Lithium Transport Across Isolated Frog Skin Epithelium

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Summary. Transepithelial Li^+ influx was studied in the isolated epithelium from abdominal skin of *Rana catesbeiana*. With Na^+-Ringer's as inside medium and Li^+-Ringer's as outside medium, the Li^+ influx across the epithelium was 15.6 $\mu A/cm^2$. This influx was considerably reduced by removal of either Na^+ or K^+ from the inside bath or by the addition of ouabain or amiloride. Epithelial K^+ or Na^+ concentration was respectively lower in epithelia bathed in K^+-free Ringer's or Na^+-free Ringer's. In conditions of negligible Na^+ transport, a 20 mM Li^+ gradient (out→in) produced across the short-circuited epithelium a Li^+ influx of 11.8 $\mu A/cm^2$ and a mean short-circuit current of 10.2 $\mu A/cm^2$. The same Li^+ gradient in the opposite direction produced a Li^+ outflux of only 1.9 $\mu A/cm^2$. With equal Li^+ concentration (10.3 and 20.6 mM) on both sides of the epithelium, plus Na^+ in the inside solution only, a stable Li^+-dependent short-circuit current was observed. Net Li^+ movement (out→in) was also indirectly determined in the presence of an opposing Li^+ gradient. Although Li^+ does not substitute for Na^+ as an activator of the $(Na^+ + K^+)$-ATPase from frog skin epithelium, Li^+ influx appears to be related to Na^+-K^+ pump activity. It is proposed that the permeability of the "outer barrier" to Na^+ and Li^+ is regulated by the electrical gradient produced by electrogenic Na^+-K^+ pumps located in the membrane of the deeper epithelial cells.

It is generally accepted that active Na^+ transport across skin epithelium consists of at least two basic processes. First, Na^+ gains access to the epithelial cells across an "outer barrier" and second, it is transported from the cells to the inside medium across an "inner barrier". Initially, it was thought that the translocation across the "outer barrier" was a passive phenomenon and that active transport only occurred across the "inner barrier" and involved a 1:1 interchange of Na^+ for K^+ in a nonelectrogenic fashion [17]. The pump at the "inner barrier" was assumed to be closely related to the $(Na^+ + K^+)$-ATPase system found in single cells. This is supported by the fact that ouabain causes inhibition of the transepithelial active Na^+ transport [16]. Furthermore, in toad bladder, there is a remarkable agreement between the inhibition curves

for enzyme activity and transport [5]. The presence of $(Na^+ + K^+)$-ATPase in frog skin was first demonstrated by Bonting *et al.* [15].

More recently, a considerable amount of experimental evidence has shown that the "outer barrier" does not behave in a purely passive way [2, 4, 12, 13, 19, 22] and it has been proposed that an active mechanism is responsible for the entry of Na^+ into the epithelial cells [18, 26]. One of the characteristics of the "outer barrier" is that Na^+ and Li^+ compete with one another for entry [4, 18] into the epithelial cells.

Candia and Chiarandini [9] have shown that, if Na^+ is present in the inside solution, a net Li^+ movement occurred across the frog skin that lasted for several hours. This Li^+ transport was inhibited by ouabain. They suggested that the $(Na^+ + K^+)$-ATPase system may provide the energy for the Li^+ transport but need not be the carrier itself. This interpretation was in line with predictions in a model suggested by Candia [7] in which an electrical coupling exists between the energy source at the "inner barrier" and a rectifier at the "outside barrier".

The transepithelial Li^+ transport could also be explained (in a simpler manner) by assuming that Li^+ could replace Na^+ in the stimulation of the $(Na^+ + K^+)$-ATPase system. However, this possibility is considered very unlikely since it has been recently found that Li^+ does not substitute for Na^+ in the activation of an ouabain-sensitive $(Na^+ + K^+)$-ATPase from frog skin epithelium [24].

In this paper we provide evidence that in the isolated skin epithelium: a) Na^+ and K^+ requirements are common to Na^+ transport, Li^+ transport, and the enzyme system; b) Unidirectional Li^+ influx is significantly higher than Li^+ outflux. Their difference is equivalent to the short-circuit current (SCC) in the absence of an appreciable net Na^+ transport; and c) Li^+ can be transported in the absence of and against an electro-chemical gradient provided that Na^+ is present in the inside bathing medium. A model showing a possible coupling of the energy from the $(Na^+ + K^+)$-ATPase system to the Li^+ transport is presented.

Materials and Methods

Most of the experiments were performed using the abdominal skin epithelium isolated from *Rana catesbeiana*. The epithelium was obtained from large animals weighing about 600 g kept in a container with tap water at room temperature (21–23 °C).

For the isolation of the epithelium, a method derived from that of Aceves and Erlij [1] and of Rajerison *et al.* [21] was used. A detailed description can be found in a previous publication [24]. Additional experiments were performed using the abdominal skin from *Rana pipiens*.

Table 1. Composition of solution (values given in millimoles/liter)

No.	Solution	Na^+	Li^+	$Choline^+$
1	Na-Ringer's	103	0	0
2	Li-Ringer's	<0.1	103	0
3	Choline-Ringer's	<0.1	0	103
4	Li 10-Choline-Ringer's	<0.1	10.3	92.7
5	Li 20-Choline-Ringer's	<0.1	20.6	82.4
6	Na 10-Choline-Ringer's	10.3	0	92.7
7	Na 20-Choline-Ringer's	20.6	0	82.4
8	Na 68-Choline-Ringer's	68.7	0	34.3
9	Na 82-Choline-Ringer's	82.4	0	20.6
10	Li 10-Na-Ringer's	92.7	10.3	0
11	Li 20-Na-Ringer's	82.4	20.6	0
12	Li 34-Na-Ringer's	68.7	34.3	0

Each solution contained, in addition to the above, the following in mM: K^+ 2.5; Ca^{++} 1.8; Cl^- 105.5; $gluconate^{2-}$ 1.8; and Tris-HCl 2.0. The pH was 7.4.

Electrical Measurements

The epithelium was mounted in Lucite chambers that permitted electrical measurement across an area of 0.5 cm². (In the experiments with *Rana pipiens* chambers with a cross-sectional area of 2 cm² were used.) The chamber and the mounting procedure is described in a previous publication [8]. The voltage and current bridges were polyethylene tubing filled with 3% agar dissolved in Ringer's. Voltage bridges were connected to the recording equipment (Electrometer Model 200B, Keithley Instruments, Cleveland, Ohio; and recorder Model EU 20, Heath Co., Benton Harbor, Michigan) by means of calomel cells. The SCC was measured with an automatic voltage-clamp system. The composition of the various Ringer's solutions used to bathe the epithelium is described in Table 1.

Measurements of Na^+, Li^+, and K^+ Concentration

The concentration of these cations was determined by flame photometry (Ependorf Model 700, Brinkmann Instruments, Westbury, N.Y.). When Na^+ was measured in Li^+-Ringer's and Li^+ and Na^+-Ringer's, special care was taken to avoid error due to interference by the other ion.

Determination of K^+ Content in the Skin Epithelium

The skin epithelium isolated from one piece of skin was divided into 3 or 4 portions. One portion was bathed in a K^+-free Ringer's for 130 min. A second portion was bathed in regular Ringer's for 60 min and then transferred to a K^+-free Ringer's for another 70 min. The remaining one or two portions were used as controls and were bathed for the entire period of 130 min in Ringer's with 2.5 mM K^+ concentration. The bathing solutions were periodically renewed and large enough so that K^+ lost from the epithelium did not increase the K^+ concentration. At the end of the incubation the extended layer of epithelium was photographed, gently bottled, weighed, and dissolved in nitric acid (0.05 N). This solution

was slowly and completely evaporated at 60 °C. The solid residue was redissolved in distilled water and the K^+ concentration was determined. K^+ content is expressed per unit area and dry weight.

Determination of Na^+ Content in the Skin Epithelium

One obvious difficulty in the determination of the intracellular Na^+ concentration is to avoid including Na^+ that is in the extracellular space. We did not attempt to measure Na^+ content in control conditions (epithelium bathed in Na^+-rich Ringer's) but only after the epithelium was incubated in choline-Ringer's (Na-free) for 85 min. The remaining Na^+ content was compared to values in the literature for control conditions. This permitted us to evaluate the effect produced by removal of Na^+ from the inside solution on the epithelial Na^+ concentration. The technical procedure for the Na^+ concentration measurement was the same as described for K^+.

Measurement of Li^+-Originated Short-Circuit Current[1] Simultaneously with Unidirectional Li^+ and/or Na^+ Fluxes

Several experimental conditions were designed in which Na^+ and Li^+ fluxes were measured across the isolated epithelium. Each side was bathed with solutions containing variable concentrations of Na^+, Li^+, and choline$^+$ whereas the concentration of other ions, pH and osmolarity was kept constant. For any given solution or mixture of solutions the addition of Na^+, Li^+, and choline$^+$ concentrations was equal to 103 mM (see Table 1). Unidirectional Na^+ fluxes were measured by adding ^{22}Na to one solution (about 2 µCi/ml) and taking periodic samples from the other. Specific activity in the labeled side was constant throughout the experiment, and the activity in the "cold" side was 1% or less of that in the labeled compartment. The radioactivity was measured with a well scintillation detector and a Packard spectrometer. When Li^+ fluxes were determined only one solution contained Li^+ while the opposite bathing solution was kept free of Li^+ or with a Li^+ concentration 1% or less of the Li-rich solution. Li^+ fluxes were calculated by periodically sampling the initially Li-free solution and determining the Li^+ concentration in the samples.

Results

K^+ Content in the Isolated Epithelium: Effect of K^+-free Medium

The outside barrier of the skin is almost impermeable to K^+. It is expected that the epithelial cells will lose K^+ when bathed from the inside in a K^+-free medium. From the data of Candia and Zadunaisky [11] (using the whole skin) it can be predicted that in 2 hr the skin will lose about 0.64 µequiv of K^+ per cm^2 of surface. To compare the decline

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[1] The reader should note that we use the term SCC to refer to the current measured when the potential difference (p.d.) across the epithelium is zero without regard to the chemical composition of the solutions bathing the epithelium. Thus, when the skin is bathed with different solutions, a fraction of the SCC may reflect passive diffusion rather than an active process.

Table 2. Effect of potassium removal from the inside solution on short-circuit current and potassium content of the isolated frog skin epithelium

Outside solution	Inside solution	SCC (μA/cm^2)	K content	
			(μequiv/cm^2)	(μequiv/ mg dry wt)
		Na flux		
NaCl Ringer's	NaCl Ringer's (control)	16.1 ± 1.2 (9)	0.60 ± 0.08	0.30 ± 0.04
NaCl Ringer's	NaCl Ringer's (K-free 70′)	10.2 ± 1.3 (9)	0.50 ± 0.05	0.25 ± 0.03
NaCl Ringer's	NaCl Ringer's (K-free 130′)	8.8 ± 1.2 (9)	0.43 ± 0.05	0.22 ± 0.03
		Li flux		
LiCl Ringer's	NaCl Ringer's (control)	15.6 ± 1.3 (7)		
LiCl Ringer's	NaCl Ringer's (K-free 70′)	10.2 ± 1.1		
LiCl Ringer's	NaCl Ringer's (K-free 120′)	4.8 ± 0.6 (7)		

Each value is the mean $\pm$ SE.

of the SCC with the decline in intracellular K$^+$ content, three pieces from the same epithelium were incubated in a K$^+$-rich medium for 130 min and in a K$^+$-free medium for 70 and 130 min, respectively. These results are shown in Table 2. K$^+$ content is significantly lower (as paired data) in epithelia incubated in a K$^+$-free medium and there is a good correlation between the SCC and the epithelial K$^+$ content.

Na$^+$ Concentration in the Isolated Epithelium: Effect of Na$^+$-free Medium

Ten pieces of epithelium from different frogs were exposed to Na$^+$-free (choline) Ringer's for 85 min. Periodic washouts were made to maintain the medium free of Na$^+$. The Na$^+$ content in the epithelia at the end of the incubation was 0.00738 ± 0.00126 μequiv/cm^2. Assuming the Na$^+$ compartment in the epithelium to be 50–100 μ thick, the concentration per volume would be 1.476–0.738 μequiv/cm^3, respectively. This concentration is a small fraction of the previously reported Na$^+$ concentration in normal skin epithelium (18–35 mM [26], 16 mM [18], 24.7 mM [1]).
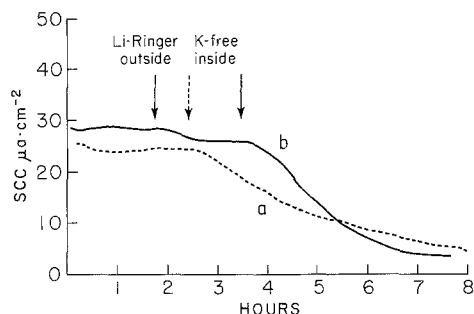
Fig. 1. Effects of K^+-free Ringer's in the inside solution on the SCC of the isolated frog skin epithelium. *a*) Na^+-Ringer's in the outside. Broken arrow indicates change of the inside medium from a K^+-rich to a K^+-free Ringer's. *b*) First arrow indicates change of the outside medium from a Na^+-Ringer's to a Li^+-Ringer's. Second arrow indicates change of the inside medium from a K^+-rich to K^+-free Ringer's

Sodium and Lithium Short-Circuit Current: Effects of K^+-free Ringer's and Ouabain

The SCC was measured in nine preparations bathed on both sides with Na^+-Ringer's. After at least 45 min of stable SCC, the inside medium was replaced with a K^+-free Na^+-Ringer's and periodic washouts were made to insure a K^+ concentration of less than 0.10 mM. This produced a slow decline of the SCC and p.d. as illustrated in Fig. 1*a*. After 130 min in K^+-free Ringer's, the SCC was about 55% of the control and continued to decline to reach zero in about 9 hr. To study the effect of K^+-free Ringer's on the Li^+ transport, another series of experiments was done in the following manner. The SCC was measured in epithelia bathed on both sides with Na^+-Ringer's. After at least 30 min of stable SCC the outside medium was replaced with a Li^+-Ringer's and periodic washouts were made with this solution to insure a Na^+-free solution on the outside. The SCC with Li^+-Ringer's in the outside was usually 10–20% smaller than the control SCC in Na^+-Ringer's. This is different from the observation of Candia and Chiarandini in whole skins [9] where no difference between Na^+ and Li^+ SCC was found. After the Li^+-SCC was stable for at least 45 min the inside medium was replaced with a K^+-free Na^+-Ringer's and the K^+ concentration in the inside was maintained below 0.10 mM. The SCC declined to about 30% of the control Li^+-SCC in 130 min (Fig. 1*b*).

Table 2 summarizes these results. The lack of K^+ in the inside medium definitely decreases the SCC when the skin is bathed on the outside with either Na^+-Ringer's or Li^+-Ringer's.

Table 3. Effect of potassium removal and 50% dilution of the Ringer's on electrical parameters
of the isolated skin epithelium

	p.d.$_c$ (mv)	p.d.$_k$	p.d.$_d$	I$_c$ (μA/cm^2)	I$_k$	I$_d$	R$_c$ (KΩ cm^2)	R$_k$	R$_d$
	43	27	54	24.0	12.0	14.8	1.79	2.25	3.64
	52	32	61	24.0	12.0	15.5	2.08	2.66	3.93
	29	22	51	16.0	10.0	13.6	1.81	1.20	3.75
	27	25	40	18.0	10.5	15.0	1.50	2.38	2.67
	51	38		18.8	11.2	16.2	2.71	3.39	
	34	25	37	20.0	12.8	16.0	1.70	1.95	2.31
Mean	39	28.2	48.6	20.1	11.4	15.2	1.96	2.47	3.26
SE	4.28	2.34	4.45	1.33	0.43	0.39	0.21	0.21	0.32

p.d.: potential difference
I: SCC
R: electrical resistance
c: control
k: K-free
d: K-free plus 50% dilution with distilled water
I$_d$ and p.d.$_d$ were stable after 10 min and remained constant for more than 1 hr. Readings
were made within this period.

Ussing [25] had argued that the inhibition of the SCC caused by the
lack of K$^+$ might be due to cell shrinkage as the cells lose K$^+$ to the
bathing solution. He showed that after the Na$^+$ transport had been inhib-
ited in a K$^+$-free medium, dilution of the K$^+$-free Ringer's to 0.5 strength
with water restored the SCC as the skin swelled.

We made six such experiments with the isolated epithelium. The skin
epithelium was bathed in regular Na$^+$-Ringer's and a stable control SCC
and p.d. were established. Then, the inside solution was replaced by a
K$^+$-free Na$^+$-Ringer's (by means of three thorough washouts). About
80–90 min later a lower and stable SCC and p.d. were measured. Following
this, both inside and outside Ringers's were diluted to 0.5 strength with
distilled water. The experiment was continued for 60–90 min. A partial
recovery of the SCC was observed but it never reached the control value.
The p.d., on the other hand, immediately rose in each case to values
higher than the control. Table 3 shows results in detail. As previously
seen in Table 2, K$^+$-free Ringer's produces a 50% reduction of the SCC.
However, dilution of the Ringer's restores the SCC to only 75% of the
initial level.

The effect on the electrical resistance produced by these changes in
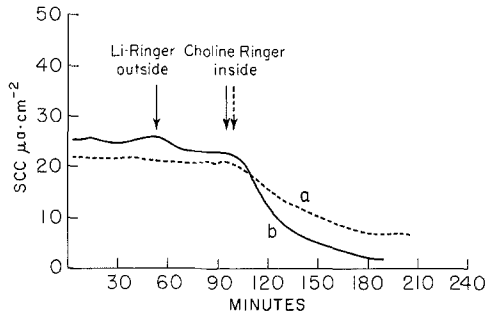solution is worth noting. The first change to a K$^+$-free Ringer's increased

Fig. 2. Effects of Na^+-free Ringer's in the inside solution on the SCC of the isolated skin epithelium. *a*) Na^+-Ringer's in the outside. Broken arrow indicates change of the inside medium from a Na^+-rich to a Na^+-free Ringer's. *b*) First arrow indicates change of the outside medium from a Na^+-Ringer's to a Li^+-Ringer's. Second arrow indicates change of the inside medium from a Na^+-rich to a Na^+-free medium
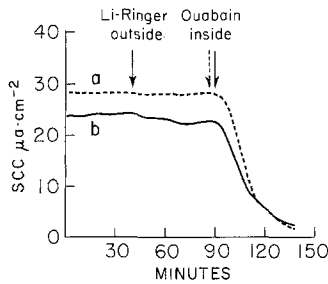

Fig. 3. Effects of ouabain (10^{-4}M) on the SCC of the isolated skin epithelium. *a*) Na^+-Ringer's in both sides. Broken arrow indicated addition of ouabain. *b*) Na^+-Ringer's in both sides. First arrow indicates change of the outside medium to a Li^+-Ringer's. Second arrow indicates addition of ouabain

the resistance as would be expected by an inhibition of the pump or cell shrinkage. However, the dilution of the Ringer's produced an additional and considerable increase in resistance in all cases. This is difficult to reconcile with Ussing's suggestion that dilution produces an exact reversal of the K^+-free effect. Our results support the view that the removal of K^+ has an intrinsic effect on the pump.

Another series of experiments was done in which the Na^+-Ringer's bathing the inside surface was replaced by a Na^+-free choline$^+$-Ringer's with the same K^+ concentration. The solution on the outside was either Li^+-Ringer's or Na^+-Ringer's. In both cases, the removal of Na^+ from the inside solution produced a sharp decline of the SCC (Fig. 2*a, b*). Rabito *et al.* also found that the SCC is a function of the Na^+ concentration in the inside bathing medium [20]. These results show that

the presence of both Na$^+$ and K$^+$ in the inside solution is necessary to maintain a stable SCC regardless of whether Na$^+$ or Li$^+$ is producing the SCC. In a few experiments, ouabain (10^{-4}M) was added to the inside medium (Na$^+$-Ringer's) while the outside medium was either Na$^+$ or Li$^+$ Ringer's. In both cases, ouabain produced an immediate decline of the SCC (Fig. 3 *a*, *b*).

Unidirectional Li$^+$ Fluxes and Short-Circuit Current. Li$^+$-Originated Short-Circuit Current in the Absence of Li$^+$ Gradient

It has been previously shown [9] that a Li$^+$ influx essentially equal to the SCC can be maintained for several hours across the isolated frog skin provided that the inside bathing solution contained Na$^+$. However, the Li$^+$ flux was down its chemical gradient and, although it was affected by ouabain and oxytocin (in the same fashion as the active Na$^+$ transport), there was not conclusive proof that metabolic energy was utilized for its transport. In this study, we measured both unidirectional Li$^+$ fluxes in equivalent conditions. For measuring the Li$^+$ influx, solution #5 (Table 1) was used on the outside and solution #9 on the inside of the skin. For measuring the Li$^+$ outflux, solution #11 was on the inside and solution #7 on the outside. In this manner, the Na$^+$ concentration was, in both cases, 82.4 mM on the inside (required for preserving the transport properties of the epithelium) and near zero on the outside. At the same time, the Li$^+$ gradient was 20.6/0 mM in the direction of the Li$^+$ flux determination. Thus, the Li$^+$ gradient was symmetrical although the solutions were not identical. Results from 19 experiments are shown in Table 4.

The Li$^+$ influx, 0.44 µequiv/hr cm^2, is 6 times larger than the outflux and their mean difference 0.35 µequiv/hr cm^2, is equal to 93% of the SCC measured in the Li$^+$ influx experiments. The SCC in the Li$^+$ outflux experiments is Na-originated and only shows that the transport system was functioning during the Li$^+$ outflux experiments.

Table 4 also shows that amiloride reduces the transepithelial Li influx and outflux to about 0.03 µequiv/hr cm^2. Unlike the SCC, the Li$^+$ influx does not reach this low value immediately. This delay is probably due to the time required for the Li$^+$ present in the cells to leak out to the inside solution. Fig. 4 shows one experiment of this kind.

In some of these experiments, after the last amiloride period, ^{22}Na was added to the inside medium and the Na$^+$ outflux was determined.

Table 4. Unidirectional Li fluxes and short-circuit current across isolated skin epithelium. Effect of amiloride 10^{-4} M

Successive 30-min periods	Li influx	SCC[a]	Li outflux	SCC[b]
	(μequiv/hr cm^2; $n=15$)		(μequiv/hr cm^2; $n=15$)	
Control-1	0.45 ± 0.03	0.41 ± 0.03	0.07 ± 0.01	0.82 ± 0.13
Control-2	0.44 ± 0.03	0.38 ± 0.03	0.07 ± 0.01	0.84 ± 0.14
Control-3	0.39 ± 0.02	0.35 ± 0.03	0.08 ± 0.02	0.81 ± 0.15
Amiloride-4	0.24 ± 0.03	0.01 ± 0.01	0.02 ± 0.01	0.04 ± 0.02
Amiloride-5	0.08 ± 0.02	0.00	0.01 ± 0.01	0.00
Amiloride-6	0.05 ± 0.01	0.00	0.03 ± 0.02	0.00
Amiloride-7	0.03 ± 0.01	0.00		

[a] The SCC indicated was obtained from the same experiments in which the Li^+ influx was measured. The SCC represents Li^+ movement. Na^+ concentration in the outside solution was less than 0.2 mM.
[b] The SCC indicated was obtained from the epithelia in which the Li^+ outflux was measured. In these experiments, the SCC represented Na^+ transport.
Each value is the mean $\pm$ SE of corresponding periods from number of experiments (n). In 6 experiments, the Na^+ outflux was determined with ^{22}Na after the amiloride-7 period and a value of 0.053 ± 0.009 μequiv/hr cm^2 was found.
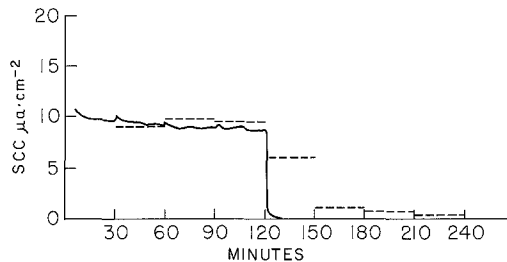Amiloride was added at the end of control-3 period.

Fig. 4. Continuous recording of short-circuit current (solid line) and average Li^+ influx for seven 30-min periods (dashed line) across isolated frog skin epithelium. Outside bathing medium was solution #5. Inside bathing medium was solution #9. Samples for Li^+ determination were taken every 30 min. Amiloride (10^{-4} M) was added at the 120-min mark

In all cases, the Na^+ outflux was between 0.02 and 0.08 μequiv/hr cm^2 (less than 8% of Na influx) indicating the tightness of the epithelium and absence of appreciable edge damage. This type of experiment was also done using the intact abdominal skin of *Rana pipiens* and the results were the same.

In addition to these experiments, six more were done in which the epithelium was bathed on the outside with solution #5 and on the inside with solution #11, so that the Li^+ concentration was the same on both sides (20.6 mM). Na concentration in the outside was less than 0.2 mM.
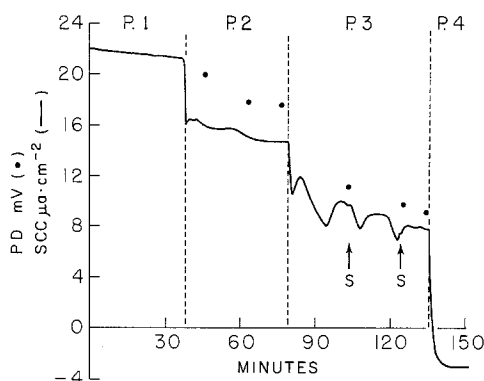
Fig. 5. SCC of the isolated frog skin epithelium. *Phase 1:* NaCl-Ringer's on both sides. *Phase 2:* Inside bathing medium was solution #10 and outside bathing medium was solution #6. SCC current decreases due to the lowering of Na$^+$ concentration. *Phase 3:* Inside bathing medium is the same as in Phase 2 and outside bathing medium was solution #4. A further decrease in SCC is observed. This smaller SCC is, however, stable for more than 2 hr while the Li$^+$ concentration was 10.3 mM in both sides. *S* indicates sampling. Na$^+$ concentration in the outside was <0.22 mM. *Phase 4:* Inside solution is the same as in Phases 2 and 3 and the outside bathing medium was a choline Cl-Ringer's (solution #3). SCC reverses due to the Na$^+$ gradient from the inside. A Na$^+$ gradient was also present during Phases 2 and 3

The SCC in these experiments was 9.6 ± 1.0 µA/cm^2 which is similar to the net movement of Li$^+$ determined by unidirectional fluxes. After a period of 1 to 3 hr, the Ringer's on the inside was replaced by solution #9 containing no Li$^+$. After this change, the SCC did not change and the Li$^+$ influx measured was within 10% of the SCC. This result suggests that the Li$^+$ influx and the SCC originated by this flux is rather independent of a change in Li$^+$ concentration in the inside solution between 0 and 20 mM.

The existence of a stable SCC in the absence of an electrochemical gradient for Li$^+$ (in conditions of negligible Na$^+$ transport) was also observed with a Li$^+$ concentration of 10.3 mM on both sides of the epithelium.

One such experiment, out of a total of 12, is shown in Fig. 5. They were divided into four phases. In the first phase, the epithelium was bathed with normal Na-Ringer's on both sides (Na$^+$ concentration 103 mM). An average stable p.d. and SCC of 89 mV and 22.7 ± 1.9 µA/cm^2 were obtained. In the second phase, the bathing solutions were #10 on the inside and #6 on the outside. Stable p.d. and SCC during this phase were, respectively, 62 mV and 13.3 ± 1.2 µA/cm^2. In the third phase, the outside solution was replaced by solution #4. During this phase, the

Li^+ concentration was the same (10.3 mM) on both sides of the epithelium. Nevertheless, a SCC was measured for 3 hr or longer. This SCC was only $6.2. \pm 0.4$ μA/cm^2 compared to the Na^+ current of 13.3 μA/cm^2 in the second phase. Paired comparison of SCC between second and third phases showed a statistically significant decrease ($p < 0.001$) of 7.1 μA/cm^2. Periodic washouts of the outside solution were made during this third phase and samples showed Na^+ concentrations of 0.22 μequiv/ml or less. The SCC in the third phase could either be inhibited by ouabain or stimulated by oxytocin. Other experiments were continued into a fourth phase. During this phase, the outside solution was choline-Ringer's (Na^+- and Li^+-free). Samples of the outside solution showed a Na^+ concentration of 0.21 μequiv/ml or less. The p.d. and SCC immediately reversed due to the opposing Na^+ gradient. This suggests that the SCC in the third phase was a minimum estimate of Li^+ transport. In most experiments, periodic oscillations of the type described by Finkelstein [14] (with a period of about 8 min were observed during the third phase). These experiments show that Li^+ (at 10.3 mM concentration) is transported in the absence of a Li^+ gradient and further suggests a direct or indirect coupling to an active mechanism.

Lithium Flux Against a Chemical Gradient and Unidirectional Na$^+$ Influx

One possible type of coupling for Li^+ movement is by a mechanism of co- or counter-transport to the movement of Na^+. With a very low Na^+ concentration in the outside solution the Na^+ outflux probably exceeds the influx and the net Na^+ flux is opposite to the net Li^+ flux. Thus, the possibility exists that the Li^+ influx is driven by the Na^+ gradient in a counter-transport fashion. With a larger Na^+ concentration in the outside solution (about 2 mM) the net Na^+ movement, although small, is in the direction of the net Li^+ movement. In this case, the Li^+ influx may be coupled to the Na^+ movement in a co-transport fashion. It is clear that there must be a Na^+ concentration in the outside solution at which the net transepithelial Na^+ movement changes direction. This concentration is undoubtedly between 0.1 and 2 mM. Thus, it was of interest to study the Li^+ influx while the outside Na^+ concentration was maintained within this range. This was done in the following manner. The epithelium was mounted in the chamber and the outside face was bathed with solution #5, while the inside face was bathed with solution #8. The p.d. was short-circuited. After stabilization of the SCC a known
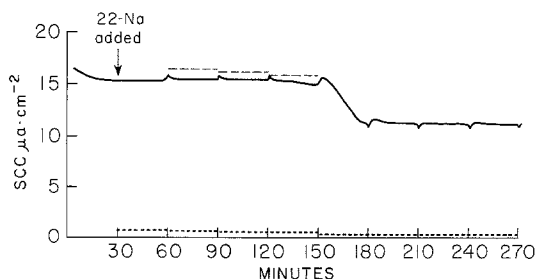
Fig. 6. Continuous recording of short-circuit current (solid line), average Li$^+$ influx (dashed line) and average Na$^+$ influx (dotted line) across isolated frog skin epithelium. Samples for measurement of ^{22}Na activity and determination of Li$^+$ concentration (when possible) were taken every 30 min. The outside bathing medium was, for the entire length of the experiment, solution #5 with a small amount of Na (0.1 mM) and ^{22}Na label added. The inside bathing medium was: before the 150-min mark solution #9, and after the 120-min mark solution #12

amount of NaCl was added to the outside medium so that the Na$^+$ concentration could be adjusted to values varying (in different experiments) between 0.1 and 1.4 mM. This produced no change or a small (up to 5%) increase in the SCC. Following this, ^{22}Na was added to the outside solution. Afterward, samples were taken from the inside solution every 30 min for 2 hr so that the Li$^+$ and Na$^+$ fluxes were determined simultaneously with the SCC (Fig. 6). The Li$^+$ influx closely matches the SCC while the Na$^+$ influx is only a small fraction of it. In a second phase of these experiments the Ringer's in the inside face was quickly replaced by solution #12. After this change, the inside Li$^+$ concentration was 34.3 mM. Li$^+$ influx could no longer be measured but SCC and Na$^+$ influx were measured for four more periods. The SCC decreased to a new stable value and the Na$^+$ influx remained very low. These results are shown in Table 5.

The most feasible interpretation for the discrepancy between the SCC and Na$^+$ influx is to assume that the Li$^+$ influx continued at a level similar to the SCC and could account for it. This Li$^+$ transport was occurring against a chemical gradient (20.6/34.3). The Li$^+$ and Na$^+$ outflux were not measured in these experiments but they have been measured in other experiments and represented no more than 0.09 µequiv/hr cm^2 for a wide range of conditions including the one described here. The Li$^+$ transport we observe has no relation to variations in outside Na$^+$ concentration from near zero to 1.42 mM. Also, the Na$^+$ movement in either direction is very small compared to the net Li$^+$ transport. Thus, a simple coupling of Li$^+$ transport to Na$^+$ movement (either co- or counter-transport) cannot explain these results.

Table 5. Li Influx, short-circuit current, and Na influx across isolated skin epithelium down and against a Li concentration gradient

Experi-ment	Na (mM)		Li (mM)		Li influx[a]	Na influx[a]	SCC[a]
	out	in	in	out	(μequiv/hr cm^2)		
1L	1.30	82.4	0	20.6	0.68	0.08	0.69
1L	1.30	68.7	34.3	20.6	—	0.06	0.45
2L	0.10	82.4	0	20.6	0.73	0.005	0.65
2L	0.10	68.7	34.3	20.6	—	0.003	0.36
3L	0.40	82.4	0	20.6	0.59	0.021	0.56
3L	0.40	68.7	34.3	20.6	—	0.015	0.32
4L	1.42	82.4	0	20.6	0.90	0.075	0.62
4L	1.42	68.7	34.3	20.6	—	0.045	0.46
5L	0.44	82.4	0	20.6	0.63	0.025	0.65
5L		68.7	34.3	20.6	—	0.014	0.41

[a] Each number in these columns is an average from 3 to 4 periods. Additional description in the text.

Discussion

It is generally accepted that the energetic requirements of Na$^+$ transport across frog skin epithelium are provided by the (Na$^+$ + K$^+$)-ATPase system. The main evidence for this is that ouabain, a potent inhibitor of the Na$^+$ pump and of (Na$^+$ + K$^+$)-ATPase in other tissues, also inhibits transepithelial Na$^+$ transport across frog skin. Since Li$^+$ does not activate the frog skin epithelium (Na$^+$ + K$^+$)-ATPase [24], it is clear that the observed transepithelial Li$^+$ transport cannot be explained by a direct Li$^+$ activation (replacing Na$^+$), of the (Na$^+$ + K$^+$)-ATPase. Nevertheless, the (Na$^+$ + K$^+$)-ATPase system seems to be related to the Li$^+$ transport. The main support for this argument is that Li$^+$ transport is inhibited by ouabain and by Na$^+$- or K$^+$-free Ringer's. Candia and Chiarandini [9] have measured the Li$^+$ flux before and after ouabain inhibition and found a good correlation with the SCC. The Li$^+$-originated SCC can also be stimulated with oxytocin and depressed by amiloride. These results suggest that the transepithelial Li$^+$ flux from the outside to the inside follows the same pathway as the active Na$^+$ transport flux.

The results in Tables 4 and 5 are conclusive in showing that an equivalent Li$^+$ gradient (20 mM) produces an influx that is six times larger than the outflux. The difference between these two flux values corresponds to the measured SCC. Therefore, either without or against a Li$^+$ gradient,

the SCC can only be explained by a net inward Li$^+$ transport. The Li-originated SCC does not change when the Li$^+$ concentration in the inside medium is raised from 0 to 20.6 mM, but it did decline when the concentration was raised to 34.3 mM. However, the Na$^+$ concentration was reduced during the latter change and this may be the cause of the decline.

Leblanc [18] reported that in epithelia bathed with Na$^+$-Ringer's on the inside and Na$^+$-free, Li$^+$-Ringer's on the outside a SCC proportional to the Li$^+$ concentration was measured. He also found that the so-called intracellular Li$^+$ accumulation has a saturation level of about 30 mM. This saturation is attained with an outside Li$^+$ concentration of 10 mM and remains unaltered upon increasing the outside Li$^+$ concentration. This saturation curve is similar to that reported for Na$^+$ [12]. The intracellular Na$^+$ concentration in skin epithelium is also in the range of 18–35 mM [1, 18, 26]. Leblanc [18] concluded that the permeability of the outer barrier might be controlled by cell metabolism. Zerahn [26] found that dinitrophenol reduced the epithelial Na$^+$ content while ouabain increased it. Both Zerahn and Leblanc proposed that an active transport step may exist at the level of the outer barrier. Moreno *et al.* [19] have found that ouabain produces a considerable inhibition (comparable to that produced by amiloride) of the Na$^+$ uptake at the outer barrier. Their conclusion was that either the Na$^+$ pool does not exist, ouabain acts directly on an active step at the outer barrier, or the Na$^+$ permeability of the outer barrier is controlled by the epithelial Na$^+$ concentration. We have found that the Na$^+$ content of epithelium incubated in Na-free medium is very low (0.007 µequiv/cm^2). On the other hand, ouabain increased the intracellular Na$^+$ concentration in both the isolated skin epithelium [1, 23] and isolated cells from the skin epithelium [27]. Although these two experimental conditions have opposite effects on the epithelial Na$^+$ content, an inhibition of the Na$^+$ and Li$^+$ transport is observed in both cases. Thus, the intracellular Na$^+$ concentration cannot be the regulator of the permeability of the outer barrier.

The transepithelial Li$^+$ transport is dependent on the presence of Na$^+$ and derives energy from the (Na$^+$ + K$^+$)-ATPase system without activating the enzyme. However, a simple counter-transport between the Na$^+$ outflux (due to the Na$^+$ gradient) and the uphill Li$^+$ movement is unlikely. This is supported by the fact that ouabain considerably reduces Li$^+$ influx without having a significant effect on the Na$^+$ outflux. Thus, the type of coupling remains obscure.

As a working hypothesis a qualitative interpretation of these results follows. A schematic representation of frog skin epithelium is shown in
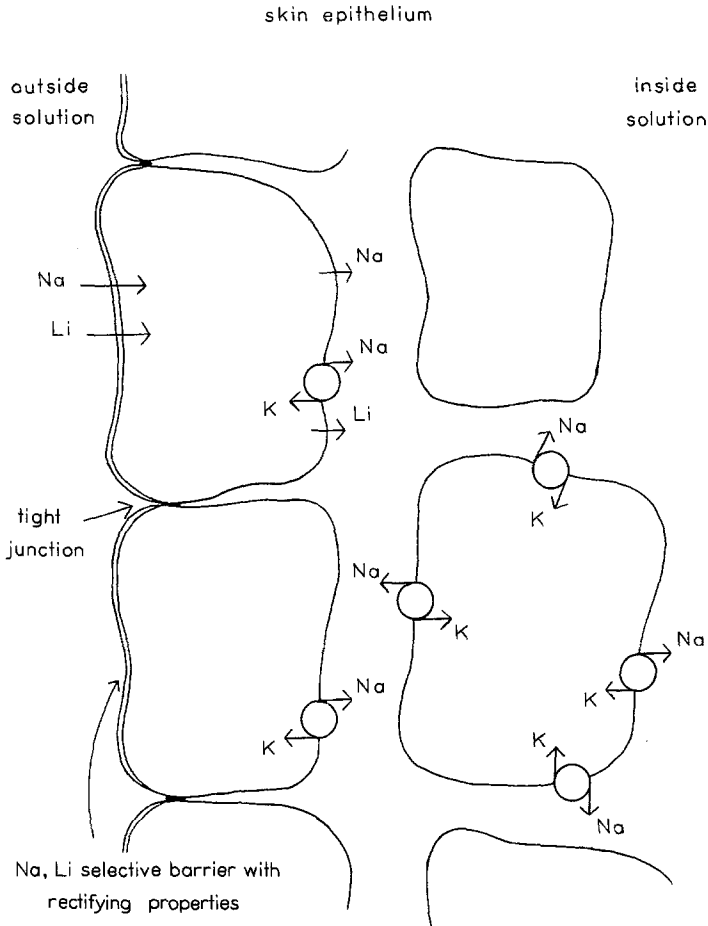
skin epithelium

Fig. 7. Schematic diagram of the frog skin epithelium to describe hypothetical mechanisms of Na^+ and Li^+ transport—discussed in the text. Arrow ($\rightarrow$) indicates diffusion due to electrochemical gradient. Circle with two arrows represents a Na:K pump

Fig. 7. The outermost active cell layer acts as a selective permeability barrier to Na^+ and Li^+ [4] and also possesses diode-type rectifying properties for these cations [7, 9]. This barrier seems to be the rate-limiting step of the transepithelial Na^+ flux [3, 10, 13], and is affected by amiloride and oxytocin. When properly electrically biased as a result of metabolic activity of Na^+:K^+ pumps present elsewhere in the epithelium, this barrier will allow Na^+ and Li^+ from the outside solution to passively enter the outermost cell layer driven by the electrical gradient although against a concentration gradient. The leak of Na^+ or Li^+ from this layer back to the outside solution is negligible [10]. The paths of Na^+ and Li^+ movement from this point to the inside solution may be different. Na^+

may either diffuse or be pumped into the intercellular spaces by the Na$^+$:K$^+$ pump. Since the Na$^+$ flux is larger than the K$^+$ uptake across the inner barrier [11], it is conceivable that a fraction of the Na flux proceeds by diffusion. If Na$^+$:K$^+$ pumps are symmetrically distributed in the membrane of other cell layers, they cannot participate in the transepithelial transport unless they also possess asymmetrical characteristics as the first active layer. Ouabain will affect the Na$^+$:K$^+$ pumps and the electrical bias across the "rectifying barrier" and as a consequence of this, Na$^+$ entry from outside will decrease. However, intracellular Na$^+$ concentration will increase due to leakage from the inside solution. Operationally, the Na$^+$ transport pool will be the Na$^+$ content of the cell layer or layers with asymmetrical characteristics. The intracellular Na$^+$ is then expected to be larger than the Na$^+$ transport pool. Na$^+$ pumped into the intracellular spaces by the first active layer could reach the inside solution either by diffusing into additional deeper cells and ultimately be pumped by these cells, or simply by diffusing along the intercellular spaces. Li$^+$, on the other hand, cannot be pumped by the Na$^+$:K$^+$ pumps, so it must proceed by diffusion. This requires that the Li$^+$ concentration in the active layer be larger than in the intercellular spaces. This will set limitations on the conditions under which a net Li$^+$ transport can occur. Low Na$^+$ concentration in the intracellular compartment will stop the Na$^+$:K$^+$ pump and the entrance of Li$^+$ across the outer barrier. Ouabain will produce a similar effect. With 10–20 mM Li$^+$ concentration in both the inside and outside solutions (and presumably in the intercellular spaces) the concentration of Li$^+$ in the active cell layer (30 mM or more) is larger than in the intercellular spaces and a net transport of Li$^+$ can be observed in the absence of an electrochemical gradient. Even though Li$^+$ may not be translocated by a "pump" its movement depends upon metabolic energy, related to the Na$^+$:K$^+$ pump. The energy required for the net Li$^+$ movement is derived from the electrical gradient created across the outer barrier by the Na:K pump.

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