PROTON-TRANSPORTING ATPASE ACTIVITY IN CRUDE HOMOGENATES OF GILL TISSUE FROM THE LITTLE SKATE, *RAJA ERINACEA*

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Gills of teleost fish are the major site of ion regulation and acid-base balance. The transport of salts is often linked to the movement of acid-base relevant ions like H^+ and HCO_3^- (Heisler, N. in *Physiology of Fishes*, D.H. Evans, Ed., pp. 343-378, CRC Press, Boca Raton, FL, 1993). While the presence of proton-transporting ATPases has been demonstrated in the gills of freshwater trout (Lin, H. and Randall, D.J., J. Exp. Biol. 180:163-174, 1993) and seawater mudsucker (Kultz, D. and Somero, G.N., J. Exp. Biol. 198:1883-1894, 1995) there have been no comparable investigations of the elasmobranchs, where regulation of salts occurs predominantly via a rectal gland. Experiments described below examined the gills of *Raja erinacea* for proton-transporting ATPase activity *in vitro*, as defined by several inhibitors.

Specimens of little skate *Raja erinacea* were collected by commercial fishermen from Frenchman Bay and stored in tanks with running seawater until use. Animals were killed by pithing the brain and spine with a wire. The gills were irrigated with running seawater (12 - 15° C), the heart was exposed and gills were perfused via intubation of the conus with ice-cold Ca^{+2}-free Elasmobranch Ringer's Solution (Ca^{+2}-free ERS; in mM, Na^+, 258; Cl^-, 269; HCO_3^-, 8; Mg^{+2}, 6.5; urea, 350; TMAO, 72) with 5U ml^{-1} heparin to clear the gills of blood. Gills were dissected free, rinsed twice in Ca^{+2}-free ERS, and blotted. Gill scrapings were homogenized (1g:5ml) in ice-cold medium (300mM sucrose, 2mM EGTA, 1mM dithiothreitol, 100mM Tris HCl, 0.1% deoxycholate, pH 7.3), centrifuged at 13k x g for 3 min., and the supernatant assayed for ATPase activity (at 15° C., via an assay linked to lactate dehydrogenase and pyruvate kinase: LDH/PK) or frozen (-80° C). The assay medium (after Kultz and Somero, 1995) contained 30mM imidazole, 125mM NaCl, 20mM KCl, 4mM $MgCl_2$, 5mM Na_2EDTA, 0.4mM NaN_3, 2mM phosphoenolpyruvate, 1mM Na_2ATP, 0.5mM NADH and 10ul LDH/PK mix (Sigma Chem. Co.). Protein was assayed using the Bradford method (Bio-Rad). Data were analyzed using one-way ANOVA followed by paired t-tests (Bonferroni Method, Fig. 1) or by Dunnett's Method (Fig. 2).

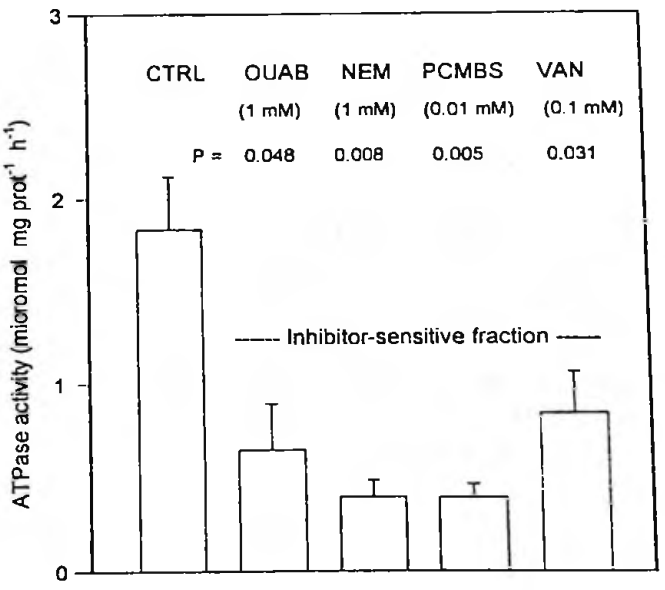
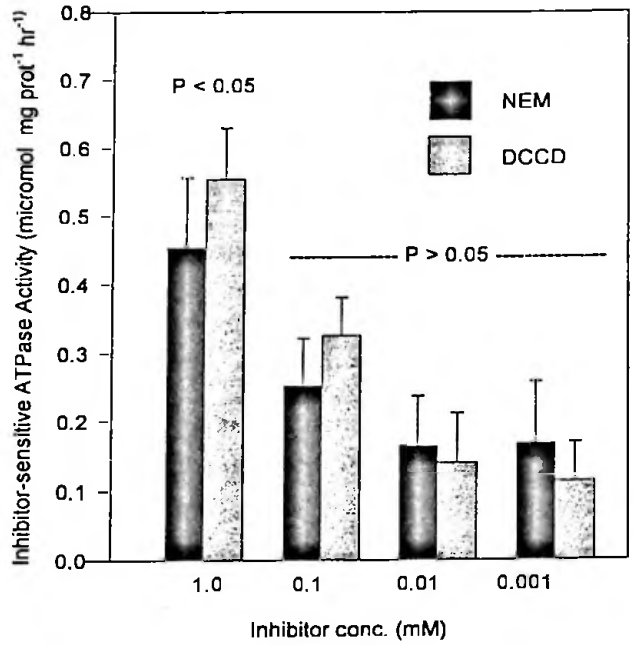


Figure 1. Control level and inhibitor-sensitive components of gill ATPase activity in *Raja erinacea*. P value is reported for paired comparison to control (n = 6).

Figure 2. Inhibitor-sensitive components of gill H^+-ATPase activity, measured in the presence of ouabain (1 mM). N = 6.



The results of the first series of experiments are presented in Figure 1. The assay mixture contained all of the substrates necessary for ATPase activity; however, EDTA/Ca^{+2}-free medium will minimize Ca^{+2} -ATPase activity and azide will inhibit F-type (mitochondrial) ATPase. The ouabain-sensitive component comprised about 30% of the remaining ATPase activity. N-ethyl maleimide (NEM)-, p-chloromercuribenzenesulfonate (PCMBS)-, and vanadate-sensitive components represented 21, 22 and 45%, respectively of control ATPase levels. In a second series of experiments the dose responses to NEM and DCCD (dicyclo hexylcarbodiimide) were examined in the presence of ouabain (1 mM) to eliminate Na^+-K^+-ATPase activity. Results of this experiment are reported in Figure 2. Both NEM and DCCD at 1mM significantly inhibited ATPase activity. The inhibitor-sensitive fractions of activity declined as dose decreased. However, inhibitor-sensitive components of activity at concentrations of 0.1 mM or less of NEM and DCCD were not significantly different from zero.

These ATPase assays on crude extracts of gill tissue were performed *in vitro*, in the presence of sodium azide, an inhibitor of F-type (mitochondrial) ATPase, and conditions that would minimize Ca^{+2} -ATPase activity. Na^+-K^+-ATPase activity (ouabain-inhibitable) comprised about 30% and H^+-ATPase (PCMBS, DCCD and NEM-inhibitable), 22% of the remaining ATPase activity. Thus, about 50% of the ATPase activity is unaccounted for. Lin and Randall (1993) speculate that it may represent Cl^-/HCO_3^- ATPase activity. NEM at 10 uM should be sufficient to inhibitor vacuolar ATPase (Pedersen, P.L. and Carafoli, E., Trends Biochem. 12:146-150, 1987). ATPases involving phosporylation (e.g. P-type ATPases) like Ca^{+2} -ATPase, Na^+-K^+-ATPase and plasma membrane type H^+-ATPase require higher levels of inhibitor and are vanadate-sensitive (Forgac, M. Physiol Rev. 69:765-796, 1989; Nelson, J. Exp. Biol. 172:19-27, 1992). DCCD inhibits mitochondrial and vacuolar H^+-ATPase at very low concentrations (less than 1 and 10 uM, respectively). Plasma membrane H^+-ATPase requires up to 100 uM (see Lin and Randall, 1993). PCMBS, which blocks H^+-ATPase in bovine medulla at 0.01 mM (Gluck, S. and Al-Awqati, Q., J. Clin. Invest. 73:1704-1710, 1984) was also an effective blocker of ATPase activity here and in the trout (Lin and Randall, 1993).

The results presented here are qualitatively similar to those of Lin and Randall (1993) for the freshwater trout, although the levels of NEM-sensitive activity measured here were about

one-third of those for freshwater-acclimated trout, but comparable to those of the seawater trout. Enzyme levels reported here and by Lin and Randall (1993) may be underestimated, since vesicles formed in crude homogenates may have basolateral ATP-binding domains either external or internal to the vesicle, with only the former being accessible to the assay medium. Kultz and Somero (1995) found that Na^+-K^+-ATPase and H^+-ATPase activities from seawater *Gillichthys* gill homogenates were more than doubled in vesicles permeabilized by alamethicin. Vanadate, an inhibitor of P-type ATPases, inhibited a large component of gill ATPase activity, most of which was probably ouabain-sensitive, since the vanadate-sensitive component was not significantly different from the ouabain-sensitive component (Fig. 1, paired data, $P = 0.083$). Experiments to determine if NEM and vanadate inhibition overlapped were not performed. Since concentrations of 1 mM NEM and DCCD were required to inhibit significantly ATPase activity, these data suggest that H^+-ATPase is the membrane type. Based on similar results, Lin and Randall (1993) reached the same conclusion for the trout, although in a later paper, immunolocalization showed that H^+-ATPase activity in the trout gill was antigenically similar to H^+-ATPase in mammalian kidney and brain, and therefore of the vacuolar type (Lin, H. *et al.*, J. Exp. Biol. 195:169-183, 1994).

These results are the first to demonstrate that H^+-ATPase activity, defined by NEM, DCCD and PCMBS inhibition, occurs in the gills of *Raja erinacea*. Preliminary results suggest that H^+-ATPase activity might be of the plasma membrane type, although immunological localization is called for to demonstrate definitively cell location and enzyme type. Supported by NSF IBN 9507456 (GAK) and NSF REU BIR9322221 to MDIBL (JB, TW).