Binding of $[^3H]$Ouabain to Split Frog Skin

The Role of the Na,K-ATPase in the Generation of Short Circuit Current

PETER M. CALA, NEALE COGSWELL, and LAZARO J. MANDEL

From the Department of Physiology and Pharmacology, Duke University Medical Center, Durham, North Carolina 27706. Dr. Cala's present address is Department of Human Physiology, School of Medicine, University of California, Davis, California 95616.

ABSTRACT The binding of $[^3H]$ouabain to the serosal side was studied in a chambered preparation of frog skin, free of connective tissue, while the short circuit ($I_e$) was concurrently monitored. Both ouabain binding and $I_e$ inhibition proceeded as hyperbolic functions of time. A plot of the number of ouabain molecules bound vs. the corresponding values of $I_e$ inhibition (percent) yielded a straight line, yet showed that one-third of the binding occurred before any inhibition of $I_e$. Upon separation of the skins into two groups based upon initial $I_e(I_{sat})$—high, >20 $\mu$A/cm$^2$ and low, <10 $\mu$A/cm$^2$—we observed two distinct populations. The high $I_{sat}$ skins bound very little ouabain before inhibition of $I_e$ whereas low $I_{sat}$ skins bound one-half of the total number of sites before exhibiting any inhibition of $I_e$. These observations strongly suggest that (a) the Na,K-ATPase is directly involved in the generation of $I_e$, and (b) at low $I_e$, inhibition of some pumps by ouabain causes a "recruitment" of other pumps to increase their turnover rate and maintain $I_e$ relatively unaffected. In addition, the binding of ouabain also displayed various characteristics that were consistent with known properties of the Na,K-ATPase: (a) increased intracellular K/Na concentrations, whether achieved through the addition of amiloride or removal of Na from the outside medium, led to a significant decrease in ouabain binding rate relative to paired controls; and (b) ouabain binding, either with normal or decreased intracellular Na, was significantly reduced in the presence of elevated K in the serosal bathing medium. Finally, the number of ouabain molecules bound to the frog skins was not correlated with their initial $I_e$ values, indicating that the spontaneous skin-to-skin variation in $I_e$ was not related to the number of functional pump sites but, rather, to their turnover rate.

INTRODUCTION

In 1958, Koefoed-Johnsen and Ussing introduced their now "classical" model for active Na transport across the frog skin featuring a passive Na entry step followed by active extrusion through a serosally located pump; this latter step was considered to occur through the Na,K-ATPase. Equating the Na,K-ATPase with the "Na pump" in frog skin was highly speculative at the time and, over the years, only indirect evidence has supported this idea. Although the presence of...
Na,K-ATPase in frog skin (Bonting and Caravaggio, 1963; Kawada et al., 1975) and the ouabain sensitivity of $I_e$ (Nakajima, 1960) are well established, it is not clear that the Na,K-ATPase is directly involved in the generation of $I_e$. Alternatively, it may be argued that the Na,K-ATPase of frog skin is functioning to maintain cell volume and ion composition (Tosteson and Hoffman, 1960), and is thus providing the proper environment for Na extrusion by another pump. Attempts to relate Na extrusion through the serosal border with K entry through the same border produced mixed results: a 3:1 stoichiometry of Na to K could be measured under some conditions, but this relationship broke down when the external Na concentration was reduced and in the presence of amiloride (Biber, Aceves, and Mandel, 1972); thus, the existence of an active, serosally located Na-K exchange system in frog skin and its role in the generation of $I_e$ is still unclear.

Investigators studying cellular systems (Dunham and Hoffman, 1971; Brading and Widdicombe, 1974; Baker and Willis, 1972) have made use of the tritium-labeled cardiac glycoside ouabain as a powerful tool in gaining understanding of kinetic and molecular events associated with active Na-K transport. Because ouabain is a potent inhibitor of the Na,K-ATPase (Shatzman, 1953; Albers et al., 1968; Schwartz et al., 1968; Schwartz et al., 1975), and because that enzyme has a high binding affinity for ouabain, it has been possible to expose biological membranes and membrane fragments to $[^3H]ouabain$, wash unbound inhibitor from the incubation media, and monitor ouabain binding to the Na,K-ATPase as a function of enzyme inhibition. Thus, investigators have been able to calculate the number of pump sites, their rate of turnover, and homogeneity and, in addition, determine the basis for differences in pump rate from tissue to tissue (Dunham and Hoffman, 1971). Such studies are fairly difficult even in relatively homogeneous cell suspensions, as a result of ouabain interactions with membrane components other than the Na,K-ATPase, i.e., nonspecific binding. Studies of ouabain binding to epithelia are therefore, complicated by an extensive cellular organization, which is characterized by irregular extracellular spaces and, in most cases, thick layers of connective tissue. As a result, extracellular and adsorbed ouabain may constitute a major fraction of the total amount associated with the tissue, severely limiting the resolution of the specifically bound fraction.

This communication reports the results of experiments in which the binding of $[^3H]ouabain$ to the serosal side was studied in a chambered preparation of frog skin while the short circuit current was simultaneously monitored.

To obviate a large source of error due to nonspecific binding, the present studies were performed on frog skins from which all connective tissue had been removed after treatment with collagenase. In addition, an extracellular space marker ($[^14C]mannitol$) was used to correct for extracellularly distributed, yet unbound, ouabain. Data are presented which demonstrate that at least 80-90% of the bound ouabain is specifically interacting with Na,K-ATPase. A high correlation is observed between ouabain binding and inhibition of $I_e$, providing direct evidence for the involvement of the Na,K-ATPase in active transepithelial Na transport. Further, alterations in the ionic milieu are shown to affect both
ouabain binding and $I_{sc}$ inhibition in a manner consistent with known properties of the Na,K-ATPase.

**MATERIALS AND METHODS**

*Procedure for Splitting and Mounting of Skins*

Skins were excised from the abdomen of bullfrogs (*Rana catesbiana*) and split from connective tissue by utilizing a modification of the method of Aceves and Erlij (1971). Briefly, the skins were tied as a diaphragm over the orifice of a glass chamber, serosal side inward. The inside of the chamber was filled with regular Ringer to which crude collagenase (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 200 μg/ml had been added, while the entire chamber was placed in a beaker filled with regular Ringer which bathed the external surface of the skin. A hydrostatic pressure (~30 cm of H₂O) was applied to the collagenase containing Ringer bathing the serosal side of the skin. The skins began to blister in ~1 h, at which time the skins were removed and the connective tissue was separated from the epithelium. The skins thus split, were mounted as flat sheets in a lucite chamber fitted with Sylgard washers (Dow Corning Corp., Midland, Mich.) to minimize edge damage; the exposed skin area was 0.785 cm². Structural support for this thin epithelium was provided by a sheet of filter paper mounted between the washer and the external surface of the skin. The serosal surface was in direct contact with the bathing medium. Each half of the chamber held 1.0 ml of bathing medium which was well stirred and oxygenated with water-saturated air at 22°C. The open circuit potential and $I_{sc}$ were monitored as previously described (Mandel and Curran, 1972). It was found that in this preparation short-circuited skins deteriorated at a more rapid rate than those which were open-circuited; therefore, the studies to be described were performed upon skins which were open-circuited except for the short time periods when $I_{sc}$ was measured.

Three bathing solutions were utilized in these experiments; their compositions are listed in Table I. The regular Ringer solution containing NaCl was utilized for the incubation with collagenase; the low-K and high-K solutions containing Na₂SO₄, were utilized during the remainder of the experimental procedures.

*Procedure for Measuring the [¹⁴C]Mannitol Space and [³H]Ouabain Binding*

The skins were allowed to equilibrate in low-K Ringer until their electrical characteristics stabilized (~45 min). To begin an experiment, [¹⁴C]mannitol (2.25 μCi) and [³H]ouabain (4.5 μCi) were added to the serosal medium and allowed contact with the skins. At the end of a predetermined interval, 20-μl aliquots of both bathing solutions were taken and placed in glass scintillation vials. The chambers were aspirated free of bathing media and were connected to solution reservoirs (Schultz and Zalusky, 1964) such that the total circulating volume in each half-chamber was 12 ml. The half-chamber bathing the outside of the skin was refilled with low-K Ringer while the half-chamber bathing the serosal surface, previously containing [³H]ouabain and [¹⁴C]mannitol, was filled with high-K Ringer. The serosal wash medium was replaced three times at 15-min intervals. Thus, the serosal surface of the skin and chamber previously exposed to 1 ml of tracer medium was washed for 45 min in a total volume of 36 ml of high-K, tracer-free medium. The elevated K was utilized in the washing solution because pilot experiments demonstrated that 30 mM K had a significant effect in depressing the rate of ouabain binding and $I_{sc}$ inhibition during the wash period. We were, consequently, able to increase our time resolution of ouabain binding and $I_{sc}$ inhibition. The percent inhibition of $I_{sc}$ was calculated by dividing $I_{sc}$ just before washing by the $I_{sc}$ recorded just before the addition
of ouabain and mannitol to the serosal bathing medium. It was important to obtain the $I_{sc}$ before washing in that the wash procedure often produced mechanical damage to the skins with consequent deterioration of $I_{sc}$.

At the conclusion of the wash period the chambers were dismantled and the washers were removed with the skin in place. Using a sharp punch with an inside diameter of 1 cm and the washer as guide, that portion of skin exposed to bathing media (0.785 cm$^2$) was removed. The skin was then placed in a preweighed glass scintillation vial and heated overnight in an oven at 70°C. The vials were then removed from the oven, placed in a dessicator, allowed to equilibrate at room temperature, and weighed, and the weight of dry cell solid (dcs) was determined by difference. [$^3$H]Ouabain and [$^{14}$C]-mannitol were extracted by placing the dried skins in 0.5 ml of 1 N NaOH for 24 h followed by neutralization with an equal volume of 1 N HCl.

After extraction and neutralization, 20 ml of Bray's solution was added to the scintillation vials containing the skins and the bathing media and was counted by an Intertechnique liquid scintillation spectrometer equipped with an external standard (model SL 50, Intertechnique Ltd., Fairfield, N. J.). Using the external standard in the channels ratio mode, quench corrections were made for all samples and efficiencies were calculated based upon quench curves established for each new batch of counting cocktail. The range of efficiency for the [$^3$H]

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>COMPOSITION OF RINGER SOLUTIONS</th>
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<tbody>
<tr>
<td>NaCl Ringer</td>
<td>K Ringer</td>
</tr>
<tr>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>112 mM NaCl</td>
<td>56 mM Na$_2$SO$_4$</td>
</tr>
<tr>
<td>2.5 mM KHCO$_3$</td>
<td>2.5 mM KHCO$_3$</td>
</tr>
<tr>
<td>1 mM CaCl$_2$</td>
<td>15 mM K$_2$SO$_4$</td>
</tr>
<tr>
<td>56 mM sucrose</td>
<td>56 mM sucrose</td>
</tr>
</tbody>
</table>

associated with the skins was 17-24% while the $^{14}$C efficiency was consistently $\approx 30\%$. Calculation of extracellular space utilizing [$^{14}$C]-mannitol required an accurate determination of the disintegrations per minute found in the skin relative to those in the bath. Inasmuch as precise measurement of extracellular space was not dependent upon exact knowledge of the mannitol specific activity, mannitol was added to the bathing media as undiluted stock solution obtained from the supplier (New England Nuclear, Boston, Mass.). On the other hand, knowledge of the specific activity of [$^3$H]ouabain was of primary importance in calculating the number of ouabain molecules associated with a given number of disintegrations per minute. A value for the specific activity of [$^3$H]ouabain was provided by the supplier (New England Nuclear); however, the precision of this value is uncertain. In order to decrease (halve) this uncertainty, half of the ouabain added to the serosal side was added as tracer stock whereas the remainder was freshly prepared unlabeled ouabain.

The total number of ouabain molecules per mg of dry cell solids present in the skin was calculated by the equation:

$$A = \frac{X_{om} \cdot \left( \frac{100}{E_{om}} \right) \cdot N}{\left[ O \right]_m \cdot V \cdot \left( E_{om} \right) \cdot W},$$

where

$A$ = total ouabain molecules present in the skin per milligram dcs

$X_{om}$ = number of ouabain molecules extracted from the skin

$N$ = number of disintegrations per minute

$[O]_m$ = concentration of ouabain in the media

$V$ = volume of media

$E_{om}$ = efficiency of counting of ouabain
\( X_{os} \) = counts per minute of \([3H]\)ouabain in skin, corrected for \(^{14}\text{C}\) spillover

\( E_{os} \) = efficiency at which the \(^{3}\text{H}\) in the skin was counted

\( X_{om} \) = counts per minute of \([3H]\)ouabain in bathing medium, corrected for \(^{14}\text{C}\) spillover

\( E_{om} \) = efficiency at which the \(^{3}\text{H}\) in the medium was counted

\( W \) = the skin weight in milligrams dcs

\( N \) = Avogadro's number: \( 6.02 \times 10^{23} \)

\( V \) = volume of medium counted in liters

\([O]_m \) = the ouabain concentration in the serosal medium.

The extracellular space was calculated with the use of the following equation:

\[
S = \frac{100}{X_{ms}} \cdot \frac{E_{ms}}{V} \cdot \frac{X_{mm}}{100} \cdot \frac{E_{mm}}{W}
\]

where

\( S \) = space in liters occupied by \([^{14}\text{C}]\)mannitol in the skin (liters/milligram dcs)

\( X_{ms} \) = counts per minute of \([^{14}\text{C}]\)mannitol in the skin

\( E_{ms} \) = efficiency at which the \(^{14}\text{C}\) in the skin was counted

\( X_{mm} \) = counts per minute of \([^{14}\text{C}]\)mannitol in the bathing medium

\( E_{mm} \) = efficiency at which the \(^{14}\text{C}\) in the medium was counted

\( V, W \) = as above.

The number of ouabain molecules bound to the skin after correction for those trapped in the extracellular space was calculated from

\[
B = A - (S \cdot [O]_m \cdot N)
\]

where \( B \) = number of ouabain molecules bound per milligram dcs.

**RESULTS**

**Determination of Optimal Experimental Conditions for Specific [3H]Ouabain Binding to Split Frog Skin**

In attempting to relate specific \([3H]\)ouabain binding to the inhibition of active transport, it was absolutely crucial to select a ouabain concentration which would lead to minimal nonspecific binding. The experiments shown in Fig. 1 were performed with this end in mind. The figure depicts ouabain "binding" (uncorrected for extracellularly trapped and nonspecifically bound ouabain) and simultaneously measured inhibition of \(I_{sc}\) at ouabain concentrations from \(10^{-6}\) to \(10^{-4}\) M, after a 60-min exposure to the drug. It is readily apparent that above \(5 \times 10^{-6}\) M ouabain, further increases in concentration produced relatively slight increases in inhibition of \(I_{sc}\), yet large increases in apparent ouabain binding. Thus, at ouabain concentrations >\(5 \times 10^{-6}\) M, interactions between ouabain and the frog skin are primarily nonspecific in nature: i.e., ouabain is binding to membrane sites which are not involved in generating \(I_{sc}\). The binding experiments could probably have been performed at \(5 \times 10^{-6}\) M, yet as seen from the graph in Fig. 1, we would have been working close to the region where the nonspecific interactions appeared to be dominant. In addition, inasmuch as binding rate is proportional to concentration, the analysis of binding vs. \(I_{sc}\) inhibition data may have been complicated by ouabain binding to
the epithelia during the wash period. As concerns ouabain concentrations \(<10^{-6} \text{ M}\), we would certainly have expected to decrease any nonspecific interactions, yet at \(5 \times 10^{-7} \text{ M}\) ouabain a 60-min exposure produced only a 10% decrease in \(I_{sc}\). Consequently, exposure times necessary for high levels of inhibition would have had to be much longer than 90 min. Associated with long exposure of skin to ouabain is the uncertainty as to what is producing decreases in \(I_{sc}\): ouabain binding or spontaneous decay. Thus, the chosen concentration of \(10^{-6} \text{ M}\) ouabain represents a compromise because at this concentration the rates of binding and inhibition are slow enough to be conveniently measured and still proceed at a rate which is high relative to that of the spontaneous decay of \(I_{sc}\). In addition, it will be demonstrated elsewhere in this publication that the upper limit for nonspecific binding at \(10^{-6} \text{ M}\) ouabain is 17% of the total, a value which will not detract from the arguments to be made herein.

Although it would appear that \(10^{-6} \text{ M}\) ouabain leads to relatively small amounts of nonspecific binding, it is doubtless that some trapping of \([^{3}H]\) ouabain in the extracellular spaces occurs leading to elevated estimates of bound drug. Thus, we decided to correct for trapped (yet unbound) ouabain using \([^{14}C]\) mannitol as an extracellular space marker (Biber, Aceves, and Mandel, 1972; Biber, Cruz and Curran, 1972). In addition, autoradiographic studies of frog skin (Wong and Biber, 1974) showed that mannitol remained exclusively in the extracellular space. Fig. 2 shows the volume of distribution for \([^{14}C]\) mannitol on the serosal side of the skin as a function of time. It is apparent from these data that \(\approx30 \text{ min}\) of exposure is necessary for mannitol to equilibrate with the extracellular compartment(s). To test mannitol's suitability
as a marker for extracellular ouabain, we utilized the virtual irreversibility of the ouabain binding reaction of the Na,K-ATPase to perform the following experiment: a high concentration ($5 \times 10^{-5}$ M) of cold ouabain was initially added to the serosal side of the skin for 45 min to obtain complete inhibition of the $I_{sc}$ and saturation of all binding sites with cold ouabain. Subsequently, [$^3$H]ouabain and [$^{14}$C]mannitol were added to the serosal side for 60 min to measure the extracellular distribution of both molecules. The results of this experiment (Table II) demonstrate that ouabain, in the absence of binding, has the same extracellular volume of distribution as mannitol. Therefore, we

\begin{table}
\centering
\caption{Extracellular Space Measured with [$^{14}$C]Mannitol and [$^3$H]Ouabain after 60 Min Exposure to $5 \times 10^{-6}$ M Ouabain}
\begin{tabular}{lcc}
\hline
Extracellular space & Mannitol & Ouabain \\
\hline
$\mu$g & 0.37±0.06 & 0.32±0.06* \\
\hline
\end{tabular}
\end{table}

$n = 6$.

* ± SE.

1 Under physiological conditions, ouabain binds so tenaciously to the Na,K-ATPase that significant dissociation of the ouabain-Na,K-ATPase complex requires hours of incubation in ouabain-free medium (Baker and Willis, 1972; Boardman et al., 1972; Glynn, 1964). In the present experiments, no degree of washing could reverse ouabain binding which had already occurred.

\textbf{Figure 2.} Mannitol space as a function of time for skins bathed in low-K Ringer with mannitol added to the serosal bathing media only. The values are mean ± SEM for six experiments.
conclude that [14C]mannitol is a good marker for extracellular ouabain. One implication of this conclusion is that the time for ouabain equilibration through the extracellular space is also \( \approx 30 \text{ min} \). This is an important consideration for the understanding of ouabain binding kinetics. The average correction for ouabain trapped in the extracellular medium was \( 22 \pm 2\% \).

One of the problems encountered early in this experimental project was that of stopping binding of extracellular ouabain during the wash period. When the skins were washed in normal NaCl Ringer or low-K Ringer (both containing 2.5 mM K) ouabain binding appeared to continue during the wash period as judged by two criteria: (a) the \( I_\text{sc} \) continued to decline during the washing period; and (b) there was little observable difference in ouabain binding as a function of incubation time in the ouabain-containing solution. To minimize this problem we made use of the fact that the rate of ouabain binding to the Na,K-ATPase is decreased by the presence of elevated K (Boardman et al., 1972; Baker and Willis, 1972; Glynn, 1964; Skou, 1965; Whittman and Wheeler, 1970); thus washing with high-K Ringer (32.5 mM K) considerably decreased the rate of ouabain binding during the wash period. Consequently, we were able to increase our temporal resolution of binding and \( I_\text{sc} \) inhibition. It is difficult to estimate quantitatively the amount of ouabain binding still occurring during the high-K Ringer wash; however, qualitatively, it was observed that no further decay of \( I_\text{sc} \) occurred after the wash was initiated, thereby substantiating the fact that ouabain binding had been minimized.

The effectiveness of the high-K Ringer in decreasing the rate of ouabain binding was tested in a series of three paired experiments comparing the binding and \( I_\text{sc} \) inhibition obtained in high-K Ringer to that obtained in low-K Ringer utilizing skins from the same frog. These results showed that a 60-min exposure to \( 10^{-6} \text{ M} \) ouabain in high-K Ringer inhibited the \( I_\text{sc} \) on the average \( 73 \pm 15\% \) less and bound \( 66 \pm 12\% \) less ouabain than a similar ouabain exposure in low-K Ringer. Assuming that the "protective" effect of high external K is specific for the Na,K-ATPase, we may conclude that at least two thirds of the observed binding in low-K Ringer is specific for this enzyme.

**Binding of [aH]Ouabain and \( I_\text{sc} \) Inhibition**

Once optimal experimental conditions were attained, a series of experiments was performed to study the kinetics of ouabain binding and \( I_\text{sc} \) inhibition, as well as the relationship between them. Fig. 3 shows ouabain binding (corrected for extracellular contribution) as a function of time after serosal exposure to \( 10^{-6} \text{ M} \) ouabain in low-K solution followed by washing in high-K solution. Each point represents a minimum of six experiments. Although ouabain binding appears to saturate as a function of time, a formal kinetic description is complicated because of (a) the uncertainty about the concentration of ouabain in the vicinity of the binding sites during the first 30 min of exposure (cf. Fig. 2 and Table II) and (b) the slow dissociation rate constant of ouabain from the Na,K-ATPase.

Fig. 4 shows the kinetics of \( I_\text{sc} \) inhibition of the same skins as those in which ouabain binding was monitored in Fig. 3. These data demonstrate that the kinetic behavior of \( I_\text{sc} \) inhibition is similar to that of binding. Upon comparison
FIGURE 3. Ouabain binding to split frog skin as a function of time. All values are corrected for unbound extracellular ouabain. Experiments were performed in low-K Ringer at 10^-6 M ouabain. The values are mean ± SEM for six experiments.

FIGURE 4. Inhibition of I_SC in split frog skin as a function of time after ouabain (10^-6 M) addition. These data were obtained simultaneously with those presented in Fig. 3. The values are expressed as mean ± SEM for six experiments.

of the two curves, however, it is apparent that binding precedes inhibition, the disparity being greatest initially. The results of Figs. 3 and 4 were incorporated into one graph of binding vs. inhibition, shown in Fig. 5, to test the relationship between these two variables. Because of the uncertainty concerning the ouabain
concentration in the extracellular space at times <30 min, only the points at times 30 min and greater were plotted in this figure. A linear correlation between ouabain binding and $I_{sc}$ inhibition is evident from Fig. 5; the line through the points was calculated by regression analysis and has a correlation coefficient $>0.99$. From these data we can conclude that ouabain binding and $I_{sc}$ inhibition are functionally related to each other. The nonzero intercept in Fig. 5 suggests that about one-third of the ouabain binding sites is occupied before any inhibition of $I_{sc}$, representing ouabain binding sites that are unrelated to inhibition of $I_{sc}$. This intercept could arise from a variety of circumstances, which are fully elucidated in the discussion to follow.

Based upon the preceding results it appears that we are able to distinguish interactions of ouabain with the Na,K-ATPase from nonspecific interactions of ouabain with the frog skin. However, a direct relationship between the Na,K-ATPase (ouabain binding) and the generation of $I_{sc}$ is yet to be established. The major argument against a direct relationship between ouabain binding and inhibition of $I_{sc}$ is this lack of temporal correspondence. Fig. 5 shows that one-third of the total number of ouabain binding sites is occupied before any inhibition of $I_{sc}$. Possible explanations for this observation are as follows:

![Graph of ouabain binding as a function of $I_{sc}$ inhibition.](image-url)
(a) the Na,K-ATPase is not directly involved in the generation of $I_{sc}$;

(b) the lag of “inhibition” behind “binding” is an artifact which is a consequence of ouabain binding that occurs during the wash period;

(c) at sub-maximal levels of $I_{sc}$, ouabain binding to the Na,K-ATPase will result in increased rates of turnover by uninhibited enzyme until turnover rate is maximized and further binding of ouabain must necessarily lead to inhibition of $I_{sc}$;

(d) all nonspecific binding occurs immediately (as would be the case if there is cellular damage).

As all these possibilities were considered, (c) became the most likely, especially in light of unpublished observations with aldosterone-treated skins showing no ouabain binding before inhibition of $I_{sc}$. Because, on the average, the aldosterone treated skins had initial $I_{sc}$ values which were about three times higher than the mean values of skins presented in Figs. 3, 4, and 5, we decided to investigate whether different patterns of binding vs. inhibition were apparent as a function of initial $I_{sc}$; hence, binding-inhibition data were separated according to $I_{sei}$. These data are presented in Fig. 6 A and B, which presents ouabain binding and $I_{sc}$ inhibition values associated with skins having $I_{sei} < 10$ $\mu$A/cm$^2$ and $> 20$ $\mu$A/cm$^2$, respectively. Linear regression lines calculated from these data show two distinct patterns of ouabain binding vs. $I_{sc}$ inhibition. Although the skins with high $I_{sei}$ (Fig. 6 B) bind no ouabain before inhibition of $I_{sc}$, those skins with $I_{sei} < 10$ $\mu$A/cm$^2$ (Fig. 6 A) bind 50% of the total number of ouabain molecules before exhibiting any inhibition of $I_{sc}$. This analysis of the results strongly supports (c); that is, ouabain binding at low $I_{sc}$ does not initially inhibit the current because uninhibited pumps can maintain the $I_{sc}$ constant. At high $I_{sc}$, any binding of ouabain to a pump results in an inhibition of $I_{sc}$. Therefore, the close correspondence between ouabain binding and $I_{sc}$ inhibition data obtained from skins with high $I_{sc}$ suggests a direct relationship between the Na,K-ATPase and $I_{se}$. In addition, the lack of temporal correspondence between ouabain binding and $I_{sc}$ inhibition of low current skins appears to be a manifestation of variable enzyme turnover rates.

All other possibilities may be rejected by the following arguments: first, (a) is unlikely in the face of the high correlation between binding and $I_{sc}$ inhibition shown in Figure 6 B. Next, (b) could contribute to the observation that binding precedes inhibition, because $I_{sc}$ is measured just before washing the skins whereas binding may proceed throughout the wash period. However, the contribution of this effect should not be appreciable if one considers that the rate of ouabain binding is directly related to the rate of enzyme turnover. Thus, the skins with high $I_{sc}$ should exhibit a more rapid rate of Na,K-ATPase turnover and, therefore, ouabain binding. If the nonzero intercept in Fig. 5 were the result of binding during the wash period, the skins with high $I_{sei}$ should, as a result of greater turnover rate, bind more ouabain during the wash period and, therefore, show a greater discrepancy between ouabain binding and $I_{sc}$ inhibition than skins with low $I_{sei}$. Inasmuch as high current skins exhibit a closer correspondence between ouabain binding and $I_{sc}$ inhibition than low current skins we must reject (b). Finally, (d) is unlikely because it would require that the amount of nonspecific binding vary with $I_{sc}$. If the nonspecific binding
were due to cellular damage, for example, an increased mannitol space would also be expected; no significant differences in mannitol space were found between high and low current skins. Rejection of all these other possibilities enhances the validity of the interpretation discussed above that the temporal separation between binding and inhibition is a manifestation of a "recruitment" phenomenon whereby binding and inhibition of one site produces a compensatory increase in the turnover rate of uninhibited sites.

Effects of Amiloride and Low External Na on Ouabain Binding

Having established the ability to measure ouabain binding to Na,K-ATPase of frog skin, it was of interest to determine the effects of specific active transport modifiers on ouabain binding. Amiloride was chosen because it inhibits trans-epithelial Na transport by blocking Na entry into the skin from the external bathing medium (Salako and Smith, 1970a; Biber, 1971); yet, the drug does not interfere with Na,K-ATPase activity (Baer et al., 1967). Paired experiments were performed utilizing skins from the same frog, comparing the amount of ouabain binding (in 60 min) in the presence and absence of 10⁻⁴ M amiloride in the external solution (in low-K Ringer). The results, shown in Table III, demonstrate that the presence of amiloride reduces the 60-min binding by 45%. The effect of amiloride in similar paired experiments is shown in Fig. 7 and expressed as fractional ouabain binding as a function of time. The fractional inhibition of binding due to amiloride appears to decrease asymptotically,

![Graph](image-url)
indicating that the effect of amiloride is to decrease the rate of ouabain binding without altering the total number of binding sites. This action of amiloride could be caused by a variety of circumstances, some of which would be: (a) a direct action of amiloride on the Na,K-ATPase in this tissue; (b) altered kinetic behavior of the Na,K-ATPase in response to amiloride-induced increases in cellular [K]/[Na]; (c) increased extracellular (serosal) K as a result of K loss from the cells, which would inhibit the rate of binding.

The next series of experiments was performed in an attempt to more clearly establish the basis for amiloride's effect upon ouabain binding. The effects of decreased intracellular Na on ouabain binding were tested in one series of experiments by studying ouabain binding to skins whose outer surface was

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Internal [3H]ouabain</th>
<th>External 10^-4 M amiloride</th>
<th>[3H]ouabain bound in 60 min (mol/cm²*mg)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^-8 M [3H]ouabain</td>
<td></td>
<td></td>
<td>1.32±0.02×10^12†</td>
<td>4</td>
</tr>
<tr>
<td>10^-8 M [3H]ouabain+30 mM K</td>
<td></td>
<td>10^-4 M amiloride</td>
<td>0.72±0.01×10^12‡</td>
<td>4</td>
</tr>
<tr>
<td>10^-8 M [3H]ouabain</td>
<td>Na_o = 0</td>
<td></td>
<td>0.66±0.08×10^12‡</td>
<td>5</td>
</tr>
<tr>
<td>10^-8 M [3H]ouabain+30 mM K</td>
<td>Na_o = 0</td>
<td></td>
<td>0.22±0.04×10^12‡</td>
<td>5</td>
</tr>
</tbody>
</table>

* The number of molecules bound has been corrected for extracellular [3H]ouabain.
† Mean ± SEM.

**Figure 7.** Effect of external amiloride on ouabain binding as a function of exposure time to 10^-8 M ouabain. Values are expressed as binding in the presence divided by that in the absence of amiloride, with all other conditions being identical. The control and experimental skins were paired tissues from the same animal. All values are expressed as mean ± SEM for four experiments.
bathed in distilled water. Decreasing the external Na concentration appears to lead to a decreased intracellular Na concentration (Cereijido et al., 1964; Cereijido et al., 1968). A 60-min incubation with \( ^{3}H \)ouabain and low-K Ringer on the serosal side and distilled water on the outside produced an amount of binding that was 50% of the control (Table III), yet not significantly different from that obtained with amiloride and low-K Ringer bathing the outside. Thus, the action of amiloride on ouabain binding is replicated by removing Na from the external solution, suggesting that this action of amiloride is the result of a reduction in intracellular Na. However, another possibility (see [c] above) is that depressed Na entry results in a decreased rate of pump cycling with a consequent increase in [K] in the intercellular spaces (Valenzeno and Hoshiko, 1976) which, in turn, inhibits ouabain binding. To test this possibility, a series of experiments was performed in which the serosal incubating solution was high-K (32.5 mM K) media containing \( 10^{-6} \) M \( ^{3}H \)ouabain and the external solution was distilled water. This treatment (Table III), produced the lowest amount of binding (after a 60-min exposure to \( ^{3}H \)ouabain) found in this investigation: 0.22 \( \times 10^{9} \) molecules/mg dcs or one-third of the value obtained with low-K Ringer as the serosal medium and distilled water as the outside bathing solution. The two-thirds decrease in binding produced by the high-K Ringer under these circumstances is almost identical in magnitude to that produced when the external solution contains 112 mM Na (see above). Therefore, it appears that the action of high serosal K is independent of external Na concentration and, inferentially, it may be concluded that low intracellular Na inhibits ouabain binding by a mechanism that does not involve loss of cellular K into the extracellular (serosal) compartment.

In measuring the volume of extracellular space with \( [^{14}C] \)mannitol, we observed that this space was 63 ± 28% \((n = 10)\) larger in the absence of external Na and 68 ± 26% \((n = 10)\) larger in the presence of external amiloride than the extracellular space observed when the external solution is low-K Ringer. This increase in extracellular volume is probably the result of a decrease in cellular volume resulting from Na, K, and osmotically obliged water loss.

**Ouabain Binding as a Function of Initial \( I_{sc} \)**

Values for ouabain binding to frog skin and simultaneously measured \( I_{sc} \) have been presented. In addition, experiments have been performed which alter \( I_{sc} \) and monitor the corresponding changes in the kinetics of binding. In performing these studies we used skins with initial \( I_{sc} \) in the range of 5-45 \( \mu A/cm^{2} \). We, thus, asked the question: what is responsible for this wide variation in \( I_{sc} \)? To determine the relationship between the absolute number of pumping sites and \( I_{sc} \), we plotted ouabain binding at 80-100% inhibition (○) and 70-80% (X) inhibition as a function of initial \( I_{sc} \). The results are presented in Fig. 8. These data show that the number of molecules of ouabain bound is independent of initial \( I_{sc} \), yet dependent upon the percentage of \( I_{sc} \) inhibition produced by ouabain. Thus, it seems that normal skin-to-skin variations in \( I_{sc} \) are due to differences in the turnover rate of the Na,K-ATPase rather than the total number of Na,K-ATPase molecules present.
DISCUSSION

One of the major obstacles to a quantitative study of drug-receptor interactions is the binding of the drug to membrane components other than the receptor. The present study has attempted to evaluate the specificity of [3H]ouabain binding to frog skin on the basis of $I_\infty$ inhibition. By correlating binding of ouabain with a physiological phenomenon we have attempted to establish its effect upon that process. Care was taken to establish the specificity of ouabain binding to the Na,K-ATPase; this involved several steps which may be summarized as follows:

(a) selecting a ouabain concentration that would minimize nonspecific "binding"; from the experiments shown in Fig. 1, the concentration chosen was $10^{-6}$ M;

(b) selecting an appropriate extracellular marker to correct for free oua-
bain present in the extracellular space(s); [\(^{14}\)C]mannitol appears to be a good choice, as shown in Table II, in that mannitol and ouabain appear to be distributed in the same extracellular space;

(c) correlating ouabain binding with \(I_{sc}\) inhibition to establish the relationship between ouabain interaction with the epithelium and a physiological effect;

(d) performing experiments under conditions known to alter the kinetics of ouabain binding to the Na,K-ATPase and demonstrating alterations in ouabain binding and \(I_{sc}\) inhibition which are consistent with known behavior of the Na,K-ATPase.

The effects of high K on ouabain binding to frog skin should give a minimum estimate for the number of ouabain binding sites that are Na,K-ATPase, because it seems reasonable to assume that high K should have no effect on nonspecific binding. The presence of high K inhibits binding at 60 min by about two-thirds, whether the external Na concentration is 112 mM or zero. Therefore, in both of these cases, at least two-thirds of the ouabain binding is to the Na,K-ATPase and no more than one-third of the binding could be nonspecific. The depressed rates of ouabain binding in Na-free or amiloride-containing external media are probably a reflection of inhibited Na,K-ATPase turnover caused by an increased ratio of intracellular K/Na, as is discussed later. Therefore, it is reasonable to assume that the experimental condition that resulted in the lowest binding, namely, no external Na and high serosal K (Table III), provides a value for the maximum nonspecific ouabain binding to frog skin: \(\approx 20\%\) of the total. The true value for nonspecific binding is probably even lower (<16% of total) inasmuch as high-K Ringer only decreases the rate and not the maximal amount of ouabain binding.

The above considerations demonstrate that at least 80–90% of \(^{3}\)Houabain binding to split frog skin is specific for Na,K-ATPase. Assuming that one ouabain molecule is bound to each enzyme molecule, we can obtain a measure of the amount of ATPase present in this tissue: \(=1.3 \times 10^{12}\) molecules/mg per dcs. This number is within the range of that obtained for other tissues (Baker and Willis, 1972; Brading and Widdicombe, 1974). Inasmuch as the average weight of tissue used was 1.52 mg and this corresponds to a surface area of 0.785 cm\(^2\), we obtain 1.95 mg/cm\(^2\) and 2.1–2.5 \(\times 10^{13}\) sites/cm\(^2\). If we assume that all these sites are directly involved in active Na transport (see discussion of Fig. 6 A and B in Results), we can calculate a range of turnover rates for the Na,K-ATPase in this tissue based on the observed range of initial \(I_{sc}\). In that the \(I_{sc}\) equals the active Na flux in this tissue (Ussing and Zerahn, 1951), turnover rates for Na transported through the pump may be calculated as follows:

\[
t = \frac{(I_{sc}/F) \times N \times 60}{\text{sites/cm}^2},
\]

where \(t\) is turnover rate in molecules/site per minute, \(F\) is Faraday constant, and \(N\) is Avogadro's number.

The calculated turnover rates range from 830 molecules/site per min when \(I_{sc} = 5 \mu\text{A/cm}^2\) to 7,500 molecules/site per min when \(I_{sc} = 45 \mu\text{A/cm}^2\). The turnover
rates cited above fall within the range of pump turnover rates calculated for frog skin (Aceves and Wright, 1977) and other tissues (Brading and Widdicombe, 1974; Baker and Willis, 1972; Dunham and Hoffman, 1971).

Recently, two other groups of investigators (Mills et al., 1977; Aceves and Wright, 1977) also found evidence that ouabain binding was correlated with inhibition of $I_e$. Both groups also observed sensitivity of ouabain binding to extracellular $K$, as found in the present investigation. In addition, Mills et al. (1977) found that $[^3H]ouabain$ was distributed throughout all layers of the epithelium leading to the conclusion that these layers functioned as a syncytium with regard to active $Na$ transport. The present observation of a linear correlation between ouabain binding and $I_e$ inhibition with a zero intercept in skins with high $I_e$ indicates that all Na,K-ATPases are involved in active $Na$ transport, thus supporting the idea of the functional syncytium. Further evidence for the syncytial nature of the frog skin may be obtained from our experiment using amiloride. These data show that addition of amiloride to the outside bathing medium depresses the rate of ouabain binding to the frog skin (Fig. 7, Table III). Inasmuch as $Na$ entry into the frog skin is almost exclusively via the amiloride sensitive pathway (Moreno et al., 1973; Rick et al., 1975), the presence of amiloride in the outside bathing media would lead to a reduction of $Na$ in the "transport pool" (Dörge and Nagel, 1970; Salako and Smith, 1970b) and, therefore, it is reasonable to expect that the alteration in ouabain binding kinetics caused by amiloride is the result of changes in cellular $Na$. This interpretation is supported by the results obtained in $Na$-free external solutions, in which ouabain binding is inhibited to the same extent as with amiloride. Thus, it would seem that the effect of external amiloride on ouabain binding is the result of some event(s) associated with depressed $Na$ entry, rather than a more direct effect of amiloride on the Na,K-ATPase. Recent studies of intact epithelia and disaggregated cells (Valenzeno and Hoshiko, 1976) have shown that $K$ lost from the intracellular compartment does not leave the intracellular spaces but is recycled. That a building of $K$ in the intracellular spaces cannot explain the effect of amiloride on ouabain binding kinetics can be seen from data in Table III and the associated discussion (see Results). Therefore, we conclude that the decreased rates of ouabain binding produced by amiloride and the absence of external $Na$ are explicable in terms of intracellular events which result in a decreased turnover rate of the Na,K-ATPase. In this regard, other investigators (Salako and Smith, 1970b; Dörge and Nagel, 1970) have shown that the addition of amiloride or removal of $Na$ from the external medium results in a decrease in the amount of $Na$ present in the $Na$ transport pool. Yet the effect of this decrease in total $Na$ upon the intracellular $Na$ concentration is still unclear because it depends upon accurate measurement of the extracellular volume. Using inulin as a space marker, Nagel and Dörge (1970) found a 5% increase (not significant) in extracellular volume upon addition of amiloride; however, various investigators have demonstrated that this marker does not accurately measure the extracellular space (Cereijido et al., 1968; Biber, Aceves, and Mandel, 1972; Biber, Cruz, and Curran, 1972). The present results, using mannitol as an extracellular space marker, show that in
the presence of amiloride in the outside bathing media there is a 68% increase in extracellular volume after a 60-min exposure to the drug. Because Salako and Smith (1970b) have shown that total tissue water remains unaltered under these conditions, the increase in extracellular volume must be accompanied by a decrease in intracellular water content. Utilizing the 3:1 ratio of intracellular to extracellular water measured by Nagel and Dörge (1970) as a rough approximation, the 68% increase in extracellular volume would produce a sufficiently large decrease in intracellular water content to maintain the intracellular Na concentration unchanged. Thus, although it is clear that the treatments described above lead to decreases in cellular Na content, changes in cellular Na concentration may be minimized by virtue of osmotically obliged cell water loss; however, if Na and water leave the cells the K concentration will rise causing an increase in the ratio of K to Na concentrations in the cell. An increase in the intracellular ratio of K to Na, whether obtained by decreased cellular Na concentration or increased cellular K concentration, will produce a well known inhibitory effect upon the Na,K-ATPase (Sachs and Welt, 1967; Knight and Welt, 1974; Sachs, 1970; Sachs et al., 1974). Thus, we propose that decreased rates of ouabain binding in Na-free and amiloride-containing media (Fig. 7, Table III) are reflections of decreased pump turnover rate in response to an increase in cell [K]/[Na] (Knight and Welt, 1974).

The effect of transepithelial potential (PD) on binding also needs to be considered because the PD value was substantial during most binding experiments whereas it was zero in the presence of amiloride or the absence of external Na. According to Nagel (1976), addition of amiloride to the external solution causes profound changes in the potential observed across the external membrane but leaves the serosal potential virtually unaffected under open circuit conditions. Therefore, a change in transepithelial potential by itself would not be expected to influence ouabain binding significantly.

The kinetics of ouabain binding are difficult to express mathematically even in cellular systems where the ouabain concentration at the membrane surface is known with certainty. In the present experiments, ouabain binding appears to increase markedly between 15 and 30 min (Fig. 3). This may be a reflection of diffusion barrier(s) presented to ouabain by the frog skin. In support of this point, under comparable conditions, saturation binding does take ≈30 min longer for frog skin than red blood cells (Dunham and Hoffman, 1971) or disaggregated cells from frog skin (Zylber et al., 1975). Finally, this delay is consistent with the time it takes mannitol and presumably, ouabain to equilibrate with the extracellular space.

The spontaneous variation of $I_{sc}$ observed from skin to skin does not appear to be correlated with the amount of Na,K-ATPase present in each skin. As shown in Fig. 8, no relationship exists between the number of ouabain binding sites and the magnitude of the $I_{sc}$. The spontaneous variations in the rate of active Na transport ($I_{sc}$) are a reflection of different turnover rates for the Na,K-ATPase and not the amount of enzyme present. The skin-to-skin variation in $I_{sc}$ must, therefore, be due to another step in transepithelial Na transport, probably the entry of Na from the external solution into the intracellular transporting compartment. The results of various studies suggest that the rate-determining
step for active Na transport in frog skin is the entry step (Curran et al., 1963; Mandel and Curran, 1973; Rick et al., 1975), and the present findings are consistent with this type of model. We are presently studying the effects of various ionic environments and pharmacological agents on both the rate and total amount of ouabain binding to split frog skins. Although the present study establishes the feasibility of studying transport of alkali metals in frog skin in conjunction with ouabain binding, it is clearly only a first step toward understanding ionic interactions with epithelial membrane components.

In summary, we have presented data demonstrating the feasibility of using split frog skin and an extracellular marker to gain sufficient resolution to isolate specific interactions of [3H]ouabain with the Na,K-ATPase from those interactions which are nonspecific. Furthermore, the relationship between ouabain binding and inhibition of $I_{sc}$ are strong evidence for the direct involvement of the Na,K-ATPase in the generation of the short circuit current. The results of experiments in which the ionic composition of extra- and, presumably, intracellular compartments are altered show that the ouabain binding (and, therefore, inhibitory) sites respond in a manner predictable from studies of the Na,K-ATPase in cellular and homogenized preparations of the enzyme. Finally, it appears that normal skin-to-skin variations in $I_{sc}$ are the result of differences in turnover rate of the Na,K-ATPase rather than the amount of enzyme present.

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