LOCALIZATION OF A PLASMA MEMBRANE Ca^{++}-PUMP IN GILL CHLORIDE CELLS AND KIDNEY DISTAL TUBULE CELLS OF THE AMERICAN EEL, ANGUILLA ROSTRATA

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In mammals, Ca^{++}-pumping ATPases of cell plasma membranes are necessary for the control of intracellular calcium (Schatzmann, H. Curr. Top. Membr. Transp. 6:126-168, 1975; Penniston, J.T., et. al. In Calcium-binding Proteins: Structure and Function. Academic Press, Inc., NY, pp. 23-30, 1980). These enzymes form a labile phosphorylated intermediate and have an apparent molecular weight of ≈ 140,000 daltons (Schatzmann, H. Curr. Top. Membr. Transp. 6:126-168; Sarkadi, B, et al. J. Biol. Chem. 261:9552-9557, 1986). The plasma membrane Ca^{++}-pump from human erythrocyte membranes has been extensively studied and characterized (Penniston, J.T., et al. In Calcium-binding Proteins: Structure and Function. Academic Press, Inc., NY, pp. 23-30, 1980; Niggli, V., et al. J. Biol. Chem. 254-9955-9958, 1979). In addition, polyclonal and monoclonal antibodies against the purified erythrocyte Ca^{++}-pump have been used to help define the tissue and cellular distribution of plasma membrane Ca^{++}-pumps (Verma, A., et al. Archives. Biochem. Biophys. 215-345-354, 1982; Borke, JL, et. al. J. Clin. Endocrinol. Metab. 67:1299-1304, 1988).

Gill filament cell plasma membranes were prepared in order to demonstrate the presence of a phosphorylated intermediate of the Ca^{++} pump and to show an immunoreactive band of the appropriate molecular weight. Gill filaments from 5 seawater eels were washed and centrifuged at 1000 rpm for 7 minutes in 100 ml of 50 mM Tris HCl (pH 7.2) containing 100 mM KCl, 5 mM $MgCl_2$, 5 mM benzamidine, 0.5 M PMSF, 50 μg/ml trypsin inhibitor and 0.5 mM DTT. The supernatant fluid was decanted, and four volumes of the same buffer were added to the pellet, and the suspension was homogenized two times on ice with a Brinkman (Westbury, NY) Polytron, at setting 8 for 30 seconds. The homogenate was centrifuged at 12,000 x g for 10 minutes and the supernatant was saved. The supernatant was then centrifuged at 112,500 x g for 1 hour. The pellet was brought up in 500 μl of 0.25 M sucrose in 10 mM Tris buffer pH 7.2, frozen in liquid nitrogen, and stored at -70°C for several weeks before using. The frozen gill membranes were thawed at 4°C and 10 μl were removed and assayed for protein by the dual wavelength method (Ehresmann, B., et. al. Analyt. Biochem. 54:454-463, 1973).

To examine La^{+++}inhibition of Ca^{++}-pump phosphorylation, gill membrane samples (12.6 μg) were placed into three tubes containing 200 μl of 60 μM K-HEPES (pH 7.0) plus 150 μM KCl. The first tube also contained 200 μM EGTA. The second tube contained 40 μM $CaCl_2$, and the third tube had 40 μM $CaCl_2$ and 100 μM $LaCl_3$ added. Each reaction was started by adding 0.5 μM γ^{32}P-ATP. The reactions were stopped after 1 minute by adding 50 μl of 60% TCA, 1 μM ATP and 10 mM K_2HPO_4. Membranes were washed and repelleted 3 times at 12,000 rpm at 4°C. After washing, 50 μl of 0.15 M Tris HCl pH 6.8 containing 2% SDS, 10 mM EDTA, 60% sucrose, and 0.014% bromophenolblue was added to each pellet. The pH of each sample was adjusted to neutrality by adding 2 μl of 1.7 M Tris base. The entire sample was run on a slightly acidic 7.5% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) as described by Sarkadi (Sarkadi, B., et. al. J. Biol. Chem. 261:9552-9557, 1986).

For Western blot analysis, gill membrane samples were electrophoresed on a 7.5% SDS-PAGE gel as described above. Lanes containing proteins of known molecular weight were also electrophoresed. Gel lanes containing these marker proteins were cut from the gel and stained with 0.5% Coomassie blue. Other lanes were transferred to nitrocellulose paper using a Bio-Rad (Richmond, CA). Transblot apparatus by the method of Towbin (Towbin, H., et. al. Proc. Natl. Acad. Sci. USA 76:4350-4354, 1979). A 1:1000 dilution of monoclonal antibody 5F10 was used for detection of Ca^{++}-pump epitopes on the nitrocellulose as previously described.

Ca^{++}-pump was localized in kidney and gill tissue from 3 seawater-adapted eels. Tissues were fixed by immersion in 10% phosphate-buffered formalin. Fixed tissues were dehydrated through a graded series of ethanols and xylene, infiltrated with paraffin and made into blocks for sectioning. Immunohistochemistry was carried out as previously described (Borke, J.L., et. al. J. Clin. Invest. 80:1225-1231, 1987). A 1:1000 dilution of monoclonal antibody 5F10, prepared against human erythrocyte plasma membrane Ca^{++}-pump, was used for immunohistochemical localization of Ca^{++}-pump epitopes in kidney and gill. The detection method used to localize this antigen was the avidin-biotin technique described by Hsu (Hsu, S., et. al. J. Histochem. Cytochem. 29:577-580, 1981). In some sections, nuclei were counterstained with Mayer's hematoxylin solution (0.1% aqueous). In other sections, the tissue was post-stained by the periodic acid-Shiff's technique as described by MacManus (McManus, J. Stain. Technol. 23:97-108, 1948).

A La^{+++}-stabilized phosphorylated protein intermediate of Mr $\approx$ 140,000 was found in eel gill membranes. Stabilization of this phosphorylated intermediate was both Ca^{++} and La^{+++} dependent. A second La^{+++}-stabilized protein band of Mr $\approx$ 100,000 is also shown to be Ca^{++} and La^{+++} dependent. This protein probably represents a proteolytic product of the plasma membrane Ca^{++}-pump or it may represent a second La^{+++}-sensitive protein not found in mammals.

Western blot analysis of nitrocellulose immobilized eel gill membrane proteins showed binding of the anti-erythrocyte Ca^{++}-pump antibody 5F10 to a band of Mr $\approx$ 140,000. Other bands at 90 kDa and 76 kDa which also bind 5F10 may represent proteolytic fragments of the pump, and are similar in molecular weights to fragments of the human erythrocyte Ca^{++}-pump which have undergone tryptic digestion (Sarkadi, B., et. al. J. Biol. Chem. 261:9552-9557, 1986; Zurini, M., et. al. J. Biol. Chem. 259:618-627, 1984; Benaim, G., et. al. J. Biol. Chem. 259:8471-8477, 1984).

Immunoperoxidase staining of eel gill filaments also shows positive staining with antibody 5F10 (Figure 1). In this tissue, the chloride secreting cells at the base of each lamellae stain positive for Ca^{++}-pump. Staining is present throughout these cells and shows no polarity. This is probably due to the tubular system of the basal membrane which fenestrates throughout the cytoplasm. These cells play an important role in the regulation of ion fluxes into the gill circulation and have been compared in this regulatory function to the cells of the kidney tubule.

Immunohistochemical studies of eel kidney also show staining with monoclonal antibody 5F10. In this tissue only the basolateral membrane of the cells of the distal tubule stain positive for Ca^{++}-pump (Figure 2). These tubules are characterized by a sparse brush border. PAS and 5F10 double staining of eel kidney sections shows that the Ca^{++}-pump positive tubules have

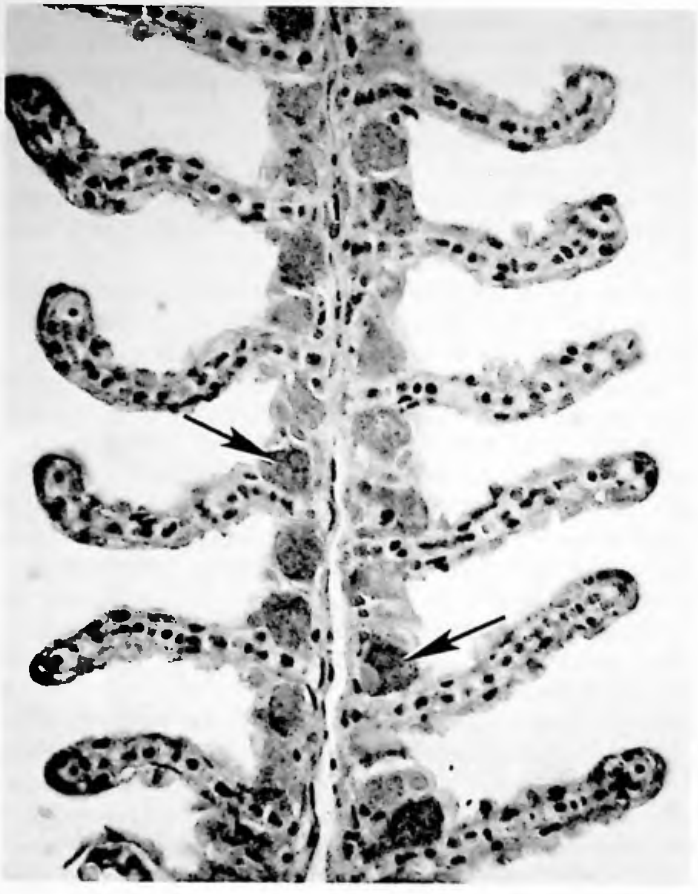

Figure 1. Immunohistochemical staining of eel gill with monoclonal antibody 5F10. Positive staining for Ca^{++}-pump seen in chloride cells (arrows). Magnification x 640.

Figure 2. Immunohistochemical staining of eel kidney with monoclonal antibody 5F10. Positive staining for Ca^{++}-pump seen in basolateral membranes of distal tubule cells (arrows). Magnification x 400.

PAS-negative luminal surfaces (data not shown). In contrast, tubules which have PAS-positive staining on their luminal surface do not stain with 5F10. Control sections of gill and kidney in which 5F10 was replaced with ascites fluid without 5F10 did not show specific staining of chloride cells or distal tubule basement membrane (data not shown).

The presence of Ca^{++}-pump epitopes in these tissues suggests that the plasma membrane Ca^{++}-pump may be involved in the adaptation of euryhaline fish to changes in Ca^{++} ion concentrations in their environment.

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