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Evaluation of Transport Pathways for Na^+ across Frog Skin Epithelium by Means of Presteady-State Flux Ratio

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Summary. A method is described that makes it possible to separate the sodium fluxes through the isolated frog skin into sets characteristic of the cellular and the paracellular pathway, respectively. If there are two significant pathways for the ion, and if they differ with respect to flux ratio as well as mean passage time, the flux ratios for the individual pathways can be obtained from a set of inward and outward tracer fluxes, covering the time from the addition of the tracers until the achievement of constant fluxes in both directions.

Key Words flux ratio · frog skin · isotopes · sodium

Introduction

If the movement of an ion across a membrane is the result of electrodiffusion, the ratio between its oppositely directed unidirectional fluxes is solely determined by the difference between its electrochemical potentials in the two bathing solutions. The relation between the unidirectional fluxes, measured with radioisotopes, and the electrochemical potential difference across the membrane is described by the flux ratio equation, which was derived by Ussing (1949, 1978) and Teorell (1949). The equation is valid not only for homogeneous membranes but also for barriers where conductance and potential vary in an arbitrary fashion along the transport path (Ussing, 1949, 1978).

In the original derivation of the flux ratio equation it was assumed that the unidirectional fluxes had to be steady-state fluxes, an assumption that is readily fulfilled if the unidirectional fluxes for the ion in question are measured across a single lipid membrane. But for a system consisting of multiple layers, as is the case for an epithelium, it can take several minutes until steady state is obtained. Recently it has been shown by Sten-Knudsen and Ussing (1981) that the flux ratio equation is valid even when the unidirectional fluxes are measured before the isotope concentration profile through the system is stationary. The flux ratio equation under nonstationary conditions was derived for a system which consists of layered barriers of different composition and with arbitrary but time-invariant potential and resistance profiles. Apart from extending the flux ratio equation to include presteady-state unidirectional fluxes, the analysis also shows that irrespective of the mode of transport, whether it is electrodiffusion, solvent drag or active transport, the presteady-state flux ratio is identical to the steady-state flux ratio. The analysis implies that if a substance can cross a membrane, say an epithelium, along different pathways which at the same time exhibit different flux ratios and different time constants, i.e., mean passage times, then the flux ratio under nonstationary conditions will vary with time (Ussing, 1978; Sten-Knudsen & Ussing, 1981). This property of the flux ratio has been used in the present study to examine if sodium crosses the frog skin along more than one pathway. Furthermore, a mathematical treatment is presented, which makes it possible to separate the unidirectional fluxes of a given substance, assuming that the ion only crosses the skin along two different pathways. Part of this work has been published previously in preliminary form (Ussing, Eskesen & Lim, 1981).

Materials and Methods

Presteady-state flux ratio of the ionic species has to be determined from the measured unidirectional isotope fluxes before they have come to steady state, i.e., when the isotope concentration is being built up in the tissue.

The ideal way to perform the experiments would have been

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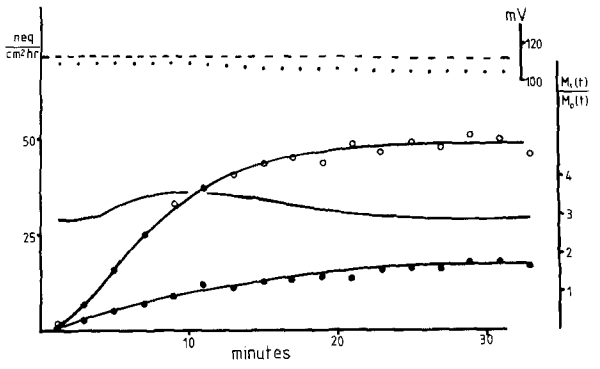

Fig. 1. Presteady-state influx (open circles), efflux (filled circles), and the flux ratio (full line without experimental points) for sodium. Influx and efflux were measured on paired halves of whole skin from *Rana temporaria*. The outside solution was 10 times diluted Ringer, and the inside solution normal Ringer. The dotted line is the spontaneous potential for the influx experiment, and the dashed line is the spontaneous potential for the efflux experiment. *Ordinate,* left: flux calculated in nanomoles per cm^2 and hour. *Ordinate,* upper right: skin potential; far right: flux ratio (influx/efflux). *Abscissa:* time in minutes

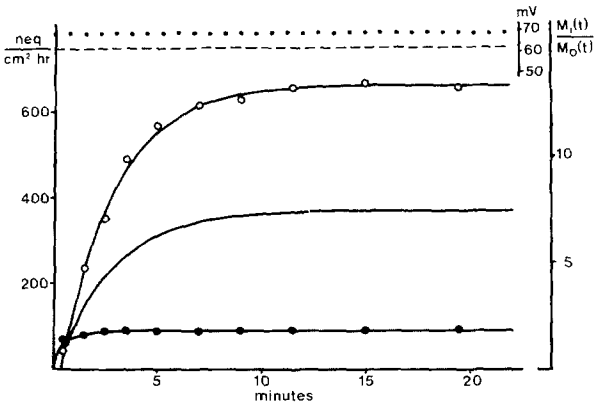

Fig. 2. Influx (open circles), efflux (filled circles), and flux ratio (full line without experimental points) for sodium. Efflux and influx were measured consecutively on the same piece of isolated epithelium. Both bathing solutions were normal Ringer. The dotted line was the spontaneous potential difference when influx was measured and the dashed line the potential difference when efflux was measured. *Ordinate,* left: flux calcuated in nanomoles Na per cm^2 and hour. *Ordinate,* right (top): potential difference; far right: flux ratio (influx/efflux)

to add the two different sodium isotopes ^{22}Na and ^{24}Na simultaneously on either side of the same skin and follow their appearance on the opposite sides.

However, this method has practical limitations, because the determination of small amounts of one isotope on a large background of another presents technical difficulties. Instead, two alternative methods were used. For whole skins, influx and efflux were measured simultaneously on each of two identical skin halves from the same frog. Since, however, two identical halves of isolated frog skin epithelium could rarely be obtained, efflux was first measured with ^{24}Na and subsequently influx was measured with ^{22}Na. Sodium-24 was allowed to decay before ^{22}Na was counted.

The experiments were performed on whole frog skins and on isolated epithelia of frog skins during the winter half of the year. The frogs (*Rana temporaria*) were kept in tap water at 5°C.

The whole skins were cut into symmetrical halves and mounted in identical flux chambers. Two types of chambers were used: either a conical type with air lifts for mixing (area 7 cm^2, volume 20 ml) (Koefoed-Johnsen, Ussing & Zerahn, 1952) or flat chambers with magnetic stirring (area 7 cm^2, volume 4.5 ml). The skins were allowed to stabilize with respect to potential and transport properties for 2 hr with the bathing solutions intended for isotope runs. The potential was monitored automatically during the experiments, and skin pairs that did not maintain constant and virtually identical potentials were discarded. Na influx and Na efflux, respectively, were measured simultaneously on one of the two skin halves. At time zero, tracer (^{24}Na) was added to one of the bathing solutions and samples were taken from the opposite solution at 2-min intervals for 30–40 min, until the fluxes had reached steady-state values. Each sample was replaced by an identical amount of isotope-free Ringer in order to keep the volumes constant during the run. The radioactivity of the samples and standards were determined in a Packard scintillation counter. Ringer solution used for whole skin (in mM): 113.4 Na^+, 1.9 K^+, 1 Ca^{++}, 115 Cl^-, 2.4 HCO_3^-.

Epithelia were isolated after collagenase treatment (Aceves & Erlij, 1971) as modified by Johnsen and Nielsen (1982). They were mounted in chambers with an exposed area of 1.5 cm^2. The bathing solutions (2.5 ml on each side) were stirred mechanically with a bent metal wire connected to a battery-driven motor. Vigorous stirring secured complete mixing and kept the solutions aerated without damage to the epithelia. The unidirectional efflux was measured first with ^{24}Na. The bathing solutions were then replaced by isotope-free Ringer, before the unidirectional influx was measured with ^{22}Na. Samples were taken every minute during the first 6 min after addition of isotope, whereafter the time between consecutive samplings was gradually increased until the steady-state unidirectional flux had been obtained. Each sample was replaced by isotope-free Ringer. At the end of each experiment a sample was withdrawn from the "hot side" for determination of the specific activity of that solution. Both isotopes were counted in a Packard scintillation counter. Ringer solution used for epithelium, "split-Ringer" (in mM): 115 Na^+, 2.5 K^+, 1 Ca^{2+}, 1 Mg^{2-}, 1 HPO_4^{2-}, 117 Cl^-, 2.5 HCO_3^-. pH was adjusted to 7.8 with NaOH. The hypotonic Ringer was prepared by diluting the Ringer to the desired Na-concentration with distilled water. Before use, glucose was added to all "split-Ringer" solutions to a final concentration of 5 mM.

Results

The figures are chosen in order to show the different types of results that have been obtained in these experiments and to illustrate how each type is interpreted. All experiments have been performed at open potential, which was recorded simultaneously. Fig. 1 shows an experiment which has been performed on whole skin with the apical side bathed in 1/10 normal Ringer. The serosal side was bathed in normal Ringer. The figure shows the

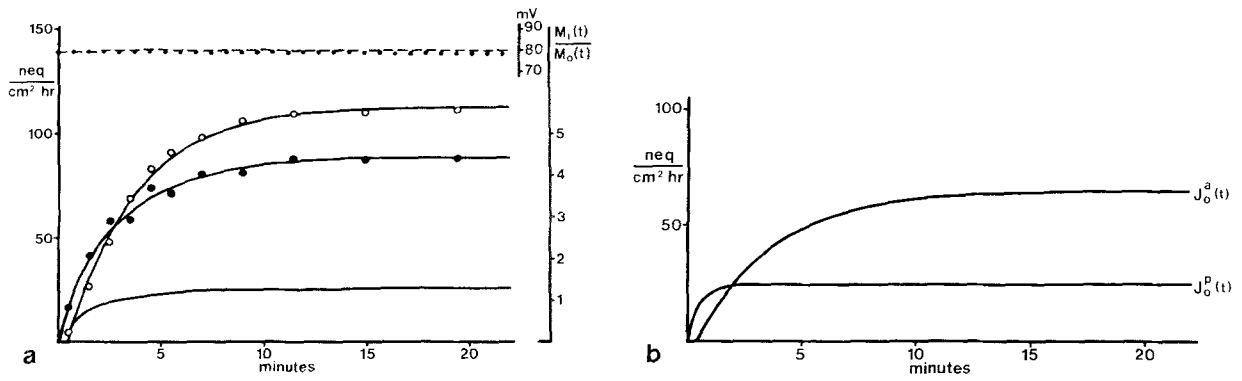

Fig. 3. (*a*) Influx (open circles), efflux (filled circles), and flux ratio (full line without experimental points) for sodium. Influx and efflux were measured consecutively on the same piece of epithelium. The outside solution was 10 times diluted Ringer, and the inside solution was normal Ringer. The dotted and dashed lines show the spontaneous potential difference during the influx and efflux experiment, respectively. *Ordinate*, left: flux calculated in nanomoles Na per cm^2 and hour. *Ordinate*, right (top): potential difference; far right: flux ratio (influx/efflux). (*b*) The two components in which the efflux curve from *a* can be separated. J_o^a was calculated from Eq. (10). $J_o^a(t) = J_o^a(1 - \exp(-k(t - t_o)))$, where $k = 0.300$ min^{-1} and $t_o = 0.46$ min. k and t_o were determined from the exponential fit program on the influx curve in *a* by nonlinear regression. $J_o^p(t) = M_o(t) - J_o^a(t)$

open-circuit potential of the two skin halves, the flux ratio and the experimentally determined unidirectional influx and efflux. The flux ratio is calculated from smooth curves drawn by eye through the points representing the experimentally determined unidirectional fluxes. The flux ratio is practically constant with time, indicating that, for that particular skin, there is one dominating pathway for sodium. Since net transport of sodium is against the electrochemical gradient, the pathway must be cellular, where sodium is transported actively. Such time-invariant flux ratios were observed for some skins, which showed very high spontaneous potentials. However, the majority of the experiments (8 out of 11) with whole skins showed a flux ratio which increased with time, and the same was the case for all isolated epithelia.

Figure 2 is an example of a flux ratio experiment on frog skin epithelium, where both bathing solutions are normal "split-Ringer." The experimental values for the time-dependent unidirectional influx are fitted to a monoexponential curve of the form $A + B \exp(kt)$ by the methods of least squares. The line through the experimental points for Na efflux was drawn by eye. The flux ratio was determined as above. In the experiment in question the flux ratio varies with time. Initially, it is less than one, and it increases toward an asymptotic value. In the upper part of Fig. 2 it can be seen that the open potential has remained stable throughout the experiment, and the conductance and the driving force through each barrier that sodium has to pass are therefore considered to be constant with time. For this reason the time dependence of the flux ratio indicates the existence of at least two different pathways with different mean passage times and different flux ratios. Extrapolation of the flux ratio to zero time gives a ratio much less than one, which is in agreement with a transport path where the process of electrodiffusion is dominating. This pathway is therefore considered to be extracellular. With time the flux ratio increases to a value that far exceeds (100 times) that predicted for electrodiffusion. This value is compatible with a flux through a cellular pathway, where sodium is transported actively. Comparison of the experimental curves of influx and efflux reveals that the unidirectional efflux has reached a steady-state value after about 3 min, at a time where the unidirectional influx is only about half maximal. It is therefore concluded that the transport path for the major part of sodium efflux is different from the transport path for sodium influx.

Figure 3 depicts another flux ratio experiment with Na on the isolated epithelium of the frog skin with hypotonic "split-Ringer" (1/10) at the apical side and normal "split-Ringer" at the basolateral side. Again the flux ratio increases with time, indicating that sodium can be transported along at least two different pathways with different mean passage times and different flux ratios. With the assumption that sodium can only cross the epithelium along two different pathways, it is, however, still possible to get an estimate of the flux through each of these from the experimental data given in the figure.

Mathematical Separation of Fluxes through Different Transport Pathways

A set of equations has been derived in order to separate fluxes through a "fast" pathway from fluxes

through a "slow" pathway. By a fast and a slow pathway are meant pathways with a shorter and longer passage time, respectively. As will appear from the derivation, the final equations can only be used under conditions where one can neglect the fraction of the unidirectional influx which is due to electrodiffusion. Other experimental conditions may require assumptions that lead to a different set of equations. To perform the calculations below it has not been necessary to use a computer. But an alternative way is to use a computer to fit the experimentally-determined time-dependent unidirectional fluxes to a series of functions which fulfill initial and steady-state conditions (*see below*). This method was used by Lim and Ussing (1982) to get an estimation of the fluxes through the different pathways in the rabbit corneal endothelium. Since the flux ratio for the individual pathways is time invariant, the time dependent unidirectional fluxes in the opposite directions through the fast pathway can be written

$$j_i^p(t) = J_i^p(1 - g(t))$$

and

$$j_o^p(t) = J_o^p(1 - g(t))$$

and, similarly, the time-dependent unidirectional fluxes through the slow pathway are

$$j_i^a(t) = J_i^a(1 - f(t))$$

and

$$j_o^a(t) = J_o^a(1 - f(t)).$$

$j_i^p(t), j_o^p(t), j_i^a(t)$ and $j_o^a(t)$ are the time-dependent unidirectional fluxes, and J_i^p, J_o^p, J_i^a and J_o^a are the unidirectional steady-state fluxes. i and o refer to influx and efflux. The notation p has been chosen for the fast pathway, since the experiments at time zero showed a flux ratio which was compatible with passive fluxes. Likewise a refers to the slow pathway, where the fluxes are active. $g(t)$ and $f(t)$ are arbitrary monotonic decreasing functions of t, that have to fulfill the following conditions:

$(g(0), f(0)) = (1,1)$ for $t \to 0$
and $(g(t), f(t)) = (0,0)$ for $t \to \infty$.

$g(t)$ and $f(t)$ may be exponential functions like $\exp(-kt)$ or combinations of such functions, but as will be seen later the solution is independent of the shape of $g(t)$ and $f(t)$. The experimentally measured time-dependent unidirectional influx $M_i(t)$ is given by

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

and, similarly, the time-dependent unidirectional efflux $M_o(t)$ is given by

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

$g(t)$, which is the time dependent function belonging to the fast pathway, approaches zero faster than $f(t)$. Rewriting Eqs. (1) and (2) for the period when $g(t) = 0$, while $f(t)$ still varies with time leads to the following

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

The flux ratio experiments have been performed under conditions where $J_i^p < J_o^p$, the latter again being smaller than J_i^a. Thus in Eq. (1a), J_i^p can be disregarded for values of t sufficiently large. The flux equations at $t = \tau$ and at steady state ($t = \infty$) can now be written:

$$M_i(\tau) = J_i^a(1 - f(\tau)) \tag{3}$$
$$M_o(\tau) = J_o^p + J_o^a(1 - f(\tau)) \tag{4}$$
$$M_i(\infty) = J_i^a \tag{5}$$
$$M_o(\infty) = J_o^p + J_o^a. \tag{6}$$

Equation (3) divided by Eq. (5) yields:

$$1 - f(\tau) = \frac{M_i(\tau)}{M_i(\infty)}. \tag{7}$$

Insertion of Eq. (7) into Eq. (4) gives

$$M_o(\tau) = J_o^p + J_o^a \frac{M_i(\tau)}{M_i(\infty)}. \tag{8}$$

J_o^p from Eq. (6) is inserted into Eq. (8)

$$M_o(\infty) - M_o(\tau) = J_o^a \left(1 - \frac{M_i(\tau)}{M_i(\infty)}\right) \tag{9}$$

which finally gives

$$J_o^a = \frac{M_i(\infty)(M_o(\infty) - M_o(\tau))}{M_i(\infty) - M_i(\tau)}. \tag{10}$$

From Eq. (6)

$$J_o^p = M_o(\infty) - J_o^a. \tag{11}$$

According to the above, J_i^p was negligible compared to J_i^a. But a value for J_i^p can be calculated (assuming electrodiffusion) from the flux ratio equation

$$J_o^p/J_i^p = (c_i/c_o)\exp((\psi_i - \psi_o)F/RT)) \quad (12)$$

where $\psi_i - \psi_o$ is the potential difference between inside and outside and c_i and c_o are the concentrations of the ion in the inside and outside medium, respectively.

Granted that the conditions for the derivation are fulfilled, Eq. (10) should give identical values for J_o^a irrespective of the time that is chosen. When the analysis is applied to experiments on whole skin, the most uniform values are obtained between 6 and 10 min. J_o^a is determined as the average of the values calculated from the smooth curves with 1-min interval in that period. On epithelium J_o^a is determined as the average from values calculated between $t = 3$ and $t = 6$ min with an interval of 0.5 min. Here it is assumed that the flux through the fast pathway has reached a steady-state value after 3 min. This time has been chosen, because total separation of the flux through a "fast" efflux pathway from the flux through a slow influx pathway, as in Fig. 2, shows that the efflux has reached a steady-state value after 3 min. When the method to separate fluxes is applied on the experiment shown in Fig. 3*a*, the two curves shown in Fig. 3*b* are obtained. They illustrate the time-dependent unidirectional efflux through the slow pathway, $j_o^a(t)$, and through the fast pathway $j_o^p(t)$.

Discussion

The only assumptions made in the derivation of the flux ratio equation under nonstationary conditions are that the transport parameters of the ionic species are constant during the experiment (Sten-Knudsen & Ussing, 1981). The fulfillment of this requirement is ascertained by equilibration of the skin with the appropriate bathing solutions for at least 1 hr. The open-circuit potential was monitored and only those experiments where the potential remained constant and practically identical for influx and efflux were used.

The method has been tested on skin as well as on isolated epithelium and the examples shown have been chosen so as to describe the essential aspects of the method. There are advantages as well as disadvantages associated with the two methods used for whole skin and epithelium, respectively. With respect to the method used for whole skin, one disadvantage is that influx and efflux are measured on paired skin halves and not on the same skin. In control experiments (parallel Na-influx and Na-efflux measured on paired skin halves) it could be shown that there was no significant difference between steady-state fluxes and between the time at which the fluxes are half maximal for paired skin halves. It may therefore be safe to state that the time-variant flux ratio is the result of at least two different transport pathways with different flux ratios just as well as a time-invariant flux ratio is due to one dominating transport pathway for the given ion. Besides the epithelial layer, the frog skin consists of a layer of connective tissue, corium, which is 2–3 times thicker than the epithelium and where the diffusion constant for Na is reduced compared to the diffusion constant in water (Hoshiko, Lindley & Edwards, 1964). This leads to an increase in the mean passage time for the ion to cross the skin compared with the mean passage time for crossing the epithelium. From this it follows that the relative difference in mean passage time for ions, which are moving along a slow and a fast pathway, will be reduced for whole skin in comparison to epithelium. It was therefore to be expected that the difference between the mean passage times of the two transport pathways could be more pronounced for isolated epithelium than for whole skins, and Figs. 2 and 3*b* show that this may indeed be the case. Isolated epithelia were discarded unless the open potential was practically the same before and after stripping, and remained high and constant throughout the experiment. That, nonetheless, isolated epithelia appear to be slightly more leaky than comparable skins can be seen by comparing individual values of the calculated Na conductance for the fast (extracellular) pathway in whole skin (1.2, 0.2, 0.4 and 0 μS cm^{-2}) with those of epithelium (0.9, 3.7, 4.9, 1.5, 5.6, 3.0, 10.6 and 4.5 μS cm^{-2}). In all the above experiments, the outside solution consisted of Ringer diluted 10 times.

As already mentioned, transport pathways with the same flux ratio, as, for example, paracellular pathways, pathways due to damaged cells, and cellular pathways exhibiting simple electrodiffusion, cannot be separated from each other by the present method, but this is immaterial if the purpose is to obtain the flux ratio for the active transport pathway.

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