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Coupled Transepithelial Sodium and Potassium Transport across Isolated Frog Skin: Effect of Ouabain, Amiloride and the Polyene Antibiotic Filipin

Robert Nielsen

Institute of Biological Chemistry A, 13 Universitetsparken,
DK 2100 Copenhagen Ø, Denmark

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Summary. Addition of the polyene antibiotic filipin (50 μM) to the outside bathing solution (OBS) of the isolated frog skin resulted in a highly significant active outward transport of K^+ because filipin *per se* increases the nonspecific Na^+ and K^+ permeability of the outward facing membrane. The K^+ transport was calculated from the chemically determined changes in K^+ concentrations in the solution bathing the two sides of the skin. The active transepithelial K^+ transport required the presence of Na^+ in the OBS, but not in the inside bathing solution (IBS), and it was inhibited by the Na^+, K^+-ATPase inhibitor ouabain. The addition of Ba^{++} to the IBS in the presence of filipin in the OBS resulted in an activation of the transepithelial K^+ transport and in an inhibition of the active Na^+ transport. This is in agreement with the notion that Ba^{++} decreases the passive K^+ permeability of the inward facing membrane. In the presence of amiloride (which blocks the specific Na permeability of the outward facing membrane) and Ba^{++} there was a good correlation between the active Na^+ and K^+ transport. It is concluded that the active transepithelial K^+ transport is carried out by a coupled electrogenic Na–K pump, and it is suggested that the pump ratio (Na/K) is 1.5.

In symmetrical cells (such as erythrocytes, muscle, and nerve) it is generally accepted that the low intracellular Na^+ concentration and the high intracellular K^+ concentration are maintained by a coupled electrogenic $Na^+ - K^+$ pump (Thomas, 1972).

The high intracellular K^+ concentration in and the active transcellular Na^+ transport across polar cells in epithelia are, according to the two membrane hypothesis, accomplished by the same mechanism, namely, a coupled, ouabain-sensitive, Na – K pump located at the inward-facing membrane (Koefoed-Johnsen & Ussing, 1958). Only indirect evidence has supported this idea. The involvement of K^+ in transepithelial Na^+ transport by frog skin was demonstrated by Huf and Wills, 1951. They showed that Na^+ transport was drastically reduced when K^+-

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free bathing solutions were used. Koefoed-Johnsen, 1957, has shown that the addition of ouabain to the isolated frog skin resulted in an inhibition of the Na^+ transport, and Cala, Cogswell and Mandel, 1978, have shown that there is a linear correlation between the number of ouabain molecules bound to the isolated epithelium and the corresponding inhibition of Na^+ transport.

The effect of ouabain on Na^+ transport and K^+ accumulation has been investigated in frog skin, toad- and rabbit urinary bladder. From studies on toad bladder and frog skin, DeLong and Civan (1978) and Valenzeno and Hoshiko (1977) suggest that there is no coupling between the cellular K^+ accumulation and net Na^+ transport, whereas Lewis, Wills and Eaton (1978) from studies on rabbit bladder suggest that a $Na^+ - K^+$ exchange mechanism is responsible for the high cellular K^+-concentration.

If a coupled $Na^+ - K^+$ pump is responsible for the trans-epithelial Na^+ transport, one might expect to observe a correlation between the rate of K^+ uptake from the inner medium into the cells, and the rate of Na^+ extrusion in the opposite direction. Many attempts have been made to measure K^+ fluxes (measured as $^{42}K^+$ uptake from the inside bathing solution) and to establish correlation with transepithelial Na^+ transport. Although different methods have been developed for measuring $^{42}K^+$ influx into the epithelial cells of frog skin, toad bladder, and other tissue, only a few of the published data are supportive (Biber, Aceves & Mandel, 1972; Finn & Nellans, 1972), whereas many experimental results suggest that no fixed coupling exists between K^+ uptake and Na^+ extrusion (Essig & Leaf, 1963; Curran & Cereijido, 1965; Frazier & Vanatta, 1971; Biber *et al.*, 1972; Candia & Zadunaisky, 1972; Giebisch, Sullivan & Whittenbury, 1973; Robinson & Macknight, 1976; Nellans & Schultz, 1976).

The isolated epithelium (from frog skin) consists of four to six layers, therefore $^{42}K^+$ ions have to diffuse through a narrow interspace system before they reach all the cells. When K^+ diffuses through a narrow interspace system the $^{42}K^+$ in the interspace is not in equilibrium with $^{42}K^+$ in the bathing solution (Harris & Burn, 1949). This lack of isotope equilibration results in an unknown degree of recycling of the ions (K^+, $^{42}K^+$). Such a recycling of the ions could completely mask the presence of a small K^+ pool with a high turnover rate, and consequently $^{42}K^+$ influx measurements on isolated epithelium and tissue slices may be difficult to interpret. Because of these complications, it was attempted to measure the net K^+ flux without using $K^+ - {}^{42}K^+$ exchange.

It has been shown that the addition of polyene antibiotics to the outside bathing solution resulted in an active outward K^+ transport in frog skin (Nielsen, 1971; Nielsen, 1972; Bakhteeva & Natochin, 1975), and in rabbit colon (Frizzell & Turnheim, 1978). It was therefore investigated whether this active outwards K^+-transport was coupled to the inward transepithelial Na^+-transport. The data presented here show that after the addition of the polyene antibiotic filipin to the outside bathing solution there is a substantial outward transport of K^+ (measured as the change in the chemical amount of K^+ in the bathing solutions). This K^+ transport is inhibited by amiloride and ouabain, and it requires the presence of Na^+ in the outside bathing solution, but not in the inside bathing solution.

Materials and Methods

The experiments were performed on male and female frogs *(Rana temporaria)*. The frogs were kept partially immersed in tap water at about 4 °C. The skin was dissected and divided into two symmetrical halves, one used as a control and the other for the experiment. The skins were mounted in perspex chambers (area 7 cm^2, volume 5 ml), and bathed in stirred Ringer's solution. Na-Ringer's solution was composed of (in mM): Na^+, 115.0; K^+, 2.5; Ca^{++}, 1.0; HCO_3^-, 2.5; and Cl^-, 117; pH = 8.2; in Tris and choline Ringer's solution all Na^+ was replaced either with Tris or choline. The K^+ content of solutions and tissues was determined with an atomic absorption spectrometer. To determine the K^+ content in the tissue, the skin was cut out of the chamber and blotted with filter paper. The skin was weighed and then extracted for at least 12 hr with 5 ml 0.1 M HNO_3.

The short-circuit experiments were performed according to the method of Ussing and Zerahn (1951), using an automatic voltage clamp apparatus, in which a slave potentiometer coupled to the recorder compensated for the current dependent potential drop between the potential bridges (SCC · R); SCC is the short-circuit current, and R is the resistance of the Ringer's solution between the bridges.

The filipin Lot u-5956 was a gift from the Upjohn Company, Kalamazoo, Mich.

Symbols

P_{out}^K: K^+ permeability of the outward facing membrane.
P_{in}^K: K^+ permeability of the inward facing membrane.
ISCC: integrated short-circuit current.
Na_{net}: net amount of Na^+ transported across the skin during the incubation period.
K_{net}: net amount of K^+ transported across the skin during the incubation period.
ΔK_i: the measured decrease in the total K^+ content of the IBS.
ΔK_o: the measured increase in the total K^+ content of the OBS.
K_i^{loss}: the net K^+ loss from the skin to the IBS.
K_o^{loss}: the net loss from the skin to the OBS.
K^{back}: the net K back transport. At the start of the experiment K^{back} is zero, but during the incubation a K^+ concentration gradient is built up across the skin; it is this concentration gradient which drives the net K back transport.

Results

Previous experiments have shown that addition of filipin to the outside bathing solution (OBS) of the isolated frog skin increases the nonspecific permeability of the outward facing membrane (Nielsen, 1977). To investigate whether the addition of filipin resulted in an active outward K^+ transport across the frog skin, two symmetrical skin halves were incubated under short circuited conditions, one in the presence of filipin and the other without filipin (the control). After two hours of incubation, the Ringer solutions were removed and replaced by new solutions and the incubations were continued. The K^+ concentration of the Ringer solution added was 2.68 ± 0.10 mM. Table 1*a–b* shows the measured K^+ concentration of the Ringer solution after the incubation.

After incubation of the skin under control conditions, the K^+ concentration of the bathing solutions were found to be slightly greater than at the start of the incubation (Table 1*a–b*). This increase in the K^+ content of the solutions must be due to a loss of K^+ from the control skin half. In the presence of filipin, the K^+ content of the inside bathing solution was found to have decreased during incubation, while the K^+ content of the outside had increased (Table 1). The changes were found to be larger during the second incubation period.

Thus, during incubation with filipin there was a significant movement of K^+ from the inside to the outside bathing solution. Since the skin halves were short-circuited during the experiment and the solutions added to both sides were identical, this (transport of K^+) must be due to an active K^+ transport mechanism in the skin.

The measured decrease in the K^+ content of the inside bathing solution (IBS) during the entire incubation was 3.68 μeq, and the measured increase in the K^+ content of the outside bathing solution was 5.71 μeq (Table 1*e*). The discrepancy between these two figures is due to a loss of (2.03 μeq) potassium from the skin. When the amounts of K^+ lost from the skins are added to the amounts found in the skin after incubation, it appears that the two skin halves contained identical amounts of K^+.

It has been shown that the addition of amphotericin B to the outside bathing solution enhanced the $^{42}K^+$ outflux more than the $^{42}K^+$ influx (Nielsen, 1971). Therefore, the effects of filipin and amphotericin B on the active outward K^+ transport were compared. From Table 2 it appears that filipin is a much more potent inducer of active outward K^+ transport than amphotericin B.

Table 1. Effect of 5×10^{-5} M filipin on the K^+ content of the Ringer solutions bathing the inside and the outside of the isolated frog skin

	Time (hr)	Unit	Control			5×10^{-5} M filipin		
			Inside	Outside	Δ loss	Inside	Outside	Δ loss
(a)	0–2	mM	2.78±0.17	2.72±0.08		2.50±0.13	3.07±0.20	
(b)	2–4	mM	2.77±0.14	2.75±0.09		2.10±0.13	3.50±0.14	
(c)	0–2	μeq	0.24±0.28	0.21±0.12	0.45	−0.83±0.15	1.86±0.28	1.03
(d)	2–4	μeq	0.45±0.15	0.15±0.16	0.60	−2.85±0.14	3.85±0.24	1.00
(e)	0–4	μeq	0.69	0.36		−3.68	5.71	
(f)	Wet wt	mg	262±28			253±26		
(g)	Potassium content	μeq	8.39±0.75			7.43±0.45		
(h)	Potassium content + loss	μeq	(8.39+0.45+0.60)=9.44			(7.43+1.03+1.00)=9.46		

The filipin was added to the outside bathing solution. Values are the means±SE of six experiments. Lines *a* and *b* show the K^+ concentrations of the Ringer solutions after the incubation. Lines *c*, *d*, and *e* show the change in the K^+ content of the inside and outside bathing solutions. Line *f* shows the wet wt of the skin halves after the incubation. Line *g* shows the K^+ content of the skin halves after the incubation. Line *h* shows the total K^+ content of the skin halves computed from the K^+ content of the skin halves and the K^+ loss. The K^+ loss from the skin halves (Δ loss) is calculated from the change in the K^+ content in the bathing solutions (Δ outside + Δ inside).

Table 2. Effect of 5×10^{-5} M filipin and 5×10^{-5} M amphotericin B on the change in the K^+ content of the Ringer solution bathing the inside and outside of the isolated frog skin

Unit	Amphotericin B		Filipin	
	Δ inside	Δ outside	Δ inside	Δ outside
μeq	−0.32±0.49	0.79±0.52	−2.25±0.45	4.16±0.47

The experiments were performed on short-circuit symmetrical skin halves. The skin halves were preincubated 1 hr with filipin or amphotericin B in the outside bathing solution before the experiment was started. The skin halves were then incubated for 2 hr. Δ inside and Δ outside are the change in the K^+ content of the bathing solutions.

Effect of Na on the K Transport

To investigate whether the active transepithelial K^+ transport required the presence of Na^+ in the OBS, experiments were performed where all Na^+ in the OBS was replaced by choline. The change from Na^+ to choline in the OBS resulted in a nearly complete inhibition of

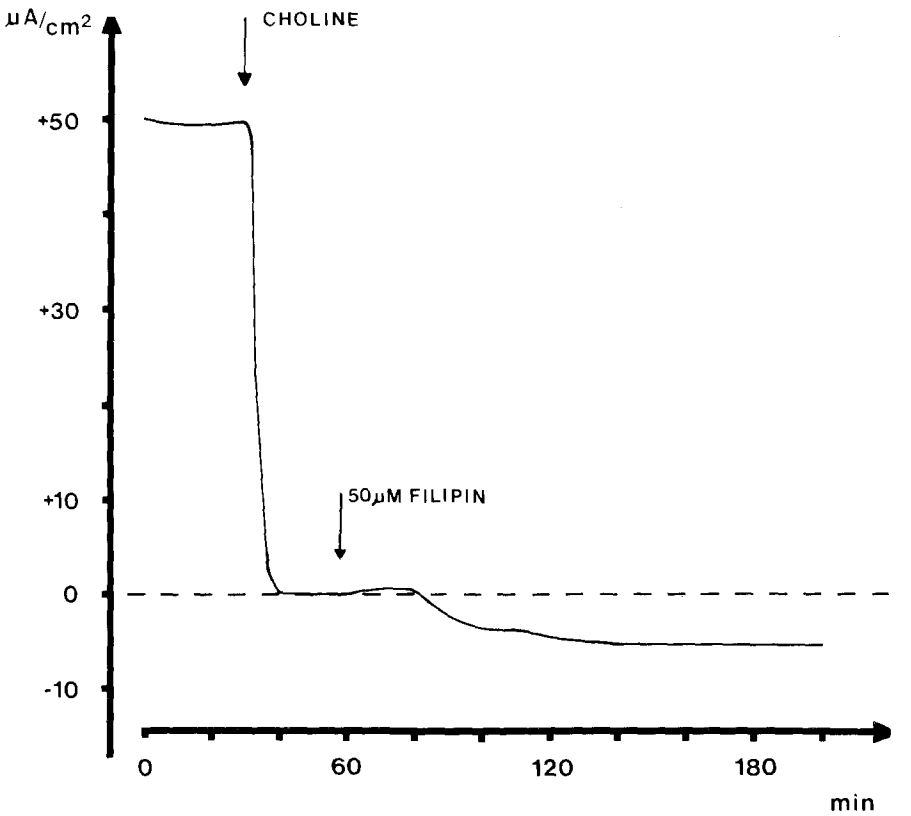

Fig. 1. At the arrow *choline*, the OBS were changed from Na Ringer's solution to choline Ringer's solution. At the arrow *filipin*, filipin was added to the OBS to give a concentration of 50 μM. (——), short-circuit current (μA/cm²)

the SCC. The subsequent addition of filipin resulted (in 7 out of 12 expts.) in the onset of a small reversed SCC (Fig. 1). Addition of filipin to the OBS increases the nonspecific transepithelial permeabilities (Nielsen, 1977). Therefore the reversed SCC is probably driven by a bi-ionic potential created by the oppositely directed choline and Na^+ gradients across the skin. Thus the reverse SCC is equal to the Na^+ outflux minus the choline influx. The replacement of Na^+ with choline in the OBS resulted also in a complete inhibition of the net K^+ transport across the skins and in an increased loss of K^+ from the skins to the bathing solutions (Table 3). Although the substitution of Na^+ in the OBS with choline reduces the net movement of K^+ from the IBS to the OBS to zero, the possibility exists that the removal of Na^+ from the OBS might result in a loss of K^+ from the skins to the IBS; a part of this K^+ could thereafter be transported to the OBS by the K^+-pump. Therefore, the

Table 3. Effect of the absence of Na^+ in the outside bathing solution on the active K^+ transport

Time (min)	n	Control + Na			Exp − Na		
		Δ inside (μeq)	Δ outside (μeq)	Δ loss (μeq)	Δ inside (μeq)	Δ outside (μeq)	Δ loss (μeq)
0–90	6	−1.32±0.28	1.84±0.34	0.52	0.23±0.20	0.72±0.16	0.95
90–180	4	−1.03±0.14	1.31±0.52	0.28	0.29±0.19	0.45±0.20	0.74

Filipin (50 μM) was added to the outside bathing solution of both skin halves. The control skin half was incubated with Na-Ringer in both bathing solutions. The exp. skin half was incubated with choline-Ringer's solution on the outside with Na-Ringer's solution on the inside. Δ inside and Δ outside are the change in K^+ content of the inside and outside bathing solution. Δ loss is calculated from (Δ outside + Δ inside).

possibility exists that a small active transpithelial K^+ transport could be masked by the loss of K^+ from the skin. To investigate this possibility, a series of experiments was performed, where the nonsteady-state $^{42}K^+$ in- and outflux were measured in symmetrical skin halves. The experiments were carried out under short circuiting, with the same K^+ concentration in the IBS and the OBS. The flux ratio equation is also valid for nonsteady-state fluxes provided the fluxes are measured in a period which is small compared with the rate of the permeability changes in the skin (Ussing, 1978). Even if the permeability of the system changes during the flux measurements, one can find the correct flux ratio at the time when the isotope was added, by extrapolation of the flux ratios to this time (Ussing, 1978). According to the flux ratio equation for non-steady-state fluxes, the flux ratio should be equal to 1 for an ion which moves passively (Ussing, 1978). The flux ratio found (Table 4) is not significantly different from 1; therefore the removal of Na^+ from the OBS resulted in an inhibition of the active transepithelial K^+ transport.

The replacement of Na^+ in the IBS with Tris or choline resulted in a 30–70 % inhibition of the SCC, and in an enhanced loss of K^+ from the skin to the bathing solutions (Table 5). The nonsteady-state $^{42}K^+$ flux ratio under these conditions was very significantly different from 1 (Table 6). Therefore, an active outward transport of K^+ took place, but when the K^+ transport was measured as changes in the K^+ concentrations of the bathing solutions (Table 5) the K^+ transport was masked by the loss of K^+ from the skins to the IBS. Thus the active outward transport of K^+ required the presence of Na^+ in the OBS, but not in the IBS.

Table 4. Flux ratio of $^{42}K^+$ in the absence of Na^+ in the outside bathing solution

Exp. no.	Min $^{42}K^+$ added after filipin	Time			
		0–30	30–60	60–90	90–120
		Flux ratio			
1	10	0.98	1.13	1.11	1.12
2	5	1.19	1.38	1.02	0.86
3	5	1.44	1.29	1.31	0.95
4	0	0.85	0.84	–	–
5	10	1.01	1.28	1.16	1.04
6	100	1.04	1.01	1.06	0.74

The $^{42}K^+$ influx and efflux were measured simultaneously on symmetrical skin halves. The skin halves were incubated with Na-Ringer's solution on the inside, and with choline Ringer's solution on the outside. Filipin (50 μM) was added to the outside. $^{42}K^+$ was added 0–100 min after the filipin. The first sample (time 0 in the table) was taken 2 min after the addition of $^{42}K^+$. The flux ratio is calculated from efflux/influx.

Table 5. Effect of the absence of Na^+ in the inside bathing solution on the net K^+ transport

Time (min)	n	Control + Na^+			Exp − Na^+		
		Δ inside (μeq)	Δ outside (μeq)	Δ loss (μeq)	Δ inside (μeq)	Δ outside (μeq)	Δ loss (μeq)
0–90	8	−0.32±0.14	0.87±0.20	0.55	2.13±0.58	0.96±0.10	3.09
90–180	8	−0.66±0.17	1.21±0.21	0.55	0.74±0.17	0.82±0.12	1.56

Filipin (50 μM) was added to the outside of both skin halves. The control skin half was incubated with Na-Ringer's solution on both the inside and the outside. The exp. skin half was incubated with Na^+-free (choline or Tris) Ringer's solution on the inside, and with Na-Ringer's solution on the outside. Δ inside and Δ outside are the changes in the K^+ content of the inside and the outside bathing solution. Δ loss is calculated from (Δ outside + Δ inside).

The observation that no active outward transport of K^+ could be detected in the absence of Na^+ in the OBS, indicates that the passive Na^+ permeability of the inward facing membrane of the K^+ transport compartment must be smaller than the Na^+ permeability of the outward facing membrane, otherwise one would expect that Na^+, which had moved from the IBS into the transport compartment, should activate the K^+ pump. This observation is in agreement with the notion that the inward-facing membrane of the transport compartment is impermeable for free Na^+ (Koefoed-Johnsen & Ussing, 1958).

Table 6. Flux ratio of $^{42}K^+$ in the absence of Na^+ in the inside bathing solution

Expt. no.	Inside	Min ^{42}K added after filipin	Time (min)			
			0–30	30–60	60–90	90–120
			Flux ratio			
1	Tris	25	5.42	5.35	4.95	3.71
2	Tris	5	2.57	2.51	2.14	1.57
3	Choline	0	5.69	3.41	1.94	1.68
4	Choline	5	15.29	12.41	10.71	6.79
5	Choline	30	5.89	7.91	4.89	4.13
6	Choline	10	7.08	22.79	–	–

The $^{42}K^+$ influx and efflux were measured simultaneously in symmetrical skin halves. The skin halves were incubated with Na-Ringer's solution on the outside and with Tris or choline Ringer's solution on the inside. Filipin (50 μM) was added to the outside. ^{42}K was added 0–30 min after the filipin. The first sample (time 0 in the table) was taken 2 min after the addition of ^{42}K. The flux ratio is calculated from efflux/influx.

In the absence of Na^+ in the IBS, there was a loss of 3.09 μeq K^+ from the skin to the bathing solutions (Table 5, 0–90 min); most of this K^+ was lost to the IBS. In the absence of Na^+ in the OBS there was no significant K^+ loss to the IBS, but a loss of 0.72 μeq to the OBS (Table 3, 0–90 min). This might indicate that the K^+ in the isolated frog skin is localized in two different compartments, one compartment which required the presence of Na^+ in the OBS, and another compartment which requires the presence of Na^+ in the IBS, in order to maintain a high concentration of K^+. However, another explanation of the observed K loss to the IBS in the presence of choline in the IBS could be that choline replaced K^+ in the $Na^+ - K^+$ pump or it might exchange with K^+ in the cells via passive "pathways" in the inward-facing membrane. Experiments are in progress to investigate why the substitution of Na^+ by choline in the IBS result in K^+ loss from the skin.

Effect of Ouabain

In the previous section it was shown that addition of filipin to the OBS resulted in an active outward transport of K^+. This K^+ transport is probably carried out by the Na^+, K^+ activated ATPase. Kawada, Taylor and Barker (1969) have shown that the Na, K activated ATPase from isolated frog skin is inhibited by ouabain. In the presence of ouabain in

Table 7. Effect of 0.5 mM ouabain in the active outwards K^+ transport

Time (hr)	Control			Ouabain (0.5 mM)		
	Δ inside (μeq)	Δ outside (μeq)	Δ loss (μeq)	Δ inside (μeq)	Δ outside (μeq)	Δ loss (μeq)
0–2	−0.12±0.46	0.95±0.44	0.83	4.02±0.12	0.57±0.22	4.59
2–4	−2.16±0.39	2.97±0.80	0.81	1.53±0.17	−0.10±0.12	1.43
0–4	−2.28	3.92	1.64	5.55	0.47	6.02

Filipin (50 μM) was added to the outside bathing solution of the skin halves, and ouabain (0.5 mM) to the inside bathing solution of one of the skin halves. Δ inside and Δ outside are the change in the K^+ content of the inside and the outside bathing solution. Δ loss is calculated from (Δ outside + Δ inside).

the IBS, there was a release of 0.47 μeq K from the skin to the OBS and a release of 5.55 μeq to the IBS (Table 7). In the control skin half the K^+ uptake from the IBS was 2.28 μeq and the K^+ release to the OBS was 3.92 μeq (Table 7). Thus, ouabain inhibited the net movement of K^+ from the IBS to the OBS and caused a substantial loss of K^+ from the skin to the IBS. The nonsteady-state $^{42}K^+$ flux ratio (efflux/influx) after 70 min preincubation with 50 μM ouabain and 50 μM filipin was 1.29 ±0.28 in the first 30-min period and 1.26±0.17 and 0.99±0.14 in the following 30-min periods ($n=5$). Thus the nonsteady-state $^{42}K^+$ flux ratio showed also that ouabain completely inhibited the active outward K^+ transport.

Effect of Ba^{++} on the K^+ Transport

If the active K^+ transport is carried out by a K^+ pump in the inward-facing membrane, then the rate of the active outward K^+ transport will depend on the rate of the K^+ pump and the ratio P^K_{out}/P^K_{in}. P^K_{out} is the K^+ permeability of the outward-facing membrane and P^K_{in} is the K^+ permeability of the inward-facing membrane. A component which decreases P^K_{in} should therefore increase the active outward K^+ transport.

Ba^{++} has been shown to decrease the K^+ conductance in frog heart (Hernsmeyer & Sperelakis, 1970), in frog muscle (Henderson, 1974), and in frog skin (Nagel, 1978).

The addition of 5 mM Ba^{++} to the IBS in the presence of 50 μM filipin in the OBS resulted in an increase in the removal of K^+ from the IBS

Table 8. Effect of 5 mM Ba^{++} in the inside bathing solution on the active K^+ transport

	Time (min)	Ba^{++} (mM)	Δ inside (μeq)	Δ outside (μeq)	ISCC (μeq)	ISCC+ΔK_i (μeq)	$\frac{ISSC+\Delta K_i}{\Delta K_i}$
(a)	0- 90	0	-1.61 ± 0.20	3.61 ± 0.36	15.44 ± 4.52	17.04 ± 4.40	10.58
(b)	0- 90	5	-3.78 ± 0.17	5.55 ± 0.63	8.60 ± 3.87	12.37 ± 3.76	3.27
(c)	90-180	0	-2.64 ± 0.37	3.87 ± 0.29	8.42 ± 1.17	10.97 ± 1.30	4.16
(d)	90-180	5	-3.78 ± 0.46	4.72 ± 0.31	4.06 ± 1.15	7.54 ± 1.86	1.99

Filipin (50 μM) was added to the outside bathing solution of both skin halves and Ba^{++} (5 mM) to the inside of one of the skin halves. Δ inside and Δ outside are the change in the K^+ content of the inside and the outside bathing solutions. ISCC is the integrated short-circuit current during the incubation period. ΔK_i is the K^+ uptake from the inside bathing solution ($\Delta K_i = -\Delta$ inside). Values are the means $\pm$ SE of four experiments.

and in an increase in the rate of appearance of K^+ in the OBS (Table 8); thus Ba^{++} activated the active outward K^+ transport. The integrated SCC (ISCC) in the second incubation period (90–180 min, Table 8) was 8.42 μeq in the absence of Ba^{++} and 4.06 μeq in the presence of Ba^{++}; thus, the addition of 5 mM Ba^{++} to the IBS resulted in 50 % inhibition of the SCC.

The net Na^+ flux across the isolated frog skin is under "normal" conditions equal to the SCC (Ussing & Zerahn, 1951). However, in the presence of an active outward K^+ transport one would expect that there should be a discrepancy between the net Na^+ flux and the SCC. Therefore, a series of experiments was carried out where the Na^+ influx, the Na^+ outflux, the SCC, and the change in the K^+ concentration in the IBS and the OBS were measured. The skins in these experiments were incubated in the presence of 5 mM Ba^{++} in the IBS and with 50 μM filipin in the OBS. The net Na^+ transport under these conditions was very significantly greater than the integrated SCC (Table 9).

There was no significant difference between the Cl^- influx and outflux under these conditions (Table 10); therefore, the discrepancy between the net Na^+ flux and the SCC was not due to an active transport of Cl^-. Under steady-state conditions in the presence of an active outwards K^+ transport, one would expect (if the K^+ transport was carried out by a K^+ pump or an $Na^+ - K^+$ pump) that

$$ISCC = Na_{net} - K_{net}. \quad (1)$$

Na_{net} and K_{net} are the net amount of Na^+ and K^+ transported across the skin during the incubation and ISCC is the integrated SCC.

Table 9. Comparison of the net sodium transport and the integrated short-circuit current across the isolated frog skin

X Influx $\times t$	Y Efflux $\times t$	A Net sodium flux $\times t$ $(X-Y)$	B ISCC	$A-B$	P
		(μeq/7 cm^2/2 hr)			
27.93	12.52	15.41	11.76	3.65	$0.005>P>0.001$
ΔK inside	ΔK outside	A Net sodium flux $\times t$	C ISCC $+\Delta K_i$	$A-C$	P
		(μeq/7 cm^2/2 hr)			
−4.32	6.11	15.41	16.08	−0.68	$0.3>P>0.25$

The skins were incubated with 50 μM filipin in the OBS, and with 5 mM Ba^{++} in the IBS. The influx was measured by means of $^{22}Na^+$ and the efflux with $^{24}Na^+$; the short-circuit current was measured and recorded automatically. ISCC is the integrated short-circuit current during the incubation period (t). ΔK outside is the increase in the K^+ content of the OBS, and ΔK inside is the decrease in the K^+ content of the IBS ($\Delta K_i = -\Delta$K inside). Values are the means of 9 expts.

Table 10. Effect of Ba^{++} and filipin on the chloride influx and efflux

	Time (min)		
	0–30	30–60	60–90
	(neq $\times$ cm^{-2} $\times$ min^{-1})		
Influx	25.1 $\pm$ 3.7	24.8 $\pm$ 1.8	29.2 $\pm$ 3.4
Efflux	25.0 $\pm$ 6.6	28.3 $\pm$ 4.9	34.4 $\pm$ 4.8

In each expt. following 20 min of equilibration for the isotope ($^{36}Cl^-$), the permeability across the skin was determined. The Cl^- influx and efflux were measured simultaneously on symmetrical halves of a frog skin. Filipin (50 μM) was added to the OBS and Ba^{++} (5 mM) to the IBS. Filipin and Ba^{++} were added before the isotope. All values are the means $\pm$ SE of 5 expts.

The decrease in the K^+ content of the IBS is different from the increase in the K^+ content of the OBS (Table 9); therefore, the skin is not in a steady state. Under nonsteady-state conditions K_{net} is equal to the measured K^+ uptake from the IBS (ΔK_i), plus the K^+ loss from the skin to the IBS (K_i^{loss}), plus the net K^+ back transport (K^{back}) from the OBS to the IBS. At the start of the experiment K^{back} is zero, but during the incubation a K^+ concentration gradient is built up across the skin; it

is this concentration gradient which drives the net K back transport. Thus:

$$K_{net} = \Delta K_i + K_i^{loss} + K^{back} \tag{2a}$$

K_{net} is also equal to:

$$K_{net} = \Delta K_o - K_o^{loss} + K^{back} \tag{2b}$$

where ΔK_o is the measured increase in the K^+ content of the OBS, K_o^{loss} is the K loss from the skin to the OBS and K^{back} is the net K back transport from the OBS to the IBS.

Addition of filipin to the OBS results in a swelling of the cells (Nielsen, 1977). Therefore, during the incubation with filipin in the OBS there is an uptake of Na^+, Cl^-, and H_2O from the bathing solutions into the cells. Since the outward-facing membrane is more permeable to Na^+ than the inward-facing membrane, the Na^+ uptake from the solutions into the transport compartment would mainly take place from the OBS. The ratio between the Cl^- permeability of the inward- and the outward-facing membrane is not known under the circumstances used in these experiments, but if the Cl^- permeability of the inward-facing is not much lower than the Cl^- permeability of the outward-facing membrane, then a part of the Na^+ taken from the OBS during the swelling of the transport compartment would be balanced by an uptake of Cl^- from the IBS. An uptake of Cl^- from the IBS together with a Na^+ uptake from the OBS will give a current without transepithelial Na flux, the contribution of this to the ISCC is called Δ_{Cl}^{swell}. Therefore, when the ISCC is measured under nonsteady-state conditions it has to be corrected by this current (Δ_{Cl}^{swell}), when it is compared with the net Na transport. The K^+ back transport will also give a current without a concomitant Na^+ transport; therefore the ISCC has to be corrected by this amount, too. Furthermore, the loss of K^+ from the cells to the IBS would be balanced by a Na^+ uptake from the OBS (because the outward-facing membrane is much more permeable for Na^+ than the inward-facing membrane); this will also give a current without a concomitant Na^+ flux.

Thus under nonsteady-state conditions Eq. (1) is:

$$ISCC - \Delta_{Cl}^{swell} - K^{back} - K_i^{loss} = Na^{net} - \Delta K_i - K_i^{loss} - K^{back} \tag{3}$$

which reduce to

$$ISCC - \Delta_{Cl}^{swell} = Na_{net} - \Delta K_i. \tag{3a}$$

From the data in Table 9 it appears that ISCC is not significantly different from $Na_{net} - \Delta K_i$; therefore Δ_{Cl}^{swell} must be small[1] compared with the other terms in Eq. (3a), and consequently it is reasonably correct (under the conditions used in these experiments) to calculate the net transepithelial Na^+ transport from the following equation:

$$Na_{net} = ISCC + \Delta K_i. \tag{4}$$

From the data in Table 8 it is seen that the net Na^+ transport (ISCC $+\Delta K_i$) was 10.97 μeq in the absence of Ba^{++} and 7.54 μeq in the presence of 5 mM Ba^{++}. Thus, the addition of 5 mM Ba^{++} resulted in 31 % inhibition of the active Na^+ transport.

The Effect of Amiloride

Addition of 10 μM amiloride to the OBS of the isolated frog skin results in a complete inhibition of the active Na transport (Nielsen & Tomlinson, 1970). In the presence of filipin in the OBS the amiloride-induced inhibition of the Na^+ transport is reduced to a variable extent (Nielsen, 1977). If the active transepithelial K^+ transport is carried out by a coupled $Na^+ - K^+$ pump, then an inhibition of the transepithelial Na^+ transport (e.g., with amiloride) should result in an inhibition of the K^+ transport. To investigate whether amiloride had an effect on the K^+ transport, a series of experiments were performed where symmetrical skin halves were incubated in the presence and the absence of amiloride in the OBS. Furthermore, filipin was added to the OBS and Ba^{++} to the IBS in these experiments.

The change in the K^+ content of the two solutions was measured together with SCC. The Na^+ transport was calculated from $ISCC + \Delta K_i$ (Eq. (4)). From the K^+ uptake and the Na^+ transport in the presence and the absence of amiloride the inhibition of the K^+ uptake and the Na^+ transport was calculated in percent. The presence of amiloride in the OBS resulted in an inhibition of both the Na^+ transport and the K^+ uptake (Fig. 2). The data in Figs. 3 and 4 were taken from the skin halves which were incubated in the presence of amiloride and filipin in the OBS

[1] Although Δ_{Cl}^{swell} was small compared with the other terms in Eq. (3a), it would, if we assume that the Cl^- permeability of the inward and the outward-facing membranes was the same, account for a 50 % increase in the volume of the cells in the epithelia.

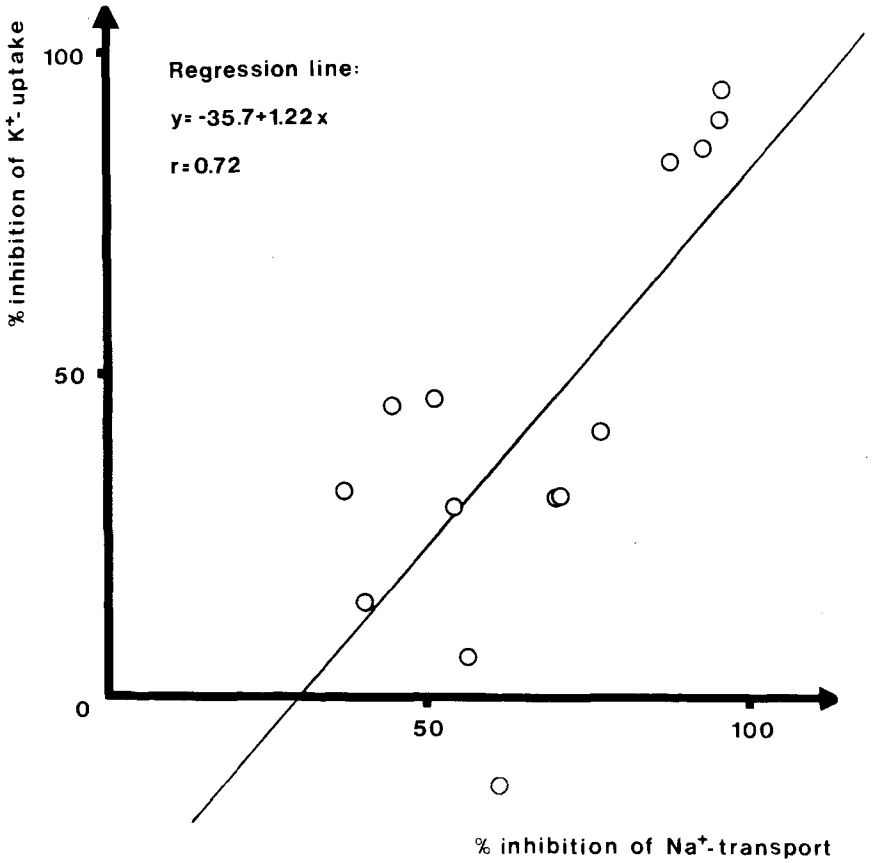

Fig. 2. Effect of amiloride on K^+ transport. The control-skin half was incubated in the presence of filipin (50 μM) in the OBS and with Ba^{++} (5 mM) in the IBS, the exp.-skin half was incubated under the same conditions but with amiloride (10 μM) in the OBS. *Ordinate:* % inhibition of K^+ uptake

$$\left(\frac{\Delta K_i^{control} - \Delta K_i^{exp}}{\Delta K_i}\right) \cdot 100.$$

Abscissa: % inhibition of Na^+ transport

$$\frac{(ISCC + \Delta K_i)^{control} - (ISCC + \Delta K_i)^{exp}}{(ISCC + \Delta K_i)^{control}} \cdot 100$$

and with Ba^{++} in the IBS; under these conditions there was a good correlation between the K^+ uptake measured and the Na^+ transport and between the K^+ release and the Na^+ transport. Thus, the data in Figs. 3 and 4 support the assumption that the transepithelial K^+ transport is coupled to the transepithelial Na^+ transport.

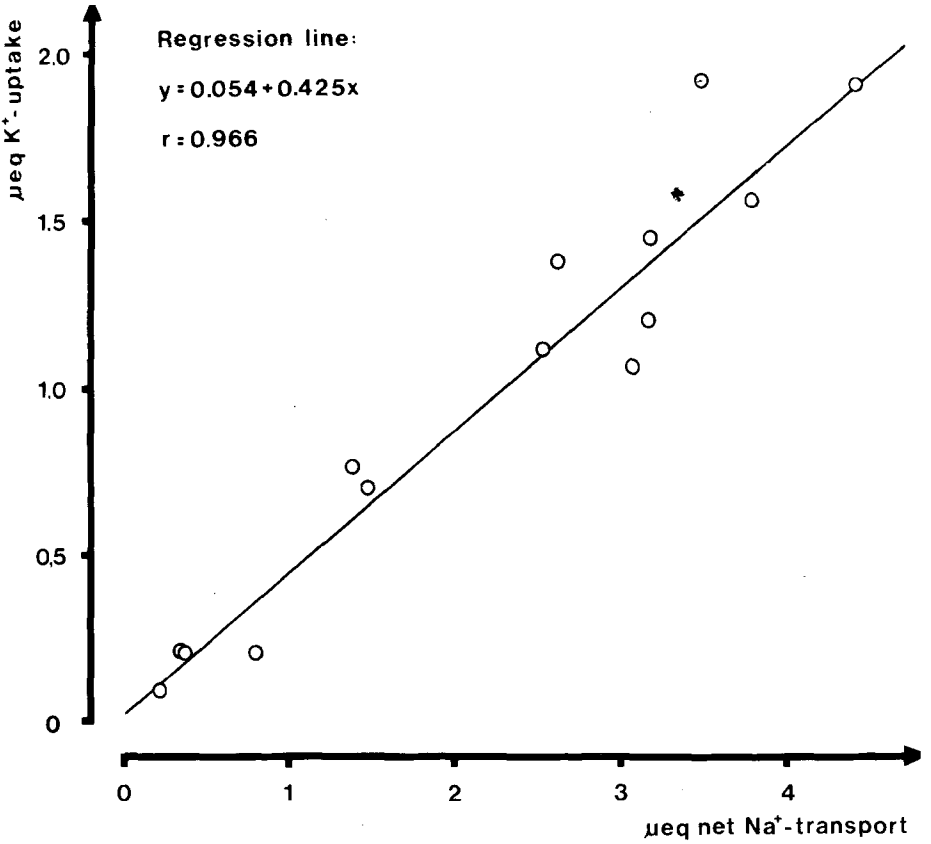

Fig. 3. Coupling between K^+ uptake and Na^+ transport. The skins were incubated in the presence of 10 μM amiloride under the conditions given in the legend to Fig. 2. *Ordinate:* K^+ uptake (ΔK_i). *Abscissa:* net Na^+ transport calculated from ISCC + ΔK_i

Discussion

According to the two-membrane hypothesis (Koefoed-Johnsen & Ussing, 1958) the origin of the electrical potential across the isolated frog skin can be explained by the fact that the frog skin is composed of an outward-facing membrane which is selectively permeable for Na^+-ions, but impermeable for K^+-ions and permeable to small anions like Cl^-. The inward-facing membrane is permeable to K^+ and small anions. The high intracellular K^+ concentration and the active transcellular Na^+ transport are, according to this model accomplished by the same mechanism, namely, a coupled Na–K pump located at the inward-facing membrane.

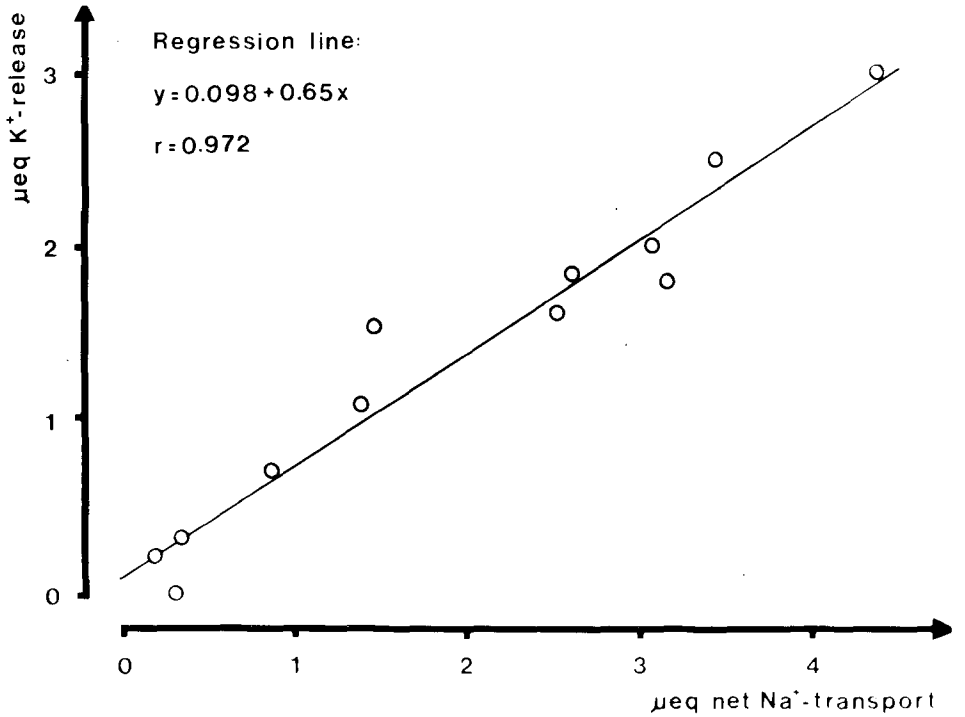

Fig. 4. Coupling between K^+ release and Na^+ transport. The skins were incubated in the presence of 10 µM amiloride under the conditions given in the legend to Fig. 2. *Ordinate:* K^+ release (ΔK_o). *Abscissa:* net Na^+ transport calculated from ($ISCC + \Delta K_i$)

Addition of the polyene antibiotic filipin to the OBS results in a progressive increase in the nonspecific permeabilities of the outward-facing membrane (Nielsen, 1977). The addition of filipin to the IBS results in a Ca^{++} mediated increase of the K^+ permeability of the inward-facing membrane (Nielsen, 1978).

The two-membrane hypothesis (Fig. 5*a*) predicts that the addition of a component which increases the K^+ permeability of the outward-facing membrane will result in an active outward K^+ transport (Fig. 5*b*). Previous experiments have shown that the addition of amphotericin B to the OBS resulted in an active outward transport of K^+ (Nielsen, 1971). However, the K^+ transport observed in the presence of amphoterecin B was small (Table 2), whereas the addition of the polyene antibiotic filipin to the OBS resulted in considerable active outward transport of K^+ (Tables 1 and 2).

The filipin-induced K^+ transport required the presence of Na^+ in the OBS (Tables 3 and 4), but not in the IBS (Tables 5 and 6), and it was

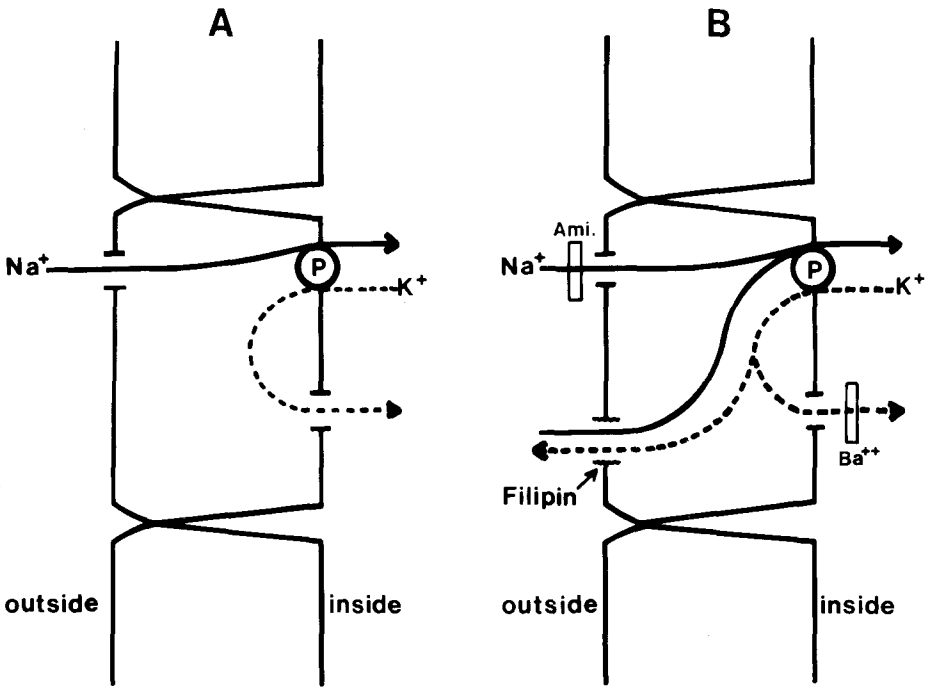

Fig. 5. (*A*): Model showing the two membrane hypothesis, (P), Na−K pump. Na^+ crosses the outside-facing membrane through the Na^+ "channel" and is pumped across the inward-facing membrane via the Na−K pump. K^+ is pumped into the cells from the IBS and moves from the cells back to the IBS via the K^+ channel in the inward-facing membrane. (*B*): Effect of filipin. Filipin induces the formation of a nonspecific "pathway" in the outwards-facing membrane; through this pathway Na^+ and K^+ can pass. *Ami*, amiloride blocks the entry of Na^+ into the cell via the Na^+ specific pathway. Ba^{++} blocks the K^+ channel.

blocked by the Na−K ATPase inhibitor ouabain (Table 7). The active transepithelial Na^+ transport also requires the presence of Na^+ in the OBS, but not in the IBS, and it is inhibited by ouabain, too. This indicates that the transepithelial Na^+ transport and the transepithelial K^+ transport are carried out by the same mechanism, namely, a coupled Na−K pump.

Addition of Ba^{++} to the IBS resulted in an activation of the active transport of K^+ from the IBS to the OBS and in an inhibition of the net transepithelial Na^+ transport (Table 8). Nagel (1978) has shown that Ba^{++} decreases the K^+ conductivitiy of the inward-facing membrane of the isolated frog skin. It has been shown previously that an increase in the K^+ permeability of the inward-facing membrane results in an increased Na^+ transport (Nielsen, 1978).

A decrease in the K^+ permeability of the inward-facing membrane should, according to the two-membrane hypothesis (Fig. 5*b*), result in an increase in the active outward K^+ transport (because it decreases the recirculation of K across the inward-facing membrane). Under steady-state conditions the net K^+ flux from the cells to the bathing solutions via the K^+ "channels" and leaks is equal to the net K^+ flux from the IBS to the cells via the K^+ pump. A decrease in the K^+ permeability of the inward-facing membrane would (under steady-state conditions) result in a decrease in the net K^+ flux from the cells and in a corresponding decrease in the K^+ flux via the K^+ pump. Therefore, if the active K^+ transport is carried out by a Na−K pump with a fixed coupling ratio, a decrease in P_{in}^{K} should result in an inhibition of the transepithelial Na transport as also experimentally found (Table 8).

Thus, the data in Table 8 support the point of view that Ba^{++} reduces the K^+ flux via the K^+ "channels" in the inward-facing membrane, and it is in agreement with the notion that the active K^+ transport is carried out by a coupled Na−K pump.

If the K transport is carried out by a coupled Na−K pump, a reduction of the Na^+ flux into the transporting cells should result in a decrease in the active outward K^+ transport. The addition of amiloride (10 μM) to the OBS results in a complete inhibition of the Na^+ transport; this inhibition is caused by an interaction of amiloride with the specific Na^+ pathway (the Na^+ channels) in the outward-facing membrane (Cuthbert & Shum, 1974). In the presence of filipin in the OBS, the addition of amiloride resulted in a variable inhibition of the Na^+ transport, because filipin forms an amiloride-insensitive Na^+ pathway in the outward-facing membrane (Nielsen, 1977). From Fig. 2 it is seen that an amiloride-induced inhibition of the Na^+ transport also resulted in an inhibition of the active outward K^+ transport. Furthermore, the data in Figs. 3 and 4 show a highly significant correlation between the transepithelial Na^+ transport and the K^+ uptake from the IBS and the K^+ release to the OBS.

Coupling between the Transepithelial Na^+ and K^+ Transport

The data in the previous section show that the transepithelial K^+ transport is coupled to transepithelial Na^+ transport. The coupling ratio (β) of the Na−K pump is:

$$\beta = \frac{J_{\text{pump}}^{\text{Na net}}}{J_{\text{pump}}^{\text{K net}}} \tag{5}$$

where $J^{Na\ net}_{pump}$ is the net Na^+ flux through the pump and $J^{K\ net}_{pump}$ is the net K^+ flux through the pump. Under short circuiting with the same Na^+ concentration on each side of the isolated frog skin, the mean net Na^+ flux through the pump ($J^{Na\ net}_{pump}$) is equal to Na_{net}/t, where t is the incubation time, and Na_{net} is the net amount of Na^+ transported across the skin during the incubation period (t). The net K flux through the pump ($J^{K\ net}_{pump}$) is equal to the net K flux from the transport compartment to the OBS via passive "pathways", plus the net K^+ flux from the transport compartment to the IBS via passive "pathways". Since the total amount of K which goes to the IBS and the OBS depends on the ratio between the K permeability of the inward and the outward-facing membranes we get:

$$t \cdot J^{K\ net}_{pump} = t \cdot J^{K\ net}_{pump} \cdot \frac{P^K_{in}}{P^K_{out} + P^K_{in}} + t \cdot J^{K\ net}_{pump} \cdot \frac{P^K_{out}}{P^K_{in} + P^K_{out}}. \tag{6}$$

The first term on the right side of Eq. (6) is equal to the amount of K which goes to the IBS and the second term is equal to the amount of K which goes to the OBS. The amount of K which goes to the OBS is equal to net amount of K transported across the skin; thus:

$$K_{net} = t \cdot J^{K\ net}_{pump} \cdot \frac{P^K_{out}}{P^K_{in} + P^K_{out}}. \tag{7}$$

By rearranging of the terms in Eq. (7), we get:

$$t \cdot J^{K\ net}_{pump} = K_{net} \left(1 + \frac{P^K_{in}}{P^K_{out}}\right). \tag{7a}$$

In Eq. (2a) it is shown that:

$$K_{net} = \Delta K_i + K^{loss}_i + K^{back}. \tag{2a}$$

By substituting Eq. (2a) into Eq. (7a), we get:

$$J^{K\ net}_{pump} \cdot t = (\Delta K_i + K^{loss}_i + K^{back}) \left(1 + \frac{P^K_{in}}{P^K_{out}}\right). \tag{8}$$

By substituting Eqs. (8) and (4) into Eq. (5), we get:

$$\beta = \frac{ISCC + \Delta K_i}{(\Delta K_i + K^{loss}_l + K^{back}) \left(1 + \frac{P^K_{in}}{P^K_{out}}\right)}. \tag{8a}$$

By using Eq. (2b) instead of Eq. (2a), we get:

$$\beta = \frac{\mathrm{ISCC} + \Delta \mathrm{K}_i}{(\Delta \mathrm{K}_o - \mathrm{K}_o^{\mathrm{loss}} + \mathrm{K}^{\mathrm{back}}) \left(1 + \frac{\mathrm{P}_{\mathrm{in}}^{\mathrm{K}}}{P_{\mathrm{out}}^{\mathrm{K}}}\right)}. \tag{8b}$$

The only terms which are known in Eq. (8a and b) are ISCC, $\Delta \mathrm{K}_i$, and $\Delta \mathrm{K}_o$. Therefore, in an attempt to estimate β from Eq. (8a) it was assumed that $\mathrm{K}_i^{\mathrm{loss}}$ and $\mathrm{K}^{\mathrm{back}}$ were small compared with $\Delta \mathrm{K}_i$. Furthermore, the experiments have to be conducted under conditions where $\left(1 + \frac{P_{\mathrm{in}}^{\mathrm{K}}}{P_{\mathrm{out}}^{\mathrm{K}}}\right)$ approaches unity. Under these conditions Eq. (8a) can be reduced to:

$$\beta = \frac{\mathrm{ISCC} + \Delta \mathrm{K}_i}{\Delta \mathrm{K}_i}. \tag{9a}$$

Addition of filipin to the OBS results in a progressive increase in the nonspecific permeability of the outward-facing membrane (Nielsen, 1977). In the presence of filipin in the OBS, β was 10.6 in the first incubation period (Table 8*a*), and 4.16 in the second incubation period (Table 8*c*). In the presence of filipin in the OBS and with Ba^{++} in the IBS (Ba^{++} decreases the K^+ permeability of the inward-facing membrane), β was 3.27 in the first period and 1.99 in the second incubation period (Table 8*b* and *c*). Figure 6 shows the results of a series of experiments where the skins were preincubated for 2 hr in the presence of filipin in the OBS and with Ba^{++} in the IBS. After the preincubation the skins were incubated for 60–120 min under the same conditions and SCC and $\Delta \mathrm{K}_i$ were measured. The full line in Fig. 6 is the theoretical line one would get if the pump ratio was 1.5 (3 Na/2 K). From Fig. 6 it appears that about half of the experimental points fits the theoretical line reasonably well whereas the other half of the experimental points fall below the theoretical line. If the transepithelial Na^+ and K^+ transport across the frog skin is carried out by a Na, K pump with a fixed coupling ratio, the data in Fig. 6 shows that the couplings ratio is 1.5 or smaller The reason why some of the experimental points in Fig. 6 fall below the theoretical line is that the (rather extreme) approximations used for the reduction of Eq. (8a) to Eq. (9a) are not fulfilled. The reduction of Eq. (8a) to (9a) is only correct in the cases where $P_{\mathrm{in}}^{\mathrm{K}}$, $\mathrm{K}^{\mathrm{back}}$, and $\mathrm{K}_i^{\mathrm{loss}}$ are zero; this assumption can only be expected to be fulfilled in borderline cases; therefore, Eq. (9a) tends to underestimate the K flux through the pump, and therefore in most cases the estimate of β will be too high.

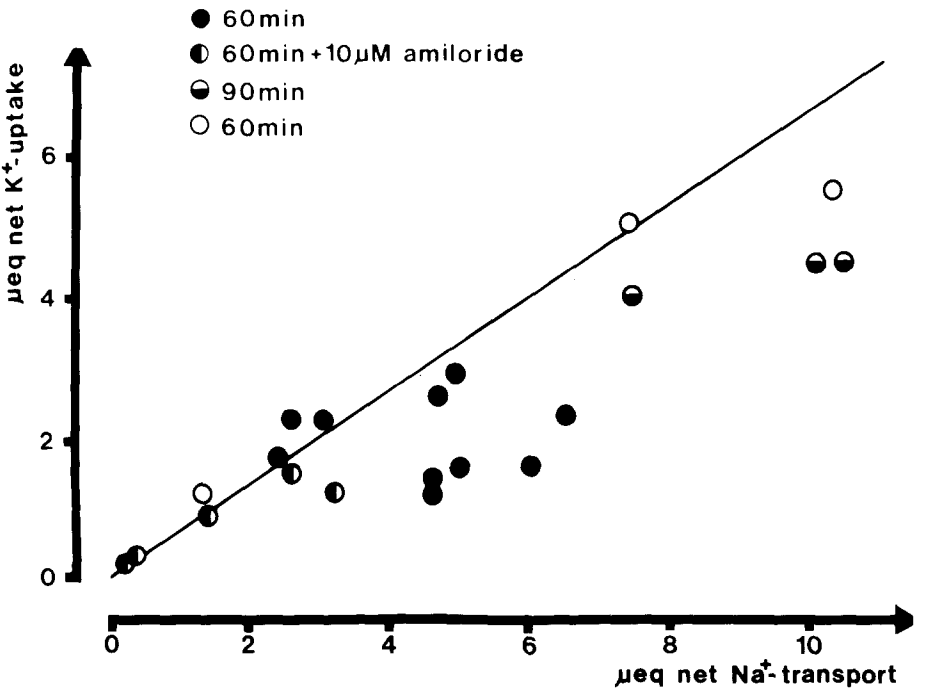

Fig. 6. The skins were incubated in the presence of Ba^{++} (5 mM) in the IBS and with filipin (50 μM) in the OBS. *Abscissa:* net Na^+ transport calculated from $\Delta K_i + ISCC$. *Ordinate:* K^+ uptake from the IBS (ΔK_i). ●, incubation period 60 min; ◐, incubation period 60 min in the presence of 10 μM amiloride in the OBS; ◒, Incubation period 90 min; ○, Incubation period 120 min

In the presence of amiloride and filipin in the OBS and with Ba^{++} in the IBS, there was a good correlation between the active Na^+ transport and the decrease in the K^+ content in the IBS (Fig. 3); the slope of the linear regression line was 0.425; according to Eq. (9a), β is equal to $1/0.425 = 2.35$. However, this estimate of β must be too high, because by using Eq. (9a) it is assumed that all K lost from the skin is lost to the OBS. In the presence of ouabain the K^+ lost from the skin was lost to the IBS (Table 7). If the K lost from the skin is lost to the IBS, then Eq. (9b) gives a better estimate of β than Eq. (9a).

$$\beta = \frac{ISCC + \Delta K_i}{\Delta K_o}. \tag{9b}$$

Equation (9b) can derived from Eq. (8b) if it is assumed that K^{back} and the loss of K from the skin to the OBS (K_o^{loss}) is small, and that $(1 + P_{in}^K/P_{out}^K)$ approaches unity. The experiments in Fig. 4 show that there is

a good correlation between the active Na^+ transport and the increase in the K^+ content of the OBS. The slope of the linear regression line (Fig. 4) is 0.65; according to Eq. (9b) this corresponds to $\beta = 1/0.65 = 1.54$. Thus, the experiments in Figs. 4 and 6 indicate that the coupling ratio between the active Na^+ and K^+ transport is 1.5 (3Na/2K).

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