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Localization of Na/K-ATPase Sites in the Secretory and Reabsorptive Epithelia of Perfused Eccrine Sweat Glands: A Question to the role of the enzyme in secretion

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Summary. Na/K-ATPase sites in both the secretory and reabsorptive epithelia of isolated and microperfused human eccrine sweat glands are localized cytologically. Localization was accomplished through autoradiography of bound ^{3}H-ouabain, a specific inhibitor of the enzyme. Ouabain binding characteristics were determined to ensure maximum specific binding. Enzyme sites are localized only on the basolateral surface of both epithelia in spite of the fact that sodium transport is reversed, i.e., secretory: blood to lumen and reabsorptive: lumen to blood. In view of these findings and in comparison with other recent observations, the role of Na/K-ATPase in secretory electrolyte transport is questioned.

Although the properties of active fluid and electrolyte movement have been studied extensively in a wide variety of transporting epithelia, a definitive localization of the cytological structures primarily responsible for active transport has not advanced much beyond logical speculation. In general, in absorptive epithelia it is believed that the primary driving force involves the transport of Na^+ coupled to a Na/K-ATPase and that this enzyme, located at one side of the cell, drives fluid transport by actively removing Na^+ which has passively diffused into the cytoplasm at the contralateral side. In this case, fluid is moved in the direction of lumen to blood. For secretory epithelia, in which the transport direction is reversed, the situation is less clear and not only has the above sequence been proposed, but alternatively it has been suggested that solute is actively transported into the cytoplasm as the primary step in the fluid movement process [6]. The cytological location of the active transport step is of prime concern to any theory on fluid transport,

but is particularly critical to understanding differences between absorptive and secretory epithelia.

Thus, it was anticipated that a simultaneous comparison of the cytological localization of the Na/K-ATPase enzyme in a secretory and absorptive epithelium would help clarify (1) the mechanisms by which these tissues transport fluids in opposite directions, apparently irrespective of cellular ultrastructure, and (2) the role of the Na/K-ATPase in fluid transporting processes.

The human eccrine sweat gland offers a unique opportunity to examine the structure-function relationships of Na/K-ATPase in both types of transporting epithelia (Fig. 1). The general structure of the gland is that of a coiled tubule (ca. 4 mm long and 40 μm in diameter), blind at one end and opening onto the epidermis at the other. The proximal half of the tubule consists of an epithelium that secretes an isotonic precursor into the distal half, which consists of an absorptive epithelium. As the precursor courses through the reabsorptive duct, Na and Cl are removed hypertonically, so that the final sweat is markedly hypotonic. Thus, the gland combines a secretory epithelium which transports salts into the lumen with an absorptive epithelium which transports salts back into the blood.

Only a few approaches toward resolving the location and role of Na/K-ATPase in fluid movement appear feasible including: (1) the isolation and separation of epithelial plasma membrane fragments [25], (2) conjugated antibody labeling of the enzyme [21, 22], (3) modified histochemical PO_4 capture techniques [11], and (4) autoradiographic localization of label ouabain, a firmly binding enzyme inhibitor [31, 32]. This latter procedure is by far the most straightforward of approaches thus far devised, and it should be noted that ouabain sensitivity is generally a criterion for enzyme activity. However, this approach poses problems in some tissues, particularly glandular structures, in that delivery of the label to all cell surfaces cannot be assumed. In view of these considerations taken with our ability to microperfuse the entire isolated gland so that all cell surfaces were exposed to label, we chose autoradiographic localization of ^{3}H-ouabain in perfused, isolated sweat glands as the procedure of preference for studying the location and function of Na/K-ATPase in the epithelia of this organ.

Due to the small size (20–30 μg) of the glands and the technical difficulty involving their dissection, it was not possible to determine extensive biochemical parameters of the inhibitor-enzyme binding complex in this tissue. However, characterization of the complex in more

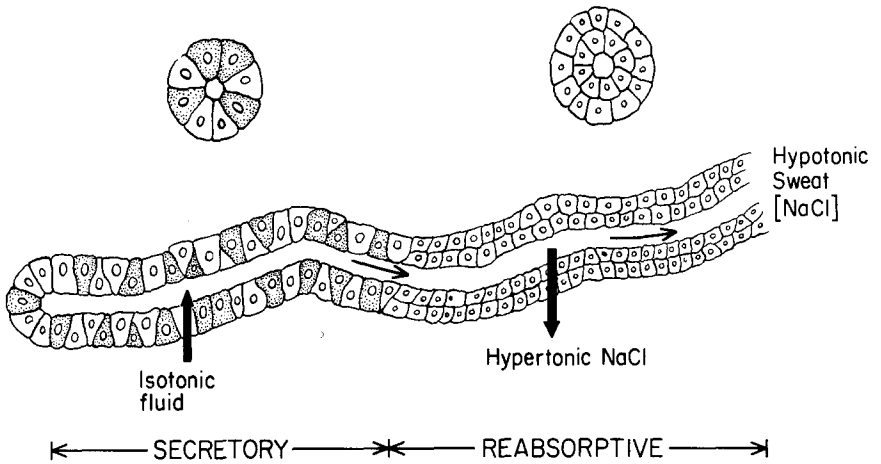

Fig. 1. Schematic diagram of human eccrine sweat gland. Isotonic sweat is secreted into the lumen of secretory tubule of the gland as indicated by the first large black arrow. The fluid thus formed moves as indicated by the small arrows through the tubule until arriving at the resorptive duct where NaCl in excess of water is removed as indicated by the second large arrow. The final sweat so modified emerges on the surface of the skin as a relatively hypotonic fluid, e.g., containing only 10–20 mM NaCl. Note that the secretory tubule consist of two distinct cell types forming a pseudo-stratified epithelium while the reabsorptive duct consists of a double layer of relatively similar cells. Although it is suggested that the two secretory cell types represent fluid transporting cells and mucus secreting cells [26], no explanation is available regarding the functional reason for a double layer in the absorptive epithelium

accessible tissues has been determined, so that, by demonstrating the same general characteristics in isolated, nonperfused glands, we were able to maximize binding conditions and minimize the possibility of nonspecific binding.

Materials and Methods

Due to the time of the technical preparation required to perfuse an isolated sweat gland, only glands used for autoradiography were microperfused. Sucrose wash-out, time-uptake, concentration-uptake, and potassium sensitivity studies, which require numerous glands, were performed on isolated, nonperfused whole sweat glands.

Source

Punch biopsy skin plugs 3.0 mm in diameter were taken from the volar surface of the forearms of Caucasian male volunteers. Prior to biopsy a small wheal was formed by the subdermal injection of 1% lidocaine in 0.6% saline for anesthesia. Plugs were placed under mineral oil immediately after removal.

Dissection

To facilitate dissection of individual glands, skin plugs were cut perpendicularly to the epidermal surface to yield slices about 1.0 mm thick. Slices were bathed in 100% O_2 bubbled, PO_4 buffered Ringer's solution (132.5 mM NaCl, 1.0 mM $CaCl_2$, 5.0 mM KCl, 2.0 mM Mg SO_4, 0.375 mM NaH_2PO_4, 2.125 mM Na_2HPO_4, 10.0 mM dextrose) pH 7.3 at 23 °C, containing 0.005% neutral red as a vital stain. After a few minutes exposure to neutral red, the sweat gland could be seen distinctly. The nuclei of the secretory tubule cells stained more intensely than their cytoplasm, while the reabsorptive duct cells stained only palely. However, the reabsorptive duct apparently actively secretes the stain, filling the duct lumen with an intensely red solution which is easily visualized.

Size Quantitation

Whole single sweat glands were dissected carefully from the collagenous matrix with Dumont No. 5 forceps sharpened to extremely fine points. The glands are so small that protein determinations and wet or dry weight measurements are impractical. Therefore, the volume of each gland was determined by transferring it into a glass measuring tube (2.5 mm I.D.) which had been pulled to a capillary tip with an internal bore (usually 200 μ I.D.) for a length of 1–2 mm. With the measuring tube filled with Ringer's solution, a gland was pushed down the tube with a thin glass rod and thereby compressed within the length of the tip. The volume of the gland was then determined as function of the length of capillary it occupied. Gland sizes were usually on the order of 20 nl. The accuracy of measurements is estimated conservatively at ±15%. After measurement, glands were transferred to appropriate incubation media.

^{14}C-Sucrose Wash-Out

The time required to rinse isolated nonperfused glands virtually free of unbound solutes was determined by preloading glands with ^{14}C-sucrose (50 μCi/ml) for 1 hr. Loaded glands were then rinsed for predetermined intervals (Fig. 2). Subsequently single glands were dissolved in 150 μl of Protosol tissue solubilizer (NEN) at 45 °C overnight. ^{14}C was counted by liquid scintillation to at least 5% accuracy.

Accuracies greater than 5% on some samples were not possible due to constraints on counting time (50 min per sample). On the other hand, the small amount of tissue produced no detectable quenching effects.

From Fig. 2, it is seen that after 2.0 min only 5% of the initial sucrose load remains. After 10 min rinsing, less than 2% remains. Therefore, all ouabain uptake studies and all tissues prepared for autoradiography were rinsed for 10 min after exposure to the labeled ouabain.

^{3}H-ouabain Uptake vs. Time and Concentration

In order to determine an adequate incubation period, glands were exposed for increasing intervals to 7.5×10^{-8}M ouabain (NEN, lot #747–170, 12 Ci/mM). As shown in Fig. 3, uptake increased with time toward apparent saturation. Although not shown in Fig. 3, binding was less after 3 hr than after 2 hr—presumably due to tissue deterioration. Since it appeared that most binding at this low concentration was accomplished within 1 hr, all subsequent studies were performed on glands exposed for 1 hr to isotope.

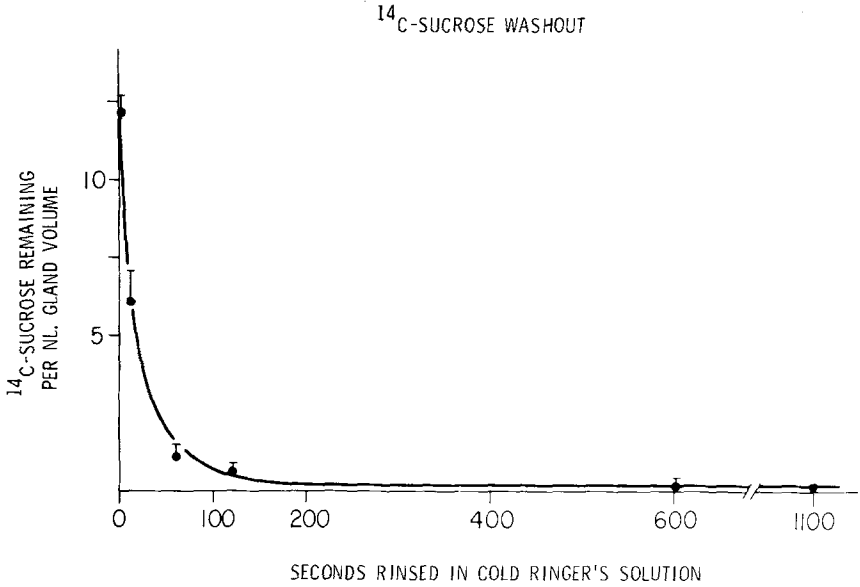

Fig. 2. Washout kinetics for sucrose. After incubation for 60 min with ^{14}C-sucrose, single glands were washed for predetermined times. The amount of label remaining in the tissue in cpm (ordinate) is plotted against each rinse time (abscissa). Vertical bars represent the SEM for 3–6 isolated glands at each time represented. Curve fitted by eye

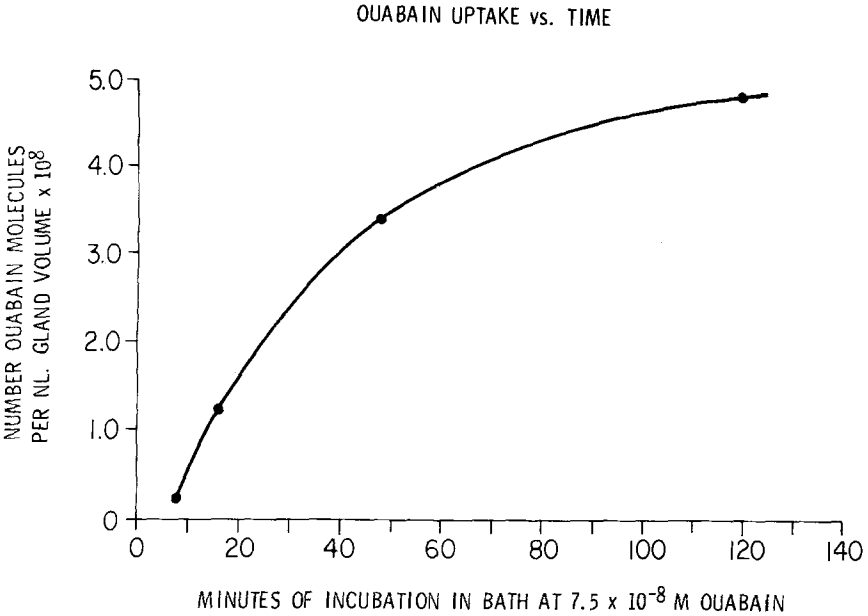

Fig. 3. Uptake of ^{3}H-oubain *vs.* incubation time. The amount of ^{3}H-ouabain taken up (ordinate) at 7.5×10^{-8} M ouabain is plotted against varying incubation times (abscissa). Each point represents the average of two glands incubated independently

Ouabain uptake as function of bath ouabain concentration was then determined. Single glands were incubated for 60 min in Ringer's solutions containing isotopically labeled ouabain in concentrations increasing by 10-fold from 5×10^{-9} to 5×10^{-5}M.

For each uptake determination, 10 to 15 glands were dissected from the same biopsy plug, and 2 to 3 glands were incubated in each concentration of ouabain. After incubation, to be certain all free solution ouabain was removed, all glands were rinsed independently for 10 min. Tritium was counted in single glands by the procedure described above under "^{14}C-Sucrose Wash-out".

Autoradiographic Localization

Nonperfused single glands were incubated in 5×10^{-6}M ^{3}H-ouabain and rinsed as described above. In order to provide a substrate matrix during freeze-drying, glands were then transferred to a Ringer's solution containing 5% dextran for 5 to 10 min before freezing in liquid propane at −170 °C. Frozen glands were freeze dried at −70 °C and 1×10^{-6} torr for 5 to 7 days. They were then fixed by exposure to OsO_4 vapors and embedded under vacuum in Spurr plastic medium [35]. Sections were cut approximately 1 μ thick, and conventional autoradiographic procedures were performed using Kodak NTB2 emulsion.

Glands perfused for autoradiography were handled identically to nonperfused glands except that during the incubation period the glands were bathed in a 2% dextran Ringer's solution but perfused with a dextran-free Ringer's solution. Both solutions contained 5×10^{-6}M ^{3}H-ouabain. Perfusion was accomplished by dissecting away the blind end of the secretory tubule from the rest of the gland. After thus obtaining access to the lumen, the gland was connected to a microperfusion pipette (8 μ diameter tip) held concentrically in an outer holding pipette (50 μ inside diameter) modified after renal tubule microperfusion apparatus [3].

Results

^{3}*H-ouabain Uptake vs. Ouabain Concentration*

The curve seen in Fig. 4 demonstrated ouabain binding over 5 orders of magnitude change in concentration. Such a large range of exposures was necessary in order to find an optimal concentration at which to autoradiographically localize the binding sites. Although the points are too few to rigorously derive binding kinetics, saturation does seem to occur at the higher concentrations which we believe reduces the possibility of the presence of a large number of nonspecific sites. At the lowest concentration, 5×10^{-9}M ouabain, accumulated label was so small as to produce count rates that were statistically undiscernible from background within available counting time [40].

However, the continuous curve formed by deriving the best fit for the observed amount bound at each concentration above 5×10^{-9}M to

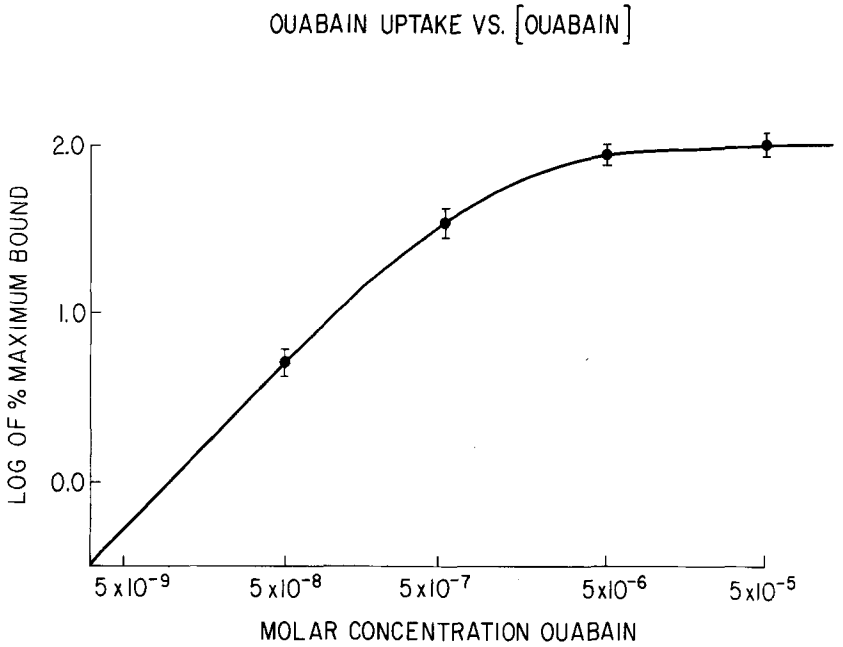

Fig. 4. Uptake of ^{3}H-ouabain concentration. The log of the percent of the maximum amount of ouabain taken up by each gland (ordinate) is plotted against decade increases of ouabain concentrations (abscissa). Vertical bars represent standard errors of 3–5 independent whole incubated for 60 min each at the indicated concentrations. The curve is generated by selecting a K_m of 9.6×10^{-7} M which gives the best fit answer for the Michaelis-Menten equation when maximum binding (100%) is taken as the total binding at 5×10^{-5} M ouabain; i.e., 3.7×10^{-9} molecules/nl gland. Since the cpm's from label bound at 5×10^{-9} M were not sufficiently above background to give a significant measurement within reasonable counting times, this point is not plotted. Vertical bars represent the SEM

the Michaelis-Menten equation leads to a K_m of 9.6×10^{-7} M assuming saturation (100%) binding at 5×10^{-5}M. Assuming the ouabain to site binding ratio is one and a tissue density of one, we extrapolate to 3.72×10^{12} sites per mg of tissue which compared somewhat higher than the values for choroid plexus obtained by Quinton *et al.* [32] and liver and kidney obtained by Baker and Willis [1].

K^+ *Sensitivity*

A rigorous criterion for specificity of binding is the sensitivity of total binding to the concentration of K^+ [10, 13, 32]. In Fig. 5, the total binding in the presence of low K^+ (5.0 mM) in a 135 mM Na-isethionate Ringer's solution is compared to that of high K^+ (140 mM K-isethionate Ringer's solution) and high K^+ less Mg^{++} Ringer's solu-

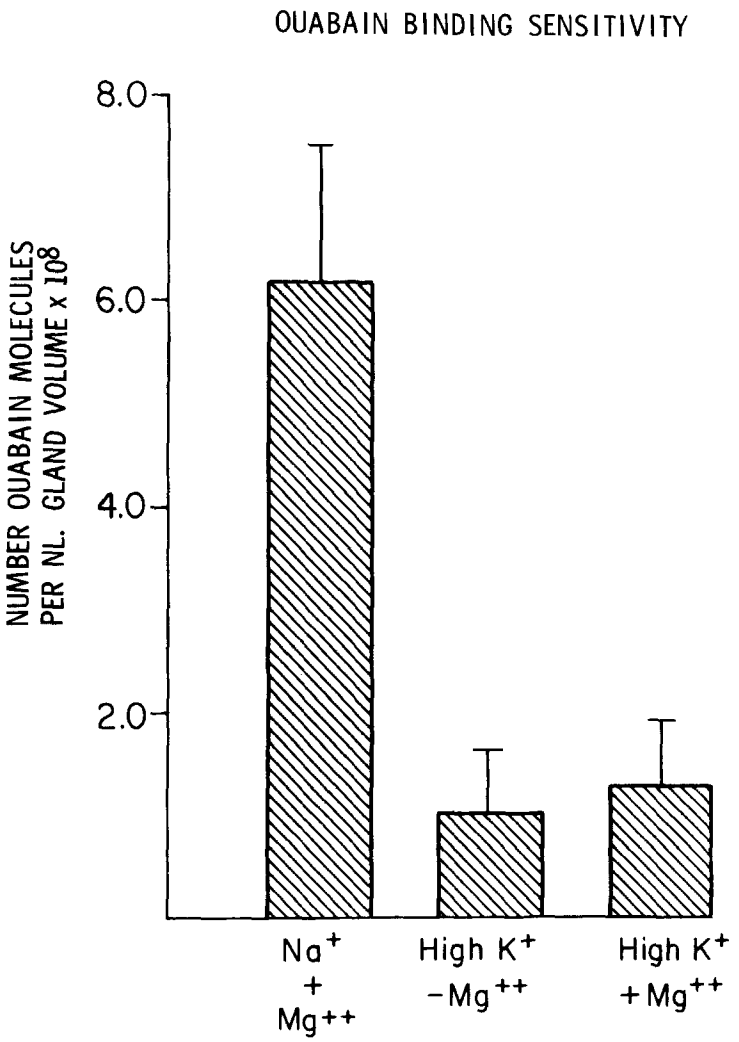

Fig. 5. Effect of K concentration on ^{3}H-ouabain uptake. The amount of ouabain at 5×10^{-7} M taken up in the presence of high K^+ and low Na^+ concentration (K^+-Mg^{++}) is compared to ouabain uptake in the presence of low K and high Na^+ with Mg^{++} ($Na^+ + Mg^{++}$) and high K^+ and low Na^+ with Mg^{++} ($K^+ + Mg^{++}$). The observed reduced binding without Mg^{++} compared to binding with Mg^{++} is expected from biochemical kinetics. All solutions contained basic ingredients of Na_2HPO_4 (2.125 mM), NaH_2PO_4 (0.375 mM), and dextrose (10 mM). High $Na^+ + Mg^{++}$ contained, in addition, Na Isethionate (135 mM) and $MgCl_2$ (2 mM). High K^+-Mg^{++} contained K Isethionate (135 mM), and high $K^+ + Mg^{++}$ contained K Isethionate (135 mM) and $MgCl_2$ (2 mM). In a single experiment at 1.1×10^{-6} M ouabain, the amount bound (molecules/nl gland) was 1.8×10^9, 9.2×10^8, and 8.3×10^8 in $Na^+ + Mg^{++}$, $K^+ + Mg^{++}$, and K-Mg^{++}, respectively

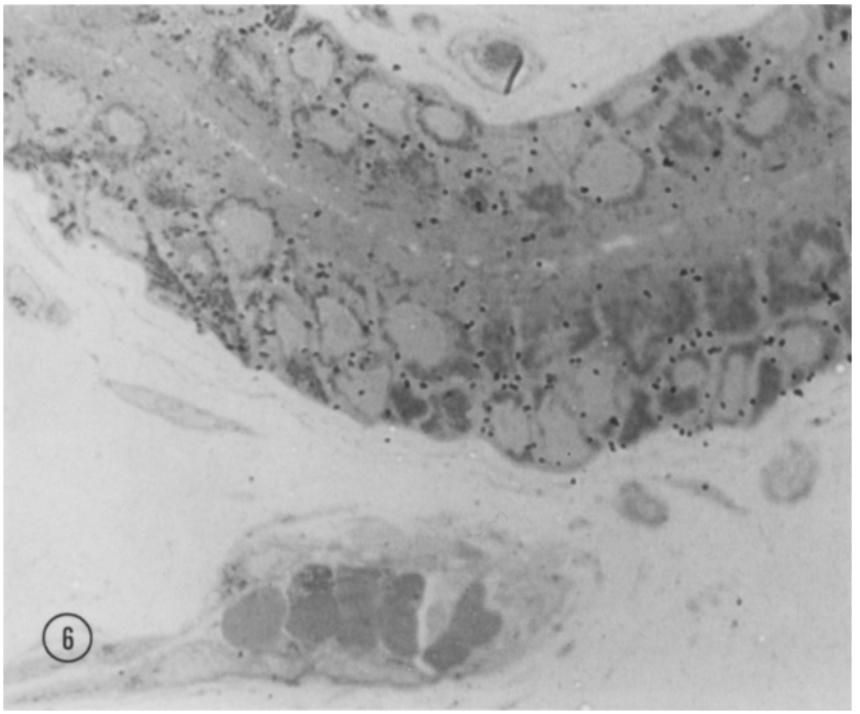

Fig. 6. Light microscope autoradiogram of ^{3}H-ouabain bound in the isolated perfused resorptive duct of the sweat gland. Note that almost all grains are situated in regions very close to the basal and lateral surfaces of cells of both layers of epithelium. In contrast, there is very little binding associated with the apical or luminal cell surface (duct sectioned longitudinally). Stained with methylene blue. 1,300 diameters

tion. In the presence of high K^+ with Mg^{++} the average binding is only about 1/5 of that in the presence of normal high Na, low K Ringer's solution. When Mg^{++} was removed from the high K^+ solution, the average binding decreased further, which is in accord with the enzyme-inhibitor binding properties [13, 23, 32].

Localization

As noted above, the sweat gland consists of two distinct tubular segments: (1) the secretory tubule, and (2) the reabsorptive duct. The distribution of labeled ouabain within the gland was determined by autoradiography of both perfused and nonperfused glands. No significant luminal binding was detected in any portion of either perfused or nonperfused glands, and contraluminal binding patterns were identical whether glands were perfused or not. In general (Figs. 6 and 7), the label was

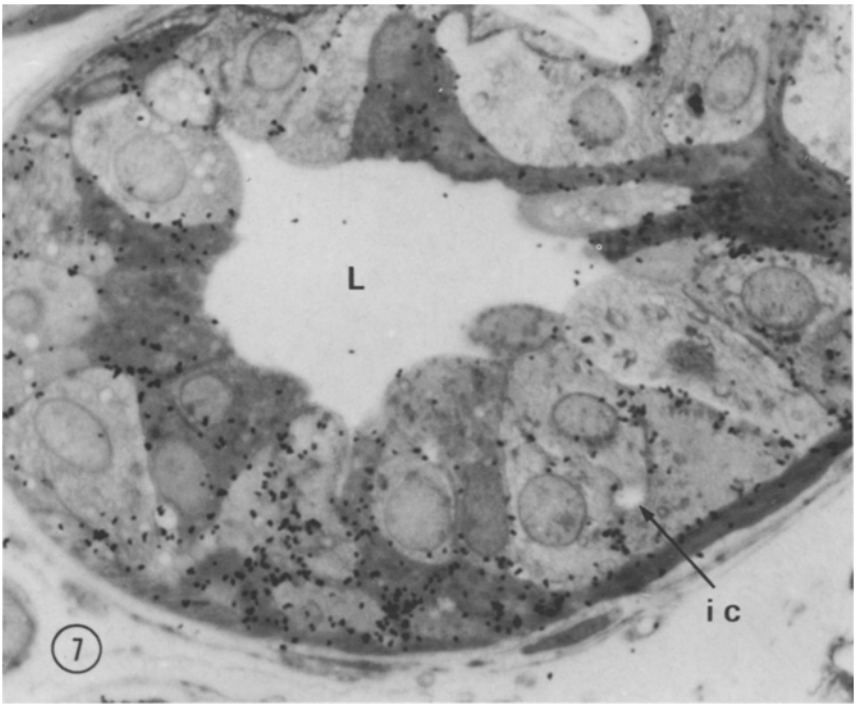

Fig. 7. Light microscope autoradiogram of ^{3}H-ouabain bound in the secretory tubule of the perfused sweat gland. Again, almost all grains are located in areas of very close proximity to the basal and lateral cell surfaces of both light and dark cells. Also, note that very few grains are associated with membranes defining the lumen (L) or intercellular canaliculi (ic). Stained with methylene blue. 1,600 diameters

located over the basal lateral surface of both reabsorptive and secretory cells.

The reabsorptive duct consists of a double layer of cuboidal epithelial cells [26, 27]. The function of this rather unique arrangement is not understood. The lumen of the duct is bordered by a relatively thick "cuticular" zone, which comprises about 1/3 of the volume of the inner layer of epithelium, and grains were observed only occasionally between cells in this region, otherwise label was distributed uniformly over the basal and lateral surface of the cells in both layers.

The secretory tubule, on the other hand, is composed of light and dark cells forming a pseudo-stratified epithelium [26]. The label in this segment of the gland was distributed predominantly throughout the intercellular spaces and along based surfaces of the epithelial cells (Fig. 7). It was not possible to determine conclusively whether the label was associated with one cell type more than the other. Although intercellular

cannaliculi have been reported [27] and are thought to be continuous with the lumen [17], no label was observed associated with this structure in the perfused secretory tubule (Fig. 7).

No other structures in the tissue showed any significant accumulation of the label.

Discussion

The validity of this study rests primarily upon the specificity of ouabain binding to Na/K-ATPase and accessibility of ouabain to these binding sites. There is considerable evidence that ouabain binds with high affinity and specificity to a single site on the Na/K-ATPase enzyme [1, 2, 7, 8, 16]. Indeed, it is accepted that one of the prime criteria for the presence of the enzyme is its cardiac glycoside sensitivity. Moreover, the binding has been demonstrated to represent a one to one correspondence between inhibitor and enzyme, with saturation kinetics [8, 16], the number of nonspecific sites, at least in the tissues investigated thus far, is relatively small [1, 7, 9]. Furthermore, it is well known that external cations affect ouabain binding [5, 10, 16, 23], and it was recently shown that this effect is due to a cation induced change in enzyme affinity for the inhibitor [10, 13, 14, 16]. These observations provide useful criteria for the specificity in autoradiographic studies [32]. Therefore, the present findings that binding demonstrates saturation (Fig. 4) and that high K^+ diminishes binding by approximately 80% (Fig. 5) meet these criteria and support the conclusion that the observed localization is specific for Na/K-ATPase. It should be mentioned that very similar results were obtained on sweat glands from cystic fibrosis patients [30].

The question of accessibility is principally a problem of diffusion to the cell surfaces. It seems reasonable to assume that ouabain diffuses readily into the extracellular spaces, since the tissue is small (about 200 μ thick) with very little connective tissue present to increase unstirred layers. On the other hand, ouabain probably would not reach the luminal surface by diffusion alone since the tight junction may offer a significant diffusional barrier [17], and diffusion along the duct through an open end would require several hours. To ensure appropriate exposure the duct was perfused with a solution of the same specific activity of ouabain as the bath. Perfusion was visually monitored by the refraction interference produced as the perfusate (containing no dextran) emerged from

the open end of the cannulated gland and mixed with the bath containing 2% dextran (83,000 mol. wt).

Localization of ouabain on the basal lateral side of the epithelia of the reabsorptive duct agrees with predictions from classical Na transport mechanisms [38] in which Na should enter the cell from the lumen passively while it is extruded by a pump in exchange for K across the basal lateral membrane into the extracellular fluid. Presumably, that pump directly involves the Na/K-ATPase found in the membranes. Although the reabsorptive duct consists of a double layer of epithelial cells, the localization pattern of the enzyme in the duct does not indicate separate functions for the two layers or suggest what role this unique morphological arrangement plays in the transport function. Even though the resolution of the localization is insufficient to determine whether there is equal binding at the apical pole of the cells of the outer layer, it is tempting to speculate that these cells possess a polarity and function in series to establish the salt gradient across the duct wall. Since no significant binding was noted either at the luminal surface or at the lateral surface in the apical cortical zone of the inner layer of cells, we surmise that this region primarily offers structural support to the duct and constitutes a relatively passive region of the cell. This view is supported by ultrastructure studies [17, 27] which show the zone to be packed with tonofilaments and virtually devoid of other cellular organelles.

The simple epithelium of the secretory coil is composed of two cell types: dark cells presumed to be responsible for "mucus" secretion and clear cells thought principally to involve fluid movement [26]. The resolution of grains clearly shows localization of the enzyme at the basal lateral cell perimeters; however, it is not possible to determine whether one cell type binds more label than the other.

The location of the binding sites in the secretory tubule is most perplexing and is emphasized diagrammatically in Fig. 8. The direction of fluid movement is from the interstitial compartment into the lumen. Presumably Na or Cl, at least, must move across the basal lateral membranes into the cell and then move out of the cell across the apical or luminal membrane. Even though the binding is clearly localized at the basal lateral membrane system and not at the apical surface, it is difficult to conclude, for at least two reasons, that the enzyme is directly responsible for the solute movement in secretion: (1) The enzyme is known to pump Na out of and K into the cell [18, 28], and ouabain is thought to bind only to the Na exiting side of the membrane [18].

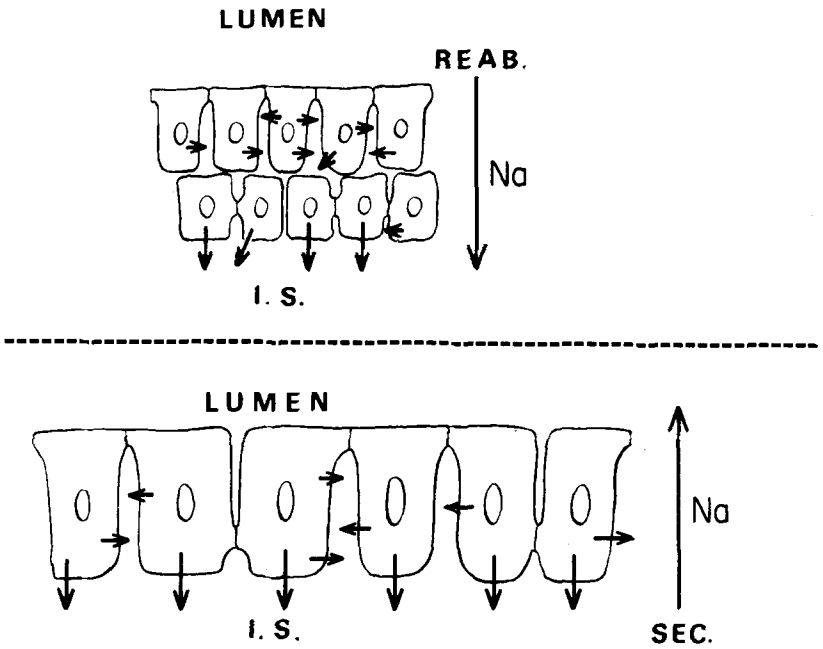

Fig. 8. Diagrammatical illustration of the localization and presumed transport direction of Na/K-ATPase compared to the direction of net Na movement in the reabsorptive (REAB.) and secretory (SEC.) epithelia of the sweat gland. In the reabsorptive duct the location and direction of the "pump" (small arrows) is compatible with the direction of net Na movement (large arrows); i.e., from lumen to interstitial space (I.S.). However, in the secretory coil, if the "pump" (small arrows) is moving Na out of the cells, it must be operating in the direction opposite to net Na movement; i.e., from interstitial space to lumen

We are not aware of any examples showing a naturally occurring reversal of this orientation. (2) If the pump were reversed, during secretion the cytoplasmic concentration of Na should approach equally with the interstitial fluid. It seems unlikely that the cell would tolerate such dramatic changes in ionic composition. Furthermore, in the avian nasal salt gland, Na/K-ATPase is located similarly on the basal-lateral membranes [11] and extraordinarily high concentrations of NaCl (600–800 mM) are secreted into the lumen, but recent studies show intracellular Na concentrations are not elevated [34].

It is most interesting to note that the only example of Na/K-ATPase localization at the apical surface of the cell is our own work [32] in the choroid plexus. In this tissue net fluid flow proceeds from the interstitium to the cerebrospinal fluid compartment, where the fluid is basically

very similar to interstitial fluid. In all other cases of true secretory[1] tissues having "backwards" transport as well as in all absorptive[2] epithelia having "forwards" transport that have been studied thus far, the localization of Na/K-ATPase has been predominantly, if not exclusively, on the serosal side of the epithelium. It is therefore tempting to suggest that there may be some correlation between the metabolic demands of the tissue and the localization and function of Na/K-ATPase. Alternatively, the enzyme may be related to more remote functions such as maintenance of cell volume, of pH, or the stimulatory mechanisms. In any case, the location of the Na/K-ATPase, like the morphological polarity of the epithelial cell is no respector of transport direction and, in fact, presents the same enigma that for years has perplexed cytologists who have tried to correlate ultrastructure with fluid movement.

If this enzyme is not directly coupled to secretory transport, then immediately the question is posed of what enzyme system is responsible for transport. There are several possible systems which might involve: (1) a Cl pump [4, 33]; (2) a separate pump for Na; (3) a separate pump coupling Na and Cl; or (4) an independent mechanism for driving fluid flow, e.g., lactic acid production [15] in sweat or a HCO_3^- or H^+ pump. It seems reasonable to expect that whatever the mechanism, ATP should be the primary source of energy. However, there is little evidence for the existence of a Cl or NaCl stimulated ATPase in animal tissues. On the other hand, there is some evidence for a HCO_3^- stimulated ATPase. Conceivably this enzyme might be involved in transporting Na^+ in either a H^+ exchange or in coupled HCO_3^- transport [20, 39]. In any case, it is not possible at this point to favor any of the alternatives, but in light of current findings, the question of their existence may be raised more seriously.

In conclusion, the active removal of electrolytes from sweat by the reabsorptive duct may be explained by a Na pump integrating a Na/K-ATPase located at the basal lateral surfaces of these cells. However, the mechanism of formation, of the primordial isotonic sweat is not known and cannot be explained in the same classical manner by a Na/K-ATPase pump; since this enzyme is also located at the basal lateral membrane and would oppose the direction of net transport (Fig. 8). Classically, it is this membrane which is predicted to be responsible for the passive

1 Nasal salt gland [11, 12], teleost gill chloride cell [36], salivary gland [29] and the human eccrine sweat gland reported here [31].

2 Intestine [35], toad bladder [24], teleost bladder [19], nephron segments [37], and again, the reabsorptive portion of the sweat gland presented here [31].

leak of Na into the cell, while the apical membrane would actively remove cytoplasmic Na against its gradient. These observations suggest (1) that at least in the secretory epithelium, Na/K-ATPase does not directly participate in the active transport step; and (2) that the enzyme may be involved in more fundamental tasks which reflect not so much the transport properties of the tissue *per se* as its metabolic requirements or other generalized functions stressed in the performance of transport activities.

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