AMILORIDE-SENSITIVE AMMONIUM ION EFFLUX FROM MEMBRANE VESICLES OF CRAB (CARCINUS MAENAS) GILL

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Ammonia excretion by branchial epithelia of teleosts and crustaceans has been attributed to both apical and basolateral membranes of ion-transporting cells within the epithelium (Kirschner, Amer. J. Physiol. 244:R429-R443, 1983; Claiborne, Evans and Goldstein, J. Exp. Biol. 96:431-434, 1982). These conclusions were based on experiments in which either whole animals were treated with an exogenous inhibitor, amiloride, or the gill was perfused with experimental media containing ouabain. In both cases, secondary consequences of perturbing other functions of the epithelium had to be ignored. Because the geometric complexity of most gills prevents experiments with microelectrodes or Ussing-type chambers, an alternative approach, studying transport by isolated membrane vesicles, has been initiated. We have shown that microsomal membrane vesicles prepared from mitochondria-rich cells of posterior gills of two euryhaline crabs, Callinectes sapidus and Carcinus maenas, are capable of ATP-dependent sodium uptake, apparently via Na^++K^+-ATPase (Fuhrman, Stansbury and Towle, Amer. Zool. 23:953, 1983; Towle, MDIBL Bull. 23:10-12, 1983). The present work was undertaken to determine whether the same preparation of vesicles is capable of transporting NH_4^+.

We developed a technique to measure NH_4^+ efflux from pre-loaded vesicles, using the NH_4^+ sensitivity of the enzyme glutamate dehydrogenase, which was supplied in the extravesicular medium along with substrates, including NADH. The in absorbance at 340 nm was measured in a thermoelectrically-regulated cuvet holder in a Beckman DU7 spectrophotometer. The data-handling capability of the DU7 was employed to calculate slopes, assemble scans, and plot results. Vesicles were prepared by sucrose density gradient centrifugation (Towle, 1983) and were loaded with intravesicular medium by dilution and centrifugation (Boumendil-Podevin and Podevin, Biochim. Biophys. Acta 728:39-49, 1983). The intravesicular medium contained 50 mM $(NH_4)_2SO_4$, 100 mM sucrose, and 10 mM Tris-HEPES (pH 7.4). The extravesicular medium designed for the detection of NH_4^+ efflux contained 10 mM Tris-HEPES (pH 7.4), 2 mM $MgSO_4$, 2 mM alpha-ketoglutarate, 2 mM adenosine diphosphate, 45 mM Na_2SO_4, 100 mM sucrose, 0.5 mM NADH (Tris salt), and 1 mg/ml glutamate dehydrogenase (Sigma G-7882). Glutamate dehydrogenase was activated by ADP and inhibited by Cl^-, in agreement with previous reports (Bidigare, Ph.D. Thesis, Texas A&M, 1981; Gilles, Int. J. Biochem. 5:623-628, 1974). Substitution of K^+ for Na^+ had no effect on the assay system. However, amiloride at concentrations above 5×10^{-5} M inhibited glutamate dehydrogenase (K_i = 1.2×10^{-4} M). This surprising effect of amiloride was taken into consideration in designing experiments with vesicles.

Amiloride concentrations below those which inhibit the assay system were consistently capable of inhibiting apparent NH_4^+ efflux from loaded vesicles (Figure 1). This inhibition occurred in vesicles obtained from either posterior or anterior gills of C. maenas, and was observed in experiments in which extravesicular Na^+ was replaced by K^+. Although Na^+ was undoubtedly present at uM quantities in the latter experiments, the results argued against a Na^+/NH_4^+ exchange mechanism.

Amiloride is thought to inhibit Na^+ uptake by ion-transporting epithelia via inhibition of apical Na^+/H^+ exchange (Kirschner, 1983). Reduction of H^+ efflux

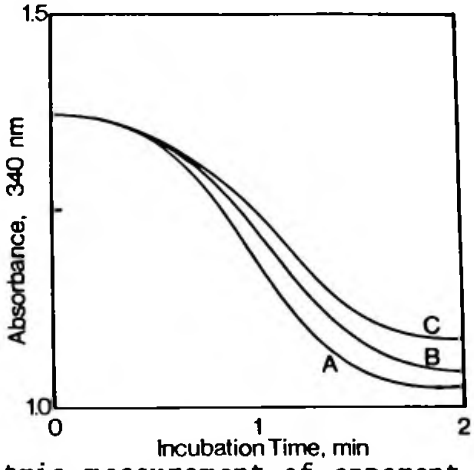

Figure 1. Spectrophotometric measurement of apparent NH_4^+ efflux from membrane vesicles of Carcinus maenas posterior gill, and inhibition by increasing concentrations of amiloride. A: control; B: $1x10^{-5}$M amiloride; C: $2x10^{-5}$M amiloride. The amiloride was dissolved in 50% ethanol and added in 2-4 ul aliquots to 0.4 ml extravesicular medium at 25 C. Ethanol itself had no effect on NH_4^+ efflux.

Table 1. Effect of intravesicular pH on apparent NH_4^+ efflux from membrane vesicles of C. maenas posterior gill. Vesicles were loaded with 50 mM $(NH_4)_2SO_4$, 100 mM sucrose, and 10 mM HEPES, pH 6.8 or 7.8 with Tris. Data are presented as means ± S.E. of triplicate determinations. Slopes were calculated from the linear portions of each scan.

	Change in absorbance/minute·mg vesicle protein		
Intravesicular pH	Total efflux	+ $2x10^{-5}$M amiloride	Difference
6.8	2.56±0.12	1.45±0.16	1.11
7.8	1.54±0.09	0.91±0.07	0.63

from amiloride-treated vesicles might prevent a shift in the NH_4^+-NH_3 equilibrium toward NH_3, decreasing the NH_3 available for non-ionic diffusion (Cameron and Heisler, J. Exp. Biol. 105:107-125, 1983). To investigate this possibility, we loaded parallel preparations of vesicles with intravesicular medium made up with 10 mM HEPES, adjusted to either pH 6.8 or 7.8 with Tris. Efflux of NH_4^+ was then measured in extravesicular medium at pH 7.4. If the apparent efflux of NH_4^+ was the result of non-ionic NH_3 diffusion, then the vesicles loaded with pH 6.8 medium should exhibit decreased efflux relative to those loaded with pH 7.8 medium, because the proportion of NH_3 at pH 6.8 should be one-tenth of that at pH 7.8 (0.2 mM vs 2 mM). Our results show that apparent NH_4^+ efflux from pH 6.8-loaded vesicles was actually greater, both in total levels and in amiloride-insensitive levels, than the pH 7.8-loaded vesicles (Table 1). The calculated difference, giving amiloride-sensitive NH_4^+ efflux, was also greater at pH 6.8. The conclusion must be that we are measuring actual NH_4^+ efflux rather than NH_3 diffusion. Although we have not eliminated the possibility of Na^+/NH_4^+ exchange, our results suggest that an amiloride-sensitive NH_4^+ channel may exist, independent of Na^+.

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