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Single-File Diffusion Through K^+ Channels in Frog Skin Epithelium

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Summary. The ratio between the unidirectional fluxes of K^+ across the frog skin with K-permeable outer membranes was determined in the absence of Na^+ in the apical solutions. The experiments were performed under presteady-state conditions to be able to separate the flux ratio for K^+ through the cells from contributions to the fluxes through extracellular leaks. The cellular flux ratio deviated strongly from the value calculated from the flux ratio for electrodiffusion. The experiments can be explained if the passive K transport through the epithelial cells proceeds through specific channels by single-file diffusion with a flux ratio exponent of about 2.5.

Key Words single-file diffusion · flux ratio · frog skin · potassium

Introduction

According to the Koefoed-Johnsen and Ussing model (1958), the apical membrane of the frog skin is selectively permeable to sodium and the basolateral membrane is selectively permeable to potassium. In some skins from *Rana temporaria,* however, it has been found that the apical membrane is potassium permeable and that K^+ permeability can be induced in the outer border with KCl Ringer on the epithelial side or when the impermeable anion gluconate replaces Cl in the Ringer solutions bathing the frog skins (Zeiske & Van Driessche, 1979; Nagel & Hirschman, 1980; Van Driessche & Zeiske, 1980; Nielsen, 1984).

Measurement of flux ratio for passive K^+ fluxes across giant axons (Hodgkin & Keynes, 1955; Begenisich & De Weer, 1980), muscle (Horowicz, Gage & Eisenberg, 1968) and red blood cell (Vestergaard-Bogind, Stampe & Christophersen, 1985) membranes shows that the value calculated for independent passive diffusion (Ussing, 1949, 1978) had to be raised to a power larger than one to obtain identity with the experimental results.

In the present investigation it is examined if this is also a property of the K^+-transport mechanism in the frog skin. It has previously been shown that potassium fluxes across the frog skin are highly potential dependent (Levi & Ussing, 1949). To distinguish cellular K^+ fluxes from extracellular fluxes the flux ratio measurements were performed under presteady-state conditions. Presteady-state flux ratio can be used to examine if a given substance crosses the epithelium along different pathways with different flux ratios. If there are two pathways the flux ratio and the unidirectional fluxes through the individual pathways can be determined (Ussing, Eskesen & Lim, 1981; Eskesen, Lim & Ussing, 1985; K. Eskesen and H.H. Ussing, *in preparation*). The method is based on the theory derived by Sten-Knudsen and Ussing (1981), who showed, if an ion following one pathway through a series of membranes with arbitrary but time invariant potential and concentration profiles, the flux ratio measured under presteady-state conditions will be identical to the flux ratio under steady-state conditions.

Materials and Methods

The experiments were done on skins obtained from *Rana temporaria,* which had been kept at 5°C in tap water. The skins were separated into symmetrical pieces and mounted in Perspex chambers with a cross sectional area of 3.14 cm^2. Transepithelial potential and clamp current were measured by automatic voltage-clamp equipment according to the procedure described by Ussing and Zerahn (1951). Skins with K^+-permeable apical membranes were selected by measuring the potential across the skin, when the outside solution was KCl-Ringer and the inside solution was NaCl-Ringer (*see below*). Assuming the skin behaved as a K^+-selective electrode, the potential would be 93 mV. However, leak pathways through leaky tight junctions or mitochondria-rich cells make the skins permeable to Na^+ and Cl^- and the resulting potential would be lower. Only skins that showed a potential higher than 30 mV were used for experiments. The skins were equilibrated in the experimental solution for about 1 hr before isotope was added. K^+ influx was measured on one

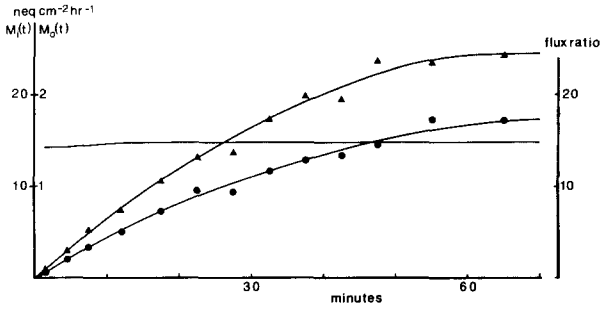

Fig. 1. Presteady-state experiment with KCl Ringer on the apical side and NaCl Ringer on the serosal side. The transepithelial potential was held at 60 mV (positive inside). Influx, ▲; efflux, ●. The solid line without experimental points is the flux ratio calculated from the smoothed curves

skin half and K^+ efflux on the other. Sampling was started immediately after addition of isotope in order to measure the flux as the isotope concentration builds up in the tissue. The isotope, ^{42}K, was delivered as a 156-mM KCl solution with high specific activity (10 mCi/ml). Isotope-free KCl solution identical to the radioactive solution was added to the "cold" bathing solutions. K^+ concentration of the bathing solutions was measured by flame photometry. The following solutions were used for the experiments (in mM): NaCl-Ringer: 115, NaCl; 2.5, KCl; 1, $CaCl_2$. KCl-Ringer: 115, KCl; 2.5, $KHCO_3$; 1, $CaCl_2$. K_2SO_4-Ringer: 57.5, K_2SO_4; 2.5, $KHCO_3$; 1, $CaSO_4$. Choline-Ringer: 115, Choline-Cl; 2.5, $KHCO_3$; 1, $CaCl_2$.

Calculation

If the flux ratio calculated from presteady-state fluxes varies with time and it can be shown that the transport parameters have been constant during the experiment, the ion must have crossed the skin along two or more pathways with different flux ratios. It is obvious that the steady-state K^+ fluxes though a leak is reached faster than the steady state through the epithelial cells, where the K^+ concentration is high. The aim is therefore to determine the flux ratio through the pathway where isotope equilibration is slow. This can be done in two ways. One method is thoroughly described in another paper (K. Eskesen and H.H. Ussing, *in preparation*), but is briefly outlined here. The other method was worked out to be able to handle experiments, where steady-state fluxes were not attained. This method can be used if isotope equilibration with the tissue is very slow. In the following it is assumed that the ion only crosses the epithelium along two different pathways. The experimentally measured time-dependent influx $M_i(t)$ is given by

$$M_i(t) = J_i^p(1 - g(t)) + J_i^a(1 - f(t)) \tag{1}$$

where J_i^p is the steady-state influx through the fast pathway and J_i^a is the steady-state flux through the slow pathway. $g(t)$ and $f(t)$ are arbitrary, monotonic decreasing functions of t that have to fulfil the following conditions: $(g(0), f(0)) = (1,1)$ for $t = 0$ and $(g(t), f(t)) = (0,0)$ for $t \to \infty$. Since the flux ratio for an ion through one pathway under presteady-state conditions is identical to the flux ratio in steady state, the time-dependent functions are identical for influx and efflux. As above, the time-dependent efflux is given by

$$M_o(t) = J_o^p(1 - g(t)) + J_o^a(1 - f(t)). \tag{2}$$

The steady-state fluxes are given by

$$M_i(\infty) = J_i^p + J_i^a \quad \text{and} \quad M_o(\infty) = J_o^p + J_o^a \tag{3 and 3a}$$

and the flux ratios through the fast, r^p, and the slow, r^a, pathways are

$$\frac{J_i^p}{J_o^p} = r^p \quad \text{and} \quad \frac{J_i^a}{J_o^a} = r^a. \tag{4 and 4a}$$

For values of $t > \tau$ $g(t) = 0$, while $f(t)$ changes with time. The unidirectional fluxes will then have the form

$$M_i(t) = J_i^p + J_i^a(1 - f(t)),\ t > \tau \tag{5}$$

$$M_o(t) = J_o^p + J_o^a(1 - f(t)),\ t > \tau. \tag{6}$$

After insertion of the steady-state fluxes into Eqs. (5) and (6) and rearranging, the flux ratio through the slow pathway will be given by

$$\frac{J_i^a f(t)}{J_o^a f(t)} = \frac{M_i(\infty) - M_i(t)}{M_o(\infty) - M_o(t)} = r^a. \tag{7}$$

In summary, the flux ratio through the slow pathway is determined by the difference between the steady-state fluxes and values of the flux curve after steady state through the fast pathway has been reached.

The other method is based on the same set of equations. From Eqs. (4) and (4*a*) we have

$$J_i^p = J_o^p r^p \quad \text{and} \quad J_i^a = J_o^a r^a. \tag{4 and 4a}$$

Inserting this into Eq. (1) leads to

$$M_i(t) - r^a J_o^a(1 - f(t)) + r^p J_o^p(1 - g(t)). \tag{8}$$

$J_o^a(1 - f(t))$ is isolated from Eq. (2) and inserted into Eq. (8).

$$M_i(t) = r^a(M_o(t) - J_o^p(1 - g(t))) + r^p J_o^p(1 - g(t)). \tag{9}$$

By rearranging this gives

$$M_i(t) = r^a M_o(t) - (r^a - r^p)J_o^p(1 - g(t)). \tag{10}$$

For $t > \tau$, $g(t) = 0$ and $(r^a - r^p)J^p = k$, where k is a constant. From this it follows that

$$M_i(t) = r^a M_o(t) - k.$$

The flux ratio for the slow pathway can be determined from the slope of the curve where $M_i(t)$ is depicted as a function of

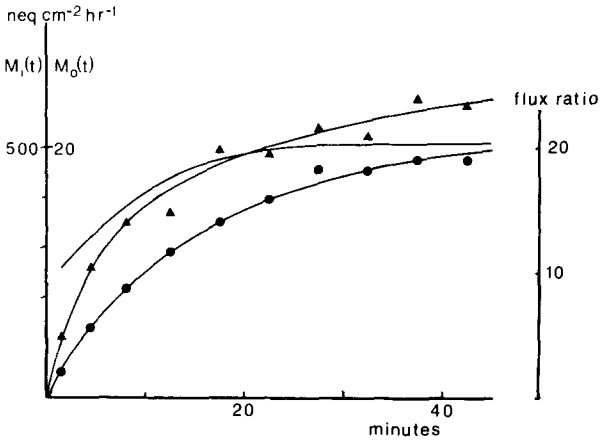

Fig. 2. Presteady-state experiment with KCl Ringer on both sides of the frog skin and with transepithelial potential held at −30 mV. Influx, ▲; efflux, ●. The solid line without experimental points is the flux ratio through the smoothed curves

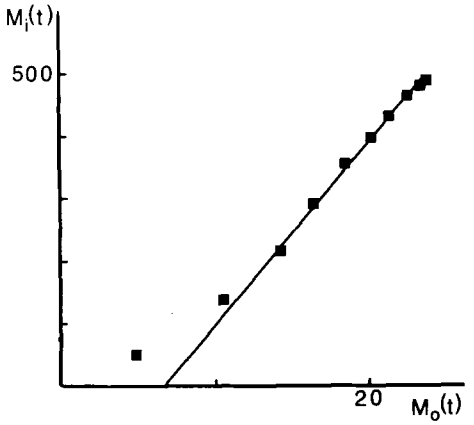

Fig. 3. In this figure values from the smoothed influx curve are depicted as a function of values from the smoothed efflux curve. According to Eq. (11) the flux ratio through the slow pathway can be determined from the slope drawn through those values where the fluxes through the fast pathway have become constant. The slope of the drawn curve is 28.8

$M_o(t)$, for the values of t where the fluxes through the fast pathway have become constant.

Results

Nielsen (1984) has demonstrated that K^+ can be transported actively outward by the Na-K pump in the basolateral membrane, when Na^+ is present in the outside bathing solution. To avoid this, all solutions bathing the apical membrane were Na^+ free.

Figure 1 shows a presteady-state experiment, where the outside bathing solution was KCl Ringer and the inside bathing solution NaCl Ringer. The transepithelial potential was held at 60 mV (positive inside). The isotope fluxes increase continuously, but the flux ratio remains constant throughout the experimental period, indicating there is one major pathway for K^+ across the epithelium.

Figure 2 shows an experiment with KCl-Ringers on both sides. Here the flux ratio varies with time, indicating the presence of more than one pathway. The steady-state unidirectional fluxes were not attained in this experiment, but the time-dependent flux ratio became constant, showing that the fluxes through a fast pathway had reached their steady-state values. The flux ratio through the slow pathway was therefore determined according to Eq. (11), which is illustrated in Fig. 3. In those experiments where the flux ratio was time variant the flux ratio through the cellular pathway was determined from the slope of the straight part of the curve that depicts the influx as a function of the efflux.

The relationship between influx and efflux for passive independent movement of ions is described in the flux ratio equation (Ussing, 1949, 1978):

$$J_{13}/J_{31} = K_1/K_3 \exp(V_m zF/RT) = \exp((V_m - V_K)zF/RT).$$

J_{13} and J_{31} are the influx and the efflux, respectively, K_1 and K_3 the activities of the outside and inside solutions, and V_m is the transepithelial potential. R, T, z and F have their usual meanings. V_K is the equilibrium potential for K^+. According to this equation the flux ratio for K^+ in Figs. 1 and 3 should be 2.97 and 3.26. However, the measured flux ratios are 14.8 and 28.8. A similar tendency is obtained for all experiments (*see* Table 1). The net flux of K^+ across the skin is always in the direction of the electrochemical gradient, indicating that the transport measured is passive (*see* Table 2). Hodgkin and Keynes (1955) showed that the movement of K^+ across the membrane of the giant axon from the cuttlefish was passive, but not independent. Neither did the experimentally determined flux ratio agree with the theoretical value of the flux ratio, but they found it necessary to raise the flux ratio equation to a power n

$$J_{13}/J_{31} = \exp((V_m - V_K)nzF/RT).$$

Using the same approach as Hodgkin and Keynes (1955), a value for n can be calculated. The results from different experiments with varied K^+ concentrations are given in Table 1.

Table 1.

	K concentration		Potential trans. epi. (mV)	Flux ratio		
	Outside (mM)	Inside (mM)		Experimental	Theoretical	Exponent (n)
Chloride-Ringers on both sides						
1	117.5	4.0	47	52	4.6	2.58
2	117.5	4.0	60	11.4	2.77	2.39
3	118.1	3.75	60	14.8	2.97	2.48
4	118.1	3.75	60	18.8	2.97	2.70
5	4.75	118.1	−60	0.073	0.426	3.06
6	3.80	118.1	−60	0.034	0.341	3.14
7	122.2	122.2	30	0.123	0.307	1.77
8	122.2	122.2	−30	28.8	3.26	2.85
Sulfate-Ringers on both sides						
9	117.5	117.5	30	0.056	0.307	2.42
10	117.5	117.5	30	0.060	0.307	2.37

Columns 1 and 2 give the K^+ concentration of the solutions on the epithelial side and the serosal side. Column 3 gives the electrical potential across the skin measured with respect to the outside compartment. Column 4 gives the experimentally determined flux ratio and column 5 the theoretical value assuming independent, passive transport. The calculated flux ratio exponent is given in column 6.

Discussion

A complicating factor in this investigation is that the flux ratio is determined for two membranes in series, the apical and the basolateral, and not through a single membrane. We must therefore discuss the results from the point of view that single-file transport occurs in one of the two membranes or in both.

For a system consisting of membranes arranged in series it can be shown (Ussing & Zerahn, 1951) that the flux ratios are multiplicative, so that the flux ratio for the whole array is the product of the flux ratios for the individual membranes. This is correct quite independent of the mechanism underlying the individual fluxes such as electrodiffusion, active transport, single filing, solvent drag, etc. Since the frog skin epithelium is a functional syncytium, we can treat it as a cytoplasmic phase with an apical and a basolateral membrane. It is reasonable to assume that the ion pump does not contribute to the exchange of potassium (due to lack of sodium). Let is first assume that the potassium channels of the two membranes are of the same nature, both groups exhibiting single filing. If the electrochemical potential of the potassium ion in the apical solution is called $\tilde{\mu}_1$, that of the cytoplasm $\tilde{\mu}_2$ and that of the basolateral solution $\tilde{\mu}_3$, we can now write using the logarithmic form of the equation ($J_{12}/J_{21} = \exp(\tilde{\mu}_2 - \tilde{\mu}_1)nF/RT$)

$$\ln(J_{12}/J_{21}) = (\tilde{\mu}_2 - \tilde{\mu}_1)nF/RT$$

and

$$\ln(J_{23}/J_{32}) = (\tilde{\mu}_3 - \tilde{\mu}_2)nF/RT.$$

Thus

$$\ln(J_{13}/J_{31}) = (\tilde{\mu}_3 - \tilde{\mu}_1)nF/RT.$$

In other words, the number n, which indicates the number of the potassium ions in single file, is the same as one could have found when studying a single membrane. In the giant axons Begenisich and De Weer (1980) demonstrated that n might be a function of potential and possibly of internal K^+ concentration. However, in the Ca^{2+}-activated K^+ channel in red blood cells such a relation could not be detected (Vestergaard-Bogind et al., 1985).

With identical channels, any difference in K permeability of the two membranes has no influence on the final result obtained for n. This is, however, not the case if only one of the membranes shows single-file transport. To simplify the problem only two types of pathways will be considered: one membrane has channels that show single-file transport and the other membrane has channels, where ion movement is described by the flux ratio equation for electrodiffusion.

Table 2.

Solutions	$V_m - V_K$ (mV)	Influx (neq/cm²hr¹)	Efflux (neq/cm²hr¹)	
Outside KCl-R	39	47.6	1.14	
Inside NaCl-R	26	70	6	
	28	24	1.7	
	28	91	4.4	
Outside Choline-R	−22	23	304	Steady state
Inside KCl-R	−27	14.5	411	Steady state
Outside KCl-R	−30	81.5	590	
Inside KCl-R	30	482	24	
Outside K_2SO_4-R	−30	13	230	Steady state
Inside K_2SO_4-R	−30	29	474	Steady state

The table shows that the net fluxes of K^+ are in the direction of the driving potential. Column 2 gives the driving potential across the skin, column 3 the unidirectional influx, and column 4 the unidirectional efflux. When indicated, it is steady-state values, otherwise it is the last experimentally determined flux value.

One possibility would be that the transport across the rate-limiting barrier was simple electrodiffusion and single-file transport occurred in the other membrane. If the ion is practically at equilibrium across the "single-file barrier," the flux ratio would be close to one and single-file transport may not be detected by the present method. Our experiments do not indicate such a relation.

Another possibility is that single-file transport occurred in the rate-limiting barrier. If equilibrium for the ion in question existed across the barrier with simple electrodiffusion, the exponent n across the whole skin would be identical to the one across the rate-limiting barrier. The combination is in fact a possibility for the present system. It is well known that the resistance of the apical membrane in the frog skin is higher than the resistance of the basolateral membrane. This may also be the case when K^+ replaces Na^+ in the bathing solutions. Furthermore, the resistance of the basolateral membrane can be eliminated by replacing Na^+ in the serosal solution with K^+. Since the experiments with high K^+ in the serosal solution show a value for n higher than 2, it can be concluded that the potassium channels in the apical membrane show single-file behavior.

A similar conclusion for the K^+ channels in the basolateral membrane, when K^+ replaces Na in the outside bathing solution, cannot be drawn. Even under these conditions the K^+ conductance in the apical membrane may be lower than K^+ conductance in the basolateral membrane. Any identity of the K^+ channels in the two membranes should be solved by other means and must await further investigations. It is, however, tempting to propose that identical K^+ channels are incorporated into the two membranes, which would also be fully compatible with our observations.

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Note Added in Proof

Cox and Helman (*J. Gen. Physiol.* **87:**485–502, 1986) have shown that K^+ transport in the basolateral membrane proceeds through single-file channels with an n equal to 2.9.