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INDUCTION OF THE CATALYTIC PROTEIN OF $(Na^+ + K^+)$-ATPase IN THE SALT GLAND OF THE DUCK

D. J. STEWART, E. W. SEMPLE, G. T. SWART and A. K. SEN

Department of Pharmacology, Faculty of Medicine, University of Toronto, Toronto, M5S 1A8 (Canada)

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SUMMARY

The $(Na^+ + K^+)$-ATPase activities in salt gland homogenates increased 3- to 4-fold after saline treatment of ducks for 3 weeks. The ATPase was purified to a specific activity of 460 and 1015 μmol P_i/mg protein per h, respectively, in control and saline-treated ducks. The catalytic protein was identified on polyacrylamide electrophoresis gels by phosphorylating the enzyme with [^{32}P]ATP. The molecular weight of the protein was estimated to be 98 000. The amount of catalytic unit increased commensurately with the enzyme activity after saline treatment. It is therefore concluded that the increased enzyme activity is due to a de novo enzyme synthesis and is not an activation effect.

Phospholipid concentration in the salt gland tissue increased 1.7-fold after the saline treatment. Significant increases occurred in the percentage of the total phospholipids as phosphatidylserine and sphingomyelin. In the partially purified $(Na^+ + K^+)$-ATPase preparation, the percentage composition of phosphatidylserine and phosphatidylethanolamine increased after saline treatment.

INTRODUCTION

$(Na^+ + K^+)$-ATPase (ATP phosphohydrolase, EC 3.6.1.3) is an integral part of the $(Na^+ + K^+)$ pump of the cell membrane [1]. The activity of the enzyme has been observed in a number of tissues to vary with the cellular rates of transport for sodium and potassium [2–7]. The correlation indicates that an important factor in determining the sodium pump capacity is the activity of the $(Na^+ + K^+)$-ATPase in the cell membrane. Activity changes in the enzyme could occur either by changes in enzyme kinetic properties, changes in the proportions of latent enzyme molecules present or changes in the total number of enzyme molecules. A 3- to 4-fold increase in the specific activity of the $(Na^+ + K^+)$-ATPase can be produced in the salt gland of the domestic duck by saline treatment [2]. The kinetic properties before and after the saline treatment appear to be similar [2]. The observed activity change could therefore result from either a reduction in the proportion of latent enzyme molecules or an increase in the total number of enzyme molecules. One way to distinguish between these alternatives is to measure the actual quantity of enzyme protein in the

tissue. As shown in this paper, the enzyme in this tissue can be purified sufficiently in two centrifugation steps to allow an estimate of $(Na^+ + K^+)$-ATPase protein by polyacrylamide disc gel electrophoresis. Also, as a part of the present investigation, the phospholipid composition of the partially purified enzyme complex was examined. A number of studies have indicated that phospholipids can reactivate partially delipidated enzyme preparations [8–16]. We were therefore interested in the possibility that an altered phospholipid composition in the salt gland might play a role in the observed enzyme activity changes. Preliminary reports of this study have been presented previously [17, 18].

MATERIALS AND METHODS

Male Pekin ducks of age 7 weeks were obtained from a local source and maintained indoors on an ad libitum supply of Purina duck chow. The saline-treated animals were given 155 mM NaCl drinking water for 3 weeks and the control animals were given tap water. At the end of the treatment period, the maximum transport rate for sodium by the salt glands was determined by the following procedure. Salt gland fluid was collected as described previously [19]. Two stomach loads (50 ml of 0.5 M NaCl) were given 20 min apart. The rate of secretion was monitored until a maximum rate was reached. Salt gland fluid samples were taken for analysis of Na^+ by flame photometry. The animals were then killed by decapitation. The salt glands were removed, weighed and cut into small cubes with a razor blade. The tissue was homogenized in 9 vols (w/v) of distilled water (0–4 °C) in a conical ground glass homogenizer at 200 rev./min. The homogenate was divided into aliquots equivalent to 200 mg tissue and lyophilized for storage at −25 °C under gaseous nitrogen. Under these conditions the enzyme retains full activity for at least 1 year. Lyophilization of the tissue was necessary to permit a high recovery of $(Na^+ + K^+)$-ATPase in the initial centrifugation (see below). With fresh tissue, almost no $(Na^+ + K^+)$-ATPase is recovered.

Preparation of low speed supernatant fraction

Each 200 mg of lyophilized tissue was homogenized in 4 ml of a buffer containing 20 mM Tris · HCl, pH 7.8, and 1 mM disodium EDTA in a Teflon-glass homogenizer. The suspension was centrifuged at $5100 \times g$ in the SS 34 rotor of the Serval RC-2 centrifuge for 10 min at 4 °C. The pellet was rehomogenized in the same volume of buffer and centrifuged as before. The pellet was discarded and the two supernatants were pooled.

Isolation of membrane fractions

The low speed supernatant was then layered onto either linear or discontinuous sucrose density gradients for further purification.

(*a*) *Linear sucrose gradients.* Either 1/19 vol. of distilled water or 1/19 vol. of 2.0 % sodium deoxycholate (final concn 0.1 %) was added to the low speed supernatant fraction. The mixture was allowed to stand for 60 min at room temperature (23 °C) and then 2.0 ml aliquots were layered over six 11.5 ml linear sucrose gradients (15–50 %, w/v) containing 0.2 mM EDTA and 10 mM Tris · HCl, pH 7.8. The gradient tubes were then centrifuged at $280\,000 \times g$ for 4 h at 4 °C (Beckman SW 40

rotor, L2-65B Utracentrifuge). The gradients were divided into 20 fractions using a device which permitted collection from 6 gradients simultaneously. The fractions were stored at −25 °C. The enzyme activity remains stable for at least 1 year under these conditions.

(*b*) *Discontinuous gradients.* The low speed supernatant was treated as above with either water or deoxycholate, then 8.0 ml aliquots were layered over discontinuous gradients consisting of 1 ml each of 50 %, 40 %, 20 %, 15 % and 10 % sucrose. The sucrose solutions contained 0.2 mM EDTA and 5 mM imidazole · HCl, pH 7.0. The gradient tubes were centrifuged at 280 000 × *g* for 1 h at 4 °C (SB 283 rotor, International B 60 Ultracentrifuge). The gradients were fractionated at half-way between each sucrose interface. The fractions were stored frozen as above.

Phospholipid analysis

(*a*) *Extraction.* Total lipids were extracted from lyophilized tissue by a modification [20] of the procedure of Folch et al. [21]. Lipids were extracted from sucrose gradient fractions using the solvent ratios of Bligh and Dyer [22].

(*b*) *Thin layer chromatography.* Individual phospholipids were isolated on 20 × 20 cm plates of silica gel H, 0.5 mm thick, by a modification of the procedure of Rouser et al. [23]. The plates were activated at 120 °C for 30 min before use. First dimension solvent was chloroform/methanol/28 % aqueous ammonia (65 : 25 : 7.5 by vol.). The second dimension solvent was chloroform/acetone/methanol/glacial acetic acid/water (8 : 4 : 2 : 2 : 1 by vol.). Both solvents contained 0.0005 % butylated hydroxytoluene as an antioxidant. The various phospholipid spots were identified by comparison with standard phospholipids and by chromatographic stains (molybdenum blue, phospholipids; ninhydrin, amino groups). The individual phospholipids were quantitated by locating the spots with iodine spray (1 % iodine in methanol), circling the spots with a needle, allowing most of the iodine to evaporate, and then quantitatively transferring the gel to 61 % perchloric acid for digestion at 180 °C for 3 h. The digest was diluted with 7 vols of water and the gel removed by centrifugation. Phosphate in the supernatant was determined by the automated method described previously [24]. To estimate the total amount of lipid phosphate spotted onto the thin-layer chromatography plate, an aliquot of the chloroform extract was spotted onto a region free of lipid and then carried through the same procedure as for the lipid spots.

Analytical disc gel electrophoresis

The separating gels were 5 mm × 70 mm and contained 7.5 % acrylamide, 0.2 % *N,N'*-methylene bisacrylamide, 0.032 % tetramethylenediamine, 0.05 % ammonium persulfate, 0.375 M Tris · HCl, pH 8.8, and 0.1 % sodium dodecyl sulfate. The stacking gels were 0.2 ml and contained 3.0 % acrylamide, 0.08 % *N,N'*-methylene bisacrylamide, 0.098 % tetramethylenediamine, 0.13 M Tris · HCl, pH 7.0, 0.05 % ammonium persulfate and 0.1 % sodium dodecyl sulfate.

The gel tubes were coated with 2 % Photoflood (Eastman Kodak). The gels were overlaid with water during polymerization. The sample was solubilized at room temperature in a mixture with final concentrations of 5 % mercaptoethanol, 2 % sodium dodecyl sulfate, 10 % sucrose and 0.002 % bromophenol blue.

Between 10 and 140 µl of the above mixture containing 5, 10 or 15 µg of pro-

tein was applied to the gels. The electrode buffer was 0.025 M Tris, 0.19 M glycine and 0.1 % sodium dodecyl sulfate (pH 8.3). The current was adjusted to 1 mA per gel for the first 1½ h, and 2 mA per gel thereafter until the tracking dye was approximately 5 mm from the bottom of the gel. The gels were fixed overnight in a mixture of methanol/glacial acetic acid/water (5 : 1 : 4, by vol.) and then stained for 1 h in 0.25 % Coomassie blue dissolved in the same solvent. The gels were partially destained for 30 min (in 20 ml of the same solvent per gel and then transferred to 7.5 % acetic acid for 30 min) before electrophoretic destaining.

To estimate the relative proportions of the various polypeptides on the stained gels, the gels were scanned at 555 or 610 nm in the linear transport attachment for the Gilford 240 spectrophotometer. The phosphorylating unit of the $(Na^+ + K^+)$-ATPase was located on the gels by ^{32}P labelling by the method of Hokin et al. [25].

Assays

Activities of $(Na^+ + K^+)$-ATPase and ouabain-insensitive ATPase were measured by the fully automated method described previously [24]. The incubation mixture contained 120 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 3 mM disodium ATP and 66 mM Tris · HCl, pH 7.4. Ouabain-insensitive ATPase was measured in the above medium with the addition of 0.5 mM ouabain. The $(Na^+ + K^+)$-ATPase was measured as ouabain-sensitive ATPase. The incubation temperature was 37 °C and the incubation time was 4 min. Protein was estimated by the procedure of Lowry et al. [26]. Correction was made for interference by sucrose, buffers or EDTA, when appropriate, by adding these substances to the albumin standards at the same concentrations as were present in the samples.

RESULTS

Table I shows the effects of saline treatment for 3 weeks on $(Na^+ + K^+)$-ATPase, sodium transport and phospholipid content of salt glands of domestic ducks. The observed changes are similar to those reported previously [2, 27]. To examine the effects of saline adaptation in more detail, partial purification of the

TABLE I

EFFECT OF SALINE TREATMENT ON SALT GLANDS OF DOMESTIC DUCK

All weights are for wet tissue. Values are mean ±S.E. The number of animals is shown in the parentheses.

	Control	Saline-treated
$(Na^+ + K^+)$-ATPase (μmol P_i/g per h)	1004 ±104 (5)	3427 ±326 (6)
Protein (mg/g)	128 ± 10 (4)	143 ± 11 (5)
Phospholipid (μmol P_i/g)	12.2± 1.3 (7)	20.4± 1.4(10)
Salt gland weight (mg/pair)	366 ± 17 (15)	1101 ± 40 (18)
Maximum Na^+ secretion (mmol/g per h)	8.3± 1.0(14)	25.4± 0.8(18)

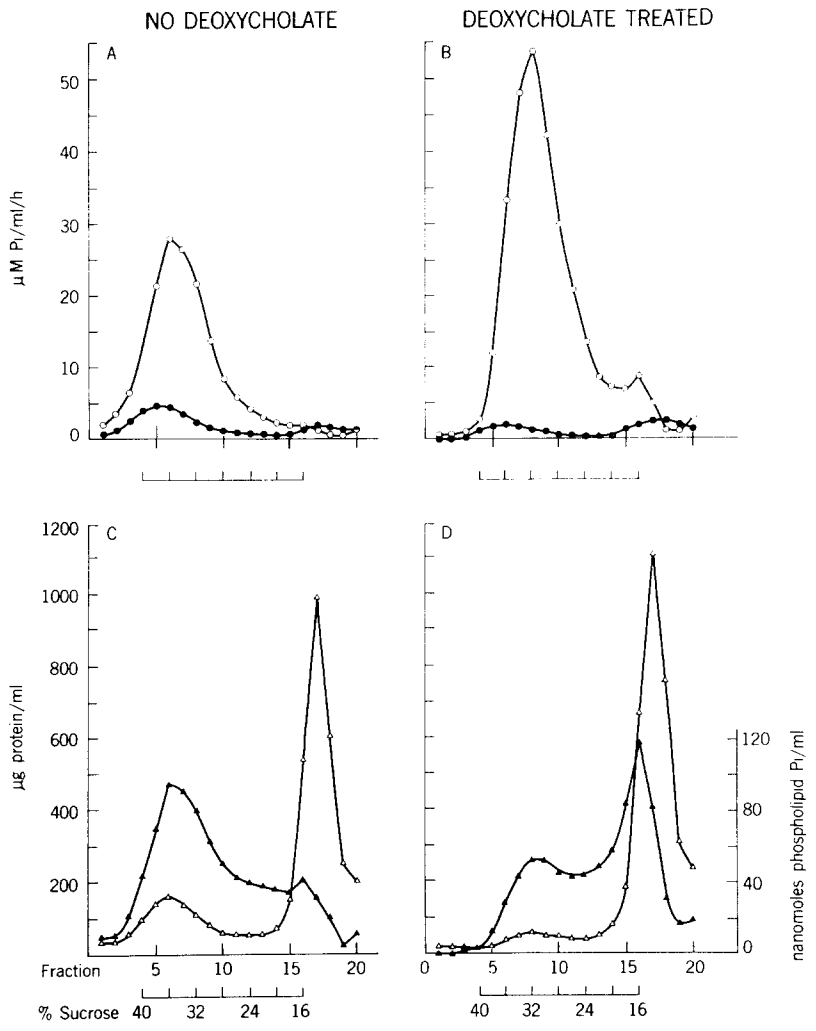

Fig. 1. Distribution of enzyme activity, protein and phospholipid on sucrose gradients. Two aliquots of the low speed supernatant fraction from salt gland of saline-adapted ducks were treated with either 1/19 vol. of water or 1/19 vol. of 2.0 % deoxycholate before centrifugation on linear 15–50 % sucrose density gradients. (●), Mg^{2+}-ATPase; (○), $(Na^+ + K^+)$-ATPase; (▲), phospholipid; (△), protein. For further details, see Materials and Methods.

membranes carrying the $(Na^+ + K^+)$-ATPase was undertaken. A membrane fraction carrying $(Na^+ + K^+)$-ATPase was isolated on linear sucrose density gradients (Figs 1 and 2). The membranes were brought to their equilibrium densities by centrifugation at 280 000 $\times g$ for 4 h. Centrifugation of the gradients for an additional 12 h does not alter the position of the enzyme peaks, although the soluble proteins do move further into the gradients. In Fig. 1, the effect of deoxycholate on ATPase, protein, and phospholipid distribution is shown.

In preparations not treated with deoxycholate, the $(Na^+ + K^+)$-ATPase sediments with a peak of activity at 36 % sucrose. A phospholipid and protein peak

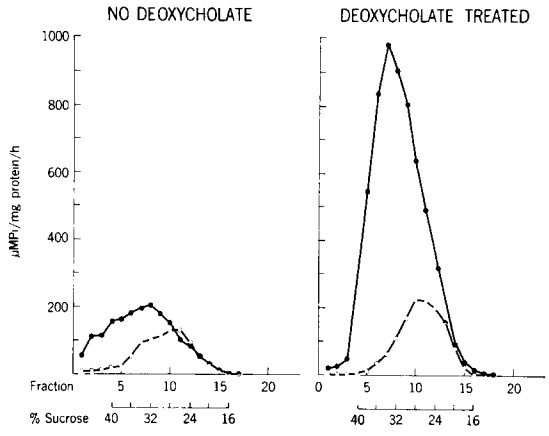

Fig. 2. Comparison of distribution on linear sucrose gradients of $(Na^+ + K^+)$-ATPase from control (○) and saline-adapted (●) ducks. For details, see Fig. 1.

also occur at the same position. Most of the Mg^{2+}-ATPase sediments with a peak of activity at 38 % sucrose and a smaller portion sediments at the top of the gradient. Most of the protein (soluble) sediments against the 15 % sucrose interface. A peak of phospholipid material moves into the gradient ahead of the soluble protein (fraction 17 vs. 16).

Treatment of the low speed supernatant fraction with 0.1 % deoxycholate produced a maximal activation of the $(Na^+ + K^+)$-ATPase (results not shown). The $(Na^+ + K^+)$-ATPase was activated nearly two-fold. The deoxycholate treatment shifted the peak of $(Na^+ + K^+)$-ATPase, protein and phospholipid from 36 to 32 % sucrose. Most of the protein and about half of the phospholipid was removed by the detergent. The protein released by the detergent appeared at the top of the gradient with the soluble proteins. The phospholipid released by the detergent appeared in fraction 16 ahead of the soluble proteins, indicating that the solubilized protein and phospholipid were no longer associated. The separation of protein and lipid components after solubilization with deoxycholate has been observed previously by Phillipott [28] with red cell membranes. The use of deoxycholate produced a significant purification of the $(Na^+ + K^+)$-ATPase, about 60 % of the protein in the enzyme peak was removed. Due to both purification and activation, the specific activity in the enzyme peak increased from 220 to 987 μmol P_i/mg protein per h. The deoxycholate treatment shifted the Mg^{2+}-ATPase peak from 38 % sucrose to 36 % sucrose. Most of the Mg^{2+}-ATPase in the peak was removed by the deoxycholate treatment. Part of the activity removed appeared at the top of the gradient, indicating solubilization. The total Mg^{2+}-ATPase recovered following deoxycholate treatment was less than the original indicating that such treatment inactivates the enzyme. The different distributions of Mg^{2+}-ATPase and $(Na^+ + K^+)$-ATPase activities indicate that these two enzyme activities are located on different membrane fragments.

Deoxycholate had the same effect on the distribution of $(Na^+ + K^+)$-ATPase, phospholipid and protein in the preparations from control salt glands (results not shown). Two significant differences do emerge in comparing membrane fractions from salt glands of control and saline maintained ducks. One difference is that the

specific activity of the $(Na^+ + K^+)$-ATPase is higher in the membrane fraction from the saline treated preparations. The other difference is that the density of the membranes carrying the $(Na^+ + K^+)$-ATPase is higher in the saline adapted salt glands. These differences are shown in Fig. 2. The membrane density would depend primarily upon the ratio of protein to phospholipid, the protein being more dense than the phospholipid. The density changes are confirmed by measurements of protein and phospholipid in the enzyme peaks. In membrane preparations not treated with deoxycholate, the ratio of protein to phospholipid in the $(Na^+ + K^+)$-ATPase peak increased from 1.17 to 1.55 μg protein/mmol phospholipid P_i after saline treatment. In preparations treated with deoxycholate, the ratio of protein to phospholipid increased from 1.05 to 1.24 μg protein/mmol phospholipid P_i after saline treatment.

Polyacrylamide gel electrophoresis

The protein composition of the membrane fractions was compared by electrophoresis. Fig. 3 shows the analytical gels of the 4 membrane preparations. The gels show almost identical banding patterns in membranes from control and saline treated birds. The catalytic protein of the $(Na^+ + K^+)$-ATPase was identified on the gels by ^{32}P labelling with [^{32}P]ATP. The protein has a molecular weight of 98 000. The catalytic protein was the major protein on all 4 gels, as determined by photometric scanning. The estimation of the amount of catalytic subunit by scanning of the polyacrylamide gels was quantitative over the range of 5, 10 and 15 μg protein applied to the gels. The estimates of the area occupied by the subunit agreed within $\pm 2\%$ at the various protein concentrations. The figures given are the average of these estimates. In membranes from the control birds, the protein accounts for 16.9

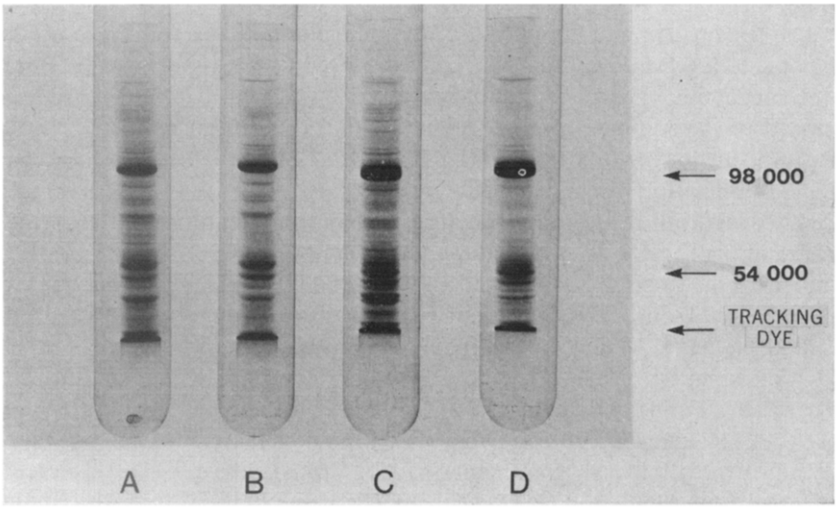

Fig. 3. Electrophoresis pattern of proteins from $(Na^+ + K^+)$-ATPase fraction of salt gland tissue. (A) Control salt gland, no deoxycholate treatment. (B) Control salt gland, deoxycholate treated. (C) Saline-adapted salt gland, no deoxycholate treatment. (D) Saline-adapted salt gland, deoxycholate treated. The protein standards used for calibration of the gels were: β-galactosidase subunit, 130 000; phosphorylase *a*, 92 500; bovine serum albumin, 68 000; pyruvate kinase, 57 000 and chymotrypsin, 25 000. 15 μg of protein were applied to each gel.

and 23.1 % of the protein on the gels of non-deoxycholate- and deoxycholate-treated membranes respectively. In the membranes from the saline-treated birds, the protein accounted for 26.6 and 43.4 % of the total, respectively. The second major protein runs as a diffuse band, has a molecular weight of about 54 000 and can be seen most clearly in the membrane preparation with the highest $(Na^+ + K^+)$-ATPase activity (Fig. 3D). Two minor proteins, running as sharper bands, are at almost the same position on the gel. The diffuse protein is probably the glycoprotein seen in highly purified enzyme preparations from dog kidney [29], rabbit kidney [30] and shark rectal gland [25]. The protein appears to increase proportionately with the catalytic protein after the saline treatment (Fig. 3B vs 3D). However, it was not possible to obtain an accurate estimate of the relative quantity of the protein because of the two other proteins running close to the same position on the gel.

Estimation of salt gland catalytic protein content

To estimate the content of $(Na^+ + K^+)$-ATPase catalytic protein in salt gland tissue before and after saline treatment, the yields of enzyme activity during purification were calculated as shown in Tables II and III. The recovery of enzyme activity on the first centrifugation step was lower in the control than the saline preparation (43.6 % compared to 70 %). Centrifugation of the low speed supernatant fraction on the discontinuous sucrose density gradients resulted in enzyme activation even in the preparations not treated with deoxycholate. The enzyme activity recovered from the sucrose density gradients was always greater than the total amount of activity applied. Recovery at the second step was calculated as a percentage recovery of the total activity present in the density gradient rather than as a percentage of the total activity applied. For example, in the control glands with no deoxycholate treatment, 412 units of activity were applied to the gradients. 551 units were recovered from all of the fractions, of which 369 were in fraction C. Thus, the calculation for percent recovery was $369 \div 551 \times 43.1 = 29.2\,\%$. The estimated recovery of enzyme was higher from the salt glands of the saline maintained birds than the glands of the control group. From these estimates of enzyme recovery and the estimates of catalytic

TABLE II

PARTIAL PURIFICATION OF $(Na^+ + K^+)$-ATPase FROM SALT GLAND OF CONTROL DUCKS

Values given are for 1 g of tissue (wet weight before lyophilization).

Fraction	$(Na^+ + K^+)$-ATPase (μmol P_i/h)	Protein (mg)	Specific activity (μmol P_i/mg protein per h)	Recovery (%)
Homogenate	944	120	7.86	100
Low speed supernatant	412	27.2	15.2	43.6
Gradient fraction C				
No deoxycholate treatment	369	1.95	189	29.2*
Deoxycholate-treated	544	1.16	472	30.8*

* Yields are calculated as 0.436 × percent of total activity in gradient in the fraction. Fraction C is the 20–40 % sucrose interface of the discontinuous gradients described in the Materials and Methods. Pooled tissue from 6 ducks.

TABLE III

PARTIAL PURIFICATION OF $(Na^+ + K^+)$-ATPase FROM SALT GLAND OF SALINE TREATED DUCKS

Fraction	$(Na^+ + K^+)$-ATPase (μmol P_i/h)	Protein (mg)	Specific activity (μmol/mg protein per h)	Yield (%)
Homogenate	3616	116	31.2	100
Low speed supernatant	2531	48.2	57.6	70
Gradient fraction B + C (no deoxycholate treatment)	2488	11.5	216	64.7*
Gradient fraction C (deoxycholate-treated)	4531	4.46	1015	50.3*

* Yields are calculated as 0.7 × % of total activity in gradient in the fraction. Fraction B is the 40–50 % sucrose interface of the discontinuous gradient. Other conditions are the same as in Table II.

protein content in the membrane preparations obtained by photometric scanning of the sodium dodecyl sulfate-polyacrylamide gels, the tissue content of catalytic protein was calculated and is shown in Table IV. The estimated gland content of the catalytic protein was 1.13 mg/g in the control and 4.72 mg/g in the saline-treated group on the basis of membrane preparations not treated with deoxycholate. The estimates with the deoxycholate-treated membrane fractions were lower at 0.87 and 3.85 mg/g, respectively. We think the lower estimate is the result of some loss of enzyme protein from the fraction containing the enzyme peak as a result of the detergent treatment. The deoxycholate, besides activating the bulk of the enzyme, also solubilizes and inactivates part of the enzyme (unpublished observations). Despite this source of error, the estimates of the increase in glandular content of catalytic protein agree closely (4.18- and 4.42-fold).

TABLE IV

ESTIMATION OF GLAND $(Na^+ + K^+)$-ATPase CATALYTIC PROTEIN CONTENT

	Catalytic protein on gel* (% total protein)	Catalytic protein in gradient fraction** (mg)	Catalytic protein in whole gland*** (mg/g)	Saline-adapted / control
No deoxycholate treatment				
Control	16.9	0.330	1.13	4.18
Saline-adapted	26.6	3.06	4.72	
Deoxycholate-treated				
Control	23.1	0.268	0.87	4.42
Saline-adapted	43.4	1.93	3.85	

* Estimated by photometric scanning of polyacrylamide gels.
** Percent catalytic protein/100 × total protein in fraction.
*** Estimated catalytic protein in fraction × 100/% recovery.

Phospholipid class distribution

The concentration of phospholipids in the salt glands increased about 1.7-fold after the saline treatment (Table I). The percentage composition of individual phospholipids in the whole glands is shown in Table V. Significant increases in the relative

TABLE V

PHOSPHOLIPID COMPOSITION OF DUCK SALT GLAND

Values are expressed as means ±S.E.

Phospholipid	Control (% of total)	Saline-treated (% of total)
Phosphatidyl-serine	4.50±0.49	6.46±0.60*
Phosphatidyl-inositol	4.70±0.20	4.67±0.28
Sphingomyelin	10.0 ±0.41	11.8 ±0.21**
Phosphatidyl-choline	35.7 ±0.93	34.1 ±0.57
Phosphatidyl-ethanolamine	30.7 ±1.0	28.8 ±0.71
Diphosphatidyl-glycerol	5.0 ±0.76	6.34±0.47
Lyso-bis-phosphatidic acid	0.41±0.05	0.31±0.05
Phosphatidyl-glycerol	0.23±0.05	0.32±0.06
Lysophosphatidyl-choline	0.95±0.09	1.34±0.16*
Phosphatidic acid	0.57±0.11	0.38±0.14
Unidentified	8.65±1.2	5.61±0.70
	$n = 7$	$n = 10$

* $P < 0.05$.
** $P < 0.001$.

TABLE VI

PHOSPHOLIPID COMPOSITION OF $(Na^+ + K^+)$-ATPase MEMBRANE FRACTION FROM SALT GLANDS

Values are expressed as mean ±S.E. No marked differences occurred between fractions treated with deoxycholate and those not treated with deoxycholate. The data from these determinations were therefore combined.

Phospholipid	Control (% of total)	Saline-treated (% of total)
Phosphatidyl-serine	6.26±0.958	9.22 ±0.132***
Phosphatidyl-inositol	6.61±0.614	4.77 ±0.344*
Sphingomyelin	13.2 ±1.87	14.2 ±0.697
Phosphatidyl-choline	35.3 ±2.83	33.6 ±1.01
Phosphatidyl-ethanolamine	25.8 ±1.18	28.8 ±0.643*
Diphosphatidyl-glycerol	4.48±1.16	4.57 ±0.620
Lyso-bis-phosphatidic acid	1.15±0.339	0.195±0.026**
Phosphatidyl-glycerol	not detectable	0.087±0.006
Lysophosphatidyl-choline	1.37±0.976	0.512±0.091
Phosphatidic acid	1.20±0.745	0.240±0.020
Unidentified	4.53±0.451	3.97 ±0.932
	$n = 4$	$n = 6$

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

content of phosphatidylserine and sphingomyelin occurred. The changes in phospholipid distribution are consistent with the observed changes in relative proportions of cellular organelles which occur following adaptation of ducks to hypertonic saline [31]. Similar class distributions were observed by Karlsson [27] for control and saline-maintained ducks.

The percentage distribution of individual phospholipids in the partially purified $(Na^+ + K^+)$-ATPase fractions is shown in Table VI. The relative proportion of phosphatidylserine was higher in the enzyme fraction than in the whole gland in both control and saline treated groups (Table V vs VI). A significant increase in phosphatidylserine content occurred in the enzyme fraction after saline treatment (Table VI). A small but significant increase in phosphatidylethanolamine also occurred. The phosphatidylinositol content of the fraction was lower after the saline treatment.

DISCUSSION

On the basis of enzyme activity yields and catalytic protein content of partially purified $(Na^+ + K^+)$-ATPase preparations from salt glands, the catalytic protein concentration in salt glands tissue is estimated to have increased about 4.2- to 4.4-fold after saline treatment. The activity increase measured in whole tissue homogenate was about 3.8-fold (total activity) or 3.9-fold (specific activity). The estimates of tissue enzyme content and the measurements of enzyme activity are in reasonable agreement. A similar increase in $(Na^+ + K^+)$-ATPase activity and catalytic protein has been reported recently to occur in the gill of the eel during adaptation to sea water [34]. The increase in $(Na^+ + K^+)$-ATPase protein concentration in the salt glands demonstrates that the change in enzyme activity observed after saline treatment is due to an increase in enzyme content rather than an activation process. Although an increase in enzyme content could result from a reduced rate of enzyme degradation, we think the observed increase in enzyme protein content is due to an accelerated rate of protein synthesis via an induction mechanism. The conclusion is supported by previous studies which show that the increase in $(Na^+ + K^+)$-ATPase in the duck salt gland is preceded by a marked increase in the tissue content of RNA [32]. At 24 h after the onset of salt gland secretion, during the period when the rate of increase of $(Na^+ + K^+)$-ATPase activity is maximal, there is an increase in the size and tissue content of polyribosomes [33]. Both observations are consistent with an accelerated rate of protein synthesis.

The increase in glandular content of $(Na^+ + K^+)$-ATPase would have the effect of increasing the transport capacity of the glands without playing a direct role in the regulation of salt secretion. Salt secretion by the glands is stimulated by cholinergic nerves [35] responding to elevated plasma osmotic pressure [19, 36]. The rate of secretion increases with the plasma osmotic pressure (ref. 37 and unpublished observations). The maximum rate of sodium excretion measured after a large salt load, correlates directly with the $(Na^+ + K^+)$-ATPase activity measured in salt gland homogenates (ref. 2 and Table I) suggesting that the glandular enzyme content is an important factor placing a ceiling on the rate of sodium excretion. The measurements of enzyme content indicate that there is little or no excess of enzyme present. In the saline treated group, 25 400 μmol of Na^+ were secreted per g of tissue per h (Table I).

As shown in Table IV, the estimated catalytic protein content of the tissue is 4.72 mg/g or 3.85 mg/g (deoxycholate-treated preparation). Assuming a coupling ratio of 3 Na^+ transported per ATP hydrolyzed [38], the enzyme in the saline-treated birds would require an in vivo activity of $25\,400 \times 1/3 \times 1/4.72 = 1794$ μmol P_i/mg protein per h, or 2199 based on 3.85 mg protein to account for maximum transport rate for Na^+. In the best preparation of enzyme obtained (1015 μmol P_i/mg protein per h, Table III) the catalytic protein accounted for 43.4 % of the total protein in the fraction. The catalytic protein therefore had an activity of $1015/0.434 = 2339$ μmol P_i/mg protein per h at 37 °C. At 41 °C, which is usually considered to be the body temperature for birds, the activity would be somewhat higher. If the enzyme was capable of the same activity in vivo as seen in partially purified membrane preparations, there would only be a small excess of enzyme present. These calculations, of course, assume that the ($Na^+ + K^+$)-ATPase system is the only mechanism actively transporting sodium in the salt gland. Support for the assumption is the direct correlation between ($Na^+ + K^+$)-ATPase activity and sodium transport and the observation of Thesleff and Schmidt-Nielsen [39] that ouabain blocks salt gland secretion.

Histological studies of the duck salt gland show a marked proliferation of the external membrane and mitochondrial membrane in the secretory cells after saline treatment [31]. The increase in phospholipid content observed in this study and previously by Karlsson [27] reflect the increased membrane content in the salt glands. A question that the present study attempted to answer was whether the increase in sodium transport that accompanied the membrane proliferation was due to more membrane, and hence transport sites, or involved a change in the characteristics of the plasma membrane. The present study shows that the cell membrane does change in at least two aspects. The ($Na^+ + K^+$)-ATPase fraction, which is a plasma membrane-enriched fraction, showed a higher content of catalytic protein relative to both non-($Na^+ + K^+$)-ATPase proteins and to phospholipids. The higher enzyme content of the membrane was also reflected as an increase in the specific gravity of the membrane observed under isopycnic centrifugation conditions. A second protein of 54 000 molecular weight also increased with the catalytic protein. This protein is probably the glycoprotein seen in highly purified ($Na^+ + K^+$)-ATPase preparations [25, 29, 30] which has been suggested by Shamoo et al. [40] to represent the sodium ionophore for the sodium pump complex. The banding pattern of the proteins in the ($Na^+ + K^+$)-ATPase fractions was the same, indicating that significant changes only occurred in the catalytic protein and in a protein that is possibly a part of the enzyme complex. The membrane fraction associated with the ($Na^+ + K^+$)-ATPase also showed a change in phospholipid composition. The percentage of phosphatidylserine and phosphatidylethanolamine increased and the percentage of phosphatidylinositol decreased. The increased phosphatidylserine content was reflected as a significant increase in glandular content of this lipid as well (Table V). Purified brain ($Na^+ + K^+$)-ATPase shows a higher content of phosphatidylserine when compared to whole tissue [14, 41]. Similarly, the ($Na^+ + K^+$)-ATPase preparations from salt glands showed a higher phosphatidylserine content in comparison to the whole tissue (Table V vs Table VI). These observations suggest that part of the membrane phosphatidylserine is closely associated with the ($Na^+ + K^+$)-ATPase. Goldman and Albers [42] have presented evidence that phosphatidylserine is a requirement for phosphorylation and dephosphorylation of the enzyme. The phosphatidylethanolamine has been

suggested by those investigators to act as a modifier which influences the affinity of the enzyme for Mg^{2+}. Studies by other investigators, however, indicate a low specificity in the phospholipid requirement for $(Na^+ + K^+)$-ATPase activity [12–14]. DePont et al. [43], for example, enzymatically replaced 99 % of the phosphatidylserine content of a partially purified enzyme preparation with phosphatidylethanolamine without any loss of enzyme activity. The present studies would indicate that phospholipids are only acting permissively to maintain the necessary environment for enzyme activity, but are not responsible for the observed changes in enzyme activity. The observed increase in $(Na^+ + K^+)$-ATPase activity after saline treatment can be entirely accounted for as an increase in $(Na^+ + K^+)$-ATPase catalytic protein.

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