

Determination of the Electromotive Force of Active Sodium Transport in Frog Skin Epithelium (*Rana temporaria*) from Presteady-State Flux Ratio Experiments

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Summary. The presteady-state influxes and effluxes of sodium across frog skin epithelium have been determined as a function of time while all electrophysiological parameters were maintained constant. The fluxes measured were resolved in the fractions which have passed a pathway through the cells and those that have used a paracellular pathway. The procedure is based on the theory that all presteady-state flux ratios have to be equal to the steady-state flux ratio if only one pathway is involved. The flux ratios for the transcellular route were used to calculate the electromotive force of the sodium pump. The calculation hinges on the assumptions (a) that both influx and efflux have to pass through the sodium pump and (b) that single file diffusion of sodium is not taking place anywhere along the path. The validity of both assumptions is discussed. Our calculated values for the electromotive force of the sodium pump E_{Na}^a vary between 146 and 200 mV, which is in agreement with the energy of the ATP/ADP system. There is a distinct indication that, as the electrochemical gradient for sodium opposing the transport is being increased, the emf increases towards an asymptotic value around 200 mV. The relation between the value of E_{Na}^a and the cellular phosphorylation potential for ATP is discussed.

Key Words E_{Na}^a · sodium · flux ratio · frog skin

Introduction

It is generally agreed that active sodium transport is energetically coupled to hydrolysis of ATP, with 3 sodium ions transported for every molecule of ATP being hydrolyzed.

In a preceding paper a method was described where the unidirectional fluxes of sodium through a cellular pathway of the frog skin could be separated from the unidirectional fluxes through a paracellular pathway (Ussing, Eskesen & Lim, 1981; Eskesen, Lim & Ussing, 1985). Thus, having the transcellular unidirectional fluxes at our disposal, it is possible to calculate the electromotive force of the active so-

dium transport mechanism, E_{Na}^a (Ussing et al., 1960), since E_{Na}^a is defined as

$$zFE_{\text{Na}}^a = RT \ln \frac{J_i^a}{J_o^a} + RT \ln \frac{(\text{Na})_i}{(\text{Na})_o} + zFV_m \quad (1)$$

where J_i^a is the unidirectional flux from the outside to the inside and J_o^a the unidirectional flux from the inside to the outside, $(\text{Na})_i$ and $(\text{Na})_o$ are the sodium activities of the inside and outside solutions, respectively. V_m is the potential difference across the skin, inside positive, and R , T , z , and F have their usual meanings. $RT \ln ((\text{Na})_i/(\text{Na})_o) + zFV_m$ represents the part of the energy from the active transport process that is conserved as electrochemical work of ion transport. Assuming that exchange diffusion or single filing do not participate in Na translocation, then the energy which is dissipated by this process is given by the flux ratio term. It is a measure of the electrochemical potential necessary to drive the net sodium current $F(J_i^a - J_o^a)$ through the internal resistance of the cellular pathway, including the pump (Ussing & Zerahn, 1951). Since FE_{Na}^a represents the work required to transport one mole of sodium ions across the skin, then $3FE_{\text{Na}}^a$ should be smaller than or equal to that of the free energy change of hydrolysis of ATP of the intracellular medium. The purpose of the present paper is therefore to calculate and compare the values for $3FE_{\text{Na}}^a$, with the values found in the literature for the free energy of ATP in order to see if the phosphorylation potential is large enough to be responsible for active sodium transport.

Theoretically E_{Na}^a might be measured as the potential which makes $J_i^a = J_o^a$. However, this requires applied potentials higher than 200 mV, which are not tolerated well by the skin. Instead E_{Na}^a has been measured under conditions where a net influx of sodium occurs. The experiments were performed under open circuit conditions, i.e., at skin poten-

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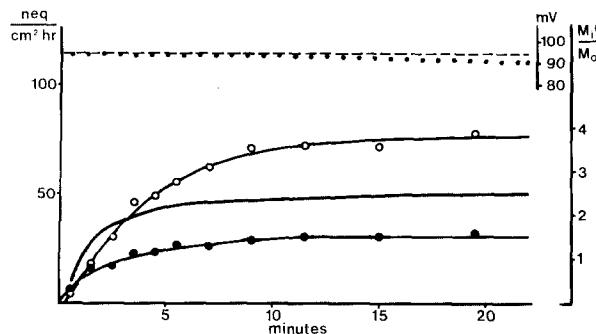


Fig. 1. An experiment on isolated epithelia with hypotonic Ringer (1/20) on the outside and normal Ringer on the inside. \circ , influx; \bullet , efflux. The curve without experimental points shows the flux ratio. At the top of the figure the transepithelial potential is shown. The experimental points of the influx curve is fitted to a monoexponential curve by the method of least squares. The influx is described by $M_i(t) = 76.74(1 - \exp(-0.2526(t - 0.3724)))$. The smoothed curve through the experimental points of the unidirectional efflux was drawn by eye. The value read from the efflux curve between 3 and 6 min were: 19.5, 20.8, 22.0, 23.1, 24.0, 24.7, 25.4 neq/cm² hr. This experiment gave a mean value for J_o^a of 20 neq/cm² hr

tials equal to those established spontaneously, and with three different sodium concentrations in the outside solution, while the inside solution was always normal Ringer.

Materials and Methods

Rana temporaria were kept at 5°C in trays with tap water. Since skins from these frogs maintain high and constant transepithelial potentials during the winter half of the year, all experiments were performed in that period. Paired skin halves or isolated epithelia were mounted in short-circuit chambers and incubated with appropriate Ringer solutions. The Ringers used for isolated epithelium differed slightly from these used for whole skin. For whole skin the serosal solution consisted of 111 mM NaCl, 1.88 mM KCl, 2.4 mM NaHCO₃, and 1 mM CaCl₂. For epithelia the serosal solution consisted of 111.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 1.0 mM NaH₂PO₄, 1.0 mM CaCl₂. pH of the latter solution was adjusted to 7.8 with NaOH. The mucosal solutions were either identical to the serosal solutions or hypotonic solutions where the serosal solution had been diluted with distilled water to 1/10 or 1/20 of the original concentration. 5 mM glucose were added to all solutions used for epithelia. The experimental procedure as well as the procedure used to separate the unidirectional sodium fluxes through the cellular pathway from the unidirectional fluxes through the paracellular pathway have been described previously (Ussing et al., 1981; Eskesen et al., 1985). The analysis requires that the time-dependent as well as the steady-state unidirectional fluxes in opposite directions are measured under conditions where the transport parameters (ionic conductances, concentrations, potential profiles, etc.) are kept constant (Sten-Knudsen & Ussing, 1981).

Table 1. Values from a presteady-state experiment on isolated epithelia with 1/10 Ringer on the outside and 1/1 Ringer on the inside^a

Time (min)	$M_i(t)$	$M_o(t)$ (neq/cm ² hr)	J_o^a
3.0	69.2	51.0	59.2
3.5	77.1	54.5	59.7
4.0	83.9	57.5	60.3
4.5	89.7	60.5	59.5
5.0	94.8	62.6	60.6
5.5	99.2	65.0	59.2
6.0	103.0	66.8	59.1
Mean of J_o^a			59.7

^a The time-dependent influx, $M_i(t)$, is described by the exponential curve: $M_i(t) = 127.1(1 - \exp(-0.2922(t - 0.3079)))$. $M_o(t)$ are values read from the smoothed efflux curve. In the table we have given those values which are needed to calculate J_o^a . The measured steady-state efflux $M_o(t) = 78$ neq/cm² hr.

DETERMINATION OF E_{Na}^a

Assuming that the Na-K-ATPase process is reversible and that the sodium pump is the only transport pathway for sodium in the basolateral membrane, we can determine the electromotive force of the active sodium transport (Ussing et al., 1960).

If the movement of sodium were purely passive, the flux ratio would be determined by the flux ratio equation

$$\frac{J_i^a}{J_o^a} = \exp(zF(V_{eq} - V_m)/RT) \quad (2)$$

where $V_{eq} = -\frac{RT}{zF} \ln \frac{(Na)_i}{(Na)_o}$, and V_m is the potential difference measured with respect to the outside solution. However, since sodium is transported actively, the flux ratio exceeds that predicted by Eq. (2), and a term, E_{Na}^a , must be included in the flux ratio equation to account for the electromotive force of the active transport mechanism. The flux ratio through the cellular pathway is then given by

$$\frac{J_i^a}{J_o^a} = \exp(zF(V_{eq} - V_m + E_{Na}^a)/RT). \quad (3)$$

By rearrangement E_{Na}^a can be obtained.

$$zFE_{Na}^a = \frac{RT}{zF} \ln \frac{J_i^a}{J_o^a} + \frac{RT}{zF} \ln \frac{(Na)_i}{(Na)_o} + V_m. \quad (4)$$

In our calculations of E_{Na}^a the activity of sodium has been calculated from Güntelberg's form of the Debye and Hückel equation.

Results

Figure 1 shows a presteady-state experiment from an isolated epithelium, where the outside solution

Table 2. The results of the individual experiments performed on whole skin bathed with normal Ringer on both sides^a

No. of exp.	Influx cellular	Efflux cellular	Efflux extra cell.	V_m	E_{Na}^a (mV)
1	372	139	0	131	156
2	184	39	1	115	154
3	637	146	0	118	155
4	201	77	34	101	125
5	194	37.4	13.5	110	152
6	345	17	23.4	73	149
7	235	69	14	102	133
Mean of E_{Na}^a				146	
Standard deviation				± 12	

^a E_{Na}^a have been calculated according to Eq. (4).

was 1/20 Ringer and the inside solution normal Ringer. The figure contains the measured unidirectional influx and efflux, the calculated flux ratio, and the measured transepithelial potential. The time-variant flux ratio reveals that sodium passes at least two separate pathways with different flux ratios and mean passage times. To separate fluxes through cellular and extracellular pathways we use the approach outlined in previous papers (Ussing et al., 1981; Eskesen et al., 1985). There we found that the steady-state efflux through the cells, J_o^a , was given by

$$J_o^a = \frac{M_i(\infty)(M_o(\infty) - M_o(t))}{M_i(\infty) - M_i(t)} \quad (5)$$

where $M_i(\infty)$ and $M_o(\infty)$ are the measured unidirectional steady-state fluxes and $M_i(t)$ and $M_o(t)$ are the influx and the efflux at time t . This equation was derived assuming that the influx through the paracellular pathway can be disregarded under the conditions of the experiment. The times for reading $M_i(t)$ and $M_o(t)$ are chosen so that the efflux through the fast pathway has come to a steady state, while the efflux through the slow pathway is still changing with time. This condition is considered to be fulfilled after 6 min for whole skin and the fluxes from 6 to 10 min with 1 min interval are read. For isolated epithelia equilibration is faster and the fluxes from 3 to 6 min with $\frac{1}{2}$ min are read. A mean of the calculated values of J_o^a was taken to be our cellular efflux. In Table 1 we have given an example of the calculations from another experiment, where the outside solution was 1/10 Ringer and the inside solution normal Ringer.

Tables 2, 3, 4, and 5 contain the results from the

Table 3. The results of the individual experiments performed on whole skin^a

No. of exp.	Influx cellular	Efflux cellular	Efflux extra cell.	V_m	E_{Na}^a (mV)
1	195	30.4	6.6	93	193
2	43	13.1	0.9	112	195
3	22	12	2.2	116	184
4	49	16.7	0	110	190
Mean of E_{Na}^a				191	
Standard deviation				± 5	

^a The Ringer on the apical side was diluted to 1/10 of normal Ringer and the serosal solution was normal Ringer.

Table 4. The results of the individual experiments performed on isolated epithelium from frog skin^a

No. of exp.	Influx cellular	Efflux cellular	Efflux extra cell.	V_m	E_{Na}^a (mV)
1	121.4	19	5	92	192
2	141	21	21	98	199
3	113.2	64	24	79	146
4	115.4	47	8	92	168
5	93	38	36	118	194
6	127.1	60	18	110	182
7	143.7	52	63	106	185
8	188.5	57	24	91	174
Mean of E_{Na}^a				180	
Standard deviation				± 17	

^a The Ringer on the apical side was diluted to 1/10 of the normal Ringer and the serosal solution was normal Ringer.

Table 5. The results of individual experiments performed on isolated epithelium, where the Ringer on the apical side was diluted to 1/20 of the normal Ringer and the serosal solution was normal Ringer

No. of exp.	Influx cellular	Efflux cellular	Efflux extra cell.	V_m	E_{Na}^a (mV)
1	76.7	20	10	93	197
2	59.7	13.8	6.2	93	200
3	86.3	12	17	84	204
Mean of E_{Na}^a				200	

individual experiments. Each table shows the measured sodium influx (active, column 2). The calculated sodium efflux through the epithelial cells (column 3) and the calculated extracellular sodium efflux. The latter may be due to fluxes through paracellular pathways or damaged tissue. Due to the large difference in electrochemical potential between inside and outside solutions, the passive (paracellular) influx can be considered to be negligi-

Table 6. Effect of amiloride on the unidirectional Na efflux with 1/10 Ringer as outside solution and normal Ringer as inside solution^a

Number	Na efflux (neq/cm ² hr)		V_m (mV)
	Before amiloride	After amiloride	
1	20	3	77
2	25	5	126
3	22	5	77
4	27	5	120
5	27	11	111
6	37	20	108
7	17	5	106

^a The experiments were performed at the given transepithelial potential.

ble and is not given in the tables. Column 5 shows the transepithelial potential difference and column 6 the calculated E_{Na}^a . The results in Tables 2 and 3 are obtained from the experiments on whole skin and in Tables 4 and 5 from experiments on split skin.

The electromotive force calculated when the bathing solutions contain identical Ringers is 146 ± 12 mV ($n = 7$). Replacement of the mucosal solution with hypotonic Ringer, 1/10 of the normal Ringer increased the calculated E_{Na}^a to 191 ± 5 mV ($n = 4$). The same type of experiment performed on isolated epithelium (Table 4) gives E_{Na}^a on 180 ± 17 mV ($n = 8$). The few successful experiments with 1/20 Ringer as the mucosal solution and the normal Ringer as the serosal solution give an average of E_{Na}^a of 200 mV. Thus the highest values for E_{Na}^a are calculated for the skins with the highest Na gradient.

INHIBITORS

The basis for our analysis is that the slow transport pathway for sodium efflux is identical to the transport pathway for active uptake. A considerable fraction of sodium efflux should therefore be inhibited with amiloride. In a series of experiments where the outside solution was 1/10 Ringer and the inside solution was normal Ringer the Na efflux was measured at the frog skin's spontaneous potential until steady state was obtained. 10^{-5} M amiloride was then added, and the skin was clamped to the potential it had before addition of amiloride. The results shown in Table 6 demonstrate that Na efflux is inhibited by amiloride. This supports our interpretation of the presteady-state experiments that under our experimental condition a Na efflux through the cells can be measured.

Addition of ouabain to toad (Larsen, 1972) and frog skin (Biber & Mullen, 1977, and own unpublished observation) under short-circuit condition results in increased efflux of sodium. Larsen proposed that ouabain changes the Na-K-exchange to Na-Na-exchange in analogy with the effect of ouabain in sheep red cells (Tosteson & Hoffman, 1960).

Discussion

In the early procedures for measuring E_{Na}^a it was assumed that all sodium ions followed one pathway (Ussing & Zerahn, 1951; Koefoed-Johnsen & Ussing, 1958). However, Ussing and Windhager (1964) pointed out that there may be a paracellular shunt, which sometimes is appreciable. Thus, earlier estimates of E_{Na}^a were lower than the true value. Different procedures have been proposed to determine the shunt conductance. Thus for sodium and chloride it can be calculated from tracer experiments (Ussing & Windhager, 1964), but other ions may contribute to the shunt conductance. Or, the cellular sodium conductance can be eliminated by the sodium channel inhibitor, amiloride (Ferreira & Hill, 1978). However, the chloride conductance of the epithelium is drastically reduced by this procedure (see Kristensen, 1978). In a previous paper (Eskesen et al., 1985) a method was described by which the part of the unidirectional sodium fluxes that pass through the cells can be separated from those that bypass them. The method does not affect the transport properties. This method is used here in order to determine the electromotive force for active Na transport. In the derivation of the relationship between flux ratio and E_{Na}^a we have made the assumption that sodium follows only one pathway through the cells and that each individual step along this pathway is governed by a process which can be described in terms of an equation like Eq. (1). Clearly, the assumption is correct for the part of the transport path covered by electrodiffusion through connective tissue, cytoplasm, etc. We must, however, discuss its validity for the passage of sodium through the cell membranes.

THE APICAL MEMBRANE

Lindemann and co-workers have shown that the transport of sodium across the apical membrane of the frog skin is restricted to specific structures, which behave as channels (Zeiske & Lindemann, 1974; Fuchs, Hviid Larsen & Lindemann, 1977; Lindemann & Van Driessche, 1978). If a channel is too narrow to allow ions to pass each other, but contains several binding sites, which may be occupied simultaneously, then the ions may proceed

through the channel in single file (Hodgkin & Keynes, 1955). Occurrence of a possible single file transport across the apical membrane would result in a flux ratio higher than predicted from the difference in electrochemical potential of the adjacent solutions, and thus the calculated E_{Na}^a would be higher than the electromotive force for active Na^+ -transport.

Recently, Benos, Hyde and Latorre (1983) found that the flux ratio exponent for sodium channels in the apical membrane of frog skin did not deviate significantly from 1. Thus they could exclude single filing of Na^+ . In our experiments the lowest E_{Na}^a were found at the highest sodium concentration, where the probability that more than one binding site in a transporting channel are occupied is highest. This makes it even more unlikely that single filing interferes with our determinations of E_{Na}^a .

THE BASOLATERAL MEMBRANE

In order to calculate E_{Na}^a it was assumed that the backflux of sodium through the cells was due to the reversal of the pump process, but it cannot be excluded that other mechanisms, involving transport of sodium, operate in parallel to the Na-K pump. The consequence of this would be that our method would yield a value for E_{Na}^a lower than the actual electromotive force for the pump process, because the total flux ratio across the basolateral membrane would then be lower than the flux ratio through the pump.

Two known pathways may bypass the pump in the basolateral membrane: Na-Ca counter transport, which operates to maintain the Ca level in the cells at a constant low level, (Taylor & Windhager, 1979), and NaCl cotransport, which is activated during volume changes in the epithelial cells (Ussing, 1982). The latter appears, however, to be dormant under normal conditions, since replacement of sodium with arginine or choline or addition of furosemide to the serosal solution does not change the cell volume. Na-Ca counter transport appears to play a significant role in epithelia in maintaining the Ca level low and though it seems to take 3 sodium to translocate 1 calcium out of the cell (Chase & Al-Awqati, 1981), it is not likely that this backflux of sodium ever reaches the same order of magnitude as is measured in our experiments, since the concentration of calcium both inside the cells and outside is in the order of 100 times less than the concentration of sodium. It has also been suggested that the basolateral membrane under certain conditions is permeable to free Na ions (see, e.g., Nielsen, 1984). But it may be argued that such a leak

must be small. From electrophysiological measurements (Nagel, 1976; Helman & Fischer, 1977) and measurements of sodium concentration (Rich et al., 1984) or sodium activities (Harvey & Kernan, 1984) it can be concluded that the electrochemical potential difference for sodium across the basolateral membrane is higher than 140 mV. An electrochemical potential difference of that size can only be maintained if a leak for ionized Na across the basolateral membrane is insignificant compared to the pump flux. It is therefore conceivable that the bulk of the back flux of sodium is through the Na-K pump.

PROPERTIES OF THE PUMP

In human red cells the pump process has been shown to be reversible by incorporation of inorganic phosphate into adenosine triphosphate (Garrahan & Glynn, 1967a; Glynn & Lew, 1970). It is therefore reasonable in this examination to assume that the Na-K-ATPase reaction is a reversible chemical process, which is governed by the law of mass action. Chemical reactions that obey the law of mass action are described by an equation like Eq. (1). But if the transport process involved exchange diffusion or single filing, the resulting flux ratio would not be a measure of the electrochemical potential necessary to drive the given current. In red blood cells extracellular Na^+ has been shown to exchange with cellular Na^+ through the pump (Garrahan & Glynn, 1967b). This property is, however, strongly inhibited by K^+ in the medium. The $K_{\frac{1}{2}}$ of cellular Na^+ for Na-Na exchange is between 15–20 mm (Garrahan & Glynn, 1967c). Since this value is a good deal above the values for cellular Na^+ in frog skin and K^+ is included in all our Ringers, Na-Na exchange through the pump is considered to be insignificant. Single filing in the direction of the driving force should be considered, if our calculated energies of E_{Na}^a were larger than expected from published values for hydrolysis of ATP (606 mV/mol ATP, Veech, Lawson, Cornell & Krebs, 1979). But this appears not to be the case and Eq. (1) is therefore considered to hold under all our experimental conditions.

According to definition then, E_{Na}^a is the energy which concerns the transport of sodium only (Ussing & Zerahn, 1951). But the transport brought about (catalyzed) by Na-K-ATPase involves recycling of potassium besides net transport of sodium (Nielsen, 1979). The amount by which $3FE_{\text{Na}}^a$ deviates from ΔG_{ATP} consequently depends on how much energy is needed to translocate potassium across the basolateral membrane. From simultaneous measurements of the activity of cellular po-

tassium and membrane potential Harvey and Kernan (1984) found an E_K of -91 mV and a membrane potential of -81 mV. Comparing electron microprobe measurements of cellular K concentration with membrane potential measurements also indicates that potassium is close to equilibrium (Rick, Dörge, von Armin & Thurau, 1978; Rick et al., 1984; e.g., Nagel, Garcia-Diaz & Armstrong, 1981). It appears therefore that the energy required for potassium recycling is small.

In our experiments we found that the average value for E_{Na}^a was 146 mV when the skin was bathed with normal Ringer on both sides, but 180 – 190 mV when the sodium gradient was increased by dilution of the outside Ringer to $1/10$ of normal Ringer. In the latter case the net transport of sodium seems to be reduced. Finally with $1/20$ Ringer as outside medium E_{Na}^a was 200 mV and net transport very low. In cytoplasmic medium ΔG_{ATP} has been determined to be 58.5 kJ/mol ATP, which when calculated in volts gives 606 mV/mol ATP (Veech et al., 1979). The values we calculate for $3FE_{Na}^a$ are either lower than or approximately equal to this theoretical maximum. ΔG_{ATP} is therefore large enough to drive the sodium transport.

Above we considered the modes of transport which may make the calculated E_{Na}^a different from the electromotive force of the active transport mechanism, and we found support for the view that these mechanisms did not interfere to any great extent with the determination of E_{Na}^a . The difference in calculated E_{Na}^a between the experiments with normal Ringer and those with hypotonic Ringer is therefore best explained by a change in phosphorylation potential, as suggested by Canessa, Labarca, DiBona and Leaf (1978). This viewpoint is in line with the well-known fact that the respiratory rate is regulated by the extramitochondrial ratio between ATP and ADP and P_i (Wilson, Owen, Mela & Weiner, 1973; Holian, Owen & Wilson, 1977). When the experiments are performed under conditions with a large net Na transport as with a high Na concentration in the outside medium, then a large amount of ATP is hydrolyzed, ATP decreases, and ADP and P_i increase with the result that ΔG_{ATP} decreases, which is in agreement with a lower value for E_{Na}^a . Net sodium transport is low and only a little ATP is hydrolyzed due to the operation of the pump when the experiments are performed under conditions where the sodium gradient across the skin is high. ΔG_{ATP} is higher and we would measure a higher E_{Na}^a . The maximal value for E_{Na}^a is, of course, obtained when the net-flux of sodium through the pump is zero.

Very early it was realized that a tight coupling existed between metabolism and active transport.

Zerahn (1956) and Leaf and Renshaw (1957) found that in frog skin 18 Na ions were transported for every oxygen molecule reduced. This ratio was found irrespective of the work that had to be performed on sodium to move it from the outside medium to the blood side. These observations have recently been confirmed on toad bladder by Canessa et al. (1978). If this is compared with the findings that the rate of oxygen consumption is regulated by the phosphorylation potential in such a way that a low phosphorylation potential stimulates the respiratory rate, it can be taken as an indirect evidence that the measured E_{Na}^a must be lower when the transport rate is high. As the net transport of sodium approaches zero (see Table 4) the measured 200 mV (57.7 kJ/mol ATP) for E_{Na}^a is practically equal to its theoretical maximum (Veech et al., 1979).

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