatin, was applied for 2 h at room temperature. Following a 5 min rinse in PBS, the primary antibody was labeled with goat anti-mouse IgG secondary antibody conjugated to rhodamine (Pierce Chemical Co., Rockford, IL). Sections were rinsed again for 5 min in PBS and mounted in gel/mount (Biomeda Corp.) under coverslips. Antibody-antigen binding was visualized with an Olympus confocal laser scanning microscope with the excitation and emission filter set for rhodamine.

Individual filaments were separated from gill pouches (dissected as above) and transferred to a hagfish Ringer's (in mM: 501 NaCl, 8.7 KCl, 5.4 $CaCl_2$, 12.6 $MgCl_2$, 3.2 Na_2SO_4 and 0.42 NaH_2PO_4) containing 25 µM DASPEI (2-(4-dimethylaminostyryl)-1-ethylpyridinium iodide, Sigma, St. Louis, MO) that was previously bubbled with a 99% O_2 / 1% CO_2 mixture of gas for ten minutes. They were allowed to incubate at 15-18 °C for one hour before rinsing two times with Ringer's lacking DASPEI. After rinsing, filaments were placed flat on a glass slide coverslip in a drop of Ringer's. Vital staining was visualized as above with the excitation and emission filter set for flourescein isothiocyanate.

Numerous, large, ovoid cells with distinct nuclei were strongly stained with DASPEI and antibody α5 (Figure 1). The mitochondria-rich cells were most abundant in the afferent multilayered epithelium (lateral side of filament) and decreased in abundance towards the efferent arterial (medial) side. Cells staining positive for the Na^+-pump showed the same distribution. No staining was observed when filaments were incubated in DASPEI free Ringer's or when sections were incubated with secondary antibody alone (data not shown).

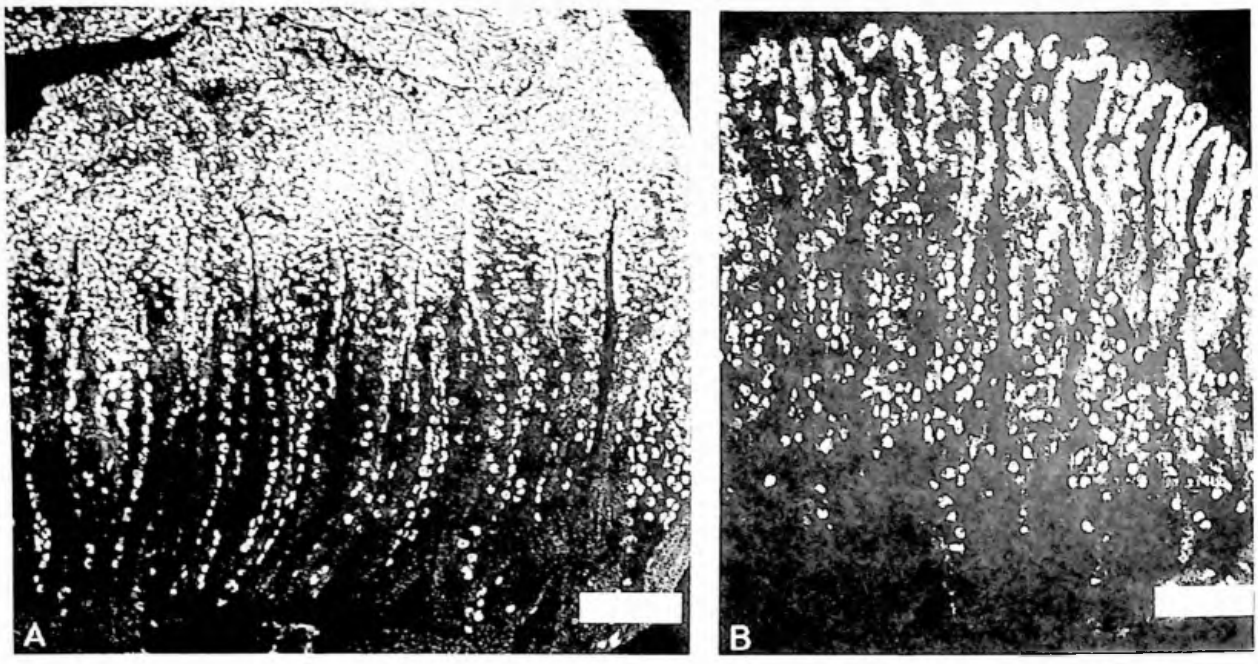

Figure 1. Confocal laser scanning micrograph of a hagfish gill filament after *in vivo* staining of mitochondria with DASPEI (A) or immunocytochemical staining for the α subunit of the Na^+/K^+-ATPase (B). (A) Image of whole filament was produced by superimposing serial sections. (A & B) The lateral (afferent arterial) side is towards the top of the images. Scale bar = 200 μm (A & B).

Based on the conserved nature of the Na^+-pump α subunit (Lebovitz, R. M. et al., *EMBO J.* 8:193-202, 1989) and the antibody's binding to a band of the appropriate size on western blots (data not shown), it is concluded that antibody α5 specifically recognizes the α-subunit of hagfish Na^+/K^+-ATPase. Calcium excretion (Forester, M. E. and J. C. Fenwick, *Gen. Comp. Endocrinol.* 94:92-103, 1994) and systemic acid-base regulation (Evans, D. H., *J. Exp. Biol.* 113:465-469, 1984) are among the functions proposed for hagfish gill mitochondria-rich cells. Evans showed that Na^+-free seawater decreases and Cl^--free seawater increases net H^+ efflux indicating the presence of Na^+/H^+ and Cl^-/HCO_3^- exchange systems (ibid.). In addition, preliminary evidence of proteins and genes homologous to Na^+/H^+ exchangers has been obtained (Edwards, S. et al., this vol.). Given the lack of extracellular Na^+ and Cl^- regulation by hagfish and their ability to increase net acid excretion following acidosis (McDonald, D. G. et al., *J. Exp. Biol.* 161:201-215, 1991), one likely function of the apparent ion exchangers is pH homeostasis. Under physiological conditions, Na^+/H^+ exchangers are driven by an inward electrochemical gradient for Na^+ across the plasma membrane. The high density of Na^+/K^+-ATPase in the gill may facilitate this exchange by setting up a Na^+ gradient and maintaining it during a pH disturbance. Future work is required to confirm the identity of Na^+/H^+ and Cl^-/HCO_3^- transporters and localize their expression relative to the Na^+-pump.

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