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Transient Potassium Fluxes in Toad Skin

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Summary. Experiments were carried out in the isolated short-circuited skin of the toad *Bufo marinus ictericus*. ^{42}K influx and efflux experiments were carried out with skins bathed on both sides by NaCl-Ringer's solution. Those fluxes showed very similar kinetics of equilibration with time and the results could be fitted by equations of a model of two intraepithelial compartments and the bathing solutions. In the steady state K influx is 3.99 $\pm$0.36 nmol cm^{-2} hr^{-1} ($n=7$) and efflux 3.62 $\pm$0.38 nmol cm^{-2} hr^{-1} ($n=7$) and are not statistically different, indicating that no net K flux is present across the epithelium. Different kinds of perturbations affecting the rates of ^{42}K discharge into the bathing solutions were studied. Immediately after addition of amiloride (10^{-4} M) to the outer solution, a sharp decline is observed in the rate of ^{42}K discharge into the bathing solution, J^K_{21}, which falls from 3.62 $\pm$0.38 nmol cm^{-2} hr^{-1} to 2.02 $\pm$0.04 nmol cm^{-2} hr^{-1} ($n=7$) 2 min after addition of the drug, followed by a partial recuperation with time. A complete Na by K substitution in the outer bathing solution induces a prompt and marked decline in J^K_{21} which is similar to that induced by amiloride. Increase in the outer bathing solution Na concentration from zero Na concentration induces a nonlinear increase in J^K_{21} and a linear relationship was observed between J^K_{21} and short-circuit current in the range of 0 to 115 mM external Na concentration. The decline in J^K_{21} induced by amiloride or by lowering external Na concentration was interpreted as being caused by electrical hyperpolarization of the external barrier of the epithelium induced by these procedures. Depolarization of the epithelial barriers by inner Na by K substitution in the short-circuited state (when the potential barriers are equal) drastically interfere with the rate of ^{42}K discharge from the epithelium into the bathing solutions. Thus, transient increases are observed both in the rate of ^{42}K discharge to the outer and to the inner bathing solutions upon depolarization of the barriers. These results indicate that at least the most important component of transepithelial K unidirectional fluxes goes through a transcellular route with a negligible paracellular component. Addition of ouabain (10^{-3} M) to the inner bathing solution induces a transient rise in the rate of ^{42}K discharge to the outer bathing solution with a peak on the order of 200% of the stationary value previous to the action of the inhibitor, followed by a return to new stationary values not statistically different from those observed previously to the effect of ouabain. The behavior of J^K_{21} upon the effect of ouabain, as suggested by comparison with predictions from computer simulation, strongly supports the notion of a rheogenic Na pump in the inner barrier of the epithelium against the notion of a nonrheogenic 1:1 Na – K pump.

Despite the lapse of almost 20 years since Koefoed-Johnsen and Ussing (1958) proposed the double-membrane model for Na transport by isolated

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frog skin, the intimate nature of the mechanism involved in Na transport across epithelial membranes is still deeply controversial, particularly the Na – K coupling at a pump level in the basolateral membrane of the epithelial cells, as is critically discussed by Finn (1976), Macknight (1977) and Schultz (1978).

A large portion of present knowledge regarding transepithelial ion transport, particularly Na transport, has been obtained from the analysis of data obtained in experiments carried out in steady state. This is comprehensible if we keep in mind that formalisms normally used for treatment of transport data require that condition. In this article, which is along the same line of our previous publication (Varanda & Lacaz-Vieira, 1978), we give emphasis to transients induced in ^{42}K fluxes by different kinds of perturbations of the steady state with the aim of obtaining more information related to the mechanisms implicated in epithelial K movements and their interactions with active transepithelial Na transport.

It has long been known that K moves across the outer border of the frog skin (Huf & Wills, 1953). These authors showed a correlation between rejection of K and transepithelial electrical potential difference, and demonstrated the existence of a relationship between Na uptake and K rejection to the external medium. Later, Nielsen (1971) showed that frog skin treated with amphotericin B secretes potassium to the external medium but that, under normal conditions, the external barrier is impermeant to potassium. Recently, in our laboratory, Procopio and Lacaz-Vieira (1977) have shown that in the isolated open-circuited toad skin bathed by Ringer's solution on the inner side and dilute solutions (in the range of 0.2 to 5.0 mM external Na concentration) on the outer side, there is a K net flux (J_K in their notation) directed from the epithelium to the outer compartment. In this external concentration range, with skin electrical potential difference varying with external Na concentration, net Na influx (J_{Na}) is a linear function of the Na electrochemical potential difference across the skin. A positive correlation was observed between J_{Na} and J_K when J_{Na} varied with external Na concentration and also when J_{Na} varied in randomly selected skins. Antidiuretic hormone stimulated J_{Na} and J_K. Sodium removal from the external solution reduced J_K almost to zero. The idea of an electrical coupling across the outer barrier was put forward to explain the dependence between Na and K fluxes. More recently, Varanda and Lacaz-Vieira (1978) have shown an insignificant steady-state K efflux (J_K^{eff}) (measured with ^{42}K) in the isolated short-circuited toad skin bathed by Ringer's solution on both sides, as compared to the simultaneously measured short-circuit current. However, they have shown that Na by K

substitution in the inner bathing solution has a profound effect on the K efflux, depending on the nature of the anion present in major proportions in the bathing solutions. The apparent K permeability (measured as $J_K^{eff}/[K]_i$, where $[K]_i$ is the inner K concentration) is of comparable magnitude in Cl^- and in SO_4^{--} media before inner Na by K substitution, and also in SO_4^{--} medium after inner Na by K substitution. However, in Cl^- medium, inner Na by K substitution increases the apparent K permeability one order of magnitude as compared to control condition before the ionic substitution. The large K effluxes observed in the steady state after inner Na by K substitution in Cl^- medium were interpreted as being a consequence of cell swelling K permeability increase of the outer barrier.

The aim of the present work was to study, in the short-circuited state, transient and stationary transepithelial K movements, the relationships between K fluxes and active Na transport, and the role of inhibitors of the active Na transport, ouabain and amiloride, on transepithelial K fluxes in order to better characterize the mechanisms and pathways involved in transepithelial K permeation and the coupling between K movement and active Na transport.

Material and Methods

The studies were carried out in modified Ussing-Zerahn chambers, according to the method previously described (Varanda & Lacaz-Vieira, 1978). To prevent the effects of skin edge damage (Walser, 1970; Helman & Miller, 1971, 1973, 1974; Biber & Mullen, 1977) on the low levels of K fluxes, the hemichambers were provided with a circular groove (4 mm wide and 0.4 mm deep) located at the internal rim of the hemichamber contact surfaces. The groove was filled with silicone grease (Dow Corning High Vacuum Grease) before mounting the skin. The silicone grease was dyed with a water insoluble dye (Sudan Black B, Allied Chemical) in order to form a visible highly viscous gasket which could be controlled during the course of the experiment for its integrity. Use of a soluble dye (Lissamine Green, Chroma Gesellschaft Schmid and Co.) in the outer compartment in some test experiments have shown that the nondamaged area of skin in contact with the silicone gasket is completely isolated from contact with the outer bathing solution. Abdominal skins of the toad *Bufo marinus ictericus* were used and the experiments performed in the short-circuited state at room temperature (20 to 25°C). A voltage clamp unit was connected to the preparation through 3 M KCl agar bridges and saturated KCl calomel half-cells (for voltage measurement) and $Cu-CuSO_4$ half-cells (for current passing). Transepithelial electrical potential difference and short-circuit current were recorded in a two-channel recorder (Varian mod. G.2500). An equilibration period of approximately 1 hr with the skin short circuited elapsed before the addition of 100 μCi ^{42}K (Institute of Atomic Energy, São Paulo, Brazil) to one of the bathing solutions (hot compartment). Every two minutes, all the solution of the compartment opposite to that receiving the isotope (cold compartment) was totally drained into counting vials for ^{42}K assay in a liquid scintillation counter (Beckman mod. LS 100) by Cerenkov effect (Moyer, 1962). Experiments performed in the short-circuited state had this

condition interrupted for 5 to 10 sec (open-circuited state) every two minutes during drainage of the cold compartment. In the kinetic experiments, sample collection started immediately following addition of the isotope to the hot compartment. In others, sampling started after an equilibration period of no less than 2 hr, time sufficient for the transepithelial ^{42}K flux to attain a steady state. The solutions used were: NaCl-Ringer's solution: 115.0 mM NaCl; 2.5 mM $KHCO_3$; 1.0 mM $CaCl_2$. When Na by K substitution was carried out in the inner solution, all Na was substituted by K on a equimolar basis. In the experiments with changes in the Na concentration of the outer bathing solution, the external Na concentrations were: 0.0, 5.5, 11.0, 15.5, 21.0, 36.6, 55.0, 73.0, and 115.0 mM, obtained by substitution of Na by K on equimolar basis. All the solutions referred to above had pH of 8.2 after aeration and osmolarity of 220 mosmol/kg H_2O. Na_2SO_4 Ringer's solution: 57.5 mM Na_2SO_4; 2.5 mM $KHCO_3$; 1.0 mM $CaSO_4$. When Na by K substitution was carried out in the inner bathing solution, all Na was substituted by K on equimolar basis. After aeration, these solutions had pH of 8.2 and osmolarity of 165 mosmol/kg H_2O. Results are presented as mean $\pm$SE. n is the number of experiments. Computer analysis was carried out in a Hewlett Packard 21 MX computer.

Results

1. Transepithelial K Fluxes

These experiments were performed to measure unidirectional K fluxes across the skin and to verify the existence of a possible K net flux from inner to outer bathing solutions in short-circuited skins bathed by NaCl-Ringer's solution on both sides. A net K flux from epithelium into the outer bathing solution of magnitude similar to that of the net Na uptake was observed in open-circuited skins bathed by dilute solutions on the outer surface, and a positive correlation was observed between this net K flux and net Na uptake (Procopio & Lacaz-Vieira, 1977). The present experiments were carried out with skins bathed by NaCl-Ringer's solution on both sides. The collection of samples from one bathing solution was begun immediately following isotope addition to the oposite bathing solution. Figure 1 *A* shows the results for efflux experiments, in which ^{42}K was added to the inner bathing solution. As can be seen, the rate of ^{42}K discharge from the epithelium into the outer bathing solution, J^K_{21}, reaches a steady-state condition approximately 100 min after addition of the isotope to the inner solution. The steady-state J^K_{21}, equivalent to J^K_{41}, is 3.62 ± 0.38 nmol cm^{-2} hr^{-1} ($n=7$) (mean of the last 10 values shown in Fig. 1 *A*). Figure 1 *B* shows the results for influx experiments, with ^{42}K added to the outer bathing solution. The rate of ^{42}K discharge into the inner compartment, J^K_{34}, also reaches a steady-state condition around 100 min from the moment of isotope addition to the outer compartment. The steady-state J^K_{34}, which is

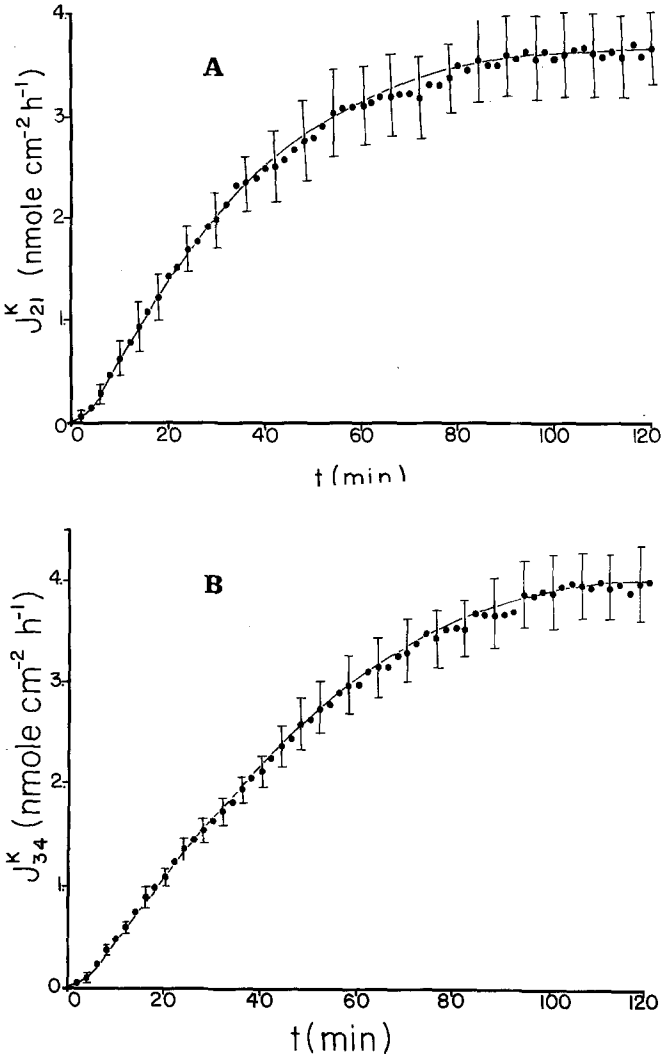

Fig. 1. Rate of ^{42}K discharge from epithelium into the bathing solutions as a function of time. (*A*): J^K_{21} is the rate of discharge into the outer bathing solution in experiments where ^{42}K was added to the inner bathing solution. (*B*): J^K_{34} is the rate of discharge into the inner bathing solution in experiments where ^{42}K was added to the outer bathing solution. Bars indicate SE. 7 skins were used for each experimental group

equivalent to J^K_{14}, is 3.99 ± 0.36 nmol cm^{-2} hr^{-1} ($n=7$) (mean of the last 10 values shown in Fig. 1*B*). J^K_{21} and J^K_{34} steady-state values are not statistically different ($P>0.5$, t test). Figure 2*A* and *B* are plots of J^K_{21} and J^K_{34} for a system of 4 compartments (two intraepithelial and the bathing solutions), according to Eqs. (A 28) and (A 20), respectively (*see Appendix*). α and β are the regression coefficients for the slow and fast exponentials,

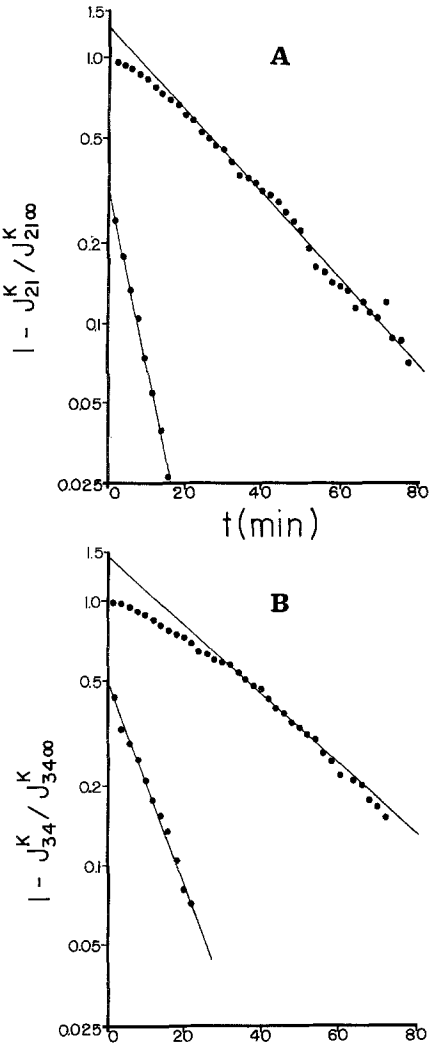

Fig. 2. Plots of the rate of ^{42}K discharge into the bathing solutions according to Eq. (A28) for J^K_{21} and Eq. (A20) for J^K_{34}. J^K_{21} is the rate of discharge into the outer bathing solution and J^K_{34}, the rate of discharge into the inner bathing solution. $J^K_{21\alpha}$ and $J^K_{34\infty}$ are steady-state values for J^K_{21} and J^K_{34}, respectively. Results presented in *A* correspond to those of Fig. 1*A* and those in *B*, to the results of Fig. 1*B*

according to the plot of Fig. 2*A* and *B*, respectively. Table 1 gives values of α and β for efflux and influx experiments. As tested by analysis of covariance (Dixon & Massey, 1969) from the comparison of influx and efflux experiments, α values and β values are not statistically different. These results and the fact that J^K_{21} and J^K_{34} steady-state values are not statistically different indicate that influx and efflux of K across the skin can be considered to be kinetically very similar.

Table 1

	J^K_{34}	J^K_{21}	$F_{observed}$	$F_{calculated}$
α	0.036	0.031	6.467	$F_{(1,17),0.99}=8.4$
β	0.155	0.086	3.693	$F_{(1,56),0.95}=4.0$

Regression coefficients for the slow (α) and fast (β) exponentials obtained by fitting J^K_{21} and J^K_{34} according to Eqs. (A 28) and (A 20), respectively (*see* Fig. 2 *A* and *B*). J^K_{21} is the rate of ^{42}K discharge into the outer bathing solution in experiments where ^{42}K was added to the inner bathing solution. J^K_{34} is the rate of ^{42}K discharge into the inner bathing solution in experiments where ^{42}K was added to the outer bathing solution. As tested by analysis of covariance, α values calculated for J^K_{21} and J^K_{34} are not statistically different. Also, β values are not different between the J^K_{21} and the J^K_{34} measurements.

2. *^{42}K Discharge into the Outer Bathing Solution*

2.1. Effect of Amiloride

Experiments were performed with NaCl-Ringer's solution bathing both sides of skin. After J^K_{21}, short-circuit current (SCC), and skin electrical potential difference (PD) had reached steady-state, amiloride was added to the outer bathing solution (10^{-4} M final concentration) and 10 consecutive samples were collected from the outer bathing solution, one every 2 min, for

Table 2

	SCC ($\mu A\ cm^{-2}$)	PD (mV)	R ($k\Omega$)	J^K_{21} (nmole $cm^{-2}\ hr^{-1}$)
Control	62.1 ± 4.5	54.1 ± 4.1	0.9 ± 0.4	3.62 ± 0.38
Amiloride	4.1 ± 0.9	11.6 ± 2.3	3.0 ± 0.3	2.02 ± 0.04
				2.22 ± 0.08
				2.28 ± 0.15
				2.32 ± 0.18
				2.50 ± 0.13
				2.55 ± 0.17
				2.56 ± 0.13
				2.78 ± 0.16
				2.75 ± 0.16
				2.73 ± 0.06

Effect of amiloride on short-circuit current (SCC), skin electrical potential difference (PD) and skin electrical resistance (R) for 7 skins where the rate of ^{42}K discharge into the outer bathing solution was measured. Amiloride was added to the outer bathing solution (10^{-4} M). All values presented are steady-state values, except for J^K_{21} in the presence of amiloride which, from top to bottom, are values ordered sequentially, 2 min apart from the moment amiloride was added to the outer bathing solution.

J^K_{21} measurement. Skin electrical resistance (R) was calculated as: $R = PD/SCC$. As can be seen, apart from the known effect of this inhibitor reducing SCC, PD and increasing R in epithelial membranes (Bentley, 1968; Crabbé, Ehrlich & Scarlata, 1968; Dörge & Nagel, 1970; Benos & Mandel, 1978), it produces a sharp initial decline of J^K_{21} immediately after its addition to the outer solution, followed by a progressive increase with time, as shown in Table 2.

2.2. Effect of Changes in the External Na Concentration

These experiments were performed with NaCl-Ringer's solution bathing the inner skin surface. The outer surface was sequentially bathed by Ringer's solution of increasing Na concentration (0.0, 5.5, 11.0, 15.5, 21.0, 36.6, 55.0, 73.0, 115.0 mM), Na being replaced by K on equimolar basis. The ionic substitution was carried out after J^K_{21} had reached stationary level. Five consecutive measurements of J^K_{21} at 2-min intervals were obtained for each external Na concentration. Figure 3*A* shows SCC and J^K_{21} as a function of the external Na concentration, $[Na]_1$. SCC displays its well-known nonlinear behavior with $[Na]_1$ (Kirschner, 1955; Cereijido *et al.*, 1964; Cirne & Malnic, 1972; Mandel & Curran, 1973; Danisi & Lacaz-Vieira, 1974). J^K_{21} also displays a nonlinear dependence on $[Na]_1$. When $[Na]_1 = 0$, J^K_{21} is 2.48 ± 0.28 nmol cm^{-2} hr^{-1} ($n=9$). This value is not statistically different from the mean of the 5 initial consecutive J^K_{21} values obtained for each experiment with amiloride (*Results*, section 2.1; Table 2) equal to 2.27 ± 0.08 nmol cm^{-2} hr^{-1} ($n=7$) ($P > 0.6$, t test). Figure 3*B* shows a plot of J^K_{21} as a function of SCC when these variables change with $[Na]_1$. A linear relationship obtains: $J^K_{21} = 0.00076$ (SCC) $+ 0.0798$, both fluxes being expressed in the same unit (μA cm^{-2}). The linear correlation coefficient is 0.93 ($P < 0.001$, $n=9$).

3. K Fluxes and the Effect of Depolarization of the Inner and Outer Facing Membranes

The aim of these experiments was to test the effect of depolarization of the inner and the outer facing membranes of the epithelial cells on the rates of ^{42}K discharge into the outer and inner bathing solutions. Depolarization of the inner membrane was accomplished by increasing K concentration in

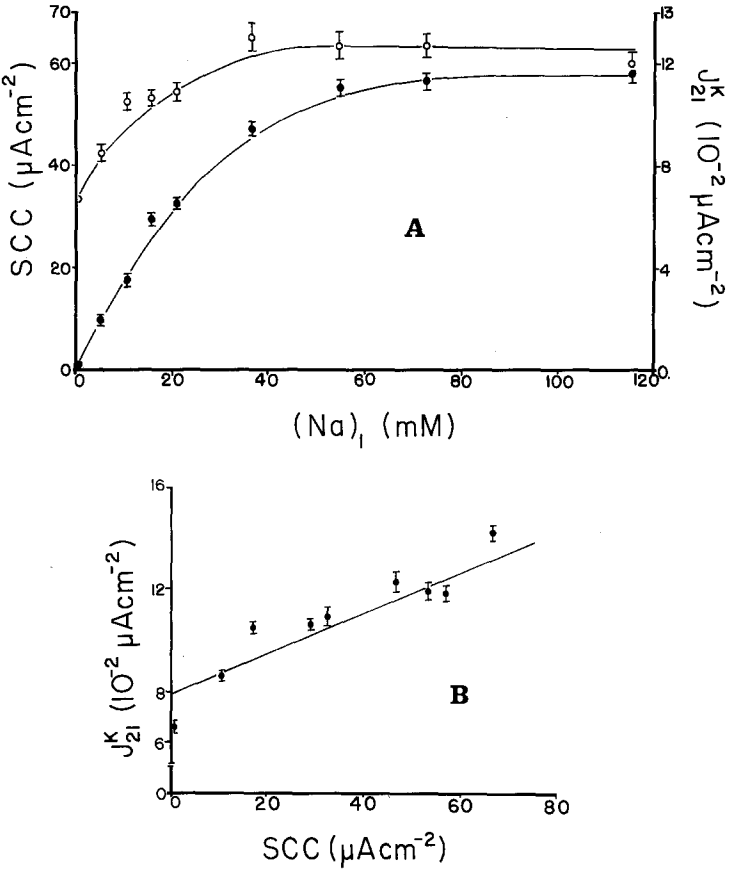

Fig. 3. (*A*): Short-circuit current (SCC) (●) and the rate of ^{42}K discharge from epithelium into the outer bathing solution (J^K_{21}) (○) as a function of Na concentration in the outer bathing solution, $[Na]_I$. Reduction of $[Na]_I$ was obtained by equimolar Na by K substitution only in the outer bathing solution, with NaCl-Ringer's solution bathing the inner skin surface. 9 skins were used in these experiments. For J^K_{21}, each point is the mean of the means of 5 consecutive J^K_{21} values obtained 2 min apart for each experiment from the moment of change in the external Na concentration. For SCC, each point is the mean for the 9 experiments of the average SCC for each external Na concentration. Bars indicate SE. In this figure, J^K_{21} is expressed in the same units (μA cm^{-2}) as the SCC. (*B*): Plot of J^K_{21} as a function of SCC when these variables change with $[Na]_I$. SCC and J^K_{21} values are presented in the same units (μA cm^{-2}). Bars are SE

the inner solution (Koefoed-Johnsen & Ussing, 1958; Fuchs, Hviid-Larsen & Lindemann, 1977) and depolarization of the outer membrane as a consequence of inner membrane depolarization in the short-circuited state due to the electrical coupling of these membranes, as can be deduced from equivalent electrical circuits (Schultz, 1972; Helman & Fisher, 1977).

3.1. ^{42}K Discharge into the Outer Bathing Solution

The control condition was carried out with NaCl-Ringer's solution bathing both sides of skin. ^{42}K was added to the inner bathing solution. After steady-state J_{21}^{K} had been reached, Na by K substitution was performed in the inner solution. The fresh KCl-Ringer's solution added to the inner compartment was a solution with no ^{42}K. Sampling of the outer compartment followed as in the control condition before the ionic substitution. Figure 4 shows, for 5 skins, the mean of the rate of isotope discharge into the outer bathing solution, J_{21}^{K}, normalized for the steady-state value, $J_{21_0}^{K}$, obtained before the ionic substitution. As can be seen, immediately following inner ionic substitution, the rate of isotope discharge to the outer medium rises sharply, followed by a slow decline with time toward zero. At the peak, attained 5 min after inner Na by K substitution, the rate of ^{42}K discharge into the outer compartment is of the order of 700% of its control stationary value.

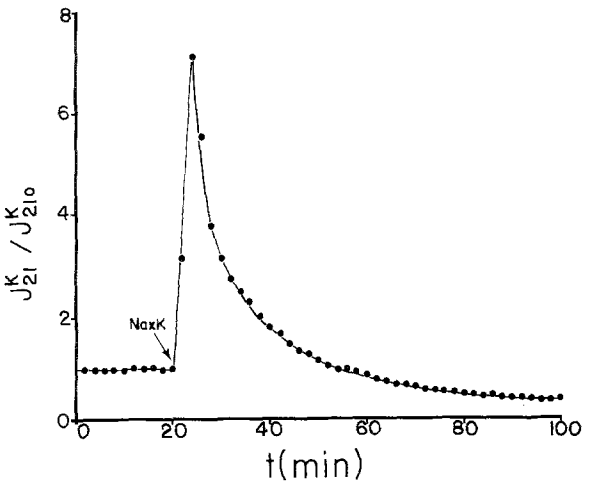

Fig. 4. Effect of outer barrier depolarization on the rate of ^{42}K discharge from epithelium into the outer bathing solution, J_{21}^{K}. $J_{21\infty}^{K}$ is J_{21}^{K} steady-state value before Na by K substitution in the inner bathing solution. This substitution was used to directly depolarize the inner membrane. Depolarization of the outer barrier was induced by the electrical coupling between these membranes due to the short-circuited condition. Each point is the mean of 5 experiments. In the course of the ionic substitution all the ^{42}K was removed from the inner bathing solution. The arrow indicates the moment of Na by K substitution in the inner bathing solution, which was carried out when J_{21}^{K} had reached the steady state

3.2. ^{42}K Discharge into the Inner Bathing Solution

The control condition was performed with NaCl-Ringer's solution bathing both sides of skin and ^{42}K added to the outer bathing solution. Na by K substitution was carried out in the inner bathing solution. The "hot"

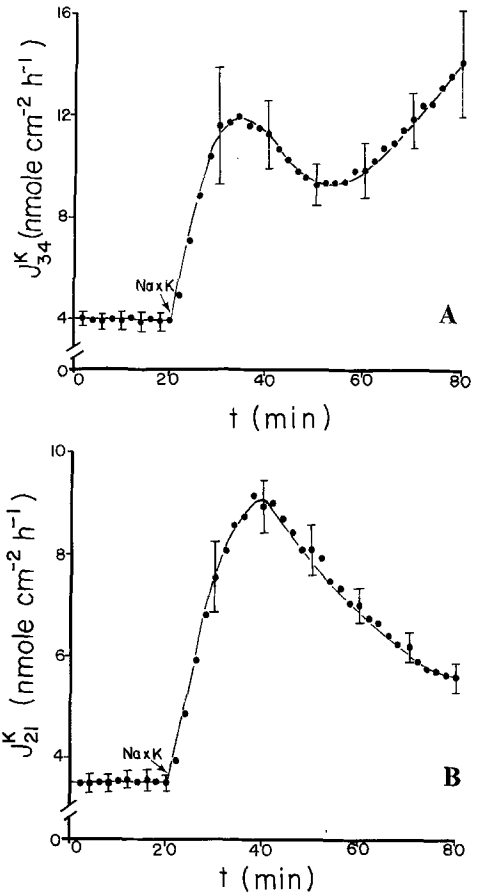

Fig. 5. Effect of inner barrier depolarization on the rate of ^{42}K discharge from epithelium into the inner bathing solution, J^K_{34}. Inner membrane depolarization was accomplished by substitution of Na by K in the inner bathing solution. The arrow indicates the moment of the substitution. (*A*): Experiments performed with Cl^- as the main anion in the bathing solutions. As can be seen, the ionic substitution induces a transient rise of J^K_{34}, which was interpreted as being produced by depolarization of the inner barrier. This transient rise was followed by a late increase with time which was interpreted as being induced by osmotic cell swelling. (*B*): Experiments performed with SO_4^{--} as the main anion in the bathing solutions. As can be noted, only the electrically induced transient is observed with no latter increase of J^K_{34} with time, which was prevented by SO_4^{--} being an impermeant anion. 7 skins were used for each experimental group. Bars indicate SE. Arrows indicate the moment of Na by K substitution in the inner bathing solution, which was carried out when J^K_{34} had reached the steady state

solution with ^{42}K was kept in the outer compartment throughout the whole experimental period. Sampling of the inner solution after the ionic substitution followed as in the control condition before the substitution, except for the composition of the inner solution which was then KCl-Ringer's solution. Figure 5*A* shows the rate of ^{42}K discharge into the inner solution for 7 skins, before and after inner ionic substitution. Inner Na by K replacement increases J^K_{34} which goes through a maximum of the order of 300% of its stationary presubstitution value, 10 min after the ionic substitution. Then, a transient decline is observed which is followed by a late and progressive rise with time.

To evaluate the role of permeability changes induced by cell swelling on the genesis of the late increase observed in J^K_{34} after inner Na by K substitution in Cl medium, as suggested by previous work (Varanda & Lacaz-Vieira, 1978) similar experiments were performed in sulfate medium. Sulfate is an impermeant anion (MacRobbie & Ussing, 1961), so it would be expected to prevent cell swelling mediated permeability changes due to inner Na by K substitution (Varanda & Lacaz-Vieira, 1978). The control condition of this experimental group was carried out with Na_2SO_4-Ringer's solution bathing both sides of skin and ^{42}K added to the outer solution. Na by K substitution was carried out in the inner solution. Sample collection followed as in the Cl^- group. Figure 5*B* shows, for 7 skins, the rate of ^{42}K discharge into the inner bathing solution before and after inner ionic substitution. Contrasting with the Cl^- group, only a transient J^K_{34} increase was observed in the SO_4^{--} group, which was followed by a progressive decline with time. At the peak, J^K_{34} is approximately 300% of the stationary value observed before the ionic substitution. The late increase in J^K_{34} observed in the Cl^- group experiments was absent in the experiments with SO_4^{--}.

4. ^{42}K Discharge into the Outer Bathing Solution and the Effect of Ouabain

These experiments were performed in order to get an insight into the role of K pumping, possibly through the action of a Na – K pump, identified as a Na – K ATPase located in the basolateral membrane of the epithelial cells (Farquhar & Palade, 1966; Mills, Ernst & DiBona, 1977) on the steady-state rate of K efflux across the whole skin. Ouabain is a known inhibitor of active Na transport in frog skin (Koefoed-Johnsen, 1957) and the species used in these experiments (Danisi & Lacaz-Vieira, 1974). Its

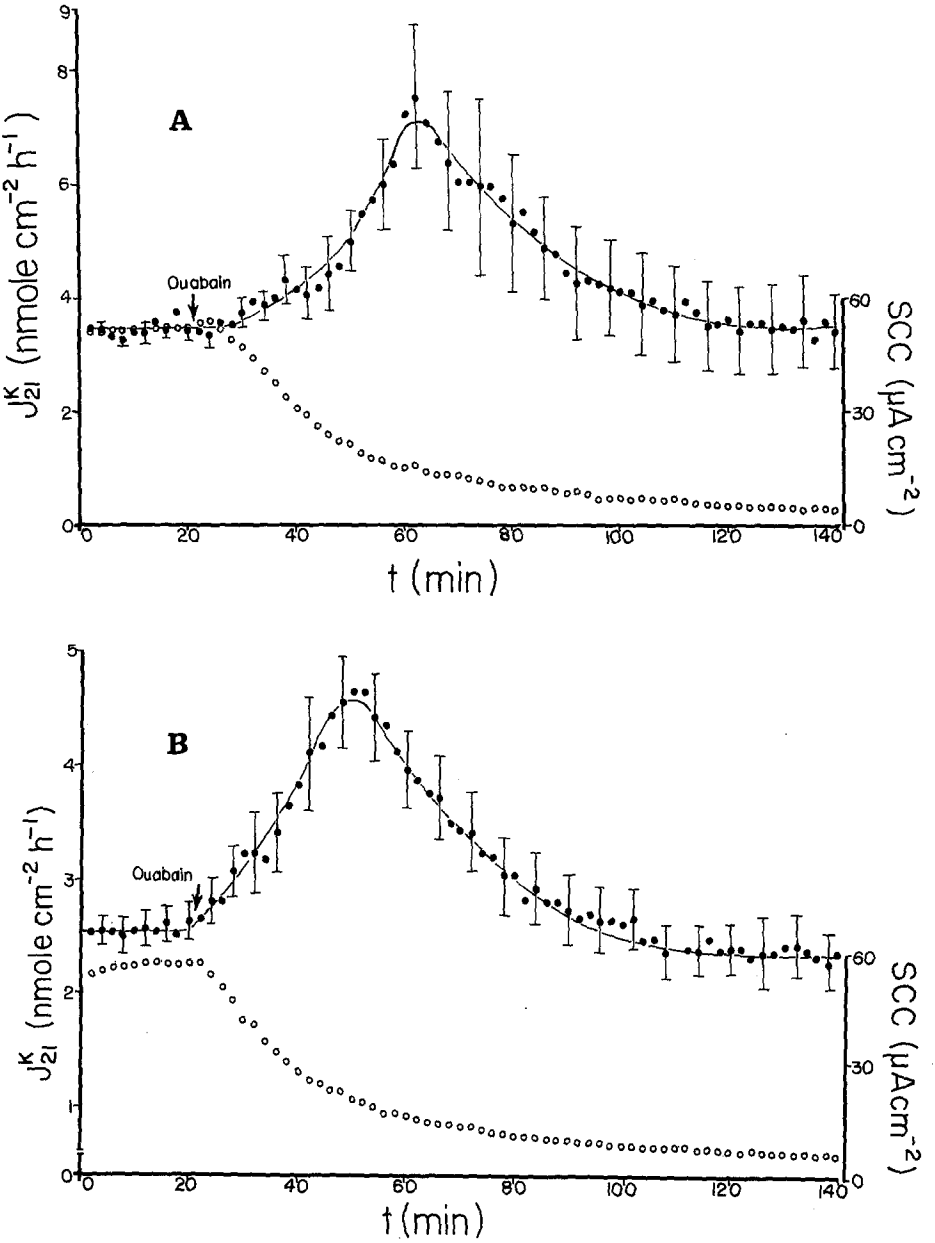

Fig. 6. Effect of ouabain added to the inner bathing solution (10^{-3} M) on the rate of ^{42}K discharge from epithelium into the outer bathing solution, J^K_{21}, and on the short-circuit current, SCC. Experiments performed with Cl^- (*A*) or SO_4^{--} (*B*) as the main anions in the bathing solutions. 4 skins were used in the Cl^- group and 5 skins in the SO_4^{--} group. Ouabain was added to the inner solution when J^K_{21} had reached the steady state. The moment of addition of ouabain is indicated by the arrow. Ouabain produced a large transient increase in J^K_{21} both in Cl^- or in SO_4^{--} media. (●) corresponds to J^K_{21} and (○) to SCC. Bars indicate SE

action is accepted to be mediated through inhibition of the Na – K ATPase, since ouabain binds to and inhibits highly purified preparations of Na – K ATPase (Kyte, 1972). Experiments were carried out in two groups of skins bathed on both sides by NaCl-Ringer's solution ($n=4$ skins) and the other by Na_2SO_4-Ringer's solution ($n=5$ skins). ^{42}K was added to the inner solution. After the ^{42}K efflux had reached steady-state, ouabain was added to the inner solution (10^{-3} M final concentration). In the NaCl group (Fig. 6*A*), the steady-state J_{21}^K is 3.44 ± 0.06 nmol cm^{-2} hr^{-1} (mean of the last 10 values before addition of ouabain) and the simultaneously measured SCC is equal to 52.3 ± 8.4 μA cm^{-2}. The effect of ouabain was to increase transiently J_{21}^K to a maximum of 7.51 ± 1.21 nmol cm^{-2} hr^{-1} (220% of the control value) 40 min after addition of the inhibitor, which was then followed by a subsequent decline to a new steady-state value of 3.46 ± 0.76 nmol cm^{-2} hr^{-1} 120 min after the addition of the inhibitor, which is not statistically different from the initial steady-state value before ouabain ($P>0.9$, t test). Aside from this effect on J_{21}^K, ouabain produced the well-known effect on SCC which was progressively inhibited to attain 4.9 ± 1.4 μA cm^{-2} 120 min after addition of the inhibitor to the inner solution (90% inhibition). Similar results were obtained with Na_2SO_4-Ringer's solution (Fig. 6*B*). Thus, in the control steady state J_{21}^K is 2.57 ± 0.13 nmol cm^{-2} hr^{-1} and SCC is 55.3 ± 9.0 μA cm^{-2}. After addition of ouabain (10^{-3} M in the inner solution) J_{21}^K increases transiently to 4.68 ± 0.74 nmol cm^{-2} hr^{-1} (180% of the control value) 40 min after the addition of the inhibitor, falling subsequently to a new steady state of 2.44 ± 0.26 nmol cm^{-2} hr^{-1} 120 min after the addition of the inhibitor, which is not statistically different from the control steady-state value before ouabain ($P>0.6$, t test). SCC fell pregressively under the effect of ouabain to attain 6.4 ± 0.8 μA cm^{-2} 120 min after addition of the drug (90% inhibition).

Discussion

The aim of this work was to study unidirectional K fluxes across the isolated short-circuited skin of the toad *Bufo marinus ictericus* bathed on both sides by Ringer's solution in order to obtain information related to pathways and mechanisms involved in the transepithelial K translocation, and also on the relationships between K movement and net active sodium transport.

Analysis of ^{42}K influx and efflux kinetic experiments (*Results*, section 1, Figs. 1 and 2) indicate that influx and efflux follow very close kinetic

patterns in regard to steady-state values, equilibration times, and parameters of exponential fitting to the equations describing a system of 4 compartments in series (2 intraepithelial and the bathing solutions – *see Appendix* and Fig. 7). At least, two different possibilities could, *a priori*, be put forward to interprete the similarities between influx and efflux kinetics. One would be to assume the existence of a single and symmetrical limiting step to K movement across the whole skin. Previous investigators have identified an important barrier to transepithelial K movement at the outer cell membranes of the most external cell layer of the *stratum granulosum* (outer barrier) (Koefoed-Johnsen & Ussing, 1958; Curran & Cereijido, 1965). The assumption of a single limiting step, however, does not explain our influx or efflux results with long equilibration times of approximately 100 min. The experimental results suggest the participation of more than one important barrier to K movement across the skin. The detection of the two regression coefficients α and β from analysis of the data, requires a second intraepithelial compartment. Therefore, we consider the epithelium, as regarded to K fluxes, as two compartments in series. We cannot, however, with the present methodology, precisely relate these compartments to the epithelium complex morphological structure of different cell layers (Farquhar & Palade, 1965; Voûte & Ussing, 1968). The data suggest that, at least, the main component of either the influx or the efflux goes through a transcellular route, crossing, therefore, the outer and the inner barriers of the epithelium with negligible paracellular component. Once crossing one of these barriers, ^{42}K ions could reach deeper cell layers through the permeable junctions between the epithelial cells (Farquhar & Palade, 1965; Ussing & Windhager, 1964). Evidence in literature supports the notion that the epithelial cells behave as a functional syncythium (Dörge, Rick & Thurau, 1976; Rick *et al.*, 1978). This assumption was incorporated in our compartmental description of ^{42}K influx and efflux kinetics by considering $k_{23}=k_{32}$, which means that the intraepithelial barrier to ^{42}K movement does not rectify ^{42}K movement and that no appreciable potential difference is present across this barrier. Two electrical potential steps of equal magnitudes can be considered to be present in the epithelium in the short-circuited state, one at the outer and the other at the inner barrier, in such a way that the cell compartment is located in a negative electrical potential well on the order of -70 to -100 mV (Nagel, 1976; Helman & Fisher, 1977), with somewhat lower values reported previously (Ussing & Windhager, 1964; Whittembury, 1964; Cereijido & Curran, 1965; Rawlins *et al.*, 1970). Therefore, for the present discussion we consider the epithelium in the short-circuited condition as behaving as two compartments in series

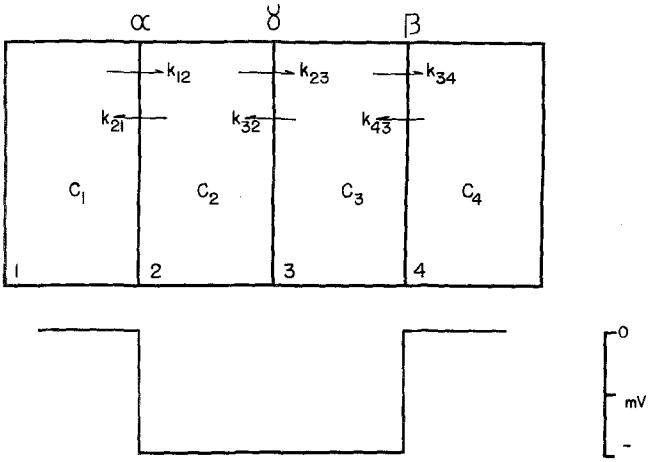

Fig. 7. Schematic representation of a 4-series compartment system which describes ^{42}K influx or efflux across the isolated short-circuited toad skin. *1* and *4* are the external and internal bathing solutions, respectively. *2* and *3* are intraepithelial compartments. C_1, C_2, C_3 and C_4 are the concentrations of ^{42}K in compartments *1*, *2*, *3* and *4*, respectively. The *k*'s are the rate constants for ^{42}K movement across the barriers α, β and γ. α and β are the outer and inner barriers of the epithelium. The lower part of the figure shows the symmetrical electrical potential well present in the epithelium in the short-circuited state

with equal electrical potential differences present at its outer and inner barriers (Fig. 7). When the steady state is attained the rates of isotope discharge into the outer bathing solution, K_{21}^{K}, or inner bathing solution, J_{34}^{K}, are constant and denoted by $J_{21_\infty}^{K}$ and $J_{34_\infty}^{K}$, respectively, and given by Eqs. (A28), (A20), (A29), (A21).

According to the model in Fig. 7, the general relationship between the rate constants in the steady state is given by Eq. (32) (*see Appendix*), which under the assumption that $k_{23}=k_{32}$ discussed above, gives

$$k_{12}/k_{21}=k_{34}/k_{43}. \tag{1}$$

This relationship implies that if an active K uptake is carried out by the basolateral membrane of the epithelial cells (Koefoed-Johnsen & Ussing, 1958; Lindley & Hoshiko, 1964; Gatzy & Clarkson, 1965) (which would be characterized by $k_{43}>k_{34}$), then $k_{12}>k_{21}$, or, in other words, it would be necessary to postulate the existence of an active K uptake at the outer barrier of the epithelium, as discussed in detail in the *Appendix*. A different possibility which is also compatible with relationship (A34) is the absence of K pumps in either the outer or the inner barriers. In this condition, a high cell K concentration would be the result of a K equilibrium across the outer

and inner barriers of the epithelium due to the electrical potential differences present across these barriers. The primary cause of the electrical potential difference would be the activity of a rheogenic Na pump located in the basolateral membranes of the epithelial cells (inner barrier).

Experiments with ouabain, as will be discussed later, strongly support the notion of a rheogenic Na pump and, consequently, of a passive K equilibrium across the cell membranes in the short-circuited state. The idea of a rheogenic Na transport across the inner barrier has gained support in literature recently.

Transient alterations of the rates of ^{42}K discharge into the bathing solutions are expected to occur in the course of changes of the electrical potential differences across the inner and outer barriers if K moves across the epithelium through a transcellular route since K electrochemical potential differences across these barriers change with the magnitude of the electrical potential difference. On the other hand, if the main component of ^{42}K fluxes follows a paracellular route, then no appreciable perturbations of $J^K_{21\infty}$ or $J^K_{34\infty}$ would be expected to occur under the effect of manipulations that alter the electrical potential difference across the outer or inner barriers. Therefore, analysis of the perturbations of the steady-state unidirectional fluxes may bring important information regarding the pathways and mechanisms involved in the transport of this ion across the epithelium. Experiments with amiloride (*Results*, section 2.1), a specific inhibitor of Na entry across the outer barrier (Bentley, 1968; Crabbé *et al.*, 1968; Dörge & Nagel, 1970; Helman & Fisher, 1977; Benos & Mandel, 1978), give strong support to the notion that K ions move through the epithelium passing across the outer barrier. This contrasts somewhat with results of Mandel and Curran (1972) in ouabain inhibited skins, showing that the main route seems to be through the paracellular spaces. Two distinct mechanisms could be suggested to explain the amiloride effect on the rate of ^{42}K discharge into the outer bathing solution, J^K_{21}. One would be to consider that amiloride would act directly on the outer barrier, reducing its K permeability, as it does for Na; the other, that the effect of amiloride would be mediated by alterations of the electrical potential difference across the outer barrier through its action on the Na channels of this barrier. Support for the last hypothesis comes from the results of Helman and Fisher (1977) showing that amiloride hyperpolyrizes the outer barrier owing to an increase in the ohmic voltage developed across this membrane. The rise in this potential barrier would transiently reduce the rate of ^{42}K discharge into the outer bathing solution. This interpretation seems plausible considering that the effect of amiloride reducing J^K_{21} is similar to the effect

on J_{21}^{K} of a nominal zero Na concentration in the external bathing solution (*Results*, section 2.2) and the observations of Helman and Fisher (1977) that amiloride or lower external Na concentration similarly hyperpolarizes the outer barrier. Thus, with 10^{-4} M amiloride in the outer bathing solution (*Results*, section 2.1) $J_{21}^{K} = 2.27 \pm 0.08$ nmol cm^{-2} hr^{-1} ($n=7$) (mean of the five initial consecutive values obtained for each skin after addition of amiloride) is not statistically different from J_{21}^{K} obtained with a nominal zero Na concentration in the outer bathing solution (*Results*, section 2.2) equal to 2.48 ± 0.28 nmol cm^{-2} hr^{-1} ($n=9$) (mean of five consecutive values ($P>0.6$, t test)). It is important to notice that the reduction observed in J_{21}^{K} after adding amiloride is a sharp transient decline followed by a subsequent partial recover with time (Table 2). This behavior contrasts with the effect of the drug on the rate of Na transport (SCC) which shows only a fast decline with time, as already documented in literature (Salako & Smith, 1969).

Helman and Fisher (1977) were able to show that in their experiments with *Rana pipiens berlindieri* amiloride or low external Na concentration hyperpolarize the outer membrane from -105 to -130 mV with amiloride and from -107 to -126 mV with low external Na concentration. From their Figs. 5 and 6 we see that the electrical potential differences were stable for short recorded intervals of no more than 2 min. They do not report stability for long periods of time, which would be very important to grant speculations regarding changes in the cell K concentration. Our results with amiloride show a sharp decline of J_{21}^{K} after addition of the drug, followed by a subsequent increase with time within the next 20 min. It is conceivable that this late increase of J_{21}^{K} after the initial decline with amiloride could be due to an increase in cell K concentration or to a decrease in the magnitude of the electrical potential difference across the outer barrier ($\Delta\Psi_o$), or both. If we consider K to distribute passively across the barriers, then, with a medium K concentration of 2.5 mM, changing electrical potential difference across the barriers to -130 mV as shown by Helman and Fisher (1977) with amiloride would cause cellular K concentration to rise to about 385 mM, which obviously seems to be a too high level. We do not claim that we have the same levels of $\Delta\Psi_o$ before or after amiloride described by Helman and Fisher (1977). However, we can compute, using the Goldman, Hodgkin, Katz equation (Eq. (2) or (3)) and assuming different values for $\Delta\Psi_o$ in the control condition ($\Delta\Psi_o'$), the new values of $\Delta\Psi_o$ after amiloride or zero external Na concentration ($\Delta\Psi_o''$) necessary to explain the observed reduction in J_{21}^{K}. From the experimental results we have: $(J_{21\ \text{amil}}^{K})/(J_{21\ \text{control}}^{K}) = 0.56$ for the first value of Table 2 and

$(J^{K}_{21\ \text{zero ext. Na conc.}})/(J^{K}_{21\ \text{control}}) = 0.57$. Therefore, we can compute the values below:

$\Delta\Psi'_o$ (mV)	$\Delta\Psi''_o$ (mV)	C^*_2 (mM)
−80	−100	120.5
−85	−105	146.3
−90	−110	177.6
−100	−117.5	237.5

We see that for lower values of $\Delta\Psi'_o$ we need lower values of $\Delta\Psi''_o$ to induce equal reductions of J^{K}_{21}. C^*_2 is the cellular K concentration that would be expected if an electrochemical equilibrium were reached with each $\Delta\Psi''_o$. If $\Delta\Psi'_o$ is in the range of −80 to −85 mV, more realistic values for the cell K concentration are expected to occur. These speculations are waiting for more direct experimental evidences. Rick *et al.* (1978) using electron microprobe analysis were able to demonstrate a significant increase in frog skin cell K concentration, though much smaller than those discussed above, when Na free solution replaced the outer bathing Na-Ringer's solution.

In order to get a better insight into the interactions between Na and K fluxes across the outer barrier as well as into the role of the electrical potential differences across this barrier on the rate of ^{42}K discharge from cell to the outer bathing solution, J^{K}_{21} was studied as a function of the external Na concentration (*Results*, section 2.2). Na was replaced by equimolar K concentrations. As shown in Fig. 3*A*, SCC displays its well-known nonlinear behavior with $[Na]_1$ (Kirschner, 1955; Cereijido *et al.*, 1964; Cirne & Malnic, 1972; Mandel & Curran, 1973; Danisi & Lacaz-Vieira, 1974); J^{K}_{21} also displays a similar nonlinear dependence on $[Na]_1$. In the range of 0.0 to 115.0 mM external Na concentration, a highly significant linear dependence is observed between SCC and J^{K}_{21} when these variables vary with $[Na]_1$ (linear correlation coefficient equal to 0.93, $P<0.001$, $n=9$) (Fig. 3*B*). From the slope of the straight line of Fig. 3*B* a ratio of 1300 net Na ions transported across the outer barrier per each K ion leaking to the outer bathing solution can be calculated. This large ratio rules out any possibility of a carrier-mediated Na−K interaction in the outer barrier, suggesting an electrical coupling as a likely candidate to explain the relationship between J^{K}_{21} and SCC. Much lower net Na/net K exchange ratios (in the range of 1.5 to 3) have been observed in our laboratory in open-circuited skins of the same animal species bathed by dilute solutions (in the range of 0.1 to 5 mM Na) on the outer surface (Procopio & Lacaz-Vieira, 1977). These lower values could

be the result of lower rates of net Na transport, due to low external Na concentration and higher rates of K fluxes from skin to the outer solution, as compared to values observed in the present experiments. A likely reason for the lower K efflux observed in the present experiments, as compared to those in the open-circuited condition (Procopio & Lacaz-Vieira, 1977) is the presence of an electrical potential difference across the whole skin in the open-circuited condition (outer solution negative to inner solution), which is a thermodynamic force favoring net K movement from inner to outer bathing solution.

With the aim of testing further the role of shifts in the electrical potential difference across the outer barrier as the determinant of transient alterations of the rate of ^{42}K discharge from skin to the outer solution, depolarization of the outer barrier was accomplished by depolarization, in the short-circuited state, of the inner barrier with high inner K concentration (Koefoed-Johnsen & Ussing, 1958; Fuchs *et al.*, 1977). Na by K substitution in the inner bathing solution is promptly followed by a sharp rise in the rate of ^{42}K discharge from skin to outer compartment (Fig. 4), subsequently followed by a slow decline with time. At the peak, attained 5 min after inner Na by K substitution, J_{21}^{K} is on the order of 700% of its control stationary-state rate before the ionic substitution. The subsequent decline observed after the peak is certainly a consequence of both stabilization of the electrical potential well in a new lower level and the removal of ^{42}K from the inner bathing solution during the substitution. Outer barrier depolarization is the most likely candidate which could be put forward to explain the fast rise of J_{21}^{K} shown in Fig. 4. From the Nernst-Planck equation and constant field assumption (Goldman, 1943; Hodgkin & Katz, 1949), an expression can be written for J_{21}^{K} and used to test the effect of changes in the electrical potential difference across the outer barrier on transient changes in J_{21}^{K}. Thus, if we assume that cell ^{42}K concentration does not alter appreciably in the first few minutes after Na by K replacement in the inner solution, then:

$$J_{21}^{K'} = P_K z_K F \Delta\psi'_{21}/RT \frac{[K]_2 \exp(z_K F \Delta\psi'_{21}/RT)}{\exp(z_K F \Delta\psi'_{21}/RT) - 1} \tag{2}$$

and

$$J_{21}^{K''} = P_K z_K F \Delta\psi''_{21}/RT \frac{[K]_2 \exp(z_K F \Delta\psi''_{21}/RT)}{\exp(z_K F \Delta\psi''_{21}/RT) - 1} \tag{3}$$

where P_K is the K permeability of the outer membrane, z_K the valence of K ion, F the Faraday constant, R the gas constant, T the absolute

temperature (298°K), $\Delta\psi_{21}$ the electrical potential difference across the outer barrier, and [K] the K concentration. 1 and 2 indicate outer and cell compartments, respectively; ′ and ″ refer to before and after inner Na by K substitution, respectively. Even though precise $\Delta\psi$ values for the present conditions were not measured, some reasonable values from literature can be used for discussion. If we accept that $\Delta\psi'_{21}$ is on the order of -100 mV (Nagel, 1976; Helman & Fisher, 1977) and that $\Delta\psi''_{21}$ is much lower, possibly within the range of few milivolts, then some rough calculations for the ratio $J^{K''}_{21}/J^{K'}_{21}$ can be carried out. Thus, with $\Delta\psi'_{21} = -100$ mV and $\Delta\psi''_{21} = -1$ mV, a value equal to 11.8 could be calculated for $J^{K''}_{21}/J^{K'}_{21}$; for the same $\Delta\psi'_{21}$ and $\Delta\psi''_{21} = -30$ mV, $J^{K''}_{21}/J^{K'}_{21}$ would be equal to 8.0. Therefore, a 7 times increase observed experimentally in J^{K}_{21} after inner Na by K replacement is well within reasonable values for a purely electrical effect on J^{K}_{21} due to depolarization of the outer barrier.

Similar effects regarding the rate of ^{42}K discharge into the inner bathing solution, J^{K}_{34}, (*Results*, section 3.2) are expected to occur following inner Na by K replacement, since both outer and inner boundaries of the epithelium are equally depolarized in the short-circuited state upon inner Na by K replacement. As seen in Fig. 5*A* and *B*, inner Na by K substitution is immediately followed by a large increase in the rate of ^{42}K discharge into the inner compartment, both in experiments performed with Cl^- or SO_4^{--} as the main anion in the bathing solutions. As compared to J^{K}_{21}, the rate of J^{K}_{34} increase is slower, possibly as a consequence of delay in a diffusion barrier due to the presence of the corium attached to the inner face of the epithelium. Since ^{42}K persists in the outer compartment after inner Na by K substitution, the decline in J^{K}_{34} that follows the peak is strong evidence that electrical depolarization of the inner membrane to a new level is the cause of the transient observed in J^{K}_{34}. It is interesting to compare the distinct behavior displayed by J^{K}_{34} in Cl^- and in SO_4^{--} media after inner Na by K substitution. In Cl^- medium, after the peak, J^{K}_{34} declines to increase again with time as shown in Fig. 5*A*. This behavior, on the contrary, is not seen in SO_4^{--} medium, where only a decline is observed after the peak (Fig. 5*B*). Osmotic cell swelling due to KCl and water movement from inner solution to cell medium is certainly the reason for the late J^{K}_{34} increase observed only in Cl^- medium. Cell swelling does not occur in SO_4^{--} medium due to the relatively high SO_4^{--} impermeability of the cell membranes (MacRobbie & Ussing, 1961). These results are in consonance with previous work of Varanda and Lacaz-Vieira (1978)

showing that inner Na by K replacement in SO_4^{--} medium does not alter significantly the steady-state K flux from inner to outer solution, as compared to control condition with Na_2SO_4-Ringer's solution on both sides of the skin. On the other hand, in Cl^- medium, the apparent K permeability across the whole skin increases one order of magnitude 90 min after inner Na by K substitution.

Interesting behavior is displayed by J_{21}^K upon the effect of ouabain added to the inner solution (10^{-3} M) (*Results*, section 4). Almost identical patterns were observed both in Cl^- or in SO_4^{--} media (Fig. 6*A* and *B*), indicating that the anion, in contrast to the experiments of inner Na by K substitution, does not play an important role in the transient J_{21}^K response to ouabain. Following addition of ouabain to the inner solution, the well-known decline of SCC is observed, with 90% inhibition 120 min after the addition of the drug. On the other hand, in contrast to the SCC continuous decline, ouabain induces a large transient increase in the rate of ^{42}K discharge into the outer bathing solution. The peak of J_{21}^K, as compared to the control steady-state values before ouabain, is 220% in Cl^- and 180% in SO_4^{--} media, 40 min after addition of ouabain to the inner solution. Following the peak, a decline with time toward a new steady state is observed. J_{21}^K steady-state values before and after ouabain are not statistically different. In Cl^- medium, J_{21}^K is 3.44 ± 0.06 nmol cm^{-2} hr^{-1} before and 3.46 ± 0.76 nmol cm^{-2} hr^{-1} after ($P > 0.9$, t test, $n = 4$). In SO_4^{--} medium, 2.57 ± 0.13 nmol cm^{-2} hr^{-1} before and 2.44 ± 0.24 nmol cm^{-2} hr^{-1} after ($P > 0.6$, t test, $n = 5$). This behavior strongly suggests that the effect of ouabain, inducing a transient increase in J_{21}^K, is mediated by a reduction in the magnitude of the electrical potential well present in the epithelium in the short-circuited state. The effect of ouabain on a nonelectrogenic Na–K pump located in the basolateral membrane of the epithelial cells would be expected, *a priori*, to have an effect opposite to that which have been observed experimentally, that is, inducing only a decline of J_{21}^K. Biber and Mullen (1977) observed both a transient and stationary increase in the rate of Na discharge from epithelium into the outer solution (J_{31}^{Na} in their notation) upon the effect of ouabain. In their opinion, these effects could be explained by reduction in the rate of reextrusion of Na across the serosal surface of the epithelial cells. Larsen (1972) has observed similar increase in J_{31}^{Na} after ouabain and explained this phenomenon somewhat differently by assuming a conversion of the Na–K ATPase system from a pump to a Na/Na exchange mechanism. In order to better discuss the effect of ouabain, let us assume that the epithelium behaves according to

the classical model (Koefoed-Johnsen & Ussing, 1958; Ussing & Windhager, 1964), with a neutral Na−K pump at the basolateral membrane of the epithelial cells and the electrical potential difference determined by a K diffusion potential, which for the sake of simplification, we take as following the Nernst equation. The use of the Nernst equation instead of the constant field equation (Goldman, 1943; Hodgkin & Katz, 1949) does not weaken our argument, since the rate of the fall of the electrical potential difference with the fall in the cell K concentration is faster with the Nernst equation than with the constant field equation. Let us assume that ouabain blocking a 1:1 Na−K pump causes not only a decline in SCC as was observed, but also reduction of the cell K concentration (Zylber, Rotunno & Cereijido, 1973), which would lead to a depolarization of the inner membrane and, due to the short-circuited condition, also depolarization of the outer membrane. The electrical potential difference across the outer membrane seems to be of a purely passive nature due to an ohmic drop across this structure, with an effective Na chemical potential difference not different from zero (Helman & Fisher, 1977). Therefore, depolarization of the outer membrane in this condition could be thought, *a priori*, to induce a transient increase in the rate of ^{42}K discharge from cell to outer bathing solution. However, in the case of a neutral Na−K pump, depolarization of the membranes could only follow the decline in cell K concentration (both cold K and ^{42}K) induced by ouabain. Thus, the rate of ^{42}K discharge from cell to the outer bathing solution would be controlled not only by the electrical potential difference across the outer barrier, but also by the cell ^{42}K concentration. If we assume that ^{42}K discharge from cell into the outer bathing solution follows the constant field equation and that the electrical potential difference across the outer barrier is equal to that across the inner barrier, which follows the Nernst equation for K, as already discussed, then it can be shown (*see Appendix*) that the expected action of ouabain, blocking a nonrheogenic 1:1 Na−K pump in the inner barrier, would be to induce only a decline with time of the rate of ^{42}K discharge into the outer solution. Figure 8*A* shows a simulation of the action of ouabain upon J_{21}^{K} through its action on a nonrheogenic Na−K pump, which shows that only a decline of J_{21}^{K} is expected to occur as a function of time after addition of ouabain. This behavior of J_{21}^{K} predicted by the nonrheogenic pump model is clearly different from that observed experimentally, since ouabain, known to reduce cell K concentration of the epithelial cells of frog skin (Zylber *et al.*, 1973), induces a transient increase in the rate of ^{42}K discharge from epithelium into the outer

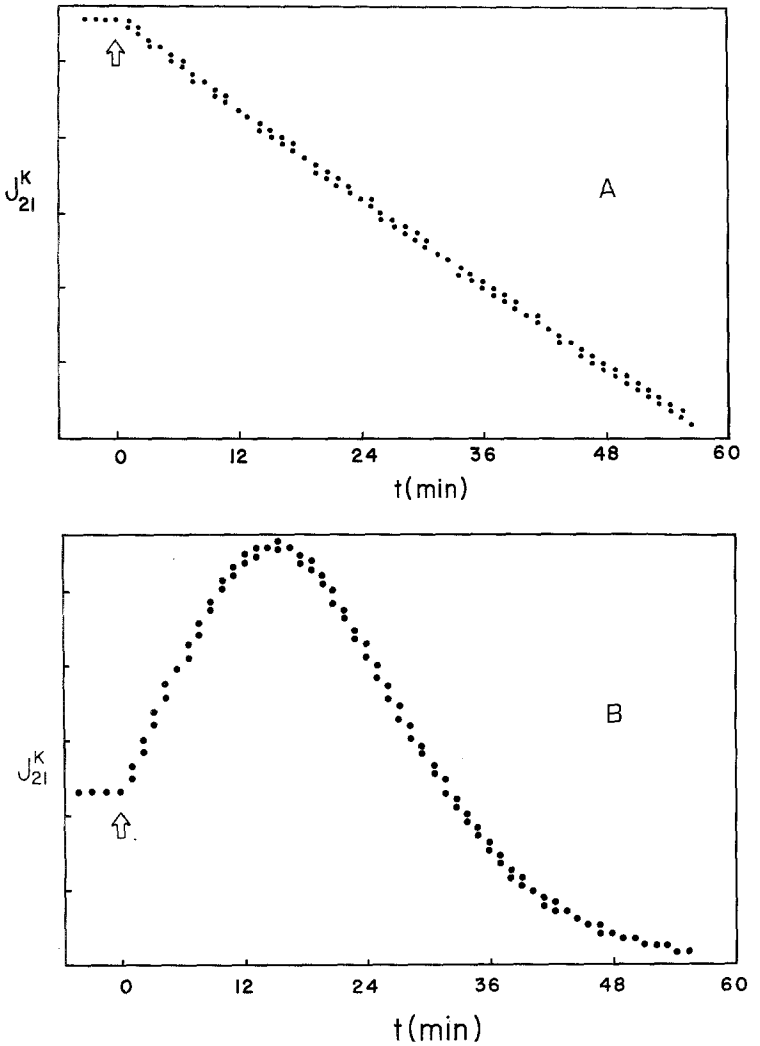

Fig. 8. Computer simulation of the effect of ouabain on the rate of ^{42}K discharge from epithelium into the outer bathing solution (*see Appendix* for details). (*A*): Simulation according to the nonrheogenic Na pump model. Parameters used are the following: $C_{20}^* = 100$ mM; $C_1^* = C_4^* = 2.5$ mM; $k = 0.035\ \text{min}^{-1}$; $q = 1$; $R = 8.3$ joule $\text{mole}^{-1}\,\text{K}^{-1}$; $T = 300$ K; $F = 96{,}500$ coulombs mole^{-1}. (*B*): Simulation according to the rheogenic Na pump model. Parameters used are the following: $P_\alpha = 0.0001$ cm sec^{-1}; $P_\beta = 0.001$ cm sec^{-1}; $\Delta\psi_o = -100$ mV; $k = 0.035\ \text{min}^{-1}$; $V_2 = V_3 = 0.002\ \text{cm}^3$; $S_\alpha = S_\beta = S_\gamma = 1\ \text{cm}^2$; $k_{23} = k_{32} = 0.001$ cm sec^{-1}; $\dot{C}_4 = 1$ (arbitrary unit of isotope concentration)

bathing solution. A model consistent with the experimental results is that of a rheogenic Na pump located in the basolateral membrane of the epithelial cells, as presented in detail in the *Appendix* (Fig. 8*B*). This rheogenic pump would be the primary source of the electrical potential

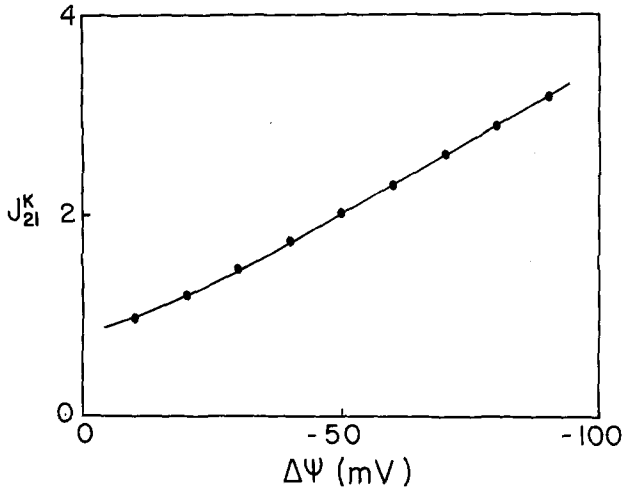

Fig. 9. Computer simulation of the effect of the electrical potential well within the epithelium in the short-circuited state on the steady-state rate of ^{42}K discharge into the outer bathing solution. Rheogenic Na pump model. Parameters used are the following: $P_\alpha = 0.0001\,\text{cm sec}^{-1}$; $P_\beta = 0.001\,\text{cm sec}^{-1}$; $k = 0.035\,\text{min}^{-1}$; $V_2 = V_3 = 0.002\,\text{cm}^3$; $S_\alpha = S_\beta = S_\gamma = 1\,\text{cm}^{-2}$; $k_{23} = k_{32} = 0.001\,\text{cm sec}^{-1}$; $\dot{C}_4 = 1$ (arbitrary unit of isotope concentration)

difference across the inner membrane and consequently to the short-circuited condition also across the outer membrane. The effect of ouabain progressively blocking this rheogenic Na pump would be, apart from reducing SCC, that of depolarizing both barriers of the epithelium. This depolarization would induce the transient ^{42}K discharge from cell to the outer solution. It might be expected that the electrical potential well would stabilize into a new lower value after ouabain had induced its full effect on the Na pump. Therefore, and as a consequence, J_{21}^{K} should attain a new steady state lower than that in the control condition, as shown in Fig. 9. The transient behavior predicted by the rheogenic Na pump model (Fig. 8*B*) was observed experimentally (Fig. 6*A* and *B*), except for the final steady-state value which did not fall below the control steady-state value. Reasons for this discrepancy are unknown. An increase in the K permeability of the outer barrier due to small swelling of the epithelial cells could possibly be the reason. The results of the experiments with ouabain do not lead to a clear conclusion on the more intimate nature of the rheogenic Na pump, if a pure Na pump or a Na−K pump with a Na/K ratio significantly greater than 1. In this last case, the electrical potential difference across the inner membrane would be of a composite nature due to the rheogenicity of the pump and to

ionic diffusion. However, for the transient increase in J_{21}^{K} induced by ouabain to be observed experimentally, the contribution of the rheogenic component of the electrical potential difference necessarily has to be significant.

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Appendix

Let us consider a 4-series compartment system (Fig. 7). 1 and 4 are the outer and inner compartments, respectively; 2 and 3 are intra-epithelial compartments. V_i is the volume of compartment i; C_i is the concentration of ^{42}K in compartment i; S_α, S_β and S_γ are the areas of membranes α, β and γ, respectively, according to Fig. 7. The k's are the rate constants for ^{42}K movement across the membranes, as shown in Fig. 7.

Influx Experiments

In the influx experiments, ^{42}K is added to compartment 1 and its rate of discharge, J_{34}^{K}, from epithelium into compartment 4 is measured (*see Methods*). Due to the low rate of ^{42}K, disappearance from compartment 1 during the experimental period, C_1 can be considered constant. On the other hand, $C_4 \cong 0$ since the inner solution is renewed every 2 min.

The following differential equations describe the rates of ^{42}K concentration change in compartments 2 and 3:

$$\frac{dC_2}{dt} = \frac{S_\alpha k_{12}}{V_2} C_1 + \frac{S_\gamma k_{32}}{V_2} C_3 - \left(\frac{S_\alpha k_{21}}{V_2} + \frac{S_\gamma k_{23}}{V_2}\right) C_2 \qquad \text{(A1)}$$

$$\frac{dC_3}{dt} = \frac{S_\gamma k_{23}}{V_3} C_2 - \left(\frac{S_\gamma k_{32}}{V_3} + \frac{S_\beta k_{34}}{V_3}\right) C_3. \qquad \text{(A2)}$$

Equations (A1) and (A2) can be written as:

$$\frac{dy}{dt} = mx - ny \qquad \text{(A3)}$$

$$\frac{dx}{dt}=a+py-bx \tag{A4}$$

where

$$m=\frac{S_\gamma k_{23}}{V_3} \tag{A5}$$

$$x=C_2 \tag{A6}$$

$$n=\frac{S_\gamma k_{32}}{V_3}+\frac{S_\beta k_{34}}{V_3} \tag{A7}$$

$$y=C_3 \tag{A8}$$

$$a=\frac{S_\alpha k_{12}}{V_2}C_1 \tag{A9}$$

$$p=\frac{S_\gamma k_{32}}{V_2} \tag{A10}$$

$$b=\frac{S_\alpha k_{21}}{V_2}+\frac{S_\gamma k_{23}}{V_2}. \tag{A11}$$

The system of differential equations (A3) and (A4) can be solved using Laplace transforms (Pipes & Harvill, 1970) yielding:

$$C_3=\frac{ma}{\alpha\beta}\left(1+\frac{\beta\exp(-\alpha t)-\alpha\exp(-\beta t)}{\alpha-\beta}\right) \tag{A12}$$

where

$$\alpha=\frac{A+(A^2-4B)^{1/2}}{2} \tag{A13}$$

$$\beta=\frac{A-(A^2-4B)^{1/2}}{2} \tag{A14}$$

and

$$A=n+b \tag{A15}$$

$$B=bn-mp. \tag{A16}$$

The steady-state ^{42}K concentration in compartment 3, $C_{3\infty}$, is obtained from Eq. (A12) when $t\to\infty$, yielding:

$$C_{3\infty}=\frac{ma}{\alpha\beta}. \tag{A17}$$

The rate of ^{42}K discharge into compartment 4, J_{34}^{K}, is:

$$J_{34}^{K} = k_{34} S_{\beta} C_3. \tag{A 18}$$

Therefore, from Eqs. (A 12) and (A 18) we have:

$$J_{34}^{K} = k_{34} S_{\beta} \frac{ma}{\alpha\beta} \left(1 + \frac{\beta \exp(-\alpha t) - \alpha \exp(-\beta t)}{\alpha - \beta}\right) \tag{A 19}$$

or

$$\left[1 - \frac{J_{34}^{K}}{J_{34\infty}^{K}}\right] = \frac{\alpha \exp(-\beta t) - \beta \exp(-\alpha t)}{\alpha - \beta} \tag{A 20}$$

where

$$J_{34\infty}^{K} = k_{34} S_{\beta} \frac{ma}{\alpha - \beta}. \tag{A 21}$$

Efflux Experiments

In the efflux experiments, ^{42}K is added to compartment 4 and its rate of discharge, J_{21}^{K}, is measured in compartment 1. For reasons analogous to those already discussed for influx, $\dot{C}_4$ is constant during the experimental period and $\dot{C}_1 \cong 0$. $\dot{C}_i$ is the concentration of ^{42}K in compartment i when the isotope is added to compartment 4 (in the influx experiments) and should be differentiated from C_i which is the isotope concentration in compaartment i when the isotope is added to compartment 1 (in the influx experiments).

If a treatment similar to that used for influx is applied to efflux, it yields:

$$\dot{C}_2 = \frac{m' a'}{\alpha\beta} \left(1 + \frac{\beta \exp(-\alpha t) - \alpha \exp(-\beta t)}{\alpha - \beta}\right) \tag{A 22}$$

where

$$m' = \frac{S_{\gamma} k_{32}}{V_2} \tag{A 23}$$

$$a' = \frac{S_{\beta} k_{43}}{V_3} \dot{C}_4. \tag{A 24}$$

α and β are given by Eqs. (A 13) and (A 14), respectively. In the steady-state, ^{42}K concentration in compartment 2, $\dot{C}_{2\infty}$, is:

$$\dot{C}_{2\infty} = \frac{m' a'}{\alpha\beta}. \tag{A 25}$$

The rate of ^{42}K discharge into compartment 1 is:

$$J_{21}^{K}=k_{21}\,S_{\alpha}\,\dot{C}_{2}. \tag{A 26}$$

Therefore, from Eqs. (A 27) and (A 23) we have:

$$J_{21}^{K}=k_{21}\,S_{\alpha}\frac{m'\,a'}{\alpha\beta}\left(1+\frac{\beta\exp(-\alpha t)-\alpha\exp(-\beta t)}{\alpha-\beta}\right) \tag{A 27}$$

or

$$\left[1-\frac{J_{21}^{K}}{J_{21\infty}^{K}}\right]=\frac{\alpha\exp(-\beta t)-\beta\exp(-\alpha t)}{\alpha-\beta} \tag{A 28}$$

where

$$J_{21\infty}^{K}=k_{21}\,S_{\alpha}\frac{m'\,a'}{\alpha\beta}. \tag{A 29}$$

From Eqs. (A 20) and (A 28) we see that influx and efflux follow a two exponential kinetic which adequately describes the experimental results, as shown in *Results*, section 1, Fig. 2*A* and *B*.

Taking into consideration the experimental results indicating that ^{42}K influx and efflux steady-state values are not statistically different (*Results*, section 1), then

$$\frac{J_{34\infty}^{K}}{C_{1}}=\frac{J_{21\infty}^{K}}{\dot{C}_{4}}. \tag{A 30}$$

Making use of Eqs. (A 29) (A 30) and (A 21), we get

$$\frac{k_{34}\,S_{\beta}\,C_{3\infty}}{C_{1}}=\frac{k_{21}\,S_{\alpha}\,\dot{C}_{2\infty}}{\dot{C}_{4}}. \tag{A 31}$$

From Eqs. (A 18), (A 25) and (A 31) and making use of (A 5), (A 9), (A 23) and (A 24), it is easy to show that:

$$k_{12}\,k_{23}\,k_{34}=k_{43}\,k_{32}\,k_{21} \tag{A 32}$$

which is valid only under the assumption expressed by the relationship (A 30) which is strongly supported by the experimental results.

If we also assume that the epithelial cell behaves as a syncythium (Dörge *et al.*, 1976; Rick *et al.*, 1978), then

$$k_{23}=k_{32}. \tag{A 33}$$

Under this assumption, relationship (A 32) gives:

$$k_{12}/k_{21}=k_{43}/k_{34}. \tag{A 34}$$

In order to discuss the transient rise in the rate of ^{42}K discharge into the outer bathing solution, J_{21}^{K}, upon the addition of ouabain to the inner compartment, we carried out computer simulation of the action of ouabain assuming two different models regarding the nature of the Na pump: one, considering a rheogenic Na pump in the inner barrier and the other, a 1:1 Na−K pump in this membrane.

Rheogenic Na Pump

We assume that the primary cause of the electrical potential difference is a rheogenic Na pump in the inner barrier and an ohmic drop due to the short-circuited condition in the outer membrane (Helman & Fisher, 1977). No K pumps are considered to be present in either the outer or the inner membranes and K ion distributes between epithelium and the bathing solutions according to the electrical potential differences present across these barriers which are equal in the short-circuited state. In the absence of K pumps, the k's could be expressed as a function of the permeability and of the electrical potential difference across the membranes, according to the constant field equation (Goldman, 1943; Hodgkin & Katz, 1949). Thus:

$$k_{12} = P_{\alpha} F \Delta\psi / RT \frac{1}{\exp(F\Delta\psi/RT) - 1} \tag{A35}$$

$$k_{21} = P_{\alpha} F \Delta\psi / RT \frac{\exp(F\Delta\psi/RT)}{\exp(F\Delta\psi/RT) - 1} \tag{A36}$$

$$k_{34} = P_{\beta} F \Delta\psi / RT \frac{\exp(F\Delta\psi/RT)}{\exp(F\Delta\psi/RT) - 1} \tag{A37}$$

$$k_{43} = P_{\beta} F \Delta\psi / RT \frac{1}{\exp(F\Delta\psi/RT) - 1} \tag{A38}$$

where P_{α} and P_{β} are the K permeabilities of outer and inner barriers, respectively, and $\Delta\psi$ is the electrical potential difference present across these barriers. R, T and F have their conventional meanings.

Computer simulation of the effect of ouabain on the rate of ^{42}K discharge from epithelium into the outer bathing solution was carried out. With ^{42}K concentration in steady state through the epithelium, the action of ouabain on a rheogenic Na pump was simulated by assuming that it blocked the pump progressively, inducing an exponential decline

of $\Delta\psi$ with time, being

$$\Delta\psi = \Delta\psi_o \exp(-kt)$$

where $\Delta\psi_o$ is the steady-state $\Delta\psi$ value before the action of ouabain and k is a constant whose value can be adjusted so that $\Delta\psi$ is close to zero at the same time SCC is near zero. Integration was performed according to the Runge-Kutta method (Pipes & Harvill, 1970). The initial boundary conditions were:

$$\dot{C}_{2\infty} = \frac{\frac{S_\gamma k_{32}}{V_2} \cdot \frac{S_\beta k_{43}}{V_3} \dot{C}_4}{\left(\frac{S_\beta k_{34}}{V_3} + \frac{S_\gamma k_{32}}{V_3}\right) \cdot \left(\frac{S_\gamma k_{23}}{V_2} + \frac{S_\alpha k_{21}}{V_2}\right) - \left(\frac{S_\gamma k_{32}}{V_2} \cdot \frac{S_\gamma k_{23}}{V_3}\right)} \tag{A39}$$

and

$$\dot{C}_{3\infty} = \frac{\frac{S_\beta k_{43}}{V_3} \cdot \left(\frac{S_\gamma k_{23}}{V_2} + \frac{S_\alpha k_{21}}{V_2}\right) \dot{C}_4}{\left(\frac{S_\beta k_{34}}{V_3} + \frac{S_\gamma k_{32}}{V_3}\right) \cdot \left(\frac{S_\gamma k_{23}}{V_2} + \frac{S_\alpha k_{21}}{V_2}\right) - \left(\frac{S_\gamma k_{32}}{V_2} \cdot \frac{S_\gamma k_{23}}{V_3}\right)} \tag{A40}$$

and the rates of ^{42}K concentration changes in compartments 2 and 3 were:

$$\frac{d\dot{C}_2}{dt} = \frac{S_\gamma k_{32}}{V_2} \dot{C}_3 - \left(\frac{S_\gamma k_{23}}{V_2} + \frac{S_\alpha k_{21}}{V_2}\right) \dot{C}_2 \tag{A41}$$

and

$$\frac{d\dot{C}_3}{dt} = \frac{S_\beta k_{43}}{V_3} \dot{C}_4 + \frac{S_\gamma k_{23}}{V_3} \dot{C}_2 - \left(\frac{S_\beta k_{34}}{V_3} + \frac{S_\gamma k_{32}}{V_3}\right) \dot{C}_3. \tag{A42}$$

k_{21}, k_{34} and k_{43} are given by Eqs. (A36), (A37) and (A38), respectively.

As can be seen (Fig. 8*B*), simulation of the behavior of J_{21}^K upon the effect of ouabain under the assumption of a rheogenic Na pump shows a transient rise of J_{21}^K which reasonably resembles the results obtained experimentally.

Nonrheogenic 1:1 Na–K Pump

Let us now take a different point of view, assuming that a K pump is present in the inner barrier oriented from compartment 4 to compartment 3 and coupled to a Na pump oriented in the opposite direction, with Na/K ratio equal to 1 (Koefoed-Johnsen & Ussing, 1958). In this

condition the rate constant k_{43} could be written as:

$$k_{43} = k_{43}^{p} + k_{43}^{a} \tag{A 43}$$

where k_{43}^{p} and k_{43}^{a} are the passive and active components of k_{43}. k_{43}^{p} as well k_{34} could be expressed according to the constant field equation, as given by Eqs. (A 38) and (A 37), respectively. Thus, if in the relationship (A 34) we introduce k_{43} given by Eq. (A 43), k_{43}^{p} given by Eq. (A 38) and k_{34} given by Eq. (A 37), then relationship (A 34) no longer holds, unless a second K pump, with a rate constant k_{12}^{a} is postulated as being located in the outer barrier, oriented from compartment 1 to compartment 2, so that:

$$\frac{k_{12}^{p} + k_{12}^{a}}{k_{21}} = \frac{k_{43}^{p} + k_{43}^{a}}{k_{34}}. \tag{A 44}$$

If the passive components (k_{12}^{p}, k_{21}, k_{43}^{p} and k_{34}) are replaced by their values given by the constant field equation, Eqs. (A 35), (A 36), (A 38) and (A 37), respectively, then Eq. (A 34) yields:

$$\frac{k_{12}^{a}}{P_{\alpha}} = \frac{k_{43}^{a}}{P_{\beta}}. \tag{A 45}$$

Equation (A 45) shows that the ratio between the pump rate constant and the permeability coefficient for K should be equal for the inner and outer membranes. As the K permeability of the inner membrane is much higher than that of the outer membrane (MacRobbie & Ussing, 1961), then a much less powerful K pump should be postulated as being present in the outer barrier. So far, no experimental evidence has been presented in favor of a K pump in the outer barrier. Its existence cannot be ruled out with the experimental data so far available in literature since the small k_{12}^{a}, as compared to k_{43}^{a} postulated in the nonrheogenic Na−K pump model, could well pass undetected. However, at low external ionic concentration in the outer bathing solution, a net K flow was observed from cell to outer medium (Procopio & Lacaz-Vieira, 1977) which is evidence (however indirect regarding the present conditions) against a K pump in the outer barrier.

Simulation of the action of ouabain on the rate of ^{42}K discharge from epithelium into the outer bathing solution was carried out according to a model assuming a nonrheogenic Na pump in the inner barrier. It was simulated that ^{42}K was added to the inner bathing solution and a steady-state condition, was reached before ouabain was added to the

inner compartment. J_{21}^{K} is given by Eq. (A26). We assume that the dependence between the concentrations of ^{42}K ($\dot{C}_2$) and of cold K (C_2^*) in compartment 2 is given by

$$\dot{C}_2 = q\, C_2^*$$

where q can be a constant or a decreasing function of time after addition of ouabain. k_{21} is given by Eq. (A36) and the electrical potential difference across the outer barrier by

$$\Delta\psi = -\frac{RT}{F} \ln \frac{C_2^*}{C_1^*}$$

where C_1^* is the concentration of cold K in the bathing solutions. If we assume the presence of a nonrheogenic Na pump in the inner barrier, then the primary effect of pump block would be of reducing cell K concentration, which we assume to decrease with time, and for the present discussion as obeying an exponential decline according to the equation:

$$C_2^* = C_{20}^* \exp(-kt)$$

where C_{20}^* is the K concentration in compartment 2 at the moment of addition of ouabain.

As can be seen (Fig. 8*A*), simulation of the behavior of J_{21}^{K} upon the effect of ouabain under the assumption of a nonrheogenic Na pump shows only a decline with time which does not agree with the experimental results.

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