SODIUM/PROTON EXCHANGE IN MEMBRANE VESICLES FROM CRAB (*CARCINUS MAENAS* AND *CALLINECTES SAPIDUS*) GILL

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Introduction

Posterior gills of euryhaline crabs possess patches of mitochondria-rich cells which are thought to be sites of transepithelial Na^+ transport from water to blood. Na^++K^+-ATPase activity is restricted to the basolateral membranes of these cells (Towle, Bull MDIBL 25:80-83, 1985) and increases upon acclimation to reduced salinity (reviewed by Towle, Amer Zool 24:177-185, 1984). Sodium uptake by intact crabs and perfused gills is inhibited by external amiloride (Cameron, J Comp Physiol 133:219-225, 1979; Lucu and Siebers, J Exp Biol 122:25-35, 1986), suggesting that apical Na^+/H^+ exchange may be working with basolateral Na^++K^+-ATPase to achieve transepithelial Na^+ movement. We have now demonstrated the presence of Na^+/H^+ exchange in crude membrane vesicles from mitochondria-rich cells of crab gill, using the pH-sensitive fluorescent dye acridine orange (Sabolic and Burckhardt, Biochim Biophys Acta 734:210-220, 1983).

Materials and Methods

Green shore crabs (*Carcinus maenas*) were collected from intertidal and subtidal areas adjacent to MDIBL, and blue crabs (*Callinectes sapidus*) were obtained from Florida. The crabs were maintained in recirculated sea water at a salt concentration of 10 ppt for at least 1 week prior to use. Crabs were placed on ice for about 30 minutes before sacrifice by bisection. Tissue regions containing mitochondria-rich cells from gills 7-9 (counting anterior to posterior) in the green crab and gills 6-7 in the blue crab were quickly dissected and immediately placed in ice cold homogenizing medium (250 mM sucrose, 6 mM disodium EDTA, 20 mM HEPES, pH 6.8 with Tris). The tissue was then blotted, weighed, and homogenized in 5 volumes of fresh homogenizing medium containing 0.1% (w/v) sodium deoxycholate. Homogenization was accomplished with a Potter-Elvehjem glass tube and teflon pestle. Homogenate was layered on continuous sucrose density gradients (10-40%, 32 ml each) and centrifuged for 30 min at 100,000 x g. The resulting gradients were inspected visually and the two membrane bands were carefully removed, corresponding to 18-20 ml from the center of each gradient. The membrane fraction was diluted 10-fold with dilution-loading-resuspension medium (56 mM sucrose, 20 mM HEPES, 100 mM sodium gluconate, pH 6.8 with Tris), and was centrifuged at 100,000 x g for 60 min to pellet the membranes. The resulting pellets were resuspended in the same dilution-loading-resuspension medium described above, producing Na^+-loaded vesicles with an internal pH of 6.8.

Assays of Na^+/H^+ exchange were conducted in a Na^+-free incubation medium containing 10 uM acridine orange, 20 mM HEPES, 36 mM sucrose and 100 mM tetramethylammonium (TMA) gluconate, pH 6.8 with Tris. Measurements of osmolarity were made to insure that the loading medium and the incubation medium were the same in this respect. Sodium-loaded vesicles were injected into a cuvet containing 2 ml of the Na^+-free incubation medium (pH_i=pH_o) and changes in fluorescence were continuously monitored using either an

Aminco-Bowman spectrofluorometer coupled to a strip chart recorder or a Gilford Instruments Fluoro IV spectrofluorometer and plotter (excitation at 493 nm and emission at 525 nm). The assay mixture was stirred throughout the experiment. The resulting pH gradient that developed across the vesicle membrane was collapsed by injection of 100 ul of 1.5 M sodium gluconate into the cuvet. The sensitivity of the Na^+/H^+ exchange system to amiloride was determined by adding amiloride (24 ul of 50 mM in 50% EtOH) to the cuvet 4-5 min after the addition of vesicles, followed by extravesicular Na^+. Appropriate amounts of ethanol were added to controls.

Experiments designed to determine if the collapse of the pH gradient was specific for Na^+ were undertaken by substituting TMA gluconate (100 ul of 1.5 M) for extravesicular sodium gluconate. In order to relate the magnitude of the fluorescence quench to the amount of protein in the vesicle preparation a dose response curve for both crab species was generated. Vesicles were added in 2-ul increments to the cuvet (up to 8 ul total) and fluorescence readings were taken after an equilibration period of at least 2 min. Total protein was determined by the method of Bradford (Anal Biochem 72:248-254, 1976) using bovine serum albumin as the standard.

Results and Discussion

When Na^+-loaded vesicles were added to the Na^+-free medium ($pH_i=pH_o$), a rapid quench of fluorescence was observed (Fig. 1), attributable to the action of the Na^+/H^+ antiporter working to exchange intravesicular Na^+ for extravesicular H^+. The decrease in intravesicular pH results in dye movement into the vesicle and consequent quenching of fluorescence, which was relieved at least partially by the reversal of Na^+/H^+ exchange upon addition of sodium gluconate to the incubation medium. The incompleteness of the relief by extravesicular Na^+ may be explained by dye binding to the vesicle membrane (Binder and Murer, J Memb Biol 91:77-84, 1986).

Addition of amiloride (from ethanol stocks) resulted in greater quenching of fluorescence due to the optical characteristics of amiloride itself (Fig. 1). More importantly, amiloride inhibited the Na^+-induced relief of quench, indicating that the Na^+/H^+ exchanger of crustacean gill is sensitive to the drug. Addition of ethanol (24 ul of 50%) to controls caused a slight increase in fluorescence (Fig. 1), but had no effect on reversal of the exchanger induced by extravesicular Na^+.

Replacement of extravesicular sodium gluconate with TMA gluconate failed to relieve quench, indicating that TMA cannot substitute for Na^+ in reversing the exchange system (Fig. 2). Addition of TMA gluconate rather produced an enhanced quench, possibly the result of an osmotic effect of the salt on vesicle size and consequent light scattering. Sodium gluconate would be expected to induce the same osmotic effect, but such an effect may be masked by the simultaneous reversal of Na^+/H^+ exchange. Relative fluorescence quench was proportional to the amount of vesicle protein added to the incubation medium, up to 100 ug protein per 2 ml medium (Fig. 3). Addition of valinomycin and K^+ had little effect on the Na^+-induced relief of quench, indicating non-electrogenic Na^+/H^+ exchange.

In preliminary experiments with K^+-loaded vesicles we found behavior similar to that observed with Na^+-loaded vesicles. Fluorescence of acridine

orange was quenched upon addition of vesicles to K^+-free incubation medium, and potassium gluconate produced a relief of quench. Whether this phenomenon represents an alternative expression of the Na^+/H^+ exchanger or a separate K^+/H^+ exchanger (Binder and Murer, J Memb Biol 91:77-84, 1986) is presently under investigation.

Our results are in basic agreement with studies of vesicles derived from mammalian kidney and intestine (Tsai, Ives, Alpern, Yee and Warnock, Amer J Physiol 247:F339-F343, 1984; Sabolic and Burckhardt, Biochim Biophys Acta 734:210-220, 1983; Barros, Dominguez, Velasco and Lazo, Biochem Biophys Res Commun 134:827-834, 1986), and suggest that the crustacean Na^+/H^+ exchange system behaves similarly to the vertebrate antiporter.

Acknowledgements

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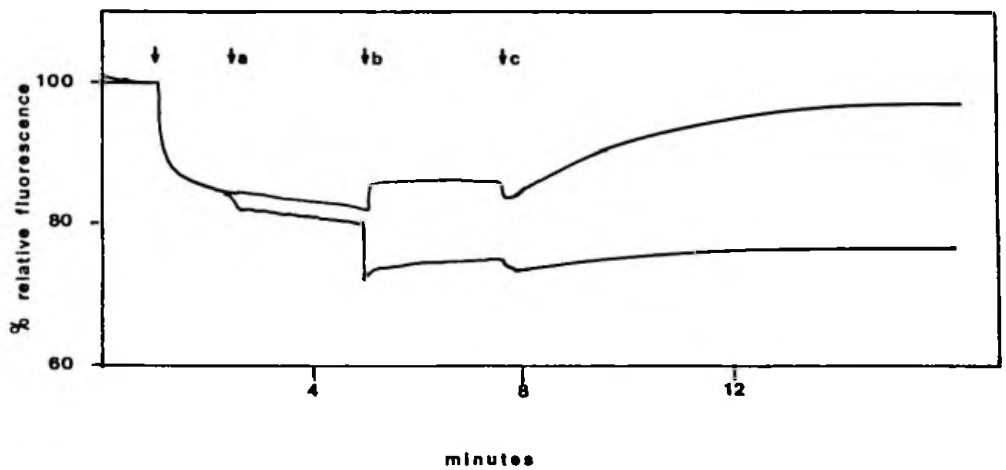

Fig. 1. Effect of amiloride (lower curve) on Na^+-induced reversal of Na^+/H^+ exchange by membrane vesicles from Callinectes gill. Sodium-loaded vesicles were added to Na^+-free medium at first arrow. At a, 4 ul of 50% ethanol or 50 mM amiloride in ethanol were added. At b, an additional 20 ul of ethanol or amiloride solution were added. At c, 100 ul of 1.5 M sodium gluconate were added.

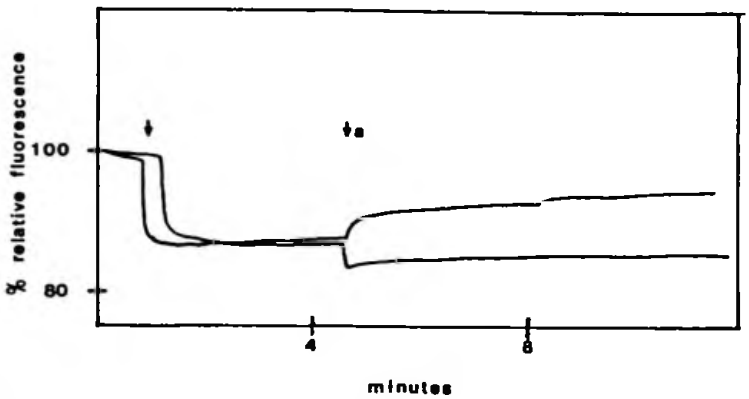

Fig. 2. Effect of TMA gluconate (lower curve) on reversal of Na^+/H^+ exchange by vesicles from Carcinus gill. At first arrow, 20 ul sodium-loaded vesicles were added to Na^+-free medium. At a, 100 ul of 1.5 M sodium gluconate or TMA gluconate were added.

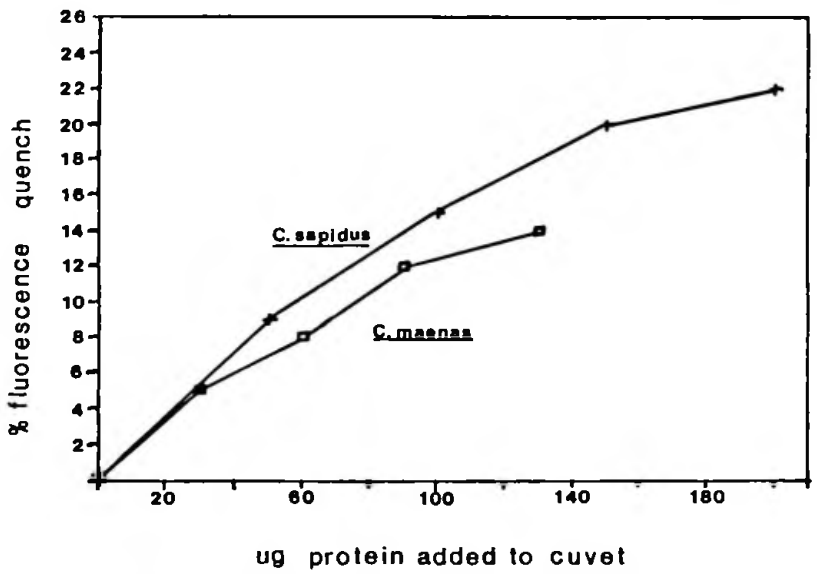

Fig. 3. Dependence of initial fluorescence quench on amount of vesicle protein added to 2 ml of Na^+-free medium.