CORRELATION OF Na⁺,K⁺-ATPase CONTENT AND PLASMA MEMBRANE SURFACE AREA IN ADAPTED AND DE-ADAPTED SALT GLANDS OF DUCKLINGS

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SUMMARY

During salt-water adaptation, an increase occurs in Na⁺,K⁺-ATPase content and surface area of the basolateral plasma membrane of the principal cell of the duck salt gland. To determine the degree to which these changes are correlated, accepted morphometric methods were used to determine numerical cell densities and plasma membrane surface densities of peripheral and principal cells. After adaptation, the plasma membrane surface area per principal cell was five times greater than in controls. Following de-adaptation, the plasma membrane content in principal cells returned to 1·9 times control levels. Two other cell constituents, mitochondria and lipid droplets, displayed similar quantitative changes. Na⁺,K⁺-ATPase content increased about fourfold with adaptation and decreased to near control levels with de-adaptation. Thus, changes in Na⁺,K⁺-ATPase content and basolateral plasma membrane surface area in adapting and de-adapting secretory epithelia of the salt gland occur nearly in parallel. These quantitative data enable Na⁺,K⁺-ATPase synthesis and degradation to be investigated in relation to membrane biogenesis.

INTRODUCTION

The avian salt gland possesses characteristics that permit its use as a model system for the study of plasma membrane biogenesis (Barrnett, Mazurkiewicz, & Addis, 1983). Significant cellular and biochemical changes involving the basolateral plasma membrane of principal secretory cells constitute a major response during adaptation (salt stress) and de-adaptation (return to fresh water) (Peaker & Linzell, 1975). Adaptive membrane amplification includes development of basal infoldings, increase in lateral plicae (Benson & Phillips, 1964; Ellis, Goertemiller, DeLellis & Kablotsky, 1963; Ernst & Ellis, 1969) and increase in levels of Na⁺,K⁺-ATPase activity (Ernst, Goertemiller & Ellis, 1967; Holmes & Stewart, 1968). De-adaptation of salt-stressed animals results in gland involution (Addis, Eager, & Barrnett, unpublished; Fletcher, Stainer & Holmes, 1967; Hossler, Sarras & Allen, 1978α; Hossler, 1982).

However, these events have not been correlated quantitatively, especially with regard to the various secretory cell stages. In other systems, ATPase content and

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Key words: Na⁺,K⁺-ATPase, plasma membrane, salt gland, morphometry.
biosynthesis are not necessarily synonymous with fluctuations in plasma membrane content (Lo & Edelman, 1976; Pollack, Tate & Cook, 1981). In this study morphometric analysis of the amount of plasma membrane in the principal and peripheral secretory cells from control, adapted and de-adapted glands is related to changes in Na⁺K⁺-ATPase content. The numerical density of mitochondria (suggested to increase during adaptation (Ernst & Ellis, 1969) and decrease during de-adaptation (Addis et al. unpublished; Hossler et al. 1978a)) as well as lipid droplets are also quantified per cell using stereological principles. The plasma membrane results form the basis for further analysis of the regulation of Na⁺K⁺-ATPase content during adaptation and de-adaptation (Merchant & Barrnett, unpublished).

MATERIALS AND METHODS

Materials

Twenty nine white 1-day-old Pekin ducklings (C & R Duck Farm, Long Island, NY) were fed on duck mash and fresh drinking water for an adjustment period of at least 15 days and then divided into three groups. Controls (11) remained on freshwater. Adapted ducklings (10) were given 1% NaCl in drinking and wading water for 10 days before being killed. Twenty days before death, the de-adapted group (8) was given consecutively 1% NaCl and freshwater (10 days each). All 29 ducklings were killed at 35 days of age. Twenty ducklings (8 control, 6 adapted and 6 de-adapted) were decapitated and their salt glands were rapidly removed for morphometric analysis and enzyme measurements.

Preparation for microscopy

Oblique slices were cut from the anterior, middle and posterior portions of all glands. Six random slices (derived from a pool of slices from both glands in each animal) were cut into 1 mm³ blocks and fixed in cold 2.5% glutaraldehyde, 2% paraformaldehyde, 0.05% (w/v) CaCl₂ and 0.1M-sodium cacodylate (pH 7.4) for 2 h. The fixed tissue blocks were rinsed (3 times) in 0.15M-cacodylate containing CaCl₂, refixed for 1 h at 25 °C in 1% osmium tetroxide in 0.1M-cacodylate, dehydrated through an ethanol series and embedded in Spurr’s (1969) medium. From seven embedded blocks/animal, three were chosen randomly for sectioning. Thick sections (2 µm) were cut with an LKB Ultratome and stained with Toluidine Blue for light microscopy. Silver sections (from areas selected on the basis of light microscopy) were placed on uncoated 200 mesh grids, stained with uranyl acetate and lead citrate, and examined with an Hitachi electron microscope. Thus, 48 control, 36 adapted, and 36 de-adapted grids were used to generate morphometric studies.

Sampling procedure

The process of sampling was influenced by the morphological organization of the gland of young ducklings as previously described (Ellis, 1965; Ernst & Ellis, 1969). To ensure adequate sampling of both peripheral and principal cells, the percentage contribution of each cell stage in control, adapted and de-adapted glands was first determined by examining randomly chosen light micrographs. These percentages were obtained by point counting positive transparencies projected onto a 240 mm × 180 mm grid at a final counting magnification of ×1040. Results obtained (10% peripheral cells in control and adapted gland; 20% in de-adapted) were then applied to the sampling of electron micrographs of principal and peripheral cells in each of the three groups of animals.

For electron microscopy, 17 tissue fields from three grids per animal were photographed (×3600) using the stratified sampling procedure (Weibel & Bolender, 1973) along with one calibration grid. To maintain the percentages of cells described above, ~10% of the micrographs (2 of 17) were of peripheral cells in the control and adapted groups and ~20% (3 of 17) were of peripheral cells in the de-adapted group. The remainder of the micrographs were of principal cells.
Morphometric analysis

To calculate membrane surface area and other cytoplasmic profiles on a per cell basis, using formulas described by Weibel & Bolender (1973), peripheral and principal numerical cell densities \( (N_v, \text{ number of cells per tissue volume}) \) were scored from positive transparencies of light micrographs projected (magnification of \( \times 2080 \)) onto a 60 mm x 60 mm test grid. The average numbers of peripheral and principal cells per test volume \( (N_v) \) were counted separately, and their individual cell densities computed on an Apple II plus computer using VisiCalc software and the following formulas: \( N_{AT} \) (cell number/test area of thickness, \( T \)) = \( N_x (Mag)^2/d^2 \), where \( d \) is a side of a test grid in \( \mu m \), Mag is the final magnification (\( \times 2080 \)) and \( N \) is the number of cells per test grid area; \( N_v \) (cell number/tissue volume) = \( N_{AT}/D + T \), where the mean particle diameter \( D = 4d/\pi \), \( T \) is section thickness (2 \( \mu m \)) and \( d \) is the mean profile diameter calculated from a histogram of 200 nuclear profiles from each cell type (7-0 \( \mu m \) for principal cell nuclei and 7-5 \( \mu m \) for peripheral cell nuclei). The mean numerical cell densities \( \pm \) the standard deviation (s.d.) of each group were calculated and analysed using the Bonferroni \( t \) test, a type of multiple comparison procedure (Glantz, 1981). These numerical cell densities \( \times \) the number of cells per \( \mu m \) were then used to calculate the plasma membrane surface area, and the number of mitochondria or lipid droplets per cell.

From 17 electron micrographs per animal, 10 were selected for scoring (maintaining appropriate ratios of peripheral to principal cells). Eighty micrographs from the controls and 60 micrographs from each experimental group were scored. Since the plasma membrane in this tissue is amplified basolaterally, particularly in the adapted group, a curvilinear test grid was required to avoid counting bias (Weibel & Bolender, 1973). This grid, containing five complete semicircles each with a 45 mm diameter, was used to count plasma membrane intersections on \( 6 \times 5 \) projections of positive transparencies made from the original \( \times 3600 \) negative (final magnification, \( \times 23400 \)). Each projected micrograph was divided into four non-overlapping areas, and plasma membrane intersecting with the test grid was counted. The average plasma membrane density \( (S_v) \) for each micrograph was computed using the formula:

\[
S_v \text{(membrane surface area/tissue volume)} = \frac{2 \times I_i \times Mag}{P_i \times T \times d/2},
\]

where \( d \) is the diameter of the curvilinear test grid in \( \mu m \), \( I_i \) is the number of intersections per test grid, Mag is the final magnification (\( \times 23400 \)) and \( P_i \) is the number of test points overlying tissue. The membrane density \( (\mu m^2/\mu m^2) \) was divided by the number of cells per \( \mu m^2 \) to yield plasma membrane surface area per cell in both peripheral and principal cells of the control, adapted and de-adapted groups. The means \( \pm \) the s.d. of each group were calculated and analysed using the Mann–Whitney Rank Sum non-parametric statistical analysis after computing the Kruskal–Wallis test statistic (Glantz, 1981).

To test the observation that mitochondria and lipid droplet profiles changed, their numbers per cell were calculated using the same formula as described above for cellular \( N_{AT} \). However, a 120 mm x 120 mm grid was used to assess the number of mitochondria per \( \mu m^2 \), and a 120 x 210 mm grid was used to count the number of lipid droplets per \( \mu m^2 \) on the same projected micrograph used to score membrane intersections (final magnification, \( \times 23400 \)). The numbers of mitochondria and lipid droplets per cell were calculated by dividing by the cellular \( N_{AT} \) (cells per area). The mean numbers of mitochondrial profiles and lipid droplets per cell were calculated and analysed using non-parametric statistics.

Biochemical studies

A 10% homogenate in 10 mM-Tris-HCl (pH 7.4) was prepared from the pooled tissue (from the same animals used for the morphometric studies) with a Brinkmann Polytron homogenizer. After filtering the homogenate through a 60 \( \mu m \) Nytex mesh (Tetko, Elmsford, NY), it was assayed in duplicate for \( Na^+ \), \( K^+ \)-ATPase and ouabain-sensitive \( p \)-nitrophenyl phosphatase \( (p\text{-NPPase}) \) specific activities as well as ouabain binding. All assays were performed on the day of killing.

The \( Na^+ \), \( K^+ \)-ATPase activity was assayed using a modification of the procedure described by Hopkins, Wagner & Smith (1976). The reaction mixture (100 mM-NaCl, 10 mM-KCl, 50 mM-Tris-HCl, (pH 7.5), 1 mM-Na2EDTA, 5 mM-MgCl2-6H2O and 25 \( \mu g \) of crude tissue homogenate, plus
or minus 1 mM-ouabain (Sigma) in a total 900 μl volume) was equilibrated at 25 °C for 5 min. The reaction was initiated by adding 100 μl of 25 mM-ATP (Sigma); after 10 min at 37 °C, it was terminated with cold trichloroacetic acid (TCA). After removal of acid-insoluble material by centrifugation, 1 ml of supernatant was assayed for inorganic phosphate (Bonting, Simon & Hawkins, 1961). Na⁺,K⁺-ATPase specific activity was taken as the difference of phosphate values obtained in the presence and absence of ouabain, and expressed per mg DNA. DNA was measured by the Burton (1956) method and protein by the method of Lowry, Rosebrough, Farr & Randall (1951).

**Ouabain-sensitive p-NPPase activity**

This activity (K-dependent enzyme phosphorylation of the Na⁺,K⁺-ATPase reaction) was determined by a modification of the method of Ernst (1972a). The reaction mixture contained 75 mM-Tris HCl (pH 7.5), 5 mM-MgCl₂, 0.5 mM-EGTA and 25 μg of homogenate in a 900 μl volume ± 1 mM-ouabain. The reaction was initiated by the addition of 100 μl of 100 mM-KCl and 50 mM-p-nitrophenyl phosphate. After 10 min at 37 °C, the reaction was terminated with cold TCA and the protein was sedimented. After addition of 1M-Tris • HCl (pH 9.5) to the supernatant, the absorbance was read against p-nitrophenol standards at 410 nm. Specific activities were expressed per mg DNA.

**[^3H]ouabain binding**

This was measured on homogenates prepared from the nine unused ducklings (three each of control, adapted and de-adapted) using a modification of the method of Hossler et al. (1978b). A crude tissue homogenate (~50 μg) was incubated at 37 °C with 3 mM-ATP, 6 mM-MgCl₂, 100 mM-NaCl, 50 mM-Tris HCl (pH 7.5), and 1 μM-ouabain containing 200 000 c.p.m. (14 Ci/mmol, New England Nuclear, NEN) in 400 μl of the reaction mixture. Background binding was determined on samples incubated in parallel at 0°C. After maximum binding (1 μM-ouabain, 30 min), the homogenate was collected on glass microfibre filters (Whatman GF/A, 2.4 cm) using a Millipore apparatus under vacuum and rinsed three times with cold buffer (50 mM-Tris • HCl, pH 7.5, 6 mM-MgCl₂, 100 mM-NaCl). Filters were dried overnight at 55 °C, cooled to 22 °C, and counted in Econofluor (NEN). Results were expressed as molecules of ouabain bound per mg DNA.

To convert enzyme specific activities and ouabain binding per mg DNA to a per cell basis, cellular DNA was determined. Cells were dissociated using the procedure described by Hootman & Ernst (1980), resuspended in phosphate-buffered saline, counted in a haemocytometer and a sample was used to measure DNA.

**RESULTS**

**Morphology**

The qualitative, morphological results of this study focus primarily upon cell surface structures. The complete morphological details of the salt gland have been reported previously (Ernst & Ellis, 1969; Peaker & Linzell, 1975). Ultrastructurally, salt-water-adapted principal cells demonstrated a dramatic increase in the basal and lateral plasma membrane as well as an increase in the number of mitochondria compared to control principal cells (Figs 1, 2). After de-adaptation (Fig. 3), the basal portion of the plasma membrane remained slightly convoluted in comparison to the flat basal membrane seen in control cells. The lateral plicae became blunted and reduced in number when compared with adapted cells. Mitochondria were fewer and distributed as in the controls. Lipid droplets increased in number with adaptation and decreased with de-adaptation. In contrast, the peripheral cells showed little, if any, irregularity of the lateral surface membrane, regardless of the osmotic conditions.
Table 1. Cell densities of peripheral and principal cells of the duck salt gland (number of cells/mm³ ± s.d.)

<table>
<thead>
<tr>
<th></th>
<th>Control (8)</th>
<th>Adapted (6)</th>
<th>De-adapted (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral (×10⁶)</td>
<td>2.1 ± 0.32</td>
<td>2.0 ± 0.26***</td>
<td>2.2 ± 0.20***</td>
</tr>
<tr>
<td>Principal (×10⁶)</td>
<td>1.5 ± 0.21</td>
<td>0.73 ± 0.30*</td>
<td>1.0 ± 0.17**</td>
</tr>
<tr>
<td>Wet weight of gland (g tissue)</td>
<td>0.13 ± 0.03</td>
<td>0.31 ± 0.05*</td>
<td>0.23 ± 0.05**</td>
</tr>
</tbody>
</table>

*P value < 0.001 for adapted versus control; **P value < 0.002 for de-adapted versus control; ***P value > 0.05.

The mean cell densities ± s.d. (number of cells/mm³ of tissue) of individual secretory cell types from 8 control, 6 adapted and 6 de-adapted animals are included. In addition, the mean wet weight of the glands (in grams) is shown. The Bonferroni test, a type of multiple comparison procedure, was used for the statistical analysis of the data. *P values < 0.05 were considered significant.

Table 2. Plasma membrane surface area per cell in peripheral and principal cells of the duck salt gland (μm²/cell ± s.d.)

<table>
<thead>
<tr>
<th></th>
<th>Control (8)</th>
<th>Adapted (6)</th>
<th>De-adapted (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral</td>
<td>590 ± 174</td>
<td>515 ± 150***</td>
<td>432 ± 200***</td>
</tr>
<tr>
<td>Principal</td>
<td>1856 ± 419</td>
<td>9522 ± 4918*</td>
<td>3541 ± 1186**</td>
</tr>
<tr>
<td>Combined cell types</td>
<td>1729</td>
<td>8621**</td>
<td></td>
</tr>
</tbody>
</table>

*P value < 0.001 for adapted versus control; **P value < 0.002 for de-adapted versus control; ***P value > 0.05.

The plasma membrane surface area per cell ± s.d. in 8 control, 6 adapted and 6 de-adapted animals are included. Of the 10 electron micrographs scored per animal, 10% were of peripheral cells and 90% were of principal cells in the control and adapted groups. In the de-adapted group, 20% of the micrographs were of peripheral cells and 80% were of principal cells. These percentages approximated the contribution each respective cell type makes to the entire secretory cell population. Accounting for these percentage contributions, the plasma membrane surface area of the entire secretory cell population was computed. Data were analysed using Mann–Whitney Rank Sum non-parametric statistics. *P values < 0.05 were considered significant.

Partially specialized cells, present in all stages and lying immediately adjacent to the peripheral cells, showed a surface intermediate between that of peripheral and principal cells.

Morphometric data

Glands of adapted ducklings contained half as many principal cells per tissue volume as glands of control ducklings; densities of principal cells in de-adapted glands were two thirds of controls (Table 1). There was no statistical difference between the number of peripheral cells per volume of tissue in the control, adapted and de-adapted
Figs 1–3
Plasma membrane morphometry, Na⁺,K⁺-ATPase

glands. The difference in numerical cell densities between peripheral and principal cells was greatest in the adapted and de-adapted glands (2.7 and 2.2 times, respectively) and least in the control gland (1.4 times). The wet weights of the adapted and de-adapted glands were, respectively, 2.4-fold and 1.8-fold greater than control glands.

Quantitative determination of membrane surface area (Table 2) revealed an average five-fold increase in the plasma membrane of adapted principal cells compared to controls, whereas the surface area of de-adapted cells was only 1.9 times that of controls. In contrast, there was no statistically significant difference in the membrane surface area of the peripheral cells in any of the three groups of animals. The ratio of the plasma membrane surface area of principal cells to that of peripheral cells was 3.1 in control, 18.5 in adapted and 8.2 in de-adapted glands.

The frequency distributions of plasma membrane surface areas of peripheral and principal secretory cells in control, adapted and de-adapted salt glands are illustrated in Fig. 4. In controls (Fig. 4A), the membrane area of principal cells had a smaller range of values compared to both experimental groups (Fig. 4B, C). The overlap between membrane surface areas of the control peripheral and principal cells (Fig. 4A) was not present in the experimental groups (Fig. 4B, C).

The principal cells responded to salt stress extensively and variably as evidenced by the heterogeneity of membrane surface area in a bimodal and possibly trimodal distribution. This resulted in large standard deviations from the mean of this population (Fig. 4B). As shown, 68% of the adapted principal cells showed a median membrane surface area of 7000 μm²/cell, 26% had a median of 15000 μm²/cell and 6% exhibited a median of 23000 μm²/cell. When the median membrane surface area of 2000 μm²/cell in the non-adapted principal cell (Fig. 4A) was compared with the median surface area of each of these three populations of adapted principal cells (Fig. 4B), an average 4-, 8- or 12-fold increase in plasma membrane surface area was calculated. Despite this range in the magnitude of increase, 70% of the cells had a

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**Fig. 1.** Electron micrograph of control principal cells. The control principal cells in this oblique section are characterized by pyramidal shape and prominent lateral plasma membrane infoldings. Basal membrane is relatively flat and abuts against the basement membrane (arrowhead). Apical membrane is a relatively small portion of the plasma membrane and exhibits few microvilli protruding into the tubule lumen (l). Junctional complexes join cell apices and occasional desmosomes are seen (arrow). The cells contain prominent Golgi complexes (G) and mitochondria are randomly dispersed within the cytoplasm. Lipid droplets and lysosomes are occasionally seen. Numerous free polysomes are scattered uniformly throughout the cytoplasm. Smooth and rough endoplasmic reticulum are not prominent features of these cells. ×15 600.

**Fig. 2.** Electron micrograph of adapted principal cells. After 10 days of adaptation, the principal cell extensively proliferates its basal plasma membrane into deep infoldings containing mitochondria, while further extending lateral plications. Golgi apparatus is present, and numerous lipid inclusions (l) are seen adjacent to mitochondria both within plications and within the hyaloplasm. l, lumen. ×15 400.

**Fig. 3.** Electron micrograph of de-adapted principal cells. Ten days after de-adaptation, the plicae in the entire basolateral region become disorganized and disrupted. This results in the establishment of large extracellular spaces containing debris, which is also found in the lumen (l). Mitochondria lose their cellular polarity established during adaptation, but lipid inclusions (l) are still present. Golgi profiles (G) remain prominent. ×15 900.
surface area of no more than 10 000 μm²/cell, accounting for the overall mean fivefold adaptive change in the plasma membrane of principal secretory cells for the entire gland. Although significant numbers of de-adapted principal cells mimicked those in the control gland, the mean and range of surface areas were greater, and sufficient numbers of cells with larger surface areas shifted the histogram toward intermediate values (cf. Fig. 4a and c).

In addition to these surface membrane changes, there was a four to fivefold increase in mitochondrial profiles (Table 3A) and a seven to eightfold increase in lipid droplets (Table 3B) over control values as a result of adaptation. The two-fold higher numerical density of mitochondria in de-adapted as compared to control principal cells was significant; the twofold difference for lipid droplets was not significant (Table 2).
Biochemical data

Correlated with the morphometric changes, the specific enzyme activities of Na⁺, K⁺-ATPase and p-NPPase (Table 4) increased fourfold over controls with adaptation and remained 1·9-fold greater than controls with de-adaptation. Similar results were

Table 3. Number of mitochondria per cell (±S.D.)

<table>
<thead>
<tr>
<th>Mitochondria/cell</th>
<th>Control (8)</th>
<th>Adapted (6)</th>
<th>De-adapted (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral</td>
<td>119±71</td>
<td>91±48***</td>
<td>100±32***</td>
</tr>
<tr>
<td>Principal</td>
<td>316±77</td>
<td>1513±659*</td>
<td>635±140**</td>
</tr>
<tr>
<td>Combined cell types</td>
<td>296</td>
<td>1371*</td>
<td>528**</td>
</tr>
</tbody>
</table>

*P value < 0·001 for adapted versus control; **P value < 0·002 for de-adapted versus control; ***P value > 0·005.

B. Number of lipid droplets per cell (±S.D.)

<table>
<thead>
<tr>
<th>Lipid droplet/cell</th>
<th>Control (8)</th>
<th>Adapted (6)</th>
<th>De-adapted (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral</td>
<td>15±10</td>
<td>28±27***</td>
<td>8±5***</td>
</tr>
<tr>
<td>Principal</td>
<td>27±8</td>
<td>219±66*</td>
<td>54±21**</td>
</tr>
<tr>
<td>Combined cell types</td>
<td>26</td>
<td>200*</td>
<td>45**</td>
</tr>
</tbody>
</table>

*P value < 0·001 for adapted versus control; **P value > 0·05 for de-adapted versus control; ***P value > 0·05.

The number of mitochondria (A) or lipid droplets (B) per cell ± S.D. in 8 control, 6 adapted and 6 de-adapted animals are included. Data were derived from the same 10 electron micrographs per animal used to compute the plasma membrane surface areas. Mann–Whitney Rank Sum non-parametric statistics were used. P values < 0·05 were considered significant.

Table 4. Enzyme specific activities and the amount of ouabain bound per cell (±S.D.)

<table>
<thead>
<tr>
<th></th>
<th>Control (8)</th>
<th>Adapted (6)</th>
<th>De-adapted (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺,K⁺-ATPase</td>
<td>5·3±4·0</td>
<td>21·5±2·0*</td>
<td>10·3±4·3**</td>
</tr>
<tr>
<td>(fmol/min per cell)</td>
<td>(8)</td>
<td>(5)</td>
<td>(6)</td>
</tr>
<tr>
<td>p-Nitrophenyl phosphatase</td>
<td>4·7±3·4 (8)</td>
<td>20·0±3·1*</td>
<td>9·1±2·7**</td>
</tr>
<tr>
<td>(ouabain-sensitive, K⁺-activated) (fmol/min per cell)</td>
<td></td>
<td>(5)</td>
<td>(6)</td>
</tr>
<tr>
<td>[3H]ouabain bound</td>
<td>8·2±2·3 (3)</td>
<td>29·6±8·3*</td>
<td>8·0±1·5***</td>
</tr>
<tr>
<td>(molecules/cell, X10⁶)</td>
<td>(3)</td>
<td>(3)</td>
<td>(3)</td>
</tr>
</tbody>
</table>

*P value < 0·001 for control versus adapted; **P value < 0·05 for control versus de-adapted; ***P value > 0·05 for control versus de-adapted.

Enzyme-specific activities and the amount of ouabain bound per cell in control, adapted and de-adapted animals (± S.D.) are included. The number of animals contributing to the mean is shown in parenthesis. Each determination was performed in duplicate. Data were analysed using the Bonferroni t test. P values < 0·05 were considered significant.
observed with ouabain binding per cell, except that the magnitude of increase was slightly less with adaptation. Also, with de-adaptation, a return to baseline was obtained (Table 4). As suggested (Hossler et al. 1978b), ouabain binding may be more accurate than specific activity in quantifying the amount of enzyme present. Calculated DNA/cell ratios ± s.d (controls, 3.6 ± 0.80 pg; adapted 4.3 ± 0.34 pg; de-adapted, 4.0 ± 0.78 pg) did not differ significantly. Therefore, an average of 4.0 pg of DNA/cell was used to convert all biochemical assays from a per mg DNA basis to a per cell basis.

To ascertain Na⁺,K⁺-ATPase density in the plasma membrane, the number of ouabain molecules bound/cell was divided by the surface area/cell (cf. Tables 2 and 4). In the control principal secretory cell, there were 4700 molecules of ouabain bound per μm² of plasma membrane, whereas there were 3400 molecules/μm² in the adapted cell and 2700 molecules/μm² in the de-adapted cell. These ratios are not significantly different from each other, an observation that could be explained in part by the consistent increase in basolateral plasma membrane surface area (~fivefold) and number of bound ouabain molecules (~fourfold) with adaptation.

DISCUSSION

This study quantifies several morphological changes occurring in the duck salt gland as a result of osmotic stress, and relates them to concomitant changes in Na⁺,K⁺-ATPase content. In response to adaptation, the principal secretory cell undergoes rapid modulation, including a fourfold increase in mitochondria, a fivefold increase in basolateral plasma membrane, a sevenfold accumulation of lipid inclusions, and a 3.5- to fourfold increase in Na⁺,K⁺-ATPase content. De-adaptation results in a significant reversal of all of these changes.

During adaptation, the observed decrease in cell density of principal cells was probably not a consequence of cell loss, since gland weight doubled during the same period. A doubling in the size of the principal cell, along with the doubling of the protein and RNA content (Holmes & Stewart, 1968), would account for this decrease in secretory cell density and increase in gland weight. Cell diameters of 8 μm for control principal cells and 15 μm for adapted principal cells, determined on dissociated cells in suspension (Hootman & Ernst, 1980), support this conclusion. Peripheral cell densities remained constant; thus, major changes in secretory cell dimensions appeared to be restricted to the principal cell stage, as suggested by Benson & Phillips (1964), Ellis et al. (1963) and Ernst & Ellis (1969). The mitotically active, immature peripheral cells did not contribute to the overall adaptive changes occurring as a result of osmotic stress. Furthermore, minimal changes in DNA synthesis and content have been documented (Hanawell & Peaker, 1975), implicating a low level of cell turnover (Hossler et al. 1978a; Hossler, 1982). Rather, the substantial accumulation of surface membrane and mitochondria (cellular up-modulation) account partly for the increase in gland size during adaptation; reversal of such changes (down-modulation) results partly in glandular involution during de-adaptation. The function of the peripheral cell appears to be only to replace slowly senescent principal cells (Ellis, 1965).
In the present study the partially specialized cells were not counted as a separate group. Often they could not be distinguished with certainty from peripheral cells or early principal cells. Indeed, no such distinct cell type exists; they represent a transitional stage through which peripheral cells traverse during maturation into principal cells (Ernst & Ellis, 1969; Levine, Higgins & Barrnett, 1972). These transitional cells were included arbitrarily in either 'peripheral' or 'principal' cell groups, and account for the overlap between surface area of these groups in the histograms presented for control and de-adapted cells. In contrast, the distinction between peripheral and partially specialized cells could be discerned with greater accuracy once adaptation had occurred, as reflected by the gap shown between the surface areas of the two cell populations in the histogram of adapted cells. These observations suggest that transitional cells, like mature principal cells, respond to cell regulators responsible for adaptive hypertrophy and for inducing membrane amplification, especially the basolateral plasma membrane.

The variability of the mean surface area of the adapted principal cell and the range of surface areas in control, adapted and de-adapted cell populations might be a reflection of the frequency and positions of these secretory cells in a tubule. Those cells having a greater surface area would be closer to a branch of the collecting duct, and those with a smaller surface area would be associated with partially specialized cells closer to the blind end. This inference, based on quantitative data, supports the model of the gland described by Ernst & Ellis (1969).

The fact that the histogram of 10-day de-adapted cells reveals a broader range of plasma membrane surface area than the control histogram indicates that the amount of plasma membrane does not return to baseline. RNA and protein content in de-adapted glands also do not return to control levels, even after 20 days of de-adaptation (Holmes & Stewart, 1969). Thus, the principal cell plasma membrane surface area in de-adapted cells may reach a new steady state, higher than the previous non-adapted state.

The contribution of the apical portion of the plasma membrane to the total plasma membrane pool was probably negligible, since a previous morphometric study showed that the ratio of apical to basolateral plasma membrane in the adapted cell was 1:1000 (Komnick & Kniprath, 1970). Qualitative information derived from numerous micrographs of control, adapted and de-adapted glands from the present study, as well as other work from this laboratory (Addis et al. unpublished; Hossler et al. 1978a; Levine et al. 1972; Mazurkiewicz & Barrnett, 1981; Mazurkiewicz, Hossler & Barrnett, 1977; Russo, Merchant & Barrnett, unpublished), support this notion. Thus, the basolateral plasma membrane of the principal secretory cell accounts for all surface membrane changes occurring in the gland during adaptation and de-adaptation.

The magnitude of the increase and decrease in the number of mitochondria per cell with adaptation and de-adaptation was similar to the changes in basolateral plasma membrane surface area per cell. This morphometric increase suggests that the increase of two mitochondrial oxidative enzymes, succinic dehydrogenase and cytochrome oxidase, with salt stress (Ellis et al. 1967; Spannhof & Jurss, 1967), and the
decrease of succinic dehydrogenase with de-adaptation (Addis et al. unpublished) represent changes in amount rather than enzymic activity. The close association of mitochondria with the plasma membrane folds, coupled with the parallel expansion of both compartments, is consistent with the ion-transporting function of the gland. Lipid droplets, mentioned by Mazurkiewicz & Barnett (1981), may represent an expanding metabolic pool for the mitochondria or a pool of substrate for the synthesis of membrane phospholipids, if they do not constitute a non-specific pathological response to stress. However, the meaning of these lipid pools is not yet understood in the salt gland under these circumstances.

Importantly, biochemical results infer that the Na⁺,K⁺-ATPase content adequately reflect the magnitude of increase and decrease in basolateral plasma membrane during adaptation and de-adaptation. Consistent with the enzyme density in principal cells of adapted glands reported here, the surface density of Na⁺,K⁺-ATPase particles in freeze-fractured purified membrane preparations from rabbit renal medulla is also 3400 molecules/μm² (Deguchi, Jorgensen & Maunsbach, 1977).

Na⁺,K⁺-ATPase activity has been localized in the avian salt gland to the basolateral membrane of the principal cell by cytochemical methods (Barnnett et al. 1983; Ernst, 1972b; Mazurkiewicz et al. 1977) and crudely confirmed by labelled-ouabain autoradiography (Ernst & Mills, 1977; Hossler et al. 1978b). Having now related the accumulation of surface membrane to the amount of change in the quantity of Na⁺,K⁺-ATPase, predictions and measurements can be made with greater certainty regarding fluctuations of basolateral membrane content in response not only to physiological stimuli, but also to specific hormones and neurotransmitters. With this information at hand, rates of biosynthesis of the α-subunit of the Na⁺,K⁺-ATPase have been investigated in control, adapted and de-adapted glands in order to assess the role played by synthesis and degradation in this event (Merchant & Barnett, unpublished). In addition, investigations into mechanisms of degradation of this membrane marker have suggested more than one mode of plasma membrane destruction (Addis et al. unpublished) and this is supported by studies involving immunocytochemical localization of the α-subunit of the enzyme (Russo et al. unpublished).

These studies were supported by grants from the NIH (AM-03688, to RJB) and EY-03239 and the Veterans Administration (to DSP). Juanita Merchant was the recipient of the Insurance Medical Scientist Scholarship Fund, Springfield, MA 01111. The authors thank Dr J. Russo for helpful criticisms and Ms P. Eager and Mrs B. Schneider for technical assistance.

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Plasma membrane morphometry, $Na^+K^+$-ATPase


*(Received 12 October 1984 – Accepted, in revised form, 8 May 1985)*