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OXYGEN CONSUMPTION BY FROG SKIN AND ITS ISOLATED EPITHELIAL LAYERS AS A FUNCTION OF THEIR SODIUM-TRANSPORTING ACTIVITY*

GABRIELA NOÉ, AGNÈS MICHOTTE and JEAN CRABBÉ

Endocrine Unit, Department of Physiology, University of Louvain (U C L) Medical School, B-1200 Bruxelles (Belgium)

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SUMMARY

The metabolic cost (in terms of oxygen consumption) of transcellular sodium transport was assessed on ventral frog skin and its isolated epithelial layers, by measuring the decrease in oxygen consumption by the tissue upon transient withdrawal of sodium from the outside solution. The same number of sodium ions was transported per molecule oxygen consumed by whole skin (17.4±2.3) and its isolated epithelium (17.3±2.4).

The metabolic cost of sodium transport could not be estimated properly when this process was blocked by amiloride or ouabain, as these drugs were found to bring about an increase in oxygen consumption by the tissue when no sodium was available for transport.

INTRODUCTION

Soon after the active nature of sodium transport by epithelia such as frog skin had been established on a rigorous basis [2], studies were undertaken to evaluate the contribution of the preparation's metabolism to this process. Thus Zerahn [3] found that 16–23 equivalents of sodium were transported across the ventral skin of *Rana temporaria* and *Rana esculenta* per mol of oxygen consumed, irrespective of the season and of sodium concentration on the outside. This investigator assumed that the tissue aerobic metabolism consisted of the sum of sodium transport-related and sodium transport-independent oxygen consumption; he determined the latter fraction after temporary withdrawal of sodium ion from the solution on the outside.

Vieira et al. [4] reported more recently a mean ratio of almost 15 for the ventral skin of *Rana pipiens*; they obtained this proportionality from the regression analysis of data corresponding to spontaneous variations in the rate of sodium transport by a given preparation vs. fluctuations in its aerobic metabolism. However, this approach

* A preliminary account of these studies has been published [1]

is of limited practicality when the preparations are functionally stable – which, needless to say, has very often distinct advantages.

The present study was meant to evaluate the relative advantages of different ways of determining the metabolic cost of sodium transport in the absence of hormonal stimulation, giving preference to manipulations exerting clear-cut, rapid, yet reversible effects When sodium transport was reduced to zero by temporary removal of the ion from the incubation solution on the outside, the metabolic cost of sodium transport could be (apparently) adequately evaluated When, however, blockade resulted from the presence of amiloride on the outside [5] or ouabain on the inside [6], residual oxygen consumption was unexpectedly high, for reasons that have as yet not been elucidated

Frog skin epithelium, isolated from underlying structures, was found to behave like whole skin in terms of the coupling between aerobic metabolism and sodium-transporting activity.

MATERIAL AND METHODS

Frogs (*Rana esculenta*) were maintained unfed in running tap water at 5–10 °C for less than 2 months prior to sacrifice. After pithing, the ventral skin was freed by dissection and transferred to Ringer's fluid (NaCl, 115 mM; $KHCO_3$, 2.5 mM; $CaCl_2$, 1 0 mM) at room temperature prior to studies which were conducted in all-glass conical chambers (incubation area: 4 cm^2; fluid contents: 2×5.8 ml) equipped with inlets and outlets for circulation of solution, with inlets for electrical potential measurement and short-circuiting [2] and for insertion of oxygen electrodes.

Similar studies were conducted on sheets of epithelium isolated from skin by a procedure combining mechanical and enzymatic treatment [7, 8].

A Clark-type polarographic electrode (Eschweiler) was used, its platinum tip covered with a fluorocarbon film (Teflon) 25 μm thick, supported on a Lucite holder. The polarization voltage was 0.6 V, corresponding to the middle of the zone characterized by complete reduction of oxygen presenting to the electrode. In these conditions, the output of the electrode in air-equilibrated Ringer's solution is 17.5 nA, which current is measured as a potential across a precision receptor (Vibron Electrometer) and recorded as a function of time on a strip chart recorder.

During the oxygen consumption measurements, which lasted for 20–40 min, circulation of the solution was interrupted and the contents of the incubation system were mixed by means of motor-driven magnets located at the top of the conical chambers Concomitantly, the preparations were maintained at zero potential by manual adjustment of current every 10 min at least. The incubation chambers were immersed in a water bath kept at a temperature of 24 °C$\pm$0.5 °C.

Despite the normal precautions, rates of lowering of oxygen tension in the closed system were larger than expected. They occurred even in the absence of tissue and were highest after flushing the system maintained under nitrogen with fresh, aerated Ringer's, suggesting an absorption phenomenon, probably on account of the electrode itself [9]. After a rapid drop over the initial hour, these rates remained almost stable, averaging 11.4 pmol $O_2 \cdot cm^{-2} \cdot s^{-1} \pm 1.8$ (S E.) ($N = 16$) for the 4 electrodes used through a 15-month period. Thus measurements were never started unless at least 1 h of exposure of the electrodes to oxygen had elapsed. (Between

experiments, they were maintained under tension in oxygen-free solutions.)

Prior to each incubation, the isolation of the chamber was ascertained by checking that pO_2 was zero after bubbling nitrogen (instead of air) in the circuit; and the linearity of the response of the electrodes to pO_2 was examined upon further aeration with 10 % O_2 (in 89.94 % N_2 and 0.06 % CO_2, v/v) and atmospheric air (20.94 % O_2).

At 24 °C and for a barometric pressure of 760 mmHg, there are 28 3 μl oxygen dissolved per ml Ringer's [10] Barometric pressure was supposed constant at 760 mmHg

Suppression of sodium-transporting activity was achieved reversibly by substituting Ringer's fluid with solutions in which NaCl was replaced with choline chloride (115 mM), magnesium chloride (57.5 mM, plus 57.5 mM sucrose) or sucrose (230 mM); or by introduction of amiloride (3,5-diamino-6-chloropyrazinoyl guanidine HCl) in incubation solution on the outside, at a final concentration of 10^{-5} M. Addition of ouabain, 10^{-5} M, to incubation fluid on the inside resulted in profound, essentially irreversible inhibition of sodium transport [11].

All incubation solutions were stored in the cold and filtered over Millipore (0.22 μm filters) so as to stem bacterial proliferation. For the same reason, antibiotics were added to Ringer's solutions: kanamycin on the inside (final concentration: 50 μg/ml) and chloromycetin* on the outside (final concentration: 1 mg/ml). As an additional precaution, incubation cells were stored in 2 % acetic acid.

To minimize residual problems arising from bacterial contamination and from oxygen uptake by the electrodes, experiments were conducted by interpolation whenever possible, i.e.: reference conditions were applied before and after a given manipulation, and the differences arising from the latter were dealt with. Measurements were carried out only after the preparations had stabilized in the conditions selected; thus the time elapsing between control periods bracketing the experimental ones was usually in excess of 1 h.

RESULTS

1. Metabolic cost of sodium transport by frog skin vs. epithelium

a) Whole frog skin. After oxygen consumption and sodium transport had been measured in reference conditions, Ringer's fluid was temporarily replaced on the outside of frog skin with sodium-free solution (see preceding section for composition) and the changes in aerobic metabolism resulting from suppression of sodium transport by mere withdrawal of the ion, were assessed. Table I summarized the pertinent data: a mean of 17.4±2 3 (S.E) sodium ions transported per molecule oxygen consumed was arrived at; there were sizable individual variations however, range extending from 5 to 35 for individual ratios.

As can be seen on Table I, the preparations were moderately stable since the decrease in sodium-transporting activity over the experimental period averaged 20 % of the initial value, i.e. 71±32 (S.E.) pmol $Na^+ \cdot cm^{-2} \cdot s^{-1}$ ($P < 0.05$). The small amplitude of this decrease is no doubt the reason why calculation of the metabolic cost

* This antibiotic, quite efficient in the conditions selected, altered the electrical properties of frog skin when present on the inside This was not so with kanamycin [12]

TABLE I

OXYGEN CONSUMPTION AND SODIUM TRANSPORT BY FROG SKIN AND ITS EPITHELIAL LAYERS TEMPORARY INHIBITION UPON REPLACEMENT OF EXTERNAL SODIUM BY MAGNESIUM

The experiments with the whole skin were conducted in December through June, with the epithelial layers, in May through July J_r, oxygen consumption in the compartments corresponding to the outer ($J_r$out) and inner ($J_r$in) surfaces of the frog skin J_{Na}, net sodium transport, derived from short-circuit current $\Delta\Psi$, electrical potential difference, in mV Usually, 15–30 min were allowed upon changes of incubation solution before the measurements were carried out (over 20–40 min) as summarized above Values are means ±S E J is given in pmol cm^{-2} s^{-1}

	Whole skin ($N = 15$)	Epithelial layers ($N = 8$)
Control		
$J_r$out	39 5± 2 6	35 3± 3 1
$J_r$in	29 2± 2 3	28.8± 2 5
J_{Na}	342 ±58	390 ±40
$\Delta\Psi$	53 9± 7 5	39 3± 6 0
Magnesium		
$J_r$out	24 4± 1 8	18 0± 1 6
$J_r$in	19 4± 1 7	15 2± 1 7
Recovery		
$J_r$out	31 1± 1 8	22 7± 1 3
$J_r$in	24 1± 2 2	20 3± 1 5
J_{Na}	271 ±45	239 ±34
$\Delta\Psi$	46 6± 5 4	20 2± 5 2

of sodium transport based on these "spontaneous" changes [4] led to a meaningless figure, of 3.1±4.8 (S E) sodium ions transported per molecule oxygen consumed.

There generally was more oxygen drawn from the solution on the outside of the skin, whether or not sodium was available for transport. When the changes in oxygen consumption resulting from temporary withdrawal of sodium from the outside are examined, so as to obviate the technical problems outlined in the Methods section, 60 % of the oxygen used in relation with sodium transport were found to originate from the outside (Table I), in keeping with published data [4].

Residual oxygen consumption (for zero sodium-transporting activity), could also be derived from the regression equation describing for all preparations the relationship between aerobic metabolism and sodium transport. This equation reads: $Y = 43.9+0.059\,X$ ($r = 0.736$) when Y stands for aerobic metabolism, in pmol $O_2 \cdot cm^{-2}$ s^{-1} and X, for net sodium transport, in pmol $Na^+ \cdot cm^{-2} \cdot s^{-1}$. There thus was agreement between the measured (43.8 pmol $O_2 \cdot cm^{-2} \cdot s^{-1} \pm 3.1$ (S.E)) and the calculated values.

b) Frog skin epithelial layers. If the part played by the oxygen-sensing electrodes is considered, aerobic metabolic needs for skin activities unrelated to transcellular sodium transport are likely to be quite low. This was evaluated directly by carrying out incubations with epithelial layers isolated from underlying structures normally present in frog skin preparations.

When the repercussions of the temporary removal of sodium from the outside

were evaluated, so as to arrive at an estimation of the metabolic cost of sodium transport, a mean of 17.3±2.4 (S.E.)Na^+ ions transported per molecule oxygen consumed was obtained (Table I). Thus isolated epithelium behaves in this respect like whole skin, save perhaps for a narrower range (8 to 31) for individual ratios.

All relevant data included in Table I were pooled for regression analysis, and oxygen consumption and sodium-transporting activity were again found to be statistically significantly related ($r = 0.674$), the regression equation reading as follows: $Y = 27.5+0.083\ X$, with X and Y having the dimensions stated above. Furthermore, as was the case with whole skin, residual oxygen consumption measured in the presence of magnesium on the outside (33.2 pmol $O_2 \cdot cm^{-2} \cdot s^{-1} \pm 3.1$ (S.E.)) corresponds reasonably well to the value derived from the regression analysis: the calculated value was only 17 % (and not significantly) lower than the measured one

When correction for the electrodes' contribution is applied, the residual oxygen consumption in epithelia measured during exposure to sodium-free solution, was one half of the value with whole skin. Both compartments of the incubation chambers contributed almost equally to the decrease, which is somewhat surprising.

Sodium-transporting activity of isolated epithelium preparations was less stable than that of intact skin; the decline averaged 151±23 (S E.) pmol $Na^+ \cdot cm^{-2}$ s^{-1} ($P < 0.001$) over the experimental period; this drop was twice as pronounced as in the case of whole skin (Table I). Therefore the ratio of the change in sodium-transporting activity to that in aerobic metabolism could be calculated for each preparation; a mean of 9.2±2.5(S.E.) sodium ions transported per molecule of oxygen consumed was arrived at, with individual values scattered over a 10-fold range (from 2 5 to 25) Aside from a large difference between this value and that of 17.3 dealt with above, there was no systematic relationship for any given preparation between the ratios calculated by the two approaches.

2 Repercussions of drug-dependent blockade of sodium transport on aerobic metabolism of frog skin

As has been said, sodium-free solutions were used to manipulate reversibly the

TABLE II

EFFECT OF AMILORIDE ON OXYGEN CONSUMPTION BY FROG SKIN

Only the total oxygen consumption is considered here Usually 15–30 min were allowed upon changes of incubation solution before the measurements (carried out over 20–40 min) summarized above Results are given in pmol $O_2 \cdot cm^{-2}$ $s^{-1} \pm$S E

Incubation conditions						
Sodium (mM)		N	Control	Amiloride	Recovery	Effect of amiloride
Outside	Inside					
115	115	13	58 3±4 8	51 7±3 6*	57 5±4 4	−6 2±2 9 ($P = 0$ 05)
0	115	3	34 9	43 6	32 8	+9.8±3 3 ($P < 0.1$)
0	0	6	21.4±7 9	31 2±6 7	26 4±5 5	+7 3±2 1 ($P < 0$ 02)

* Residual sodium transport derived from short-circuit current, averaged 26±4 (S.E) pmol · cm^{-2} s^{-1} Corresponding values in control and recovery conditions were 365±48 and 309±34, respectively, the mean decrease (15 %) was thus comparable to that seen after temporary exposure to sodium-free Ringer's (Table I)

TABLE III

EFFECT OF OUABAIN ON OXYGEN CONSUMPTION BY FROG SKIN

Total oxygen consumption is considered only Results are given in pmol O_2 cm^{-2} s^{-1} The measurements with ouabain were carried out during the third half-hour which followed introduction of ouabain At that stage, residual sodium transport derived from short-circuit current, averaged 44 ± 9 pmol Na^+ cm^{-2} s^{-1}, corresponding values in control conditions were 372 ± 65

Incubation conditions					
Sodium (mM)		N	Control	Ouabain	Effect of ouabain
Outside	Inside				
115	115	7	51 6±6 3	53 0±6 7	+ 1 5±5 1 (ns)
0	115	5	37 9±6 2	57 6±12 4	+19 7±6 9 ($P < 0$ 05)
0	0	5	25 2±6 6	35 8±9 5	+10 5±5 6 ($P < 0$ 2)

rate of transepithelial sodium transport. This could also be realized by addition of amiloride to Ringer's fluid on the outside. Oxygen consumption was less depressed in these conditions than after withdrawal of sodium (Table II). More significant perhaps: when amiloride was added to sodium-free Ringer's fluid, oxygen consumption increased, and this reversibly; this was confirmed (and established statistically) even after sodium had been removed from both incubation compartments. Oxygen consumption increased mostly (75 % of total) inside the preparations.

These unexpected results prompted an appraisal of ouabain as another pharmacological means whereby sodium transport-dependent oxygen consumption is supposedly dissociated from basal aerobic metabolism of frog skin. As seen in Table III, residual oxygen consumption by frog skin could not be depressed with this cardiac glycoside. Furthermore, treatment with ouabain of preparations incubated in sodium-free solutions resulted in an increase in aerobic metabolism. Since inhibition of sodium transport in these conditions is almost irreversible, no data could be provided for a recovery phase.

It is noteworthy that substitution of magnesium for sodium on both sides of the skin led to a further reduction of the latter's aerobic metabolism, when the metabolic activity of preparations exposed to this solution on the outside only is considered (Tables II and III).

DISCUSSION

The data presented indicate that temporary withdrawal of sodium from the solution from which the ion is transported by frog skin, provides a convenient means for estimation of the metabolic cost of sodium transport Indeed, the value of 17 sodium ions transported per molecule oxygen consumed is in good agreement with published data for control preparations [3, 4] as well as for frog skin exposed to antidiuretic hormone [3, 13, 14]. Furthermore, the value for residual aerobic metabolism of the preparations derived from regression analysis relating oxygen consumption to sodium transport, was the same as that measured after sodium was withdrawn from the solution on the outside.

Magnesium was generally substituted for sodium in the present experiments;

this ion thus appears a reliable non-toxic, non-transported substitute for sodium, allowing rapid, complete recovery of the preparation after removal*. Furthermore, when the outside-facing surface of frog skin was exposed temporarily to choline chloride ($N = 2$) or sucrose ($N = 4$) instead, residual oxygen consumption was the same as when magnesium chloride was used (mean difference: $7\,8 \pm 6.7$ pmol $O_2 \cdot cm^{-2} \cdot s^{-1}$). Another indication that exposure to magnesium Ringer's on the outside was harmless is provided by the fact that the relationship between aerobic metabolism and sodium transport by frog skin was the same before and after this manipulation, as shown by covariance analysis ($F = 1.15$).

Isolated frog skin epithelium behaved exactly like whole skin in all these respects

An important aspect of such studies lies in the definition of characteristics of individual preparations: Vieira et al. [4] had drawn attention to a large variability in the metabolic cost of sodium transport. This is in essence confirmed by the present set of data in view of the observed range for individual values (1 · 7 for whole skin; 1 : 4 for isolated epithelium), but the constancy of the ratio arrived at for a given preparation was not systematically examined here**.

When amiloride (on the outside) or ouabain (on the inside) interfered with sodium transport, oxygen consumption by frog skin was appreciably larger than when no sodium was available for transport This was found not to depend on the presence of sodium in the incubation solutions. The possible mechanisms whereby these drugs (widely assumed to interfere solely with sodium transport across the tissues studied) would influence aerobic metabolism, are not elucidated. Congruent data are however available. For amiloride· (i) the oxygen consumption by frog skin studied with the Warburg technique was not decreased upon addition of this substance [15] at a concentration (4 μM) amply sufficient to reduce sodium transport [5]; (ii) oxygen consumption (measured polarographically) by toad bladder incubated in choline-Ringer's increased by almost 30 % when amiloride, 0.1 mM, was added [16]; this is the amplitude of the effect seen here (cf. Table II); (iii) the drug exerted effects on sodium transport-related glucose metabolism in toad bladder tissue (stimulated with aldosterone) which differed from what prevails when the tissue was incubated in sodium-free solution [17].

Concerning ouabain, (i) it has been seen to induce lesions in frog skin [18]; (ii) turtle bladder aerobic metabolism was less depressed by treatment with ouabain than by incubation in choline-Ringer's [19]; (iii) oxygen consumption by resting C fibers of rabbit vagus poisoned with the glycoside was still found to react to changes in external potassium concentration [20]; (iv) treatment with ouabain of young rat soleus muscle led to an increase in caloric production by the tissue, coincident with inhibition of

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* This was already implicit in Zerahn's data [3] for 12 experiments conducted by this investigator from March through October with water temporarily substituted for Ringer's on the outside of frog skin, ratio averaged $17\,0 \pm 0\,9$, mean was $21\,3 \pm 1\,3$ for 11 experiments conducted from May through August with magnesium-containing Ringer's substituted on the outside for a solution containing Ringer's diluted 1 4 with magnesium Ringer's Mean sodium-transporting activity of frog skin in control conditions was identical for both series

** From studies on the ventral skin of *Bufo marinus*, in progress in this laboratory, variations in this ratio between preparations appear not to exceed in amplitude variations for a given preparation exposed repeatedly to magnesium Ringer's on the outside

sodium efflux [21]; (v) oxygen consumption by renal cortex slices of guinea pig was less depressed during incubation in sodium-containing medium to which ouabain (1 mM) had been added than when incubation was carried out in the absence of sodium [22]. On the other hand, ouabain depresses oxygen consumption by the ventral skin of *Bufo marinus ictericus* incubated in Ringer's [23]; one important difference with frog skin which is of possible relevance is that the inhibiting effect exerted by the glycoside on toad sodium-transporting tissues appears fully reversible [24].

It is concluded from the foregoing that the metabolic cost of sodium transport in a given frog skin preparation can be estimated adequately by temporary removal of sodium from the solution on the outside, and evaluation of the consequences for tissue oxygen consumption. In this respect, both intact skin and isolated epithelium behave similarly. Amiloride and ouabain appeared to exert on frog skin metabolic effects unrelated to mere interference with sodium transport by this tissue.

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BIBLIOGRAPHY

1 Crabbé, J , Michotte, A and Noé, G (1974) J. Physiol Lond 242, 87P–88P
2 Ussing, H H and Zerahn, K (1951) Acta Physiol Scand 23, 112–127
3 Zerahn, K (1956) Acta Physiol Scand 36, 300–318
4 Vieira, F L , Caplan, S R. and Essig, A (1972) J Gen Physiol 59, 60–76
5 Eigler, J , Kelter, J. and Renner, E (1967) Klin Wschr 45, 737–738
6 Koefoed-Johnsen, V (1957) Acta Physiol Scand 41 Sp 145, 87–88
7 Carasso, N , Favard, P , Jard, S and Rajerison, R M (1971) J Microscop 10, 315–330
8 Khatcheressian, I , Prenen, S and Crabbé, J (1976) Arch Int Physiol Bioch 84, 336–339
9 Kushmerick, M J and Paul, R J (1976) J Physiol Lond 254, 693–709
10 Dixon, M (1943) "Manometric Methods", 2nd edn , University Press, Cambridge, U K
11 Crabbé, J and Decoene, A (1974) Arch Int Physiol Bioch 82, 343–346
12 Hutchinson, J H and Porter, G A. (1972) Res Commun Chem Path Pharmacol 3, 585–594
13 Leaf, A and Renshaw, A. (1957) Biochem J 65, 82–90
14 Arczynska, W , Girardier, L and De Sousa, R C (1976) Pflugers Arch 363, 187–191
15 Salako, L A and Smith, A J (1970) Brit J Pharmacol 38, 702–718
16 Cuthbert, A W and Wong, P Y D (1971) Biochim Biophys Acta 241, 713–715
17 Beckman, B , Guertler, B , Leaf, R , Witkum, P and Sharp, G W G (1974) Biochim Biophys Acta 332, 350–357
18 Voûte, C L (1973) in Transport mechanisms in epithelia (Ussing, H H and Thorn, N A , eds) p 98, Proc Alfred Benzon Symp V, 1972, Munksgaard, Copenhagen
19 Lefevre, M. E , Cronkite, C and Brodsky, W A (1970) Biochim Biophys Acta 222, 212–215
20 Rang, H. P and Ritchie, J M (1968) J Physiol Lond 196, 163–181
21 Chinet, A , Clausen, T and Girardier, L (1977) J Physiol Lond 265, 43–61
22 Noé, G. and Crabbé, J (1975) Arch Int. Physiol Biochim 83, 343–345
23 Danisi, G and Vieira, F L (1974) J Gen Physiol 64, 372–391
24 Crabbé, J , Fanestil, D D., Pelletier, M and Porter, G A (1974) Pflugers Arch 347, 275–296