

COUPLED SODIUM-CHLORIDE TRANSPORT INTO PLASMA MEMBRANE VESICLES PREPARED FROM DOGFISH RECTAL GLAND

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The rectal gland of the spiny dogfish, *Squalus acanthias*, secretes a fluid with a high concentration of sodium and chloride, higher than plasma or seawater. Experiments on isolated perfused rectal glands have shown that the secretion of chloride occurs against a steep electrochemical gradient. The magnitude of the intracellular chloride concentration (80 Eq/L) and the transmembrane potential difference (-60 mV, cell negative) suggests that the chloride transport across the basal-lateral membrane of the rectal gland is active (Silva et al. *Am. J. Physiol.* in press 1977). Based on the sodium dependence and ouabain sensitivity of the transepithelial chloride transport, Silva et al. proposed that the transfer of chloride across the basal-lateral membranes involves a sodium/chloride co-transport system.

In order to get direct information on the transport characteristics of the basal-lateral plasma membranes of the rectal gland epithelial cells, a plasma membrane fraction rich in basal-lateral membranes was prepared and the uptake of sodium by the plasma membrane vesicles was investigated. ^{22}Na uptake was measured in preference to chloride uptake because the specific activity of the ^{36}Cl commercially available was too low to distinguish accurately any specific chloride uptake from the diffusional component. The results presented below provide evidence for the presence of a furosemide-sensitive, sodium/chloride co-transport system, in the basal-lateral cell membranes. This system might facilitate up-hill transport of chloride across intact glandular cells.

Rectal glands were removed from the spiny dogfish and perfused for 30 min through the rectal gland artery with dogfish Ringer's containing dibutyryl cyclic AMP and theophylline according to the method of Stoff et al. (*J. Exptl. Zool.* 199:443-448, 1977). The perfusion Ringer's contained in mM; Na 280; K 6; Mg 3; Ca 2.5; Cl 290; H_2PO_4 1; SO_4 0.5; HCO_3 3; urea 350; glucose 5; dibutyryl cyclic AMP 0.05; theophylline 0.1. pH 7.6 when gassed with 99% O_2 -1% CO_2 . Following perfusion, the rectal glands (1-3 gms) were minced in 2 of isolation buffer (mM): sucrose 320; EDTA 1; Tris-HEPES 20; pH 7.6; dibutyryl cyclic AMP 0.05; theophylline 0.25, and then homogenized to a 10% (w/v) homogenate in a glass/teflon homogenizing tube. The homogenate was filtered through cheesecloth and centrifuged for 10 min at 1475 x g. All procedures were carried out at 0-4°C. The supernatant was diluted to 130 ml with isolation buffer and centrifuged for 90 at 16,300 x g. The resulting pellet was composed of a soft pinkish-white layer over a tannish firmer pellet. The soft whitish layer was called the crude plasma membrane layer and was resuspended in isolation buffer by vigorous swirling, made to 12 ml, homogenized 3 times and then centrifuged 20 min at 17,000 x g. The fluffy white upper layer was then resuspended in transport buffer (mM): Mannitol 100; $\text{Mg}(\text{NO}_3)_2$ 1.2; Tris-HEPES 20; pH 7.6, homogenized 10 times and centrifuged at 35,000 x g for 20 min. Following this centrifugation, the membranes were resuspended in transport buffer to a final protein concentration of 8-10 mg/ml.

The uptake of ^{22}Na into the plasma membrane vesicles was measured by the filtration technique of Kim et al. (*J. Membrane Biol.* 21:375-395, 1975). The reaction media and membrane manipulations are described in the figure legends. After freeze-drying, Na^+-K^+ -ATPase was measured according to the method of Silva et al. (*Am. J. Physiol.* in press, 1977). Protein was determined by the method of Lowry et al. (*J. Biol. Chem.* 193:265-275, 1951).

The rectal gland, which exhibits an extremely high Na^+-K^+ -ATPase activity, is characterized by a small amount of luminal and a large amount of basal-lateral plasma membranes. (^3H)-ouabain autoradiographs have shown that the primary loci of the Na^+-K^+ -ATPase are the basal-lateral membranes (Karnaky et al. *Bull. MDIBL* 16, 1976). This enzyme was therefore used as a marker enzyme during the membrane preparation. (P)

rectal gland homogenate had a Na^+-K^+ -ATPase activity of 35.5 ± 5.4 s.e. $\mu\text{moles P}_i$ released per mg of protein per h and the plasma membrane preparation had an activity of 157.5 ± 25.9 s.e. $\mu\text{moles/mg protein per h}$. The 4.5-fold increase in enzyme activity indicates that basal-lateral plasma membranes are enriched in this fraction. The plasma membrane fraction formed vesicles as indicated by a decrease of mannitol and ^{22}Na uptake when the osmolality of the incubation medium was increased by addition of sucrose. The contribution of binding to the ^{22}Na uptake by the membranes was estimated by extrapolating uptake at infinite osmolality and was about 10% of the uptake observed at equilibrium.

As shown in Figure 1 the uptake of sodium into the vesicles was greatest in the presence of a potassium-chloride gradient directed into the vesicle. The uptake was slower when chloride was replaced by nitrate and even slower if gluconate was used instead of chloride. Osmotic effects of the different salt gradients can be excluded since the mannitol uptake under all conditions remained the same (data not shown).

The coupling observed between the movement of sodium and anions could be either direct at a molecular level via a co-transport system or indirect caused by electrical forces owing to differing permeability of the membrane to different anions; for example, chloride might diffuse more readily across the membrane as a counterion for sodium, than nitrate or gluconate. In addition, in the presence of salt gradients across the membrane of the vesicles diffusion potentials of different magnitude and direction might exist, which could also influence the movement of sodium. In order to minimize such diffusion potentials, the membranes were pre-equilibrated with KCl and KNO_3 in order to reduce further the likelihood of electrical charge across the vesicle membrane, valinomycin was incorporated into the membranes to allow charge compensating movements of potassium. Under these conditions the indirect coupling of sodium movement with the movement of anions owing to electrical forces is very unlikely. Nevertheless, Figure 2 shows that the uptake of sodium into the vesicles is greatest in the presence of chloride and strikingly reduced if chloride is replaced by nitrate. The uptake of mannitol under identical conditions was unchanged. These results strongly suggest that a sodium/chloride co-transport system is present in the plasma membranes which preferentially accepts chloride over nitrate. This correlates with data obtained from intact perfused glands where chloride is secreted actively but nitrate is not.

Additional evidence for the presence of a sodium/chloride co-transport system in the plasma membranes can be derived from studies with furosemide, which inhibits active chloride secretion by the intact perfused rectal gland (Silva et al. *Am. J. Physiol.* in press, 1977). Furosemide decreased sodium uptake by the vesicles in the presence of a chloride gradient in a dose-dependent manner. When gluconate was substituted for chloride furosemide did not inhibit sodium transport (Figure 1). The effect of furosemide on sodium uptake was also apparent when the membrane had been equilibrated with chloride and valinomycin but not in the absence of chloride (Figure 2). It should be noted that these strong inhibitory effects of furosemide on sodium movement were only observed if the membranes had been preincubated with the drug for 1 min and that mannitol uptake by the vesicles was not affected by the drug. An additional indication of carrier-mediated transport of sodium across the membranes is that in the presence of chloride, saturation of sodium uptake by increasing sodium concentrations was observed (data not shown).

These results provide direct evidence for a molecular coupling of sodium and chloride transport across plasma membranes of the rectal gland via a carrier system that is sensitive to furosemide. If this co-transport system were present predominantly in the basal-lateral plasma membranes the transcellular movement of chloride might occur in the following way. Chloride would be accumulated in the cell, entering across the contraluminal border in association with sodium, the co-transport of both ions driven by the chemical potential or the electrochemical potential that favors the entry of sodium into the cell. Movement of chloride into the lumen across the apical membrane would follow electrical driving forces for the anion by a sodium-independent mechanism. Active chloride secretion by the intact rectal gland thus could be defined as a secondary active transport process driven primarily by the Na^+-K^+ -ATPase pump which maintains the electrochemical potential difference for sodium across cell membranes.

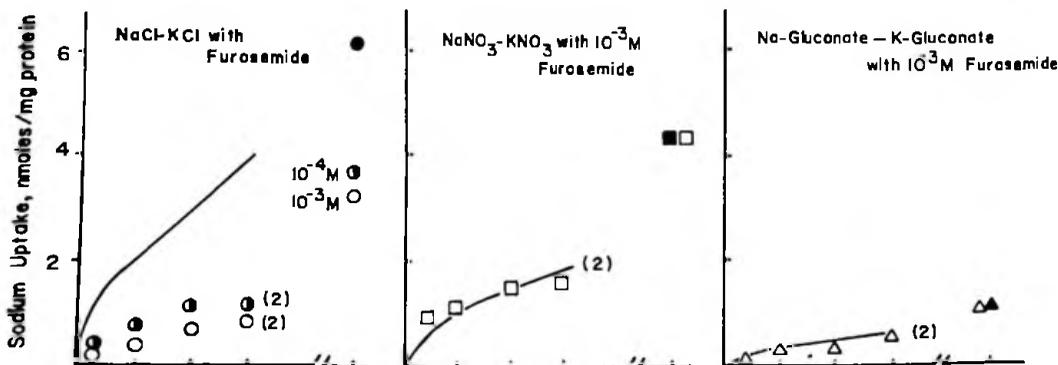
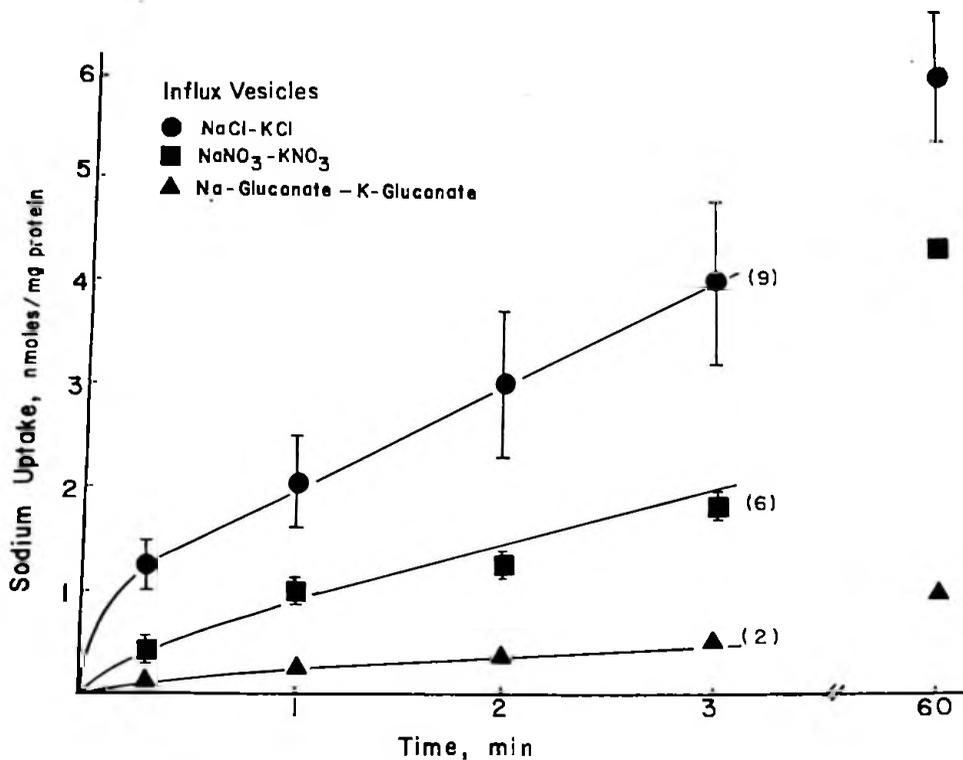


Figure 1. Sodium uptake into plasma membrane vesicles prepared from dogfish rectal gland. The vesicles were suspended in 100 mM mannitol, 1.2 mM Mg(NO₃)₂, 50 mM Tris-HEPES, pH 7.6. The incubation media contained 100 mM (¹⁴C)-mannitol, 1.2 mM Mg(NO₃)₂, 50 mM Tris-HEPES, pH 7.6 and either 2 mM ²²NaCl-98 mM KCl (circles) or 2 mM ²²NaNO₃-98 mM KNO₃ (squares) or 2 mM ²²Na-gluconate-98 mM K-gluconate (triangles). To test the effect of furosemide, the vesicles were pretreated with 10⁻³ or 10⁻⁴ M furosemide for 1 min and then uptake was studied in the presence of 10⁻³ or 10⁻⁴ M furosemide. The control uptake is shown by the solid curves and the uptake in the presence of furosemide by experimental points. The reaction was terminated at timed intervals by pipetting 20 ul of the reaction mixture into 1 ml of 240 mM mannitol, 170 mM KNO₃, 1.2 mM Mg(NO₃)₂, 20 mM Tris-HEPES, pH 7.6, 10⁻³ M furosemide and the filters were washed with the same solution. Values are means ± s.e. bars (number of experiments in parentheses).

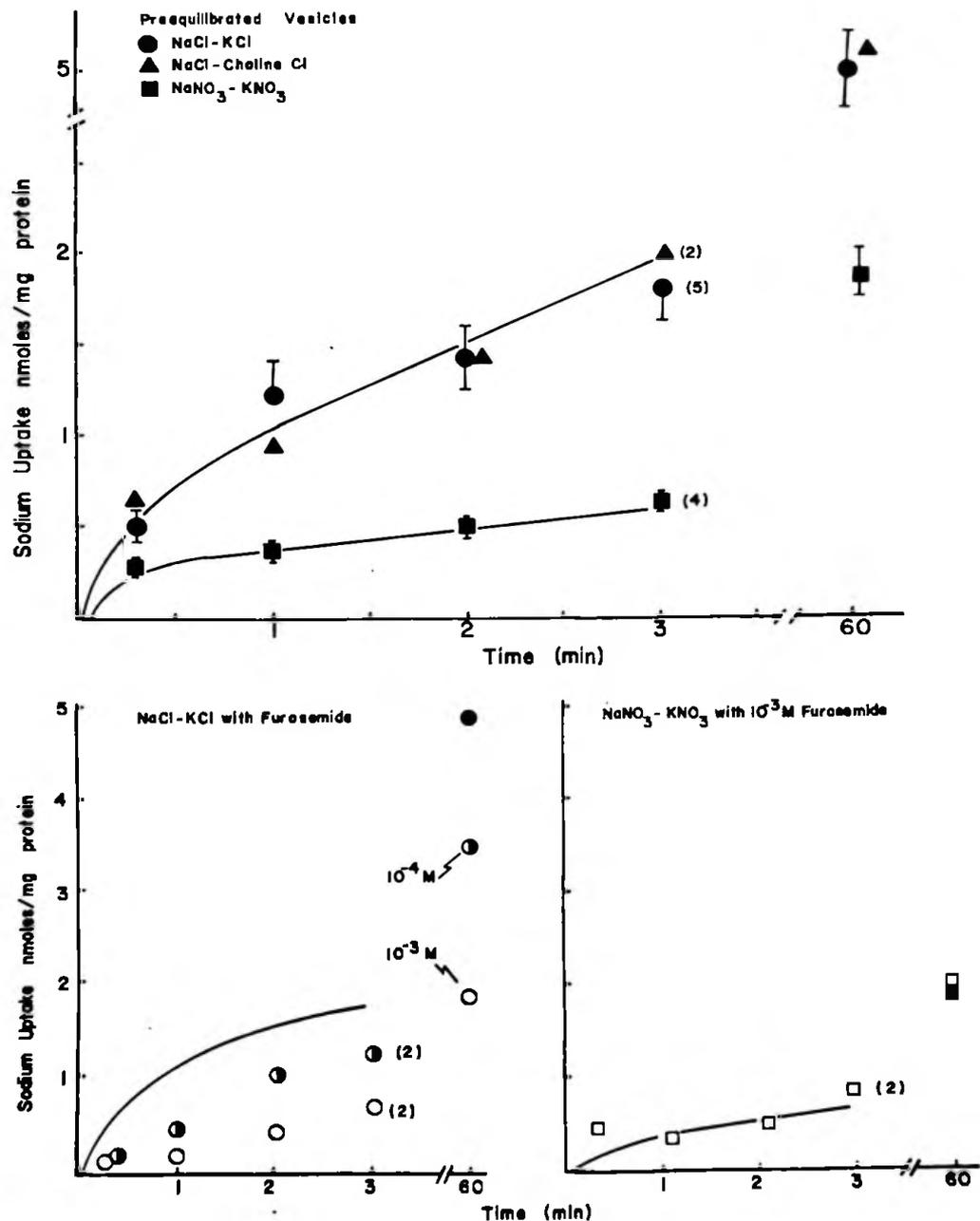


Figure 2. Sodium uptake into plasma membrane vesicles from the dogfish rectal gland preequilibrated with 100 mM mannitol, 1.2 mM Mg(NO₃)₂, 50 mM Tris-HEPES, pH 7.6 and either 2 mM NaCl-98 mM KCl (circles), or 2 mM NaCl-98 mM Choline Cl (triangles), or 2 mM NaNO₃-98 mM KNO₃ (squares) plus valinomycin (final concentration was around 1 μg/100 mg protein). The incubation was done in the same media as the pre-equilibration but containing ²²Na and (¹⁴C)-mannitol. The effect of furosemide was examined in vesicles preincubated 1 min with 10⁻³ or 10⁻⁴ M furosemide and then uptake was studied in incubation media containing 10⁻³ or 10⁻⁴ M furosemide. Values are means ± s.e. bars (number of experiments in parentheses).

Supported by NIH Grants AM 15973 and AM 18078, NSF Grant PCM 77-01146, and the Max Planck Institut für Biophysik, Frankfurt, West Germany.

DEMONSTRATION OF ACTIVE CHLORIDE TRANSPORT BY THE ISOLATED RECTAL GLAND OF THE DOGFISH, *Squalus acanthias*, BY THE USSING-ZHERAN TECHNIQUE

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The rectal gland of the spiny dogfish secretes sodium chloride through its main duct. It has been demonstrated that the rate of sodium chloride secretion is greatly stimulated by theophylline and cyclic AMP in intact glands in vitro perfused with balanced salt solution through the vascular system and with collection of fluid through the excretory duct. (see Silva et al. 1975 and 1976, this Bulletin). In a recent report (Zadunaisky and Silva 1976, this Bulletin 16, 109) we have shown that the stimulation by theophylline and cyclic AMP can be demonstrated in glands that had been opened along the length of the central duct and placed as a thick membrane between two well stirred hemichambers utilizing the Ussing-Zheran method for epithelia (1951). Under these conditions a very small potential difference is maintained by the gland duct that is stimulated to levels of 8-12 mV after addition of both theophylline (10^{-4} M) and Db cyclic AMP (10^{-5} M) to the shark Ringer solution bathing both sides of the gland. The short-circuit current after full stimulation is of the order of 16 μ Amps/cm² and its development is extremely sensitive to the presence of glucose in the bathing solutions. Furosemide proved to inhibit dramatically the short-circuit current generated by the gland.

At this time we report results of the determination of chloride fluxes with ³⁶Cl across 27 glands that were maintained under short-circuit condition during maximal stimulation with Db cyclic AMP and theophylline. Rectal glands were dissected out as previously described (Zadunaisky and Silva 1976, this Bulletin), their ends sectioned and then opened along the main secretory duct. The resulting layer was placed as a flat sheet between lucite hemichambers used before for studies of corneal transport (Zadunaisky 1966, Amer. J. Physiol. 211:506) and bathed with shark Ringers solution of the following composition: (mmoles/liter) Na 280; K 6; Mg 3; Ca 2.5; Cl 290; Phosphate 1; Sulfate 0.5; Bicarbonate 8; Urea 350; Glucose 10. After full development of a stable value for short-circuit current, ³⁶Cl Ringer was added to the mucosal (duct) side or to the serosal (capsular) side at a final concentration of 4 μ Ci/ml. After 90 minutes, sampling was started from the cold and hot side with constriction pipettes of 250 and 50 μ l respectively, at 30-minute intervals. Samples were dissolved in liquid scintillation fluid (TT-21) Yorktown Research Co. Hackensack and counted in an automatic Nuclear Chicago Liquid Scintillation Counter. Calculations for unidirectional fluxes were made as described (J. Zadunaisky 1966, Amer. J. Physiol. 211:506) on the basis of specific activity of the radioisotope determined from counting of samples and determination of chloride concentration by amperometric titration of samples of cold shark Ringers. The electrical equipment consisted of an automatic short-circuit current unit modified for low current detection. The flux from capsular to secretory side and the opposite flux were detected simultaneously in two halves of the same rectal gland.

In some instances current-voltage curves were determined in a few glands. Detection of the stimulation by Db cyclic AMP (10^{-5} M) and theophylline (10^{-4} M) and the inhibitory effect of furosemide (10^{-3} M) were tested in decapsulated glands. Decapsulation was performed under observation with a dissecting microscope and then the tissue mounted as described for the intact glands.

The results of 27 outfluxes and 25 influxes in short-circuited stimulated glands is shown in Table II. It can be observed that the flux from serosal to mucosal (capsule \rightarrow duct or outflux) was approximately 4 times greater than the passive flux of chloride in the opposite direction. The resulting net flux of 0.579μ Eq/h/cm² is statistically similar to the short-circuit current circulating across the preparation,