Ouabain on Active Transepithelial Sodium Transport in Frog Skin

Studies with Microelectrodes

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ABSTRACT Studies were done with isolated frog skin to determine the effects of 10^{-4} M ouabain on the electrophysiological parameters of outer and inner barriers of the Na-transporting cells. Microelectrodes were used to impale the skins from the outer surface to determine the intracellular voltages (V_o^{sc}) under conditions of short-circuiting and under conditions where a voltage clamp was used to vary the transepithelial voltage, V_T. From this, the electrical resistances of outer (R_o') and inner (R_i) barriers were estimated. In addition, the driving force for active transepithelial Na transport ($E_{Na} = E_i'$) was estimated from the values of V_T when the $V_o = 0$ mV (Helman and Fisher. 1977. *J. Gen. Physiol.* **69:** 571–604). Studies were done with skins bathed with the usual 2.4 meq/liter $[K]_i$ in the inner solution as well as with reduced $[K]_i$ of 0.5 and 0 meq/liter. Characteristically, the responses to ouabain could be described by an initial rapid phase (5–10 min) during which time the R_i was increased markedly and the E_i' was decreased from control values. Thereafter, during the slow phases of the response, the resistances of both outer and inner barriers increased continuously and markedly with time leading ultimately to essentially complete inhibition of the short-circuit current. Similar studies were done with skins exposed to 10^{-4} M amiloride in the outer solution. Although estimates of R_i could not be obtained under these conditions, the effects on the V_o^{sc} and E_i' were similar to those observed for the Na-transporting skins. However, the magnitudes of the effects were less and relatively slower than observed for the Na-transporting skins. The results of these studies were analyzed within the context of a proposed electrical model that takes into account the observation that the magnitude of the voltage at the inner barrier appears to exceed the equilibrium potential for K especially when transepithelial Na transport is inhibited at the apical barrier of the cells.

INTRODUCTION

Of the inhibitors of active Na transport by epithelial tissues, the action of the cardiac glycoside, ouabain, is of particular interest because of its specificity of binding to the (Na + K)-ATPase at the basolateral borders of the epithelial cells (Farquhar and Palade, 1966; Ernst, 1972; Stirling, 1972; Mills et al., 1977). In

frog skin and other epithelia ouabain causes inhibition of transepithelial Na transport as measured either isotopically or electrically as the short-circuit current, I_{sc} (Koefoed-Johnsen, 1957; Herrera, 1966; Solinger et al., 1968; Nagel and Dörge, 1971; Robinson and Macknight, 1976). In order to explain ultimately the inhibition by ouabain of the I_{sc}, it will be necessary to understand the relationship between the forces and ionic flows occurring at apical and basolateral barriers of the Na transporting cells. In this regard, the present studies were concerned primarily with an evaluation of the electrical changes occurring at these barriers as evaluated with intracellular voltage recording microelectrode techniques.

Preliminary reports of this work have appeared elsewhere (Helman and Nagel, 1977; Nagel and Helman, 1977*a*; Nagel and Helman, 1977*b*).

MATERIALS AND METHODS

Microelectrode studies were done with belly skins of *R. pipiens berliendieri* (Mogul Ed, Oshkosh, Wis.; Southwestern Scientific Co., Tucson, Ariz.) with methods described in detail elsewhere (Nagel, 1976; Helman and Fisher, 1977).

In general, the skins were short-circuited continuously with a voltage clamp and the short-circuit current allowed to stabilize for 1–3 h. The skins were bathed symmetrically with a Cl-HCO_3 Ringer (microelectrode studies) containing in millimolar: 100.0 NaCl, 2.4 $KHCO_3$, 2.0 $CaCl_2$, and 11.1 glucose or in a sulfate Ringer (studies of the I_T-V_T relationships) containing in millimolar: 57.2 Na_2SO_4, 2.4 $KHCO_3$, and 1.2 $CaSO_4$. In some studies the K concentration of the inner bathing solution was decreased from the usual 2.4 to either 0.5 and 0.0 mM by substitution of K with Na. These solutions will be referred to by the notation 2.4 $[K]_i$, 0.5 $[K]_i$, and 0 $[K]_i$. Ouabain (Sigma Chemical Co., St. Louis, Mo.) was added to the inner solution at a concentration of 10^{-4} M except in those studies of the I_T-V_T relationships where the amiloride concentration was 5×10^{-7} M. In studies of the I_T-V_T relationships, skins were mounted in chambers described previously in detail (Helman and Miller, 1971).

Chambers

In microelectrode studies, skins were mounted horizontally in chambers which were constructed to permit the solution bathing the inner surface of the skin to be changed without disturbing the position of the tip of the microelectrode. The chamber system described by Nagel (1976) and the system with essentially no hydrostatic pressure across the skin described by Helman and Fisher (1977) were used. The latter system was modified for later studies to include a stainless steel grid support at the corial side of the skin, and this permitted a subpressure of ~30 cm H_2O to be applied to hold the skin against the support and to allow continuous flow of fluid through the inner chamber throughout the entire period of study. The flow rate was usually 3–6 ml/min through a chamber volume of ~0.3 ml. Owing to the dead space between the fluid reservoir and the inner chamber, a delay of usually 30–60 s and in some cases longer (flow rates <3 ml/min) occurred before ouabain added to the reservoir reached the chamber. It should be noted that the results of studies done in the presence or absence of a hydrostatic pressure gradient and in the presence or absence of a grid were the same.

Microelectrodes

Microelectrodes were made with a horizontal puller (Narishige Scientific Instrument Lab., Ltd., Tokyo, Japan) using Omega dot glass capillaries (Frederick Haer & Co.,

Brunswick, Maine, 30-30-1) immediately before use. The tips of the electrodes were filled with 3 M KCl by capillarity and thereafter backfilled. Tip resistances ranged between 15 and 40 MΩ. In order to rule out systematic effects of KCl leakage from the tips into the cells during impalement, some tips were filled with 150 mM KCl, and the values of intracellular voltage were compared with those observed with 3 M KCl-filled electrodes. Since the values of intracellular voltage were essentially the same, all studies were done with 3 M KCl-filled electrodes. Additional studies were done with electrodes filled with either 4 M K-acetate or 3 M NH_4NO_3, and here, too, the intracellular voltages were similar. We attempted to determine the rate of K leakage using electrodes filled with 3 M KCl and the highest concentration of ^{42}K available to us (20–40 mCi/ml ^{42}K, New England Nuclear, Boston). In most electrodes the rate of K leakage was so low that an estimate of leakage rate could not be obtained despite the high level of ^{42}K activity in the pipettes. It should be noted also that the intracellular voltages remain essentially constant for considerable periods of time consistent with the belief that K leakage from the tips is negligible and at best unimportant. Moreover, reimpalement of the skin at any time during an experiment gave instantaneous voltages not different from those observed with microelectrodes used to record chronically.

Method of Procedure

MICROELECTRODE STUDIES The skins were impaled from above in a perpendicular direction with the microelectrode advanced from the outer solution. The intracellular potentials, V_o, were recorded with reference to the outer solution. A voltage clamp was used in two ways: first, to maintain the skins at a transepithelial voltage of zero thereby permitting the short-circuit current, I_{sc}, to be monitored continuously. The values of V_o for skins short-circuited will be referred to with the symbol V_o^{sc} and are identical in magnitude to those of the inner barrier, V_i, when $V_T = 0$. Second, to determine the values of E_1' that are thought also to provide estimates of E_{Na}, the voltage clamp was used to vary the V_T (pulse duration = 600 ms) in order to determine the value of V_T that brings the V_o to zero millivolts. This follows the idea proposed before that the E_{Na} can be estimated from those values of $V_T = E_1'$ at which transepithelial Na transport and the V_o are thought to be not different from zero (Helman and Fisher, 1977).

From the slope of the relationship between V_T and V_o, the fractional transcellular resistance of the outer barrier, $\%R_o$, was estimated as described in detail elsewhere (Helman and Fisher, 1977):

$$\%R_o = V_o^{sc}/E_1' \times 100 = \Delta V_o/\Delta V_T \times 100 = R_o^f/(R_o^f + R_i) \times 100. \quad (1)$$

The symbols R_o^f and R_i refer to the specific resistances of the outer and inner barriers, respectively. It should be noted that the R_o^f is determined when the V_o is negative. No attempt was made in the present studies to evaluate the R_o^b when V_o is positive (see Helman and Fisher, 1977).

Since

$$R_o^f + R_i = R_A^f = E_1'/I_{sc}, \quad (2)$$

the specific resistances in ohm·cm² could be estimated.

After the skins had been allowed to stabilize while short-circuited, the control values of V_o^{sc} were determined. It was not unusual to obtain an impalement in which the values of V_o^{sc}, $\%R_o$, and E_1' would remain stable for considerable periods of time often exceeding 1 h or more. Thus, we could measure from a single impalement control values for 10–20 min and then follow the time-course of the changes caused by ouabain (10^{-4} M) added to

the inner solution. The control values of V_0^{sc} observed among cells of a single skin were remarkably similar and in preliminary studies (not reported) a number of cells were punctured at intervals of 5–10 min throughout control and experimental periods. Despite a slightly larger scatter of the data, these studies gave the same results as obtained by continuous monitoring from a single impalement.

I_T-V_T RELATIONSHIPS Skins were short-circuited continuously, and the I_T-V_T relationships determined during control and ouabain experimental periods with methods described in detail elsewhere (see for example Helman and Fisher, 1977). From the steady-state values of transepithelial current and voltage, I_T and V_T, respectively, the slope resistances were calculated with linear regression analysis and the transepithelial voltage E_1 defined at the intersection of slope resistances R_1 and R_2 (see Fig. 1). Previous studies indicated that the values of E_1 were the same as those of the E_{Na} of Ussing and Zerahn (1951) and the same as those of E_1' as described above in studies with microelec-

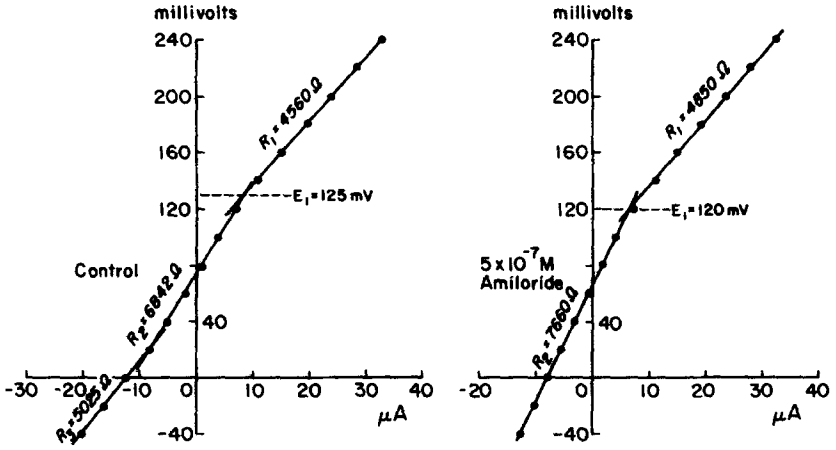

FIGURE 1. Effect of 5×10^{-7} M amiloride on $I_T - V_T$ relationship. Note change of slope resistance R_3 and linearity of slopes R_2 and R_3 after amiloride. Voltage E_1 at break remains essentially unchanged by amiloride.

trodes (Helman and Fisher, 1977). Accordingly, we have assumed this identity to be valid in the present studies, and the calculations of the resistances reflect this.

RESULTS

Studies of $I_T - V_T$ Relationships

Previous work from our laboratory (Davies, 1973; Fisher and Helman[1]) which involved studies of the transepithelial current-voltage relationships of frog skin showed that despite large decreases of the I_{sc} with 10^{-4} M ouabain, the changes of E_{Na} as estimated from the breaks at voltages E_1 (see Fig. 1) were relatively small, in the vicinity of 20%. To explain the entire decrease of the I_{sc} it was necessary to suggest that the electrical resistance to transepithelial Na transport was increased by ouabain. This suggestion was in accord with previous measurements of the effect of ouabain on the Na influx at the apical barrier of the skin (Biber, 1971; Erlij and Smith, 1973) and on the osmotic behavior of the frog skin

[1] Fisher, R. S., and S. I. Helman. Unreported results.

(MacRobbie and Ussing, 1961). Since the effects of ouabain on E_1 were somewhat unexpected, we report in detail one set of experiments.

In these studies, skins were incubated in a sulfate-Ringer solution containing 5×10^{-7} M amiloride in the outer solution. This causes, as reported previously, a partial inhibition of the I_{sc} and a complete linearization (see Fig. 1) of the I_T-V_T plots between voltages −40 mV and the E_1 (Macchia and Helman, 1974; Helman and Fisher, 1977; Macchia, 1977). Under these circumstances, the transepithelial electrical model of Fig. 2 describes the behavior of the skin. It follows directly from the slope resistances R_1 and R_2 ($\Delta V_T/\Delta I_T$) that

$$R_A^f = [(R_2R_s)/(R_s - R_2)] = E_1/I_{sc}, \tag{3}$$

and

$$R_A^b = (R_1R_s)/(R_s - R_1), \tag{4}$$

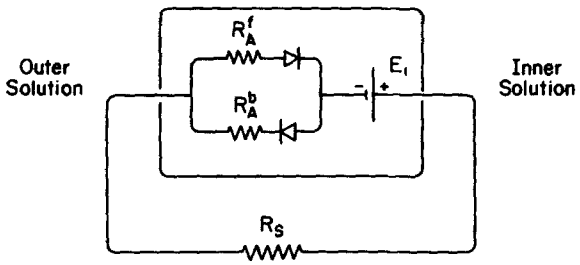

FIGURE 2. Electrical equivalent circuit of skin in the presence of 5×10^{-7} M amiloride. Current flow from outside to inside occurs via resistance R_A^f. No distinction is made here for the resistance of outer R_o^f and inner barriers R_i. $R_A^f = R_o^f + R_i$. When $V_T > E_1$, current flow is via $R_A^b = R_o^b + R_i$. When $V_T = E_1$, current flow is via the shunt pathway, R_s. $R_A^f \neq R_A^b$.

where R_s is the shunt resistance. It also follows directly that if $E_1 = E_{Na}$, the transepithelial current is via R_s alone when $V_T = E_1$. Thus, $R_s = E_1/I_1$ (Macchia and Helman, 1974; O'Neil and Helman, 1976; Helman and Fisher, 1977; Macchia, 1977).

Ouabain on E_1 and I_{sc}

A typical study consisted of the determination of six to nine control I_T-V_T relationships at intervals of 10 min. A summary of mean control parameters is given in Table I for skins exposed to 5×10^{-7} M amiloride. After 10^{-4} M ouabain was added to the inner solution, I_T-V_T relationships were determined at intervals of 5 min for up to 3 h. Within 20–40 min, E_1 fell ~20% from control and remained essentially constant thereafter (Fig. 3). The I_{sc} fell initially to ~60% of control within 60 min followed thereafter by a secondary decrease toward zero.

It should be noted for these particular studies with skins exposed to 5×10^{-7} M amiloride in sulfate Ringer that the time required for ouabain to act on the I_{sc} was relatively long (see later). The reason(s) for this are unknown but the

prolonged duration of action taken together with an especially high shunt resistance averaging 39,000 $\Omega \cdot cm^2$ permitted long-term observation of I_T-V_T relationships with excellent resolution of the breaks at E_1.

Ouabain on Transepithelial Resistances

No significant changes of the slope resistances R_1 and R_2 were observed for the first 40 min of ouabain action (Fig. 4). Between 40 and 60 min, both R_1 and R_2 decreased markedly to new stable values. As noted above, the shunt resistance was especially high, and as shown in Fig. 5, the shunt conductance, G_s, tended

TABLE I

CONTROL VALUES OF *I-V* PARAMETERS OF SKINS INCUBATED IN SO_4 RINGER IN THE PRESENCE OF 5×10^{-7} M AMILORIDE

	I_{sc}	E_1	V_{oc}	G_s	R_A^f	R_A^b	R_1	R_2
	$\mu A/cm^2$	mV	mV	$\mu mho/cm^2$	Ωcm^2	Ωcm^2	Ωcm^2	Ωcm^2
Mean	16.1	117.6	83.0	25.7	9,460	4,471	3,612	6,020
± SE	±4.9	±3.7	±11.6	±6.8	±1,504	±647	±489	±651
$n = 6$								

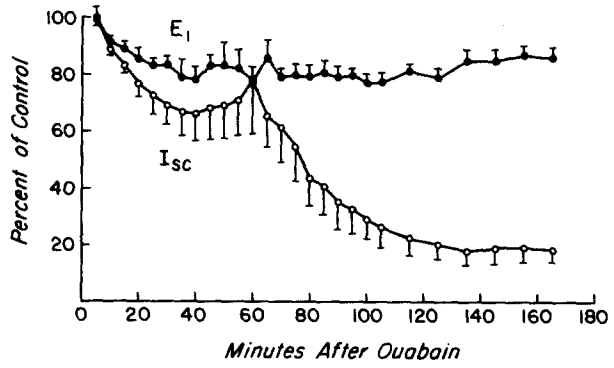

FIGURE 3. Changes of E_1 and I_{sc} with 10^{-4} M ouabain added to the inner solution of 5×10^{-7} M treated skins. Note small decrease of E_1 and initial and secondary effects on I_{sc} after 60 min. Values are mean ± SE as percentage of control.

to increase but not significantly during the first 40 min. Thereafter, the G_s increased markedly (~14 times control value) during the ensuing 40 min. Consequently, the decreases of R_1 and R_2 shown in Fig. 4 can to a large part be attributed to a large decrease of the shunt resistance, R_s. Such an observation is compatible with the finding of Biber and Mullen (1977) and this laboratory that ouabain causes large increases of the unidirectional fluxes (inside to outside) of Na and sucrose (Davies, 1973).

Calculation of the values of R_A^f and R_A^b indicated as shown in Fig. 6 that ouabain caused large but selective increases of these resistances. During the first 40–60 min, the resistances increased slowly, but thereafter the R_A^f was increased considerably more than the R_A^b.

In summarizing the results of these studies, the data can be interpreted to

indicate that ouabain causes first a small decrease of the E_{Na} as estimated from values of E_1, followed by secondary increases of the R_A^f and G_s occurring predominantly at a time when the E_1 appears rather constant.

Studies with Microelectrodes

In order to permit an assessment of the changes occurring individually at apical and basolateral membranes, intracellular microelectrodes were used to monitor the intracellular voltage. This also permitted us to obtain estimates of the specific resistances of these barriers to ion transport. Shown in Table II are the control parameters (before ouabain) of 34 skins subjected to study with microelectrodes. Of these, 12 skins were exposed continuously to 10^{-4} M amiloride and were considered to be non-Na transporting. The mean I_{sc} of the 22 Na-transporting skins was 44.2 μA/cm^2. The V_o^{sc} averaged -103.6 mV and the E_1'

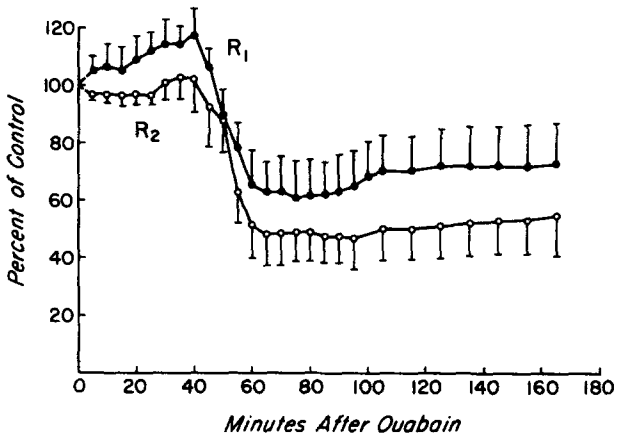

FIGURE 4. Changes of slope resistances R_1 and R_2 of the I_T-V_T relationships. Values are mean ± SE as percentage of control.

averaged 121.2 mV. The $\%R_o$ averaged 85.7% and so the outer barrier accounted for a very large fraction of the transcellular resistance. For the skins exposed to 10^{-4} M amiloride, the I_{sc} averaged 1.1 μA/cm^2, the V_o^{sc} averaged -111.2 mV, and the E_1' averaged 114.7 mV. With amiloride present in the outer solution, essentially all of the transcellular resistance could be attributed to the outer barrier since the $\%R_o$ averaged 97%. For Na-transporting skins, the R_A^f was calculated to be 2,470 Ω·cm^2, and as expected, amiloride caused the mean value of R_A^f to increase to 104,000 Ω·cm^2.

Ouabain on Na-Transporting Skins: Effects on V_o^{sc}, E_1', I_{sc}, R_o^f, and R_i

In all studies, the responses to ouabain could be characterized by two phases to be referred to as rapid and slow phases. As shown in Fig. 7 (see also Figs. 8 and 9), after a delay of a few minutes (ouabain to reach inner chamber and diffuse through corium to the inner barrier), the voltages V_o^{sc} and E_1' fell rapidly within 5–10 min to mean values of 61.9 and 64.0% of their pre-ouabain control values (Table III) for skins bathed with 2.4 $[K]_i$. Thereafter, the voltages most often

continued to decline slowly, although occasionally the E'_1, as shown in Fig. 7, tended to increase during the slow phase. The I_{sc} fell abruptly during the rapid phase to ~60.6% of control and continued to decline during the slow phase of response. At 60 min the I_{sc} had fallen to a mean of 18.3% whereas the V_o^{sc} and E'_1 remained elevated at 51.6 and 63.1% of their respective pre-ouabain control values (Table III).

According to the interpretation of the above studies of the I_T-V_T relationships, it was anticipated that, in part, inhibition of the I_{sc} with ouabain was mediated via changes of the R_A^f. To examine this premise with regard to outer and inner barriers, the R_o^f and R_i were calculated with Eqs. 1 and 2. The results of a typical experiment are shown in Fig. 7 (see also Figs. 8 and 9 at reduced $[K]_i$) and are

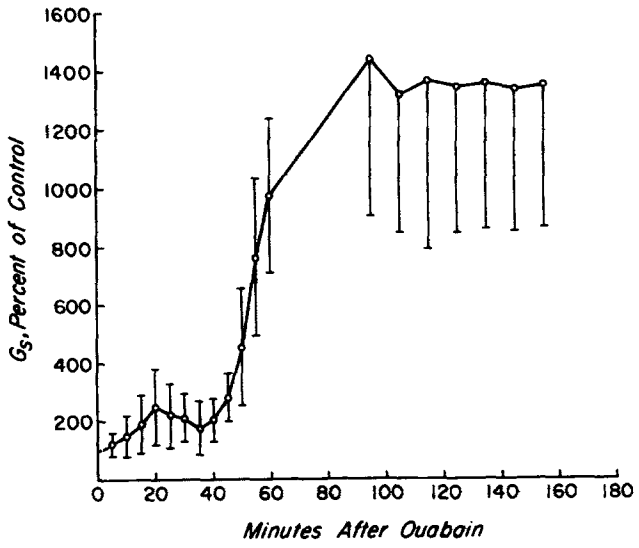

FIGURE 5. Change in shunt conductance with 10^{-4} M ouabain added to skins treated with 5×10^{-7} M amiloride. Values are mean ± SE as percentage of control.

summarized in Table III. As soon as I_{sc} began to decrease (rapid phase), the R_i increased to values considerably above control with little or no change of the resistance of the outer barrier, R_o^f. During the slow phase, both the R_i and R_o^f increased markedly to values approximately 14 and 10 times control, respectively, at 60 min for skins bathed with 2.4 $[K]_i$. Indeed, the rapid phases of the response to ouabain were associated with decreases of the voltages V_o^{sc} and E'_1 and selective increases of R_i. The slow phases were characterized predominantly by changes of the resistances of both apical and basolateral barriers. It should be recalled that since $R_o^f >> R_i$, the decrease of the I_{sc} is more likely attributed to the increase of R_o^f of the outer barrier than to the increase of R_i.

Ouabain in the Presence of Reduced $[K]_i$

Additional studies were done with ouabain after the [K] of the inner solution was reduced to either 0.5 or 0.0 mM. As shown in Figs. 8 and 9, reduction of

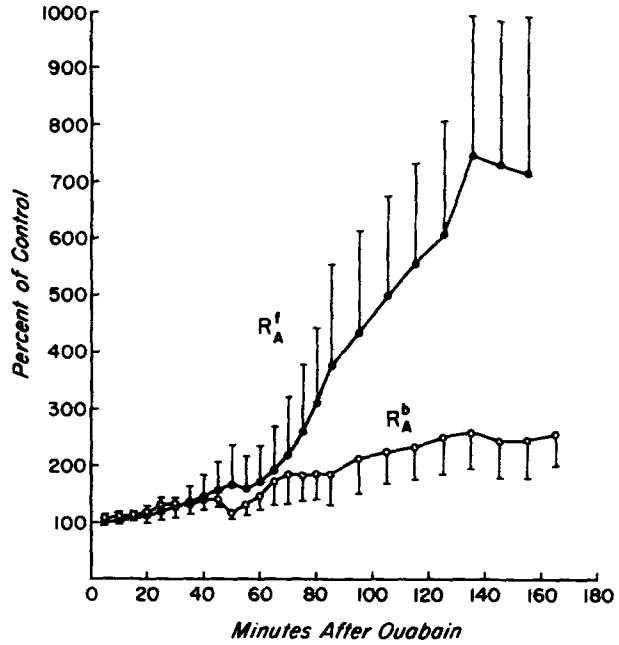

FIGURE 6. Changes of R_A^t and R_A^b with 10^{-4} M ouabain. Values are mean ± SE as percentage of control.

TABLE II

CONTROL PARAMETERS FOR SKINS BATHED WITH 2.4 $[K]_i$, Cl-HCO_3 RINGER

	I_{sc}	V_o^{sc}	E_t'	$\%R_o$
	$\mu A/cm^2$	mV	mV	
Control	44.2	−103.6	121.2	85.7
(n = 22)	±4.9	±2.6	±2.4	±1.9
	(11–113)	(−75 to −128)	(100–143)	(64.5–96.7)
10^{-4} M Amiloride	1.1	−111.2	114.7	97.0
(n = 12)	±.45	±2.7	±2.4	±1.0
	(+1 to −4)	(−99 to −129)	(99–127)	(91.4–102.0)

$[K]_i$ caused the voltages V_o^{sc} and E_t' to increase. Summarized in Table IV are the pre-ouabain control values of skins bathed with 0.5 and 0.0 $[K]_i$. As for skins bathed with 2.4 $[K]_i$, ouabain caused the voltages to fall at first rapidly followed by a slow phase of voltage decline. In these studies the rate of voltage decrease during the rapid phase was considerably faster, especially in those skins bathed with 0 $[K]_i$ (see below).[2] Correspondingly, the R_t was increased selectively during

[2] No attempt was made to regulate precisely the flow rate through the inner chamber, and consequently, the time of appearance of the ouabain in the inner chamber and its concentration buildup to 10^{-4} M was variable. In addition, because of the appreciable unstirred layer consisting primarily of the corium of the skin (~500 μm) the appearance of ouabain at the functional inner barrier would be expected to require in the order of 1–2 min depending, of course, on the thickness

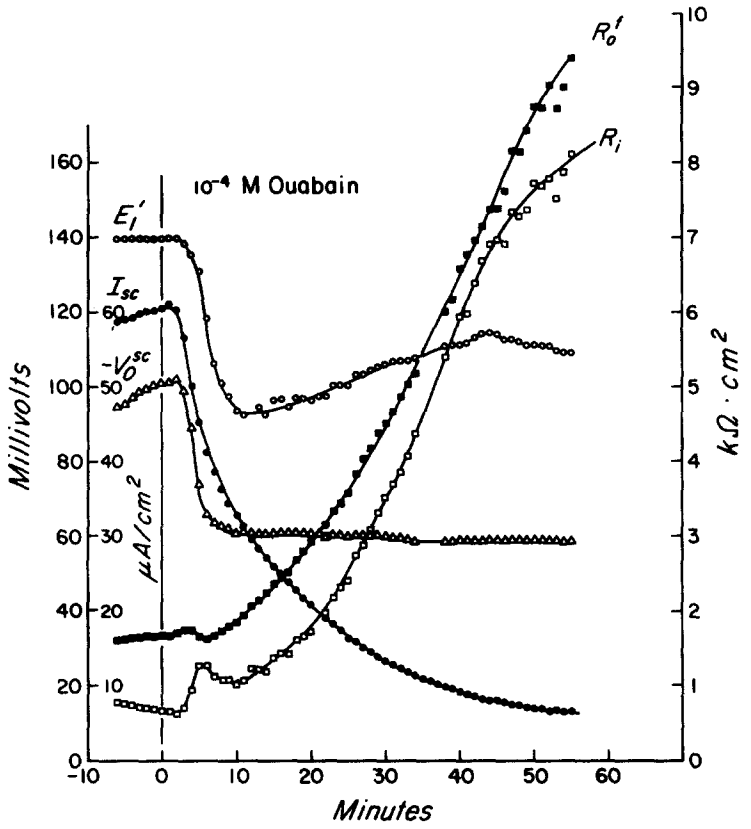

FIGURE 7. Effect of ouabain on electrical parameters of a Na-transporting skin. $[K]_i$ = 2.4 mM. Note rapid and slow phases of the responses. 10^{-4} M ouabain was added to the inner solution reservoir at time zero. The delay in response is in part attributed to the time required for the ouabain to reach the inner chamber, to mix within the chamber, and to diffuse through the corium to the functional inner barrier. Note also the immediate increase of R_i with little or no change of R_o^f.

the rapid phase with little or no change of the R_o^f, and thereafter both resistances increased markedly during the slow phases of the response to ouabain (Table III).

In order to quantitate the rapidity of the voltage responses to ouabain, we

of the corium in any particular skin and the effective diffusion constant of ouabain in this layer. Consequently, it was not surprising to observe delays usually in the range of 2–6 min before changes in electrical parameter values were observed. Because of the existence of unstirred layers, it remains unknown whether the rate of decline of the voltages reflects the rate at which the ouabain concentration increases at the functional inner barrier or the rate at which the pump sites bind ouabain. Assuming all of these factors being equal, it was observed that the rate of fall of the voltages was more rapid when the $[K]_i$ was reduced from 2.4 to 0.5 and 0 mM. Such an observation would be compatible with facts established elsewhere that the rate of inhibition of the (Na + K)-ATPase by ouabain varies with the [K] of the incubation media (see review by Glynn and Karlish, 1975).

TABLE III

EFFECT OF OUABAIN ON Na-TRANSPORTING SKINS (PERCENTAGE OF CONTROL)

	I_{sc}	V_o^{sc}	E'_i	R'_o	R_i	R'_A
			%			
2.4 $[K]_i$ (n = 6)						
Rapid phase	60.6±4.9	61.9±1.5	64.0±1.6	107±12	179±34	119±12
Slow phase, *min*						
15	45.6±5.9	60.8±1.0	64.3±1.6	161±18	210±36	166±16
30	26.5±5.7	57.8±3.1	62.6±4.7	291±55	368±76	308±60
45	21.8±7.8	55.9±4.0	63.5±5.6	568±188	718±241	621±207
60	18.3±9.5	51.6±4.1	63.1±7.5	1,039±439	1,373±648	1,145±555
0.5 $[K]_i$ (n = 7)						
Rapid phase	53.5±5.2	67.2±5.9	70.1±4.2	120±10	277±48	131±10
Slow phase, *min*						
15	39.2±4.9	65.3±6.0	67.6±4.4	160±15	301±49	170±15
30	25.6±5.8	63.1±6.6	64.7±4.8	268±40	435±85	280±40
0 $[K]_i$ (n = 5)						
Rapid phase	36.4±4.4	46.2±3.6	52.6±5.3	104±20	304±26	120±23
Slow phase, *min*						
15	30.5±3.1	47.4±3.9	52.2±4.9	156±13	367±45	172±16
30	17.2±2.7	41.7±4.7	48.5±7.2	187±16	1,052±520	256±82
45	11.0±1.9	42.2±6.6	45.4±8.5	422±114	890±297	468±144
60	8.5±2.5	40.5±3.1	47.4±8.3	513±140	1,470±752	628±218

For purposes of summary, values were taken at the end of the rapid phase and at 15, 30, 45, and 60 min after addition of 10^{-4} M ouabain to the inner solution reservoir. Means ± SE.

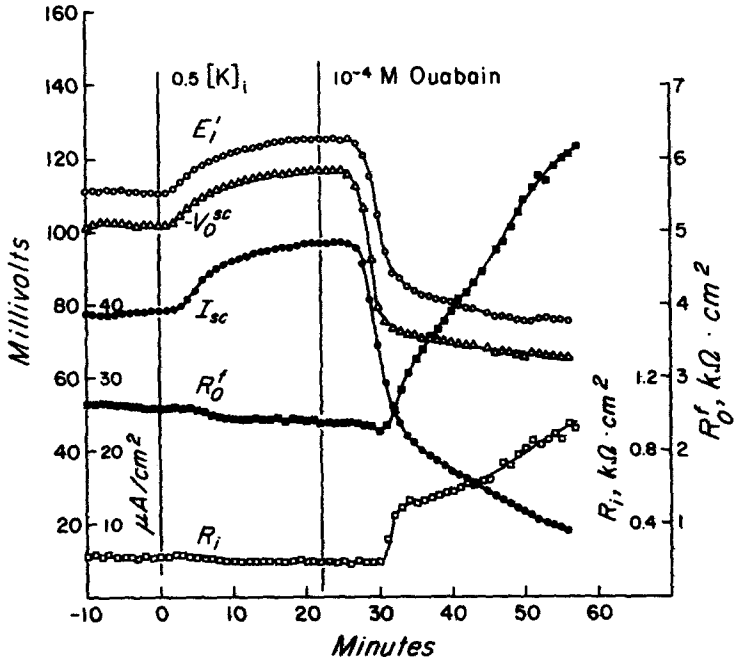

FIGURE 8. Effect of ouabain on electrical parameters in the presence of 0.5 $[K]_i$. Note rapid and slow phases and large selective increases of R_i during rapid phase.

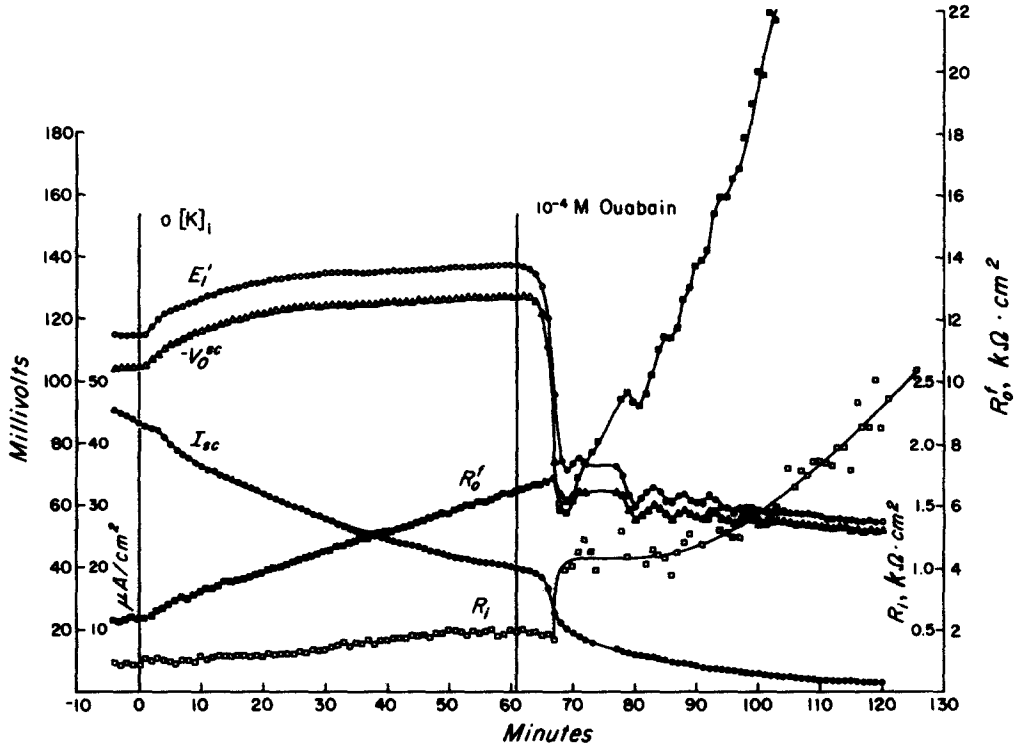

FIGURE 9. Effect of ouabain on electrical parameters in the presence of 0 $[K]_i$. In this particular experiment I_{sc} was not constant during control and experimental periods. Nevertheless, ouabain caused changes of voltage and resistance as observed before. Note rapid and slow phases and selective increases of R_i during rapid phase.

TABLE IV

EFFECT OF REDUCED $[K]_i$ ON TRANSPORT PARAMETERS

	I_{sc}	V_o^{sc}	E'_i	R'_o	R_i
	$\mu A/cm^2$	mV	mV	$k\Omega cm^2$	$k\Omega cm^2$
$n = 7$					
2.4 $[K]_i$	30.7±6.6	−107.9±4.5	120.3±4.3	4.73±1.11	0.50±0.12
0.5 $[K]_i$	31.8±5.8	−122.3±5.6	135.1±4.0	4.98±1.09	0.46±0.14
0.5/2.4	1.07±0.06	1.14±0.04	1.13±0.02	1.08±0.06	0.94±0.12
$n = 5$					
2.4 $[K]_i$	57.9±14.4	−109.9±2.5	121.1±3.5	2.32±0.49	0.18±0.33
0 $[K]_i$	41.3±16.0	−130.2±1.9	143.9±2.7	4.77±1.01	0.45±0.17
0/2.4	0.69±0.16	1.18±0.02	1.19±0.03	2.42±0.41	2.22±0.48

chose to determine the time required, τ^*, for the voltages to fall from 10–90% of the entire change of voltage observed during the rapid phase (100%). Despite the uncertainty of the meaning of τ^*, it was clear that reduction of $[K]_i$ resulted in more rapid changes of the voltage in response to ouabain (see Table V). Moreover, these studies with reduced $[K]_i$ made it especially clear that within a

period of time of, at most, a few minutes ouabain exerted a profound effect at the inner barrier leading to changes of intracellular voltage and large selective increases of the R_i.

Ouabain-Inhibitable Conductance of Inner Barrier

If we assumed that the action of ouabain on voltage and resistance is confined in the first few minutes to the inner barrier, it was possible to calculate from the changes of R_i (rapid phase) the "ouabain-inhibitable conductance." For skins

TABLE V

VALUES OF τ^*

	Amiloride-treated skins	Na-transporting skins	
	$\Delta E'_t$	ΔV^{τ}_{sc}	$\Delta E'_t$
2.4 $[K]_i$	8.7±0.9 (6)	4.3±0.4	4.7±0.5 (6)
0.5 $[K]_i$	–	3.8±0.4	4.1±0.5 (7)
0 $[K]_i$	5.2±0.9 (6)	1.8±0.1	2.6±0.5 (5)

Means ± SE (n). Values in minutes.

TABLE VI

EFFECT OF OUABAIN ON R_i (RAPID PHASE)

	2.4 $[K]_i$			0.5 $[K]_i$			0 $[K]_i$		
	C	E	E/C	C	E	E/C	C	E	E/C
	Ωcm²	Ωcm²		Ωcm²	Ωcm²		Ωcm²	Ωcm²	
	150	100	0.67	1,200	1,200	1.00	490	1,100	2.24
	670	700	1.04	630	1,200	1.90	1,100	3,100	2.82
	550	950	1.73	370	730	1.97	290	850	2.93
	600	1,300	2.17	180	530	2.94	185	650	3.51
	250	550	2.20	300	940	3.13	190	700	3.68
	620	1,800	2.90	410	1,450	3.54			
				150	730	4.87			
Mean	473	925	1.79	463	969	2.76	451	1,280	3.04
± SE	±89	±240	±0.34	±137	±124	±0.48	±171	±462	±0.26

Abbreviations: C, pre-ouabain control; E, after-ouabain (rapid phase) experimental.

bathed with 2.4, 0.5, and 0.0 $[K]_i$ solutions, the ouabain-inhibitable conductance averaged 28.4, 54.6, and 66.1% of the conductance (pre-ouabain = 100%) of the inner barrier, respectively (Table VI). Thus, a rather large fraction of the conductance of the inner barrier was ouabain sensitive.

Ouabain on Amiloride-Treated Skins

To assess directly the effects of ouabain on the voltage at the inner barrier, studies were done with skins exposed to 10^{-4} M amiloride outside. Under these circumstances, the intracellular voltage can be attributed to events occurring primarily if not alone at the inner barrier since $R_o^t >>> R_i$ and the $I_{sc} \rightarrow 0$.

The results of representative studies are shown in Figs. 10 and 11 and are summarized in Table VII. As in the above studies of Na-transporting skins,

ouabain caused the voltages to fall rapidly at first followed by a slow phase during which the voltages remained at high values for considerable periods of time. Reduction of $[K]_i$ to 0 mM caused the voltages to increase in magnitude. In six skins the mean values of V_o^{sc} and E_1' were increased from -112.3 ± 4.5 and 113.0 ± 4.5 mV to -135.3 ± 5.1 and 137.3 ± 5.2 mV, respectively. As can be seen in summary Table VII and Figs. 10 and 11, ouabain caused the voltages to decrease approximately 15–20% during the rapid phase. It was observed consistently, with amiloride-treated skins, that the rate of fall of the voltages (rapid phase) was slower than observed for Na-transporting skins. As shown in

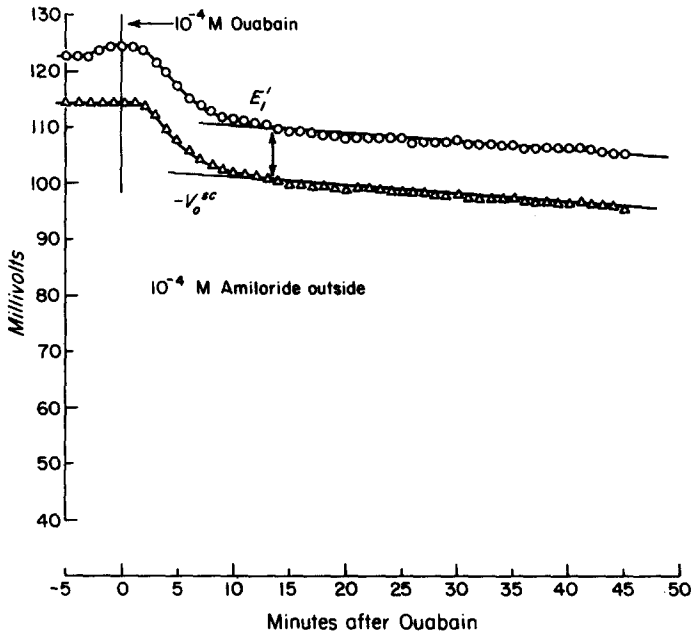

FIGURE 10. Effect of 10^{-4} M ouabain on V_o^{sc} and E_1' of non-Na-transporting skin (10^{-4} M amiloride outside). Note rapid and slow phases.

Table V, the values of τ^* averaged 8.7 min for amiloride-treated vs. 4.7 min for Na-transporting skins incubated with 2.4 $[K]_i$. Similarly, for skins incubated with 0 $[K]_i$, the mean values of τ^* were 5.2 and 2.6 min for amiloride-treated and Na-transporting skins, respectively. Although the reasons for this are not known, ouabain appeared to exert an effect on the voltages at the inner barrier (rapid phase) regardless of the rate of transepithelial Na transport.

DISCUSSION

It is well established that the glycoside ouabain acts specifically to inhibit the (Na + K)-ATPase of many cell systems thereby inhibiting active Na transport. In frog skin and other epithelia, it is assumed that, by virtue of the existence of this enzyme in these tissues, the inhibition of active transepithelial Na transport by ouabain can be attributed to the inhibition of the ouabain-sensitive Na pumps, presumably the (Na + K)-ATPase.

It is of interest to note recent reviews by Thomas (1972) and DeWeer (1975) where much discussion has centered on the nature of Na pumps in nonepithelial tissues. The observations cited in these reviews and elsewhere (Ussing et al., 1974; DeWeer and Geduldig, 1978) have led to the idea that the Na pumps

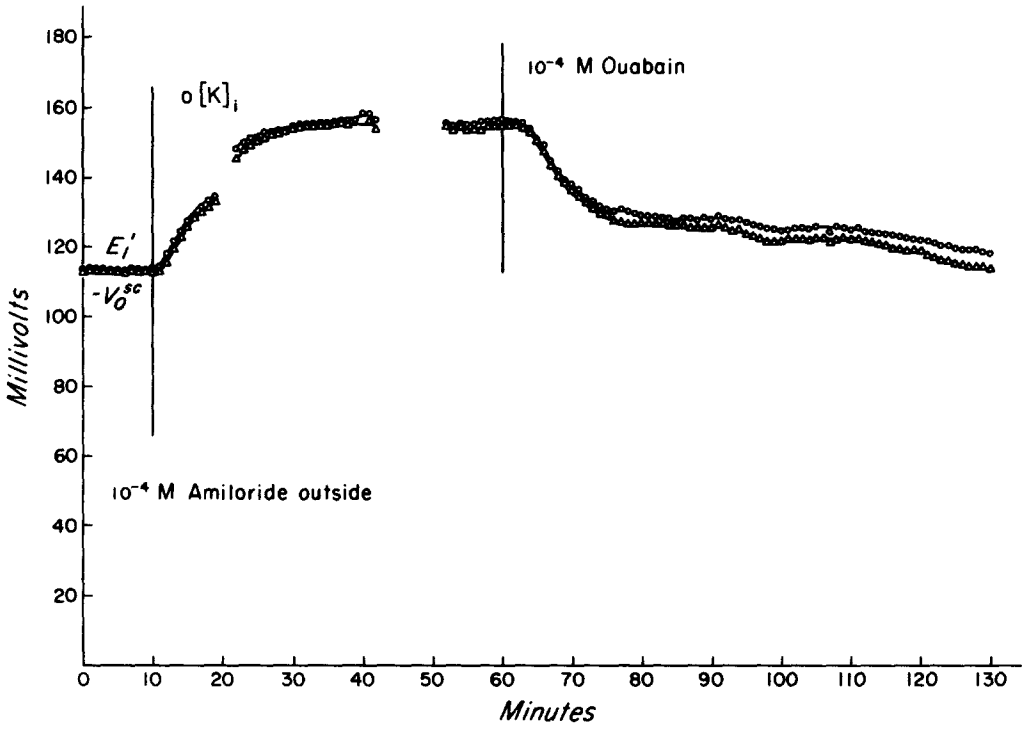

FIGURE 11. Effect of ouabain on V_o^{sc} and E_1' on non-Na-transporting skin in the presence of 0 $[K]_i$. The data were collected from three cell impalements.

TABLE VII

EFFECT OF OUABAIN ON AMILORIDE-TREATED SKINS

	2.4 $[K]_i$		0 $[K]_i$	
	V_o^{sc}	E_1'	V_o^{sc}	E_1'
			mV	
Pre-ouabain	−110.2±3.0 (6)	115.6±2.4 (6)	−135.3±5.1 (6)	137.3±5.2 (6)
10–15 min	−95.1±3.1 (6)	101.7±3.3 (6)	−109.3±6.7 (6)	112.2±6.9 (6)
30 min	−87.5±4.1 (5)	95.2±3.8 (5)	−97.2±8.8 (6)	101.3±9.2 (6)
45 min	−89.0±2.8 (4)	95.1±4.7 (4)	−87.3±10.7 (6)	91.4±10.8 (6)
60 min	–	–	−76.2±12.4 (6)	83.3±11.0 (6)

Means ± SE (n).

could indeed be electrogenic, thereby contributing to the magnitude of the intracellular voltage. Consequently, it would be expected that ouabain inhibition of the pumps would result in a decrease of membrane voltage. In this regard, the results of the present studies are in accord with the idea that the Na pumps of frog skin are electrogenic since, regardless of the state of transport, ouabain

caused substantial decreases of intracellular voltage. However, such findings taken together with the changes of membrane resistances raise several fundamental questions with regard to the nature of inner and outer barriers and the electrical coupling that occurs between these transport barriers.

Resistances of Apical and Basolateral Barriers

The specific resistances of apical and basolateral barriers were found to change when skins were treated with ouabain. Concurrent with the decreases of E'_1 and V_o^{sc} (rapid phase), the R_i was observed to increase markedly with little or no effect on the R_o^f. Such findings are consistent with the idea that ouabain inhibits a pump whose conductance contributes appreciably to the R_i. It is possible that ouabain exerts a direct effect on the K conductance, g_K (see later), of the inner barrier, but in the absence of evidence to the contrary, it would seem reasonable to believe that in frog skin the increases of R_i with ouabain, at least during the rapid phase, can be attributed to a direct action of ouabain on the pump sites. During the slow phase of the response, the resistances of both apical and basolateral membranes increased markedly and continuously with time. This finding is in line with the observations that, approximately 1 h after ouabain, the permeability to Na entry is markedly reduced (Biber, 1971; Erlij and Smith, 1973). Also, MacRobbie and Ussing (1961) concluded that ouabain caused a pronounced decrease of the passive ion permeability of the inner barrier of frog skin. It follows that, if the pumps are inhibited during the rapid phase, then the secondary increases of R_i and R_o^f during the slow phase can be attributed to changes of the conductances of other ions, most likely K at the inner barrier and Na at the outer barrier.

Electrochemical Potential of K within the Cell

According to Koefoed-Johnsen and Ussing (1958), the inner barrier of frog skin was thought to possess a neutral Na-K active exchange process that extruded Na from the cells and pumped K into the cells. Owing to a highly selective passive permeability to K, the K efflux from the cells proceeded down its electrochemical gradient so that at the steady state the rates of active K influx and passive K efflux were the same. Thus, the voltage at the inner barrier, V_i, is:

$$V_i = E_K - I_K/g_K,$$

where E_K is the Nernst potential for K, g_K is the passive conductance to K, and I_K is the current carried by K from cell to inner solution. Consequently, the V_i would be expected to be less than or equal to E_K under all transporting conditions if the pumps were neutral or electrogenic. Thus, observation of intracellular voltages less than the E_K are compatible with the existence of an electrogenic Na-for-K exchange pump that exists in parallel with a leak pathway for K.

With regard to the interpretation of the origin of the intracellular voltages of the skin and their relationship to the mechanism of the pumps, it is important to know the magnitude of E_K, especially in view of our past and present observations of high intracellular voltages in this tissue. In particular, for Na-transporting skins bathed with 2.4 $[K]_i$, the V_o^{sc} averages near -100 mV and in

nontransporting skins (amiloride or low Na outside) the intracellular voltage averages considerably higher (115–130 mV). It is not possible at present to know with absolute certainty the E_K at the inner barrier. However, if we take the highest values of intracellular K concentration, $[K]_c$, near 130–140 meq/liter, reported in the literature (Aceves and Erlij, 1971; Rick et al., 1978 *a* and *b*), and assuming an activity coefficient of unity, the E_K is calculated to be near 102 mV when $[K]_i$ is 2.4 mM. Since the intracellular voltages are essentially constant for considerable periods of time (transporting and nontransporting skins) and presumably the tissues are in a steady state, the V_o^{sc} would be expected to be less than 102 mV under all conditions. Indeed, for Na transporting skins, the values of V_o^{sc} are near the E_K and so taken at face value, given the usual uncertainties, K seems to be close to equilibrium at the inner barrier. We are, however, compelled to note that in the absence of transepithelial Na transport, where presumably the intracellular Na concentation is lowest, the skins generate their highest intracellular voltages which seem to exceed the highest values of E_K that can be expected on the basis of the K distribution across the inner membrane. It should be emphasized that acceptance of this idea requires that (*a*) the tissues exist in a steady state so that the extracellular [K] at the functional inner barrier is the same as the bulk phase, and (*b*) that estimates of intracellular [K] determined chemically reflect the activity of K within the cells. To the extent that we have not been able to uncover a systematic error in the measurement of the intracellular voltages, we are compelled to consider model systems of the inner barrier that take into account not only the expected observation that $V_i \leqslant E_K$, but also the possibility that $V_i \geqslant E_K$, especially when transepithelial Na transport is blocked at its site of entry at the apical barrier. It follows directly that, if $V_i \geqslant E_K$ at the steady state, a mechanism must exist at the inner barrier for active extrusion of K from the cells to the inner solution.

Models of Inner Barrier

Shown in Figs. 12 and 13 are mechanistic and electrical equivalent circuit models, respectively, that are consistent with the observed electrical properties of the Na-transporting cells of frog skin. The new suggestion made here is that, in part, K efflux from cell to inner solution may occur via the pump itself. As indicated in Fig. 12, the pump is electrogenic. Accordingly, the pump generates a net current since cation efflux exceeds cation influx ($r > 1$). Although it is usually thought that active Na efflux exceeds active K influx, it remains possible that, in part, a portion of the active K influx (I_K^{pi}) is recycled via the pump (I_K^{pe}) to the inner solution thereby decreasing the K flux via the leak pathway (I_K^l). To the extent that the affinity of the pump for Na far exceeds its affinity for K, the cation efflux via the pump would be mainly Na.

Although the intracellular [Na] is not known with certainty (see, for example, Rick et al., 1978 *a*), its concentration is known to fall when Na entry is blocked at the apical barrier of the cells. To the extent that a finite competition might exist between Na and K for pump-mediated efflux (especially when $[K]_c >> [Na]_c$), it would not be difficult to envision situations where the active K influx, I_K^{pi}, could be recycled exclusively via the pump, I_K^{pe}, so that the I_K^l was zero or even reversed from its usual direction. In such situations the $V_i \gtrless E_K$. Indeed, under

extreme conditions when the $[Na]_c \rightarrow 0$, the $V_i > E_K$, and this situation would correspond in our studies to skins treated with amiloride or bathed with 0 Na outside. Whether this suggestion is tenable remains to be proven, but for the moment, such a mechanism is capable of encompassing the observations that $V_i \gtrless E_K$.

We have chosen the above model, in part because it represents to us the simplest model consistent with present beliefs requiring, at most, the simple additional postulate that the affinity of the pump for Na is not absolute. Indeed, it is possible to advance more complicated theories that, in our view, are not presently warranted. Nevertheless, it will be necessary ultimately to explain the existence of high intracellular voltages that are ouabain-inhibitable under conditions where intracellular Na concentrations are likely to be quite low.

Electrical equivalent circuits of the above models are shown in Fig. 13. The

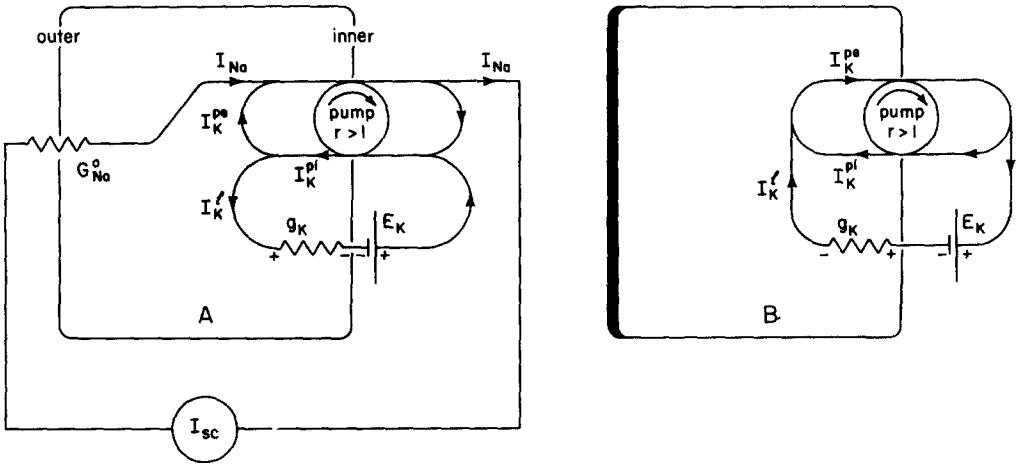

FIGURE 12. Mechanistic model of (A) Na-transporting cell and (B) non-Na-transporting cell. The pump is electrogenic with cation efflux/cation influx ratio $r > 1$. In part, K efflux is assumed to occur via the pump. At the steady state, $I_{sc} = I_{Na}$ (see text for details).

pump is modeled with a Thévenin equivalent consisting of electromotive force (EMF), E_{pump}, in series with its equivalent conductance, g_{pump}. Defining I_K^l positive as indicated by the arrows in Fig. 13, current via the pump, I^p, is:

$$I^p = I_{Na} - I_K^l, \tag{5}$$

and

$$I_K^l = I_K^{pi} - I_K^{pe}. \tag{6}$$

When $I_K^{pe} \leq I_K^{pi}$, I_K^l occurs from cell to inner solution. Thus,

$$V_i = E_K - I_K/g_K.$$

If, however, $I_K^{pe} > I_K^{pi}$, $V_i > E_K$ since I_K^l must occur at the steady state from inner solution to cell interior.

In general, for skins short-circuited, it can be shown that:

$$V_o^{sc} = E_{pump}\left(\frac{g_{pump}}{g_{pump} + g_K + G_{Na}^o}\right) + E_K\left(\frac{g_K}{g_{pump} + g_K + G_{Na}^o}\right), \quad (7)$$

and so the intracellular voltage will depend not only on the EMFs E_{pump} and E_K but also on the conductances where G_{Na}^o is the Thévenin conductance to Na of the apical barrier (see later).

In this regard, it was observed in the present studies that ouabain caused substantial increases of the resistance of the inner barrier, R_i. If the R_i can be attributed primarily to the conductances g_K and g_{pump}, then it is obvious that the pumps cannot be rheogenic (constant current-like) inasmuch as a rheogenic pump mechanism would require that $gp << g_K$. Since this appears not to be the case in frog skin, it follows that the current via the pump would be influenced by changes of intracellular voltage however mediated. If we assume that ouabain

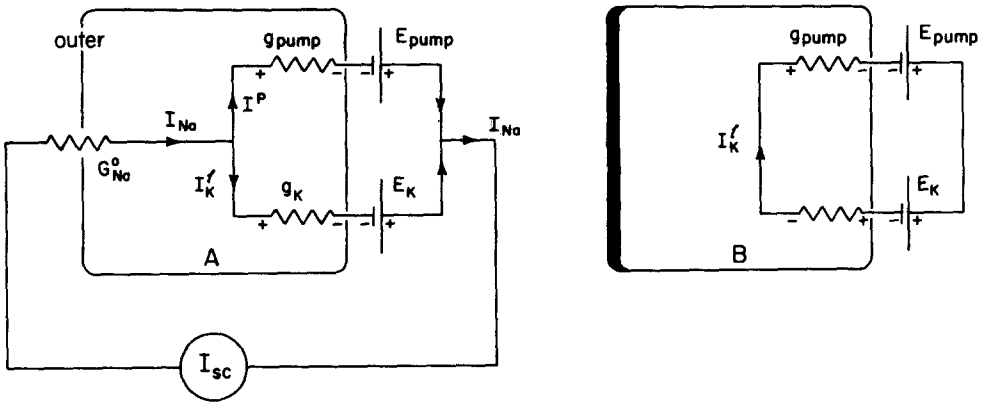

FIGURE 13. Electrical equivalent circuits for mechanistic models shown in Fig. 12. Arrows indicate direction of currents. Apical barrier is modeled with conductance G_{Na}^o. (See Helman and Fisher, 1977, and Discussion).

at 10^{-4} M binds specifically and inhibits all pump sites, it follows that the V_o^{sc} and I_{sc} after ouabain are

$$V_o^{sc} = E_K\left(\frac{g_K}{g_K + G_{Na}^o}\right), \quad (8)$$

and

$$I_{sc} = E_K(g_K + G_{Na}^o). \quad (9)$$

Clearly, such a situation is transient since the I_{sc} is carried by Na entry at the apical barrier and by K efflux via the basolateral barrier leading ultimately to a dissipation of the Na and K concentration gradients. Although no data are now available on the time-course of change of intracellular Na and K concentrations, it is well established for Na-transporting skins that the Na and K gradients fall within 60–90 min (see, for example, Rick et al., 1978 *a*). It is also noteworthy

that for non-Na-transporting skins (amiloride or 0 [Na] outside) the cells maintain their gradients for Na and K for at least 60–90 min (Rick et al., 1978*a*). In the context of the models shown here, such an observation would not be surprising by virtue of the electrical requirement that for K to leave the cells at the basolateral barrier, Na entry (current) at the apical barrier must occur in a 1:1 intracellular exchange of Na for K.[3] In this regard, it was observed that for nontransporting skins, the intracellular voltages remained at high values after ouabain consistent with the observations of the persisting Na and K gradients after ouabain reported by Rick et al., 1978*a* (see also below).

Changes of V_o^{sc} with Ouabain

It would seem straightforward to expect, because of the electrical coupling between outer and inner barrier, that changes of V_o^{sc} would be complicated not only by changes of the resistances but also by changes of the EMFs of outer and inner barriers. Such difficulties are obviated in studies of nontransporting skins (amiloride or 0 [Na] outside), where the changes of V_i are due to events occurring primarily if not alone at the inner barrier. Under these circumstances, ouabain caused the V_i to fall ~15–20% from control values (2.4 $[K]_i$) averaging near 95 mV at the end of the rapid phase. From the models shown above, the V_i before ouabain is:

$$V_i = E_{pump}\left(\frac{g_{pump}}{g_{pump} + g_K}\right) + E_K\left(\frac{g_K}{g_{pump} + g_K}\right), \tag{10}$$

and after ouabain assuming $g_{pump} \rightarrow 0$,

$$V_i \cong E_K. \tag{11}$$

Accordingly, the V_i after ouabain might, as a first approximation, estimate the E_K which in the present studies was observed to fall in the vicinity of 95–100 mV at the end of the rapid phase. With 2.4 $[K]_i$ the intracellular [K] would be expected to be near 105–127 meq/liter if E_K is 95–100 mV. It is unkown, however, to what extent and at what rate intracellular and extracellular concentrations of K change after ouabain and so influence the magnitude of V_i under Na-transporting and non-Na-transporting conditions. For the latter case, the changes of intracellular (and extracellular) [K] might be expected to be relatively small since $[K]_c$ remains elevated for considerable times (at least 60–90 min) after ouabain. However, for Na-transporting skins treated with ouabain, changes of the K gradient could be appreciable due not only to decreases of $[K]_c$ but also to increases of extracellular [K] at the functional inner barrier of the cells. For example, assuming I_{sc} of 18 $\mu A/cm^2$ and equal to the rate of K loss at the inner

[3] The models suggested here assume that the apical membrane is passively permeable only to Na and that the basolateral membrane is passively permeable only to K. To the extent that large changes of [Cl] on both sides of the tissue and large changes of [Na] in the inner solution cause little or no change of the V_o^{sc}, it is assumed at least to a first approximation that the outer barrier is primarily permeable to Na and that the inner barrier is primarily permeable to K as suggested originally by Koefoed-Johnsen and Ussing (1958). Indeed, the inner barrier of frog skin with 10^{-4} M amiloride outside yields changes of V_i that average near 65 mV/decade change of $[K]_i$ (Fisher and Helman, 1978).

barrier, it can be calculated that extracellular [K] would increase from 2.4 to 5.2 meq/liter in order for K to diffuse at this rate from the extracellular space to the inner solution through the unstirred layer of the corium of approximately 460 μm in thickness possessing a diffusion coefficient for K of 3×10^{-6} cm^2/s as estimated in separate studies by Fisher and Helman.[1] Consequently, elevation of extracellular [K] alone from 2.4 to 5.2 meq/liter would cause the E_K to decrease by about 20 mV over and above depolarization of V_i by ouabain inhibition of the pumps. This, in part, may explain why the changes of V_0^{sc} after ouabain were considerably greater in Na-transporting vs. non-Na-transporting skins. To test this idea, it will be necessary to study split skins, in the absence of the unstirred layers of the corium.

Transepithelial Driving Force

In 1951 Ussing and Zerahn proposed an equivalent circuit wherein the E_{Na} represented the Thévenin EMF for active transepithelial Na transport. It would seem clear that, because it is an equivalent transepithelial parameter, the E_{Na} is not a parameter of the pump mechanism itself. Indeed, as observed here and elsewhere, the voltages at both apical and especially basolateral barriers are sensitive to ouabain and to changes of the $[K]_i$. If we assume according to our previous observations that the E_{Na} can be estimated equally well from values of E_1 (I_T-V_T relationship) as well as E'_1 (microelectrode determinations), then it would seem clear that the E_{Na} is dependent upon the E_K and g_K of the inner barrier. Indeed, when $I_{sc} \rightarrow 0$ (amiloride or 0 [Na] outside), the Thévenin EMF of the inner barrier is equal to E'_1 and so changes of E'_1 with ouabain or $[K]_i$ would be reflected as changes of E_{Na}. Perhaps the point to be emphasized is that the E_{Na}, defined electrically, cannot be assumed to be the "EMF of the Na pump." In this regard, the E_{pump}, in our opinion, better serves this purpose.

As a final note, little is known of the mechanism of Na entry at the apical barrier of the cells. Our own studies have emphasized the existence of electrical rectification at this barrier, and taken together with the known saturable-like phenomenon for Na entry and the additional fact that the V_0^{sc} is far from the Na chemical equilibrium potential, it is difficult to know with certainty what model best serves to describe the behavior of this barrier. Our previous studies indicated that the Thévenin equivalent of the apical barrier was a simple resistor ($V_T < E'_1$), and we have used this equivalent in the present studies ($G_{Na}^0 = 1/R_0^t$). To the extent that we have not been able to obtain data in conflict with this idea, the outer barrier has been modeled with conductance G_{Na}^0, and the calculations reflect this working hypothesis.

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