PURIFICATION OF TRANSPORTING APICAL MEMBRANE VESICLES FROM THE RECTAL GLAND OF SQUALUS ACANTHIAS

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The rectal gland of the spiny dogfish Squalus acanthias shares features common to a number of Cl^- secreting epithelia (Silva, P., J. Stoff, M. Field, L. Fine, J.N. Forrest and F. Epstein. Am. J. Physiol. 233: F298-F306, 1977) and as such serves as a useful and convenient model for corneal (Zadunaisky, J.A. and M. Wiederholt, Fed. Proc. 42: 5800, 1983); colonic (Welsh, M.J., P.L. Smith, M. Fromm and R.A. Frizzell. Science 218: 1219-1221, 1982) or tracheal secretory processes (Shorofsky, S.R., M. Field, and H.A. Fozzard. Phil Trans. R. Soc. Lond. B. 299: 597-607, 1982; Smith, P.L., M.J. Welsh, J.S. Stoff and R.A. Frizzell. J. Memb. Biol. 70:216-217, 1982). One approach to the elucidation of the underlying biochemistry of specific cell membrane transporters is to study them in isolated or reconstituted membrane vesicles. In studies of the $(Na^+ + K^+)$-ATPase, basolateral membranes (Hokin, L.E., J.L. Dahl, J.D. Deupree, J.F. Dixon, J.F. Hackney and J.F. Perdue. J. Biol. Chem. 248: 2593-2605) were purified from the shark rectal gland. The preparation was adapted to the isolation of transporting basolateral membrane vesicles to study coupled, neutral Cl^- transport (Hannafin, J., E. Kinne-Saffran, D. Friedman and R. Kinne. J. Memb. Biol. 75: 73-83, 1983). The apical membrane has not been isolated owing in part to the lack of a suitable enzymatic marker to identify this fraction. On the basis of ion flux measurements, microelectrode analysis, and direct measurement of Cl^- channels using patch clamp techniques it is clear that the apical membrane of the stimulated gland may be characterized as having a high Cl^- conductance which distinguishes it from the basolateral surface (Silva, P., J. Stoff, M. Field, L. Fine, J.N. Forrest and F. Epstein. Am. J. Physiol. 233: F298-F306, 1977; Welsh, M.J. Am. J. Physiol. 245: F640-F644, 1983; Gregor, R., E. Schlatter and H. Gogelein. Pflug. Arch. 403: 446-448, 1985). Using this property, namely, conductive Cl^- transport as a marker activity we have developed a method to prepare highly resolved apical membrane vesicles from the rectal gland of Squalus acanthias.

METHOD

Rectal Gland Membrane Preparation

The plasma membrane preparation is schematically outlined in Figure 1. Spiny dogfish Squalus acanthias were obtained from commercial fisherman off the coast of Mt. Desert Island and kept in marine live cars. The sharks were used within three days of capture. The animals were sacrificed by cervical segmentation and the rectal gland was removed through a lateral incision in the abdomen. Immediately after its removal the gland was perfused through the rectal gland artery at 16°C with a

modified Ringers solution of the following composition (in mM): Na^+ 280, K^+ 6, Mg^{++} 3, Ca^{2+} 2.5, Cl^- 290, $H_2PO_4^{-1}$ 1, SO_4^{2-} 0.5, HEPES 3, urea 350, glucose 5. The perfusate also contained 0.05 mM dibutyryl cAMP and 0.25 mM theophylline to stimulate the secretory activity of the gland (Silva, P., J. Stoff, M. Field, L. Fine, J.N. Forrest and F. Epstein. Am. J. Physiol. 233: F298-F306, 1977). The gland was perfused for a period of 15-30 minutes and then removed from the perfusion apparatus and placed in ice cold 50 mM mannitol, 2.5 mM K-HEPES, 1 mM EDTA, pH 7.6. The gland was finely minced and then homogenized by hand with ten strokes in a Potter-Elvehjem homogenizer.

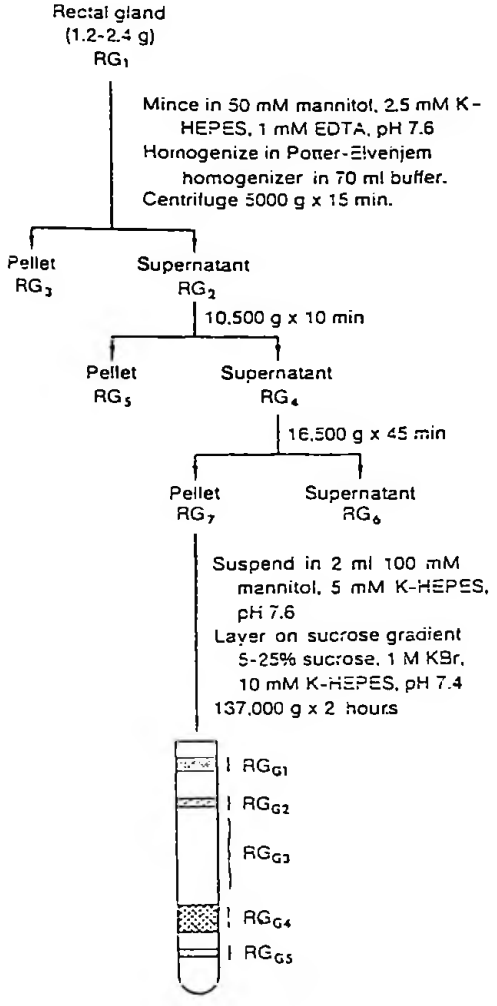

Figure 1. After a series of centrifugation steps, the pellet RG_7 was resuspended to a final volume of 2 ml in 100 mM mannitol, 5 mM K-HEPES, pH 7.6. This fraction was then layered on a 5-25% sucrose gradient prepared in 1 M KBr, 10 mM K-HEPES pH 7.6 and centrifuged at 137,000 g x 90' in a Beckman SW 27.1 ultracentrifuge rotor. The gradient yielded several distinct bands of protein as shown in the figure. The gradient was fractionated by removal of the bands with a Pasteur pipet. Each fraction was diluted with 5-10 volumes of 100 mM Mannitol, 5 mM K-HEPES and centrifuged at 180,000 g x 15'. The supernatant is discarded and the pellet resuspended in 0.2 ml of the same buffer.

Assay procedures:

The basolateral marker, ouabain sensitive $(Na^+ + K^+)$-ATPase, was assayed spectrophotometrically using a coupled assay adapted from Kagawa (Meth. Enzymol. 10: 505-510, 1967) and the mitochondrial marker succinate

dehydrogenase was assayed as previously described (King, T. Meth. Enzymol. 10: 322-335, 1967). Protein was determined by the method of Lowry (Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. J. Biol. Chem. 193: 265-275, 1951) using bovine serum albumin as a standard. Specific conductive Cl^- transport used as the marker for the apical membrane was assayed using a previously described method (Dubinsky and Monti, submitted). Briefly, anion efflux is measured with an anion specific electrode selective for either Cl^- or Br^-. In the studies reported here Br^- was substituted for Cl^- due to a greater sensitivity and stability of the Br^- selective electrode. Vesicles are equilibrated by incubation at 0° for 90′ in 100 mM mannitol, 100 mM KBr and 10 mM K-HEPES pH 7.4. An aliquot of the vesicles are put on a 0.7 x 6 cm Sephadex G-50 medium column equilibrated with 100 mM Na_2SO_4, 10 mM Na-HEPES pH 7.4 and eluted into a chamber housing the electrode. The Br^- electrode responds to the appearance of Br^- in the reaction mixture which is recorded on a strip chart recorder. The Sephadex column replaces extravesicular KBr with Na_2SO_4 creating outwardly directed K^+ and Br^- gradients.

RESULTS

A typical recording of Br^- efflux is presented in Figure 2. The predominant permeability of the apical membrane is to Br^-, thus its net efflux is limited by the law of electroneutrality. Specifically increasing the permeability to K^+ with the conductive ionophore valinomycin permitted the co-transport of K^+ and Br^- through parallel conductive pathways. Thus, the rate of specific conductive Br^- efflux is estimated from the difference of the rates of spontaneous efflux and total efflux in the presence of valinomycin. This fraction exhibited a specific activity of Br^- transport of 140 nmoles mg protein^{-1}min^{-1}. The initial rate of conductive transport is used as an index of the degree of purification.

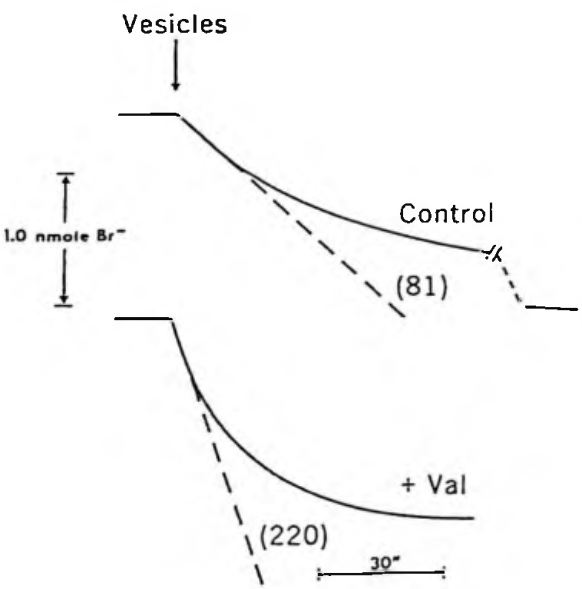

Figure 2. Br^- efflux from rectal gland membrane vesicles. To assay Br^- efflux 5 μl of the membrane suspension 1.3 mg protein ml^{-1}) was injected into the column and eluted as described in the Methods with 100 mM Na_2SO_4, 10 mM TRIS-SO_4, pH 7.4 buffer. The numbers in parenthesis are the initial rates of Br^- efflux (nmol Br^- min^{-1}mg protein^{-1}) estimated from the slope of the dashed lines. Valinomycin was added to the reaction chamber prior to the addition of the vesicles from stock ethanolic solution (1 mg/ml) to a final 1μg/ml).

The specific activities of the various marker activities are shown in Table I. Conductive Br^- flux exhibits a modest purification through the initial centrifugation steps. The major increase in the purification was observed on the sucrose gradient. The band labelled RG_{G2} had a specific activity of 296 ± 87 nmole mg protein^{-1} min^{-1} representing a 50 fold purification of the apical marker, whereas both the $(Na^+{+}K^+)$-ATPase and succinate dehydrogenase were each enriched about 0.2 fold compared to the crude homogenate. There was a 19% recovery of Br^- transport in this fraction but only 0.1 and 0.06% recovery of the ATPase and succinate dehydrogenase respectively. Thus, the preparation yields a highly enriched transporting apical membrane fraction which should provide a useful source of material for future resolution-reconstitution studies of the transport complex. (Funded by the Lucille B. Markey Foundation and the Mount Desert Island Biological Laboratory and by a Grant #GO-74 from the Cystic Fibrosis Foundation and by funds from the Bristol-Myers Company.)

TABLE I

SPECIFIC ACTIVITY OF MARKER ENZYMES

Fraction	$(Na^+{+}K^+)$-ATPase	Br^- Flux	Succinate Dehydrogenase
RG_1	59.6 ± 13.4 (n = 7)	5.9 ± 1.6 (n = 3)	4.13 ± 0.70 (n = 3)
RG_2	60.1 ± 16.0 (n = 8)	16.9 ± 2.2 (n = 3)	3.75 ± 1.23 (n = 3)
RG_3	94.7 ± 7.6 (n = 3)	10.8 ± 8.5 (n = 3)	5.2 ± 2.4 (n = 3)
RG_4	33.7 ± 19.4 (n = 3)	18.4 ± 3.4 (n = 6)	3.31 ± 0.23 (n = 3)
RG_5	79.9 ± 34.0 (n = 3)	16.0 ± 10.8 (n = 3)	6.69 ± 0.47 (n = 3)
RG_6	5.2 ± 1.9 (n = 3)	22.0 ± 22.0 (n = 3)	1.53 ± 0.92 (n = 3)
RG_7	215.0 ± 32.0 (n = 7)	n.d.	10.56 ± 1.59 (n = 3)
RG_{G2}	17.6 ± 5.7 (n = 7)	296.0 ± 87.0 (n = 6)	0.95 ± 0.89 (n = 3)
RG_{G4}	366.0 ± 37.0 (n = 8)	44.8 ± 30.0 (n = 7)	4.62 ± 0.92 (n = 3)

Specific activities of $(Na^+{+}K^+)$-ATPase, Br^- transport and succinate dehydrogenase are expressed as μmoles mg protein^{-1} hour^{-1}, nmole mg protein^{-1} min^{-1} and nmole mg protein^{-1} min^{-1}, respectively. The results are the arithmetic means ± S.E. for n preparations. Refer to Figure 1 for identities of the fractions.