ON THE DISTRIBUTION OF Na⁺-PUMP SITES IN THE FROG SKIN

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ABSTRACT

Exposure of the outside of the isolated frog skin to a Ringer’s solution, made hypertonic by the addition of mannitol, causes a rapid and sustained increase in transepithelial permeability through a structural distortion—a focal blistering—of the “tight” junctions of the outermost living cell layer. [³H]ouabain, used as an autoradiographic marker for the Na⁺-pump (Na⁺-K⁺-adenosine triphosphatase), is usually unable to penetrate the frog skin from the outside solution, but when added to a hypertonic mannitol-Ringer’s solution in the outside bath it readily penetrates the epithelium, presumably through the opened shunt pathway. Radioautographic analysis of [³H]ouabain binding sites revealed that most of the ouabain that enters from the outside solution binds to sites on the cell membranes of the stratum spinosum, as was the case when it was applied from the inside bath in an earlier study. The outer living cell layer, the first to be exposed to ouabain, does not appear to be the major site for the Na⁺-pump, and therefore, is not likely to be responsible for most of the active pumping of Na⁺. This result demonstrates that previous failure to show a high density of Na⁺-pump sites on the cells of the outermost layer, when [³H]ouabain was applied from the inside solution, was not due to the inability of the marker to reach these cells at a sufficient concentration to reveal all the pump sites. These results provide further support for a model of Na⁺-transport across the frog skin which distributes the active pump step on the inward-facing membranes of all the living cells.

KEY WORDS Na⁺-pump · frog skin · cell junctions · [³H]ouabain

The isolated, intact frog skin has long served as a model system for the study of active Na⁺-transport across epithelia. Transepithelial transport is generally viewed as a two-membrane phenomenon, and because the frog skin is a complex, multilayered epithelium, a significant amount of effort has been directed toward determining what role the various cells play in the transport process. We have recently proposed a model depicting the pathway for active transport of Na⁺ across the frog skin which places the entry step at the outward-facing membrane of the outermost living cell layer, and distributes the active extrusion (pump) step over the inward-facing membranes of all the living cells (10). This model is based on results obtained with a recently developed technique for the radioautographic localization of [³H]ouabain binding sites (12). Ouabain, a cardiac glycoside, is known to be a highly specific inhibitor.
of the enzyme Na⁺-K⁺-adenosine triphosphatase (ATPase) (11) which is usually equated with the Na⁺-pump (5).

An important detail of this hypothesis is that, although all of the living cells take part in the transepithelial movement of Na⁺, the largest fraction of the pump activity occurs in the cells of the stratum spinosum. This indicates that the cells of the outermost living cell layer (outer layer of the stratum granulosum or “outer reactive cell layer”) do not play as major a role in the pumping of Na⁺ as has been implied from previous morphological studies (14, 15).

An alternative explanation for the fact that only a small fraction of the labeled pump sites could be demonstrated on the outer living cell layer is that [³H]ouabain, applied from the inside bathing solution, may not penetrate the epithelium sufficiently to allow radioautographic detection of all available binding sites. This problem of accessibility might be overcome by exploiting the fact that amphibian skin undergoes a substantial increase in paracellular permeability when it is exposed to a sufficiently hypertonic outside bath (2, 13). In this case, with an open paracellular pathway, [³H]ouabain might be presented to the epithelial cells from the outside solution so that the outer living cell layer would be the first to be exposed to ouabain. In the present study, we have found that this is indeed the case, and that applying [³H]ouabain in this fashion confirms our previous identification of the stratum spinosum as the major site of the Na⁺-pump in the frog skin.

MATERIALS AND METHODS

The protocol for preparation and mounting of frog skins in Lucite double chambers has been previously described (10). The electrical characteristics of the tissue were studied with a square wave voltage-clamping routine similar to that employed by Civan and Hoffman (1). This technique allows simultaneous monitoring of both short-circuit current and transepithelial conductance. Paired abdominal half-skins were bathed on both outer and inner surfaces with an isotonic Na⁺-Ringer’s solution (220–225 mOsm/kg H₂O) (10), and allowed to stand until short-circuit current and conductance had reached steady-state values. Chambers were then drained and each bath was refilled with fresh Na⁺-Ringer’s solution, except that the tonicity of the outer bathing solution on one of the half-skins was raised to 400 mOsm/kg H₂O with mannitol. 30 min later, [³H]ouabain (2 × 10⁻⁶ M) was added to the solution bathing the outside of each skin, and the incubations were continued for an additional 120 min. The chambers were then drained, each side was rapidly washed three times, and the chambers were refilled with isotonic Na⁺-Ringer’s solution. This wash-out procedure was repeated 15 and 30 min later, and then the chambers were opened and pieces of skin were either processed for liquid scintillation counting or frozen in preparation for freeze-dry radioautography (10). The distribution of grains in radioautographs was quantified with a computer-assisted routine described in detail in a previous report (10). Briefly, radioautographic images were transmitted by video from the light microscope to a computer graphics terminal (Princeton 801, Princeton Electronic Products, Inc., North Brunswick, N. J.). The boundaries of epithelial cell layers and the positions of overlaid silver grains were then specified for computer analysis of distribution through a sparkpen (Computek Data Tablet, Computek, Inc., Cambridge, Mass.). Data processing was done on a PDP 11/10 minicomputer (Digital Equipment Corp., Maynard, Mass.).

In another set of experiments, paired pieces of skin were initially treated as previously described, but 30 min after application of hypertonic mannitol to the outer surface of one half-skin, both pieces of tissue were fixed by simultaneous addition of concentrated glutaraldehyde (final concentration 1%) to both solutions. Fixation was continued in the chambers for 30 min, and then the tissues were excised and fixation continued overnight (at 4°C) before postfixation in 1% OsO₄ for 1 h. Dehydration, embedding, and sectioning for light and electron microscopy were carried out as previously described (4). Tissue sections were examined in a Philips EM-200 electron microscope.

RESULTS AND DISCUSSION

It is well established that an elevation of the tonicity of the solution bathing the outer surface of the frog skin causes a rapid and substantial increase in transepithelial ionic conductance (13). As shown in Table I, this was clearly the case in the present study. 30 min after the addition of mannitol to the outer bathing solution, just before the addition of ouabain, total transmural conductance had more than doubled while the conductance of control tissue (exposed only to isotonic solutions) did not change in any significant way. There was a progressive increase in conductance throughout the 150-min exposure to hypertonic mannitol, but this effect was reversible because restoration of isotonicity to the outer bathing solution resulted in the return of conductance toward the control value within 30 min. There was a decline in the short-circuit current immediately after the addition of mannitol to the outside solution. Therefore, any inhibition due to ouabain, per se, may have been partially masked,
TABLE I

Effect of 400 mOsm Mannitol-Ringer's Solution on Short-Circuit Current and Conductance across the Frog Skin

| Time min       | SCC  | G     | SCC  | G     |
|----------------|------|-------|------|-------|
| 0 (Addition of mannitol) | 1.00 | 1.00  | 1.00 | 1.00  |
| 30 (Addition of \[^{3}H\]ouabain) | 1.20 \(\pm\) 0.11 | 1.03 \(\pm\) 0.06 | 0.84 \(\pm\) 0.20 | 2.21 \(\pm\) 0.52 |
| 60             | 1.22 \(\pm\) 0.16 | 1.16 \(\pm\) 0.09 | 0.77 \(\pm\) 0.23 | 2.44 \(\pm\) 0.44 |
| 90             | 1.20 \(\pm\) 0.16 | 1.21 \(\pm\) 0.11 | 0.79 \(\pm\) 0.20 | 2.82 \(\pm\) 0.41 |
| 120            | 1.15 \(\pm\) 0.16 | 1.22 \(\pm\) 0.11 | 0.70 \(\pm\) 0.21 | 3.06 \(\pm\) 0.39 |
| 150 (Washout of \[^{3}H\]ouabain) | 1.10 \(\pm\) 0.17 | 1.22 \(\pm\) 0.12 | 0.62 \(\pm\) 0.20 | 3.30 \(\pm\) 0.39 |
| 180            | 1.11 \(\pm\) 0.23 | 0.93 \(\pm\) 0.13 | 0.74 \(\pm\) 0.17 | 1.20 \(\pm\) 0.08 |

Ouabain bound (pmol/mg wet wt)

| Time min       | SCC  | G     | SCC  | G     |
|----------------|------|-------|------|-------|
| 0              | \(0.010 \pm 0.001\) | \(0.163 \pm 0.039\) | \(0.010 \pm 0.001\) | \(0.163 \pm 0.039\) |

Short-circuit current (SCC) and conductance (G) changes across the frog skin after exposure to either isotonic solutions on each side or a hypertonic outside:isotonic inside solution. Time zero was taken as the time of addition of mannitol-Ringer’s solution to the outside bath of one of a paired half-skin. 30 min later, \[^{3}H\]ouabain was added to the outside solution of both skins and the incubation continued for 120 min. The data is presented as the ratio of the experimental values to those at time zero (before addition of mannitol-Ringer’s solution). Thus, a value of 1.00 indicates no change, and a value greater than one indicates an increase. \(n = 5\) SEM.

Although the short-circuit current continued to decline in every case. (In the absence of ouabain, the inhibition of short-circuit current due to hypertonic mannitol was largely reversible.)

Transmission electron microscopy was performed to compare tissue fixed after exposure to the hypertonic mannitol solution on its outer surface with tissue exposed only to isotonic solutions. The only reproducible change in epithelial structure that could be associated with hypertonic solutions on the outer surface was a structural distortion of the intercellular junctions between the cells of the outermost living epithelial layer (Fig. 1). These cell contacts, *zonulae occludentes* (tight junctions, [7]) or limiting junctions (2, 3), are thus osmotically sensitive, as are those of the toad urinary bladder and skin (3) where such "blistering" has been quantitatively established to parallel the osmotically induced change in transmural conductance. The junctions were also implicated as the site of osmotically induced changes in conductance by Erlij and Martinez-Palomo (6), although they did not identify junction blistering as the mechanism upon which the phenomenon depends.  

With this evidence that paracellular permeability could thus be enhanced, it was reasonable to assume that \[^{3}H\]ouabain might be applied to the outer surface of the skin and penetrate the epithelial intercellular spaces in sufficient concentrations to permit a radioautographic localization of specific binding sites. Tissue binding studies confirmed this possibility (Table I). Skins exposed for 120 min to \[^{3}H\]ouabain on the outer surface bound 0.163 pmol/mg wet wt when the outer bath was a Ringer’s solution brought to 400 mOsm/kg \(H_2O\) with mannitol. By comparison, skins exposed to isotonic bathing media bound only 0.010 pmol/mg wet wt of \[^{3}H\]ouabain after a similar length of exposure from the outside solution. (The amount bound by mannitol-treated skins was, in fact, some 34% as much as was bound to skins exposed to a similar concentration of ouabain from the inner surface in our earlier study [10]).

A radioautograph from a piece of frog skin bathed in isotonic Na+-Ringer’s solution and exposed to \[^{3}H\]ouabain from the outside is shown in Fig. 2a. As expected from the binding data, midgut, [9]) suggest that this structural responsiveness is intrinsic to the junctions of all epithelia which maintain strong transmural osmotic gradients in vivo.

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1. Recent observations on the osmotic sensitivity of invertebrate epithelia (Planarian epidermis, [8], and crayfish midgut, [9]) suggest that this structural responsiveness is intrinsic to the junctions of all epithelia which maintain strong transmural osmotic gradients in vivo.
very few grains are found in association with the
epithelium, except within the cells of the stratum
corneum. [3H]ouabain apparently penetrates
these dead cells during the incubation period in
sufficient quantity to remain after 30 min of
washout. Fig. 2b provides a comparative view of
a piece of skin after exposure to [3H]ouabain
from the outside, but in the presence of 400
mOsm mannitol-Ringer's solution. The grains,
reflecting ouabain binding sites, are distributed
throughout the epithelium with an apparent con-
centration in the region of the stratum spinosum.
It does not appear that a large number of binding
sites are present in the outer living cell layer (the
stratum granulosum). The similarity of this finding
to earlier studies (with application of [3H]ouabain
from the inside solution, [10]) was readily quanti-
fied by computer analysis of the patterns of grain
distribution.

Fig. 3 presents data from a sampling of tissues
where grain distribution was assessed. Fields for
analysis were chosen at random from each of the
sampled experiments with the single criterion that
the outer and inner boundaries of the epithelium
were relatively parallel. The width of each bar
represents a slice of the epithelium equal to 10%
of the total thickness. The left edge of slice 1 is
equivalent to the outer edge of the stratum cor-
neum, and the right edge of slice 10 is equivalent
to the inner edge of the stratum germinativum.
The height of the bar indicates the number of
grains in that slice, expressed as a percentage of
the total number of grains over the epithelium.
Because each bar represents an equivalent area,
the height of each is also a direct index of grain
density.

It is obvious that the middle area of the skin,
ecompassing the cells of the stratum spinosum,
had the greatest number, as well as the greatest
density of grains. The stratum corneum had a
high density of grains, but this would appear to
be largely due to grains within the cells rather
than to those associated with the cell membrane.
The slices associated with the outer living cell
layer (parts of 2 and 4, and all of 3) had a
significant number of grains, but the values were
not statistically different (P > .05) from the slices
associated with the stratum germinativum (slices
8-10).

These results demonstrate that the permeability
of the paracellular pathway in the frog skin can
be increased sufficiently to allow penetration of a

![Figure 1](https://via.placeholder.com/150)

**Figure 1** Junctions between cells in the outermost living cell layer (outermost layer of stratum granulosum) of the frog skin. (a) Bathed with isotonic Na⁺-Ringer's solution on both the inside and the outside. (b) Bathed with 400 mOsm mannitol-Ringer's solution on the outside, isotonic Na⁺-Ringer's on the inside. Notice the distortion or "blistering" (•) of the apical portion of the junctional space in (b). × 30,000.
Radioautographs of freeze-dried preparations of frog skin that were exposed to [3H]ouabain from the outside solution. (a) Bathed with isotonic Na+-Ringer's solution on both the inside and outside. (b) Bathed with 400 mOsm mannitol-Ringer's solution on the outside, isotonic Na+-Ringer's on the inside. Notice the dense localization of grains in the stratum spinosum (Sp). There are fewer grains associated with the plasma membranes of the outer living cell layer (Gr), here clearly definable as a reactive cell layer. This is especially evident when the intercellular space between the outer living cells and the next deepest layer (arrow) is compared to a space between two cells in the stratum spinosum (arrowheads). Notice also the grains within the cells of the cornified layer in both (a) and (b) (Cor). × 920.
molecule the size of ouabain (formula weight of hydrated species, 728.6). As anticipated, the osmotically sensitive structures are the rate limiting tight junctions of the outer living cell layer.

It must also be concluded that failure to demonstrate the largest fraction of epithelial Na⁺-pump sites in association with the plasma membranes of the outer living cell layer (10) is not due to the restricted ability of [³H]ouabain to reach these cells from the inside solution. Even when this layer of cells is the first to be exposed to [³H]ouabain, the number of pumps revealed there is less than the number of pumps in the deeper stratum spinosum.

This evidence, taken together with the assumptions that ouabain binds to a functioning Na⁺-K⁺-ATPase, and that the actively transported Na⁺ goes through this Na⁺-pump, reinforces a model which distributes the active pump step over the inward-facing membranes of all the living epithelial cells of the frog skin.

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