OUABAIN BINDING DURING PLASMA MEMBRANE BIOGENESIS IN DUCK SALT GLAND

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SUMMARY

The conditions necessary for optimal ouabain binding in the avian salt gland were examined. Binding was enhanced by ATP and Mg²⁺ and was decreased by K⁺, but was unaffected by added Na⁺. Both maximal binding and complete inhibition of Na,K-ATPase activity were obtained at 1 x 10⁻⁴ M ouabain. Half maximal binding and half maximal inhibition of Na,K-ATPase activity were obtained at 1.7 x 10⁻⁷ M ouabain. Ouabain binding increased in parallel with increasing specific activity of the Na,K-ATPase during salt-induced salt gland specialization. The ratio of Na,K-ATPase activity to ouabain-binding sites remained constant during the salt stress as well as after removal of the salt diet. Autoradiography indicated binding to partially and fully differentiated secretory cells of the salt gland. The ouabain binding assay appeared to be a more useful indicator of membrane amplification than Na,K-ATPase activity since it is rapid, essentially irreversible, less sensitive to tissue fixatives, and quantitatively measured the number of enzyme molecules.

INTRODUCTION

The protein components of plasma membranes are in a state of constant turnover and renewal, and many have half lives of about 85 h or less (Arias, Doyl & Schimke, 1969; Widnell & Siekevitz, 1967). However the sites of synthesis of these components in the cell and the mechanisms by which they are assembled into new or existing membrane are largely unknown, except for the general fact that synthesis must occur on free or bound polysomes.

In the quest for this information, advantage has been taken of the properties of several unique tissues, such as erythrocyte ghosts, which provide a readily available source of uncontaminated plasma membranes. Since these are end stage cells with little or no synthetic capability, younger, synthetically more active cells have been studied (Koch, Gardner, Gartrell & Carter, 1975a; Koch, Gartrell, Gardner & Carter, 1975b; Lodish, 1973). Retinal rod outer segments represent a membranous compartment which is constantly renewed and for which there is an established marker protein, opsin (Papermaster, Converse & Siu, 1975).

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Our approach to membrane biogenesis has been via the study of phospholipid and protein synthesis and assembly in membranous elements which are rapidly enlarging as a result of developmental, physiological, or drug-induced events. These studies included: proliferation of smooth endoplasmic reticulum in rat hepatocytes induced by phenobarbital (Higgins, 1974; 1976; Higgins & Barrnett, 1972); the process of myelination of rat trigeminal nerve (Benes, Higgins & Barrnett, 1973); plasma membrane formation during the transition from the syncytial to the cellular phase of Drosophila embryos (Heckman, Friedman, Skehan & Barrnett, 1977); and plasma membrane biogenesis induced by salt stress in the secretory cells of the salt gland of ducklings (Barrnett, Levine & Higgins, in preparation; Levine, Higgins & Barrnett, 1972). In the latter studies evidence indicated that Golgi elements were concerned with phospholipid synthesis and suggested that the Golgi was also the site of assembly of plasma membrane-associated proteins and phospholipids since Golgi-derived vesicles fused with the baso-lateral plasma membrane of secretory cells during amplification of that structure.

The avian salt gland has unique morphological and functional properties related to Na+ transport (Ellis, Goertemiller, DeLellis & Kablotsky, 1963; Ernst & Ellis, 1969; Schmidt-Nielsen, Jorgensen & Osaki, 1958), which are advantageous for the study of plasma membrane biogenesis (Barrnett et al. in preparation; Levine et al. 1972). The baso-lateral plasma membranes of the secretory cells of ducklings, which undergo amplification in response to salt stress, contain the marker enzyme, Na,K-ATPase, and biochemical (Ernst, Goertemiller & Ellis, 1967) and cytochemical (Ernst, 1972) assays indicate that its activity increases in parallel with plasma membrane amplification. Elimination of the salt stress causes a reversal of the above processes in that both the plasma membrane area (Hossler & Sarras, in preparation) and Na,K-ATPase activity decrease (Ernst et al. 1967).

In order to use Na,K-ATPase as a plasma membrane marker protein in situations involving a net gain or loss of membrane, it is essential that the total number of enzyme molecules in the membrane be measured. Although determination of changes in enzyme specific activity provides suggestive evidence for enzyme synthesis and incorporation or loss, it does not take into account activation or inhibition of pre-existing enzyme.

Ouabain is a specific inhibitor of the Na,K-ATPase (recently reviewed by Schwartz, Lindenmayer & Allen, 1975) and binds essentially irreversibly to this enzyme on a one to one basis (Jorgensen, 1974; Kyte, 1972); thus its binding is a measure of the number of Na,K-ATPase molecules. In addition, [3H]ouabain autoradiography has been used for localization of the Na,K-ATPase in a variety of tissues (Ernst & Mills, 1975; Karnaky, Kinter, Kinter & Sterling, 1976; Mills & Ernst, 1975a, b; Quinton, Wright & Tormey, 1973; Stirling, 1972, 1976). This paper describes the conditions for optimal ouabain binding in the duck salt gland and shows that this binding is specific. In addition we demonstrate biochemically and by autoradiography that ouabain binding increases and decreases respectively during salt stress and freshwater refeeding in parallel with changes in Na,K-ATPase activity.
MATERIALS AND METHODS

Ducklings

Day-old domestic ducklings were purchased from C. and R. Duck Farm, Inc., Long Island, N.Y. and maintained on starter mash and freshwater for one week before the start of salt stress. At 7 days 1% NaCl was substituted for the drinking water and the water in the mash for the experimental ducklings. Control ducklings remained on the initial diet. In each case food and water was supplied ad libitum and the ducklings were housed in cages provided with a cycle of 16 h of light and 8 h of darkness.

Preparation of salt gland homogenates

At various times after initiation of the salt stress, experimental and control ducklings were sacrificed by decapitation and the salt glands were rapidly removed and freed from the surrounding connective tissue. Salt glands were then diced into 3-mm blocks, and washed briefly in ice-cold 0.05 M cacodylate-HCl buffer (pH 7.2) containing 0.25 M sucrose. Initially, both fresh and parafomaldehyde-fixed tissues were used until it was discovered that paraformaldehyde-fixed tissue bound more ouabain than unfixed tissue (see Results). Fixation was accomplished by immersion of buffer-washed blocks for 1 h in ice-cold 4% freshly prepared paraformaldehyde in 0.05 M cacodylate-HCl (pH 7.2) containing 0.25 M sucrose. After fixation, the tissue was washed in 3 changes (10 min each) of ice-cold 0.05 M cacodylate-HCl (pH 7.2) containing 0.25 M sucrose, and then in 3 changes (15 min each) of 0.1 M Tris-HCl (pH 7.5) containing 0.25 M sucrose. The tissue was then homogenized in a glass homogenizer (20 mg wet weight per ml of the same Tris-sucrose buffer) and assayed immediately.

Ouabain binding

Determination of ouabain-binding sites was accomplished by a modification of the method described by Hoffman (1969). Salt gland homogenates (4-10 mg wet weight of tissue) were shaken for 15 min at 37°C in an incubation medium (volume, 0.4-2.0 ml) containing 3 mM ATP, 6 mM MgCl₂, 100 mM NaCl, 1 x 10⁻⁴ M [³H]ouabain (0.1 Ci/mmol), and 50 mM Tris-HCl (pH 7.5) at 37°C. The control medium lacked ATP and MgCl₂, and contained 5 mM EDTA and 10 mM KCl. The reaction was started by the addition of homogenate and was stopped by placing the incubation tubes in a bath of crushed ice. No ouabain binding occurred at 0°C. Unbound [³H]ouabain was removed by 4 sequential centrifugations (5 min at 35,000 g). Pellets were resuspended after each centrifugation in a washing solution which was the same as the incubation medium except that it was ice-cold and lacked ATP and ouabain. During these washes, Na,K-ATPase was not solubilized and lost, as demonstrated in control experiments from which ouabain was omitted from the incubation medium; Na,K-ATPase activity was not detectable in any of the supernatants. Measurements of ³H radioactivity in the wash solutions indicated that all of the unbound [³H]ouabain was removed during the first 2 washes. The binding of [³H]ouabain was essentially irreversible since the amount bound was not decreased by including 10⁻⁶ M unlabelled ouabain in the wash solutions. The washed tissue pellets were resuspended in toluene-Triton X-100 scintillation fluid with a motor-driven pestle, and counted in an Intertechnique Model L30 scintillation counter (which had a counting efficiency for ³H of 35%). Counts were corrected using an internal standard.

Light microscopy and autoradiography

Fresh salt gland was cut into 3-4-mm³ blocks, immersed in ice-cold 5% polyvinyl alcohol in 0.05 M cacodylate-HCl buffer (pH 7.5) for 45 min, then quick frozen by immersion in liquid N₂. Sections, 6-8 μm thick, were cut in a cryostat, mounted on glass slides coated with albumin, and immersed for 1 h in freshly prepared, ice-cold 4% paraformaldehyde in 0.05 M cacodylate-HCl buffer (pH 7.2) containing 0.25 M sucrose. After fixation, the sections were washed by immersing the glass slides in 2 changes (10 min each) of 0.05 M cacodylate-HCl buffer (pH 7.2) containing 0.25 M sucrose, and then in 3 changes (15 min each) of 0.1 M Tris-HCl buffer (pH
For light microscopy the sections were stained in 1 % toluidine blue for 10 s.

For autoradiography experiments the tissue sections were flooded with either the \([^3H]\)ouabain or control media (see binding experiments) and incubated in a humidified chamber for 15 min at 37 °C. After incubation, the unbound \([^3H]\)ouabain was removed by immersing the slides in 3 changes of ice-cold wash solutions (as above) for a period of 45 min. Autoradiograms were prepared by dipping the slides in Ilford L4 photographic emulsion and allowing an exposure of 3 days to 3 weeks. Slides were developed in D-19 developer and observed by bright-field or dark-field light microscopy, either stained with toluidine blue or unstained.

Enzyme assay and chemicals

ATPase activity was determined by colorimetric measurement of Pi, released (Rendi, 1966). The assay medium for ATPase contained 3 mM ATP, 6 mM MgCl2, 100 mM NaCl, 10 mM KCl, and 50 mM Tris-HCl (pH 7.5 at 37 °C), with or without 1 mM ouabain or 5 mM EDTA. Tissue homogenates were incubated in this medium for 15 min at 37 °C. Na,K-ATPase activity was that component of total ATPase activity which required Na+, K+, and Mg2+ and was inhibited by ouabain. ATPase activity, which required Mg2+ and was ouabain resistant, was designated Mg-ATPase activity.

For K+-dependent phosphatase activity (Judah, Ahmed & McLean, 1962), tissue homogenates (0.05-1.0 mg wet weight of tissue) were shaken on a 37 °C waterbath for 15 min in a reaction volume of 1.0 ml containing 3 μmol p-nitrophenyl phosphate, 6 μmol MgCl2, 10 μmol KCl, and 50 μmol Tris-HCl (pH 7.5 at 37 °C). Controls lacked KCl and contained 1 μmol ouabain. The reaction was started by adding the substrate and stopped by adding 3 ml 0.1 M NaOH and placing the tubes on ice. After centrifuging 5 min at 7000 g, the amount of p-nitrophenol in the supernatant was determined spectrophotometrically from the absorbance at 420 nm.

The activity of 5'-nucleotidase was assayed according to the method of Michell & Hawthorne (1965). \([^3H]\)ouabain (12 Ci/mmol) was purchased from New England Nuclear. Ouabain, ATP (as the Tris salt), and p-nitrophenyl phosphate were purchased from Sigma Chemical Co. Ilford L4 autoradiographic emulsion was obtained from Polysciences.

RESULTS

Requirements for optimal ouabain binding in the duck salt gland

Ouabain binding was found to be less sensitive to tissue fixation than was Na,K-ATPase activity. The specific activity of Na,K-ATPase was inhibited 20 % after 1 h of fixation with 4 % paraformaldehyde, and 50 and 99 % inhibition occurred after fixation with 0.1 and 2 % glutaraldehyde, respectively (data not shown). These results are in general agreement with those of Ernst & Philpott (1970) who reported 30 % inhibition after 1 h fixation with 3 % paraformaldehyde and complete inhibition after 1 h fixation with 0.5 % glutaraldehyde.

Surprisingly, however, ouabain binding was enhanced by 43 % after fixation with 4 % paraformaldehyde (Table 1). As a result of this finding, we fixed tissue in 4 % paraformaldehyde prior to preparation of the homogenate for most of our studies.

Table 1 also shows that some ouabain binding occurred even after fixation in 2 % glutaraldehyde, a concentration which completely eliminated Na,K-ATPase activity.

Several ingredients of the Na,K-ATPase medium were found to strongly influence ouabain binding (Table 2). ATP and Mg2+ were required for maximal binding, but omission of the Na+ had no effect. Addition of 10 mM K+ reduced the binding 76 % from maximal levels. Only 5 % of the total ouabain was bound in the absence of in-
Ouabain binding in salt gland

Ingredients of the Na,K-ATPase medium. The same result occurred with unfixed tissue (data not shown) and this suggests that paraformaldehyde fixation did not induce artifactual binding. Preincubation of both fresh and fixed tissue with unlabelled ouabain abolished labelled ouabain binding.

Ouabain binding increased proportionally with increasing tissue content in the incubation from 4 to 20 mg, indicating that the binding assay was quantitatively reliable over the range of tissue wet weight employed (Fig. 1). Binding occurred opti-

Table 1. Effect of various fixatives on $[^3]$H]ouabain binding

<table>
<thead>
<tr>
<th>Fixative</th>
<th>$[^3]$H]ouabain bound, pmol/mg wet wt</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfixed</td>
<td>11.9</td>
<td>100</td>
</tr>
<tr>
<td>4 % paraformaldehyde</td>
<td>17.0</td>
<td>143</td>
</tr>
<tr>
<td>4 % paraformaldehyde + 0.5 % glutaraldehyde</td>
<td>4.83</td>
<td>41</td>
</tr>
<tr>
<td>2 % glutaraldehyde</td>
<td>2.84</td>
<td>24</td>
</tr>
</tbody>
</table>

Salt gland blocks (3 mm$^3$) were fixed by immersion for 1 h in ice-cold paraformaldehyde prepared fresh in 0.05 M cacodylate-HCl buffer (pH 7.2) containing 0.25 M sucrose. After fixation the tissue was washed and homogenized as indicated in Materials and methods. Ouabain binding was determined by incubating the homogenates for 15 min at 37 °C with agitation in a medium containing: 50 mM Tris-HCl (pH 7.5 at 37 °C), 100 mM NaCl, 3 mM ATP, 6 mM MgCl$_2$, and $1 \times 10^{-6}$ M $[^3]$H]ouabain (0.1 Ci/mmol). After incubation, the homogenates were placed on ice and washed 4 times by centrifugation and resuspension in the incubation medium minus ATP and ouabain and counted in toluene-Triton X-100 scintillation fluid.

Table 2. Conditions for $[^3]$H]ouabain binding in the avian salt gland

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Conditions</th>
<th>$[^3]$H]ouabain bound, pmol/mg wet wt</th>
<th>% of complete incubation medium*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salt-stressed gland</td>
<td>Complete incubation medium*</td>
<td>17.0</td>
<td>100</td>
</tr>
<tr>
<td>homogenate</td>
<td>Minus NaCl</td>
<td>17.0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Minus ATP</td>
<td>6.8</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Minus MgCl$_2$</td>
<td>13.9</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>Plus 10 mM KCl</td>
<td>4.08</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Minus MgCl$_2$ and ATP; plus 10 mM KCl and 5 mM EDTA</td>
<td>0.85</td>
<td>5</td>
</tr>
<tr>
<td>Non-stressed gland</td>
<td>Complete incubation medium*</td>
<td>3.70</td>
<td>100</td>
</tr>
<tr>
<td>homogenate</td>
<td>Minus MgCl$_2$ and ATP; plus 10 mM KCl and 5 mM EDTA</td>
<td>0.15</td>
<td>4</td>
</tr>
</tbody>
</table>

Paraformaldehyde-fixed salt gland homogenates were incubated for 15 min at 37 °C with agitation. *The complete incubation medium contained: 50 mM Tris-HCl (pH 7.5 at 37 °C), 100 mM NaCl, 3 mM ATP, 6 mM MgCl$_2$, and $1 \times 10^{-6}$ M $[^3]$H]ouabain (0.1 Ci/mmol). Other procedures were as for Table 1.
mally at 37 °C (Fig. 2) and essentially reached a maximal level after 15 min incubation at that temperature (Fig. 3). Although binding increased slowly with longer incubation times, much of this slow binding was apparently non-specific as indicated by the slope of the control binding curve.

Fig. 1. Linear increase in ouabain binding with increasing gland wet weight. Ouabain binding conditions were as described in Table 1. The values shown have been corrected for controls.

Binding increased with increasing ouabain concentrations up to 1 × 10^{-6} M (Fig. 4). Half maximal binding was obtained at about 1.7 × 10^{-7} M. These results correlated well with the results observed in Na,K-ATPase inhibition studies (Fig. 5), in which 100% inhibition was obtained at about 1 × 10^{-6} M ouabain, and the $K_i$ for ouabain (estimated by inspection) was about 1.7 × 10^{-7} M.

**Effect of salt stress on ouabain binding**

The effect of salt stress on the number of ouabain-binding sites in the salt gland was determined since this treatment has been shown to induce simultaneously an amplification of the baso-lateral plasma membrane (Ernst & Ellis, 1969) and an in-
crease in the activity of the Na,K-ATPase (Ernst, 1972; Ernst et al. 1967) in the specialized secretory cells of the salt gland.

A time course was obtained during salt stress in which Na,K-ATPase activity and ouabain-binding capacity were simultaneously measured in each salt gland (Fig. 6). Because some variation occurred among glands from different ducks, each point represents an average value obtained from repeated assays of glands from a number of ducks (see figure legend for details). There was a sizable increase in both Na,K-ATPase activity and in ouabain binding beginning at least as early as 24 h after the onset of salt stress and continuing in each case up to 7–9 days. Both activities increased 4–5 fold. Fig. 6 also shows that the number of ouabain-binding sites decreased in parallel with the decrease in Na,K-ATPase activity following a return of the ducks to a freshwater diet.

In addition, the ratio of Na,K-ATPase activity to ouabain-binding capacity remained relatively constant throughout the period of salt stress despite the large increases in both activities (Fig. 7). In contrast, the ratio of Na,K-ATPase activity to that of
Mg-ATPase rose from 0.45 at day zero to about 1.6 at day 8 of the salt stress, and fell again to 1.0 at day 9.

The disproportionate increase in Na,K-ATPase related activities is further emphasized in Table 3 in which the specific activity of the Na,K-ATPase is compared with

Fig. 3. Effect of incubation time on ouabain binding. Ouabain-binding conditions were as described in Table 1. ○, experimental values; ●, control values (lacking ATP and Mg²⁺; containing EDTA and KCl).

related and unrelated enzyme activities in the stressed and non-stressed salt gland. Ouabain binding and K⁺-dependent phosphatase are activities associated with the Na,K-ATPase system (Schwartz et al. 1975) and increased in parallel during the salt stress. However the ratios of Na,K-ATPase activity to Mg-ATPase activity and to 5'-nucleotidase activity changed dramatically during salt stressing, even though the latter 2 enzyme activities are considered to be associated with the plasma membrane. This is likely a reflection of the salt-induced morphological and functional (Schmidt-Nielsen et al. 1958) specialization of the secretory cells of the salt gland.
Ouabain binding in salt gland

Ellis et al. (1963), have provided histochemical and morphological evidence that the unspecialized, generative cells of the salt gland lie at the periphery of the gland lobes, and that the more mature, partially and fully specialized secretory cells are located

\[ \text{Fig. 4. Effect of ouabain concentration on ouabain binding. Ouabain binding conditions were as described in Table 1. O, experimental values; \bigcirc, control values (lacking ATP and Mg}^{2+}; \text{containing EDTA and KCl).} \]

\[ \text{Fig. 5. Inhibition of salt gland Na,K-ATPase by ouabain. ATPase activity was determined by incubating tissue homogenates (1-2 mg wet weight) for 15 min at 37 °C in a medium containing 3 mM ATP, 6 mM MgCl}_2; 100 mM NaCl, 10 mM KCl and 50 mM Tris-HCl, pH 7.5 at 37 °C. Control media lacked one or more of the above ingredients, or contained either 5 mM EDTA or various concentrations of ouabain. Only Na,K-ATPase activity is shown and is defined as that portion of the total ATPase activity which is dependent upon Na}^+, K}^+, \text{and Mg}^{2+} \text{and is ouabain-sensitive.} \]
progressively deeper in the lobe towards the medulla. If this pattern of specialization were true, it would be revealed by the distribution of ouabain-binding sites in the lobes of the gland, since the degree of specialization of secretory cells should be reflected by their content of Na,K-ATPase. This hypothesis was tested using \[^{3}H\]ouabain autoradiography. Fig. 8A is a cross-section of a typical lobe of a salt gland from a duckling salt stressed for 9 days. Secretory tubules radiate out from the medulla which contains one main excretory duct surrounded by connective tissue; numerous adjoining, smaller ductules connect this duct with the secretory tubules which end in acini at the periphery of the gland. Connective tissue septa containing blood vessels and nerves separate adjacent lobes. Fig. 8B is an autoradiogram of an adjacent, unstained section of the same lobe after incubation with \[^{3}H\]ouabain viewed with dark-field microscopy. Exposed silver grains appear white and are heavily deposited over

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**Fig. 6.** Effect of salt-water feeding (started at arrow) on Na,K-ATPase activity (A) and number of ouabain sites (B) in the salt gland. The conditions for Na,K-ATPase assay and ouabain binding were as described in Fig. 5 and Table 1, respectively. All values were corrected for controls and each point represents an average value obtained from salt glands from at least 5 ducklings. ○, ducklings fed salt water; ●, ducklings which were returned to freshwater.
Ouabain binding in salt gland

Fig. 7. Effect of salt water feeding (started at arrow) on the ratio of Na,K-ATPase activity to Mg-ATPase activity (○) and ouabain binding (●) in salt gland. Na,K-ATPase activity and ouabain binding were determined as described in Fig. 5 and Table 1, respectively. Mg-ATPase activity is defined as that portion of the total ATPase activity which requires Mg²⁺ and is insensitive to ouabain. Note that the ratio of Na,K-ATPase activity to ouabain binding was multiplied by a factor of 10⁻³ in order to plot both ratios on the same log scale.

Table 3. Changes in ratios of enzyme activities during salt stress

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Activity assayed</th>
<th>Specific activity (A)</th>
<th>Na,K-ATPase A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-stressed gland homogenate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na,K-ATPase</td>
<td>283 nmol Pi/min/mg</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>K⁺-dependent phosphatase</td>
<td>1.77 nmol Pi/min/mg</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Ouabain binding</td>
<td>3.70 pmol/mg</td>
<td>7.7 x 10⁻²</td>
<td></td>
</tr>
<tr>
<td>Mg²⁺-ATPase</td>
<td>6.27 nmol Pi/min/mg</td>
<td>6.45</td>
<td></td>
</tr>
<tr>
<td>5'-nucleotidase</td>
<td>1.78 nmol Pi/min/mg</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Salt-stressed gland homogenate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na,K-ATPase</td>
<td>14.2 nmol Pi/min/mg</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>K⁺-dependent phosphatase</td>
<td>11.1 nmol Pi/min/mg</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>Ouabain binding</td>
<td>17.0 pmol/mg</td>
<td>8.3 x 10⁻²</td>
<td></td>
</tr>
<tr>
<td>Mg²⁺-ATPase</td>
<td>11.8 nmol Pi/min/mg</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>5'-nucleotidase</td>
<td>1.47 nmol Pi/min/mg</td>
<td>9.7</td>
<td></td>
</tr>
</tbody>
</table>

Ouabain binding was determined as described in Table 1. Other enzyme activities were determined as described in the Materials and methods section.
Fig. 8. Distribution of ouabain-binding sites in stressed salt gland as indicated by [\(^3\)H]ouabain autoradiography. The morphology of a typical lobe is shown in the toluidine blue-stained section in A. An autoradiogram of an adjacent unstained section incubated with [\(^3\)H]ouabain and viewed with dark-field microscopy is shown in B. The inset C is a higher magnification of the area outlined in B. ct, connective tissue; bv, blood vessel; d, main excretory duct; d', small excretory duct; arrows, central secretory tubules; arrowheads, peripheral secretory tubule. A, B, × 110; C, × 275.
Fig. 9. Distribution of ouabain-binding sites in stressed salt gland as determined by \[^{3}H\]ouabain autoradiography. The morphology of the gland lobe is shown in the toluidine blue-stained section in A. An autoradiogram of an adjacent unstained section incubated with \[^{3}H\]ouabain and viewed with bright-field microscopy is shown in B. The central secretory tubules (arrows) nearest the medulla (white area) of the lobe are heavily labelled while the more peripheral tubules (arrowheads) contain progressively fewer silver grains. Other symbols are as in Fig. 8. \( \times 140 \).
the secretory tubules, but are absent over ducts, blood vessels, and connective tissue. The inset Fig. 8c is a higher magnification of a section of the lobe in Fig. 8b, which indicates that the silver grains are denser over the more central tubules than over the peripheral tubules. The gradient of ouabain binding is more pronounced in Fig. 9b which reveals only radiographic grains viewed with bright-field microscopy. Ouabain-binding sites are heavily concentrated in the more centrally located tubules; clearly these sites are less evident towards the periphery of the lobe where less-specialized cells occur. Fig. 9a, its parallel section, shows a stained cross-section of salt-stressed gland.

A higher magnification of individual tubules from the central, most reactive, part of the lobe are shown in Fig. 10b to demonstrate by autoradiography the discreteness of the [3H]ouabain-binding reaction. Silver grains cover the tubule but are absent from the connective tissue between tubules. Fig. 10a is a parallel section stained with toluidine blue. Fig. 10c is an autoradiogram of a similar tubule after incubation in the [3H]ouabain control medium (see Table 2) which lacked ATP and MgCl₂, but contained KCl and EDTA. As expected from the results in Table 2, only a few scattered exposed silver grains are detected.

DISCUSSION

Several of the above results indicate that ouabain binds specifically to the Na,K-ATPase of the secretory cells of duckling salt glands. In the first place, 95% of the ouabain bound is affected by biochemically characterized ingredients of the Na,K-ATPase medium in both fresh and paraformaldehyde-fixed tissue. There is good evidence that certain conformations of the Na,K-ATPase promote ouabain binding (Schwartz et al. 1975) and one of these is the 'high energy' phosphoenzyme (designated E₁→P) (Post, Kume, Tobin, Orcutt & Sen, 1969). E₁→P can be formed by incubation of the enzyme with ATP and Mg²⁺ in the presence of Na⁺, but is destroyed when K⁺ is added (Post et al. 1969). The present results indicate that ouabain binding in the salt gland is enhanced by ATP and Mg²⁺, and this binding is greatly reduced by the presence of K⁺. The apparent lack of Na⁺ requirement could be explained by the presence of small amounts of endogenous Na⁺ in the tissue homogenates which might be expected despite fixation and washing because of high concentrations in vivo. In this regard, Han, Tobin, Akera & Brody (1976) have recently reported that both the formation of E₁→P and the binding of ouabain to rat brain Na,K-ATPase preparations are strongly stimulated by low concentrations of Na⁺ (Kᵣ ≈ 1 mM) in

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Fig. 10. Specificity of ouabain binding in individual secretory tubules. Shown are high-magnification light micrographs of cross-sections of individual secretory tubules taken from adjacent sections of salt-stressed gland. A, a tubule which was untreated and stained with toluidine blue. B, autoradiogram of the same tubule after incubation in the [3H]ouabain-binding medium (see Table 2). Silver grains are heavy over the tubule cells (tc) but are absent over the connective tissue (ct) cells. C, autoradiogram of a similar tubule after incubation in the [3H]ouabain control medium which lacked ATP and MgCl₂ and contained KCl and EDTA (see Table 2). Only a few scattered silver grains are visible as background. × 1100.
the presence of ATP and Mg\(^{2+}\); at higher Na\(^+\) concentrations, the stimulation is less. Thus, the present results could be explained by stimulation of ouabain binding by low concentrations of endogenous Na\(^+\). Further stimulation of binding by the higher concentrations of added Na\(^+\) was not detectable.

Secondly, maximal binding (saturation of binding sites) is obtained at the same ouabain concentration (1-2 \times 10^{-6} \text{ M}) which produced complete inhibition of Na,K-ATPase activity. In addition, the concentration of ouabain which gives half maximal binding is identical to the \(K_t\) for inhibition (1.7 \times 10^{-7} \text{ M}). Ernst & Philpott (1970) reported total inhibition of the Na,K-ATPase at 10^{-4} \text{ M} ouabain and found the \(K_t\) for ouabain to be 1.26 \times 10^{-6} \text{ M}. The differences in sensitivity to ouabain is best explained by the differences in Na,K-ATPase assay conditions, including tissue preparation of the 2 experiments. We determined the optimal assay conditions at higher concentrations of NaCl, KCl, MgCl\(_2\) and a slightly higher pH. Although both sets of conditions could yield ouabain-sensitive conformations of the Na,K-ATPase, the present results confirm those of Schwartz et al. (1975) and indicate the degree of sensitivity to which ouabain binding is influenced by ingredients of the Na,K-ATPase medium.

Finally, the present experiments indicate a constant ratio of Na,K-ATPase activity to ouabain binding during the 9-day period of salt stress. Despite large increases in both activities, the ratio remained approximately 0.8 \times 10^{-3}. The 2 activities also exhibit a parallel drop when salt-stressed ducklings were fed fresh water.

Stimulation of ouabain binding by paraformaldehyde fixation was surprising and initially seemed inconsistent with the fact that aldehydes inhibited to a variable degree the activity of Na,K-ATPase. However, fixation which is known to increase tissue permeability, could expose additional, poorly accessible binding sites. In addition, paraformaldehyde fixation could favour a ouabain-binding conformation of the enzyme complex. A variety of conditions are known to yield such conformations, and these need not be conditions which lead to an active state of the enzyme (Schwartz et al. 1975).

In consideration of the potential usefulness of the duck salt gland as a model system for studies of plasma membrane biogenesis, a plasma membrane protein marker, such as the Na,K-ATPase, to monitor synthesis and assembly is useful if not crucial. At first glance the increase in Na,K-ATPase activity and ouabain binding during plasma membrane amplification does not necessarily prove new synthesis since it does not rule out activation or change in conformation of pre-existing enzyme. However, given the binding properties of ouabain to Na,K-ATPase (Jorgensen, 1974; Kyte, 1972), the increase in binding sites observed in these experiments during salt stress, unlike specific enzyme activity, is more consistent with \textit{de novo} synthesis of this enzyme. This suggestion is made more substant by the fact that the ducklings had not been previously exposed to salt. Another communication (Barrnett et al. in preparation) will indicate that the initial increases of enzyme activity to salt stress is inhibited by actinomycin or puromycin. Therefore, the increase in ouabain binding provides a reliable measure of the increase in synthesis and assembly of at least this plasma membrane protein during the period of salt stress.
A comparison of Na,K-ATPase activity with other plasma membrane-associated enzyme activities, 5'-nucleotidase and Mg-ATPase, in salt-stressed and non-stressed salt glands, provides further evidence that salt stress preferentially induces increases in selected plasma membrane proteins, namely those involved in Na+ transport. Autoradiography with [3H]ouabain indicates that this selected increase in the Na,K-ATPase parallels the scheme of secretory cell specialization suggested by Ellis et al. (1963). Cells in the peripheral tubules of the gland lobe are unspecialized and contain few ouabain-binding sites (Na,K-ATPase molecules), whereas those nearer the medulla of the lobe are progressively more specialized and contain higher concentrations of ouabain-binding sites.

If the plasma membranes of the duckling salt gland were the only subcellular compartment to undergo amplification during salt stress, the salt gland would provide an advantageous system for the study of plasma membrane biogenesis which could be conveniently controlled (Ernst & Ellis, 1969). This is not the case, however, since there is marked increase in the Golgi compartment (Levine et al. 1972); amplification of the mitochondrial compartment also occurs and shall be reported on in another paper. Since several compartments respond in this unique system, Na,K-ATPase activity and ouabain binding become important measures. The advantages of using ouabain binding as a label for plasma membrane protein biogenesis are: the number of ouabain binding sites quantitatively measures the number of molecules of an intrinsic, plasma membrane protein; ouabain binding does not necessarily require an active state of the enzyme (Schwartz et al. 1975); ouabain binding is less sensitive to fixatives (and is actually stimulated by paraformaldehyde) than Na,K-ATPase activity, and thus may be more useful in procedures involving localization with electron microscopy; ouabain is bound avidly (the $K_i$ for ouabain is $1.7 \times 10^{-7} \text{M}$) and tightly (bound [3H]ouabain is not displaced by washing with excess unlabelled ouabain) to the salt gland Na,K-ATPase; and the procedure for ouabain binding is simple, rapid, and reproducible. Although the present work is not directed toward identifying the sites of synthesis and/or assembly of Na,K-ATPase prior to plasma membrane insertion, it forms a basis for further work directed to this problem. It will be addressed first by the fine-structural localization of Na,K-ATPase with a method which utilizes an electron-dense derivative of ouabain (Mazurkiewicz, Hossler & Barnett, 1974) which has similar binding properties to the native compound.

This work was supported by grants TOIGM-105 and AM-03688, National Institutes of Health, Department of Health, Education and Welfare.

REFERENCES


Ouabain binding in salt gland


(Received 21 June 1977)