BBA 75225

THE INFLUENCE OF ANAEROBIC CONDITIONS ON SODIUM TRANSPORT AND ADENINE NUCLEOTIDE LEVELS IN THE ISOLATED SKIN OF THE FROG *RANA TEMPORARIA*

P. KRISTENSEN AND A. SCHOUSBOE

The Institute of Biological Chemistry, University of Copenhagen, Copenhagen (Denmark)

(Received August 14th, 1968)

(Revised manuscript received October 28th, 1968)

SUMMARY

1. An apparatus is described in which frog skins can be studied under anaerobic conditions, while short-circuit current is recorded until the moment of sampling.

2. An interdependence was found between the ATP/ADP ratio and short-circuit current under anaerobic conditions.

3. The efflux of sodium was increased under anaerobic conditions.

4. On the basis of the present experiments it is suggested that ATP may be involved directly in the process leading to active transport of sodium in the frog skin and that the sodium pump of that tissue may have some features in common with the sodium pump of erythrocytes.

INTRODUCTION

Although a great deal of work strongly indicates the central role of ATP in ion transport processes, the description of the regulatory role of ATP, ADP, and orthophosphate is far from complete.

The work of CALDWELL *et al.*[1] indicates that a high ATP/ADP ratio is important for maintaining the normal potassium dependence of the sodium extrusion in squid axons, and FORTE[2] found that acid secretion of the frog gastric mucosa was correlated linearly with the ATP concentration in that tissue. Furthermore, it has recently been shown by GARRAHAN AND GLYNN[3] that ATP, ADP, and orthophosphate play central roles in transmembrane transport processes in red blood cells. They presented evidence that a low phosphate potential or high concentrations of ADP or orthophosphate favour a reaction in which sodium is exchanged for sodium across the red blood cell membrane.

To our knowledge such studies have not been performed on the isolated skin of the frog. We have studied the relation between active transport of sodium and the levels of ATP and ADP in that tissue. Changes in the concentrations of the compounds mentioned were brought about by working under anaerobic conditions, as it has recently been shown that anaerobic metabolism cannot supply the skin with sufficient energy for a normal transport rate[1–6].

MATERIALS AND METHODS

The frogs (*Rana temporaria*, L.) were kept partially immersed in tap water at a temperature of about 3° and used immediately after decapitation.

Ringer's solution: 111 mM NaCl, 2.4 mM $NaHCO_3$, 1.0 mM $CaCl_2$, and 1.9 mM KCl.

Anaerobic experiments with sampling of skins for subsequent analysis of ATP and ADP. The skins were mounted in the chamber shown in Fig. 1. They were equilibrated for 1 to 2 h before short-circuiting, and 1 h later anaerobic conditions were induced by allowing a large volume of nitrogen-equilibrated Ringer's solution to run through the two half chambers. After the introduction of anaerobic conditions, the skins were frozen at various times by pushing the Ag–AgCl electrodes against the skins from both sides with two metal blocks, which had been previously cooled in crushed, solid CO_2. The Ringer's solution is forced out by the electrodes. This part of the manipulation lasts for less than 100 msec, so the Ringer's does not freeze. In preliminary experiments, where thermocouples were mounted between two skins, the temperature fell to below —16° in less than 1 sec, and to below —40° in about 3 sec. After freezing the skins were taken out and stored at about —50° for later extraction.

The aerobic control skins were mounted in an open chamber, which permits the

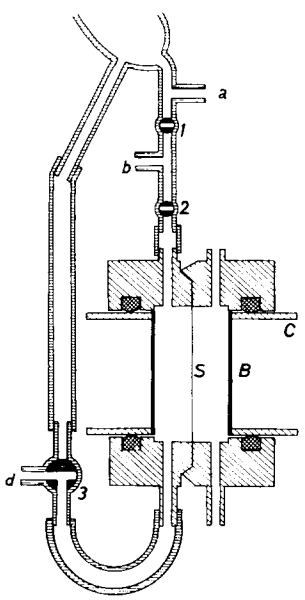


Fig. 1. Schematic drawing of the chamber used for anaerobic study of frog skins. The chamber is made of Perspex. S, skin; C, cylinders which can be pushed towards the skin from both sides. The bottoms, B, of the cylinders are silver plates which permit rapid heat transfer and may also serve as current electrodes. a, an inlet for atmospheric air; b, the outlet for Ringer's solution when changing to anaerobic conditions; d, the inlet for Ringer's solution bubbled with N_2. During the aerobic period the system is closed at d and b, and stopcocks 1 and 2 are open. When the system is flushed with N_2-Ringer's, 1, b, and d are opened. In the anaerobic period, 2 and 3 are closed to the system. Immediately before pushing the cylinders against the skin, 2 and b are opened. The holders for the cold blocks are connected to a microswitch, which will disconnect the current a few msec before freezing of the skin starts.

use of a modified WOLLENBERGER clamp[7] after removal of the Ringer's solution by suction. The area of skin frozen was 4.9 cm^2 in all experiments.

Extraction of the skin samples. Due to the large amount of connective tissue present, it is not possible to homogenize frog skin by the traditional methods. The stored skins were cooled down further with liquid N_2, broken into smaller pieces, and transferred to a cold steel capsule of the Braun Mikro-dismembrator (Braun, Germany). The capsule and its content, including a steel ball, were cooled in liquid N_2 again, so that it would remain cold during the 2-minute shaking period. After homogenization, the resulting powder was transferred to a glass homogenizer containing 1 ml ice-cold perchloric acid (0.3 M) and extracted three times with the same volume. The combined extracts were neutralized with a KOH–Tris mixture to a final pH of about 7, centrifuged, and stored at −50° for later analysis.

Analysis of ADP and ATP. Analysis of ATP was carried out with the luciferase method of RASMUSSEN AND NIELSEN[8].

Analysis of ADP was carried out after separation of the nucleotides by thin-layer ion-exchange chromatography according to the method of RANDERATH AND RANDERATH[9–11]. Standards and internal standards were run on the same plate as the samples. The spots were localized by comparison with markers containing enough nucleotide to be visible under ultraviolet light. After extraction from the respective areas, ADP was measured as ATP after its conversion by incubation with phosphoenolpyruvate and pyruvate kinase.

Short-circuit experiments and efflux measurements were performed according to USSING AND ZERAHN[12]. The anaerobic period in the efflux experiments was induced by bubbling with N_2 instead of atmospheric air. Efflux was measured with ^{22}Na.

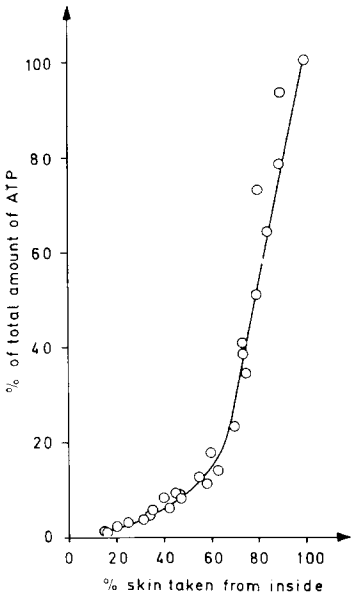
Horizontal slicing of skins was performed in a few experiments in order to localize the ATP. A technique for slicing has been described by HANSEN AND ZERAHN[13], in which the skin is cut off the frog and placed with the outside down on a slice of agar block previously cut with the microtome. After freezing the skin is cut into slices starting from the inside. The slices were extracted with cold $HClO_4$. The thickness of the slices was 14–16 μ.

RESULTS

Frog skin is not a homogeneous tissue. On the outside is a single layer of cornified cells, below which is the epithelium which carries out the active transport of sodium. The epithelium consists of about six layers of cells. Below the basement membrane there are a variety of tissues, such as muscle cells, nerves, glands, and chromatophores. On the inside there is a thick layer of connective tissue containing only a few cells. It is therefore important to know the fractions of the compounds measured in the various layers. Three skins were cut into slices parallel to the surface, as outlined in the MATERIALS AND METHODS, and their ATP contents were measured. The connective tissue, constituting about 60 % of the skin thickness, contains 15 % of the total amount of ATP in the skin, and the combined outer layers contain about 85 %, while they represent only 40 % of the total thickness of the skin (Fig. 2). It is possible to distinguish the various layers from each other to some degree because slices from connective tissue and epithelium are white and are separated by a yellow layer of chromatophores and glands.

The measurements of Table I were performed to estimate the differences between two symmetrical pieces of skin from one frog and the range of amounts of ATP found in skins from different frogs. The latter was found to be 14.0 ± 3.2 nmoles·cm^{-2} skin, while the mean of the differences between two half-skins is 2.5 nmoles·cm^{-2}.

Table II shows no difference between short-circuited and open-circuit skins, so open-circuit skins have been used as controls.



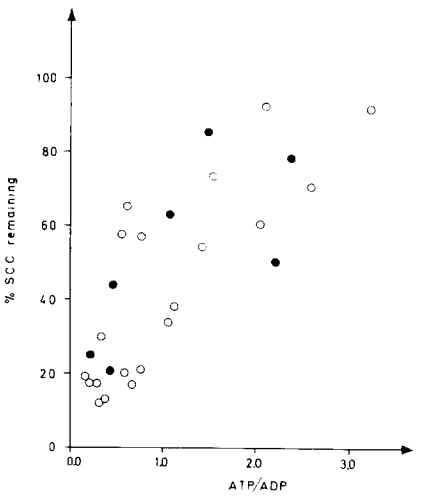


Fig. 2. Distribution of ATP in the frog skin.

Fig. 3. Relation between current and ATP/ADP ratio. ○, determination of both ATP and ADP; ●, determination of ATP and calculation of amount of ADP in anaerobic skins based on the sum of ATP + ADP remaining constant (Table III). SCC = short-circuit current.

TABLE I

DIFFERENCE BETWEEN THE AMOUNTS OF ATP PRESENT IN SYMMETRICAL HALVES OF FROG SKIN FROM THE SAME ANIMAL

Skin No.	*ATP (nmoles·cm^{-2})*		
	Half a	*Half b*	*Numerical difference*
1	13.7	11.6	2.1
2	12.6	14.8	2.2
3	12.6	11.6	1.0
4	11.6	18.8	7.2
5	20.6	21.2	0.6
6	11.9	10.8	1.1
7	17.0	11.6	5.4
8	14.8	15.9	1.1
9	12.3	15.2	2.9
10	10.1	11.6	1.5
For all pieces: mean ± S.D. = 14.0 ± 3.2			Mean: 2.5

Table III shows that the sum of amounts of ATP and ADP remains unchanged when a skin is transferred to anaerobic conditions. This means that no loss of nucleotides occurs during the treatment.

The correlation between active sodium transport, measured as short-circuit current, and the ATP/ADP ratio is given in Fig. 3.

TABLE II

COMPARISON BETWEEN SHORT-CIRCUITED SKINS AND OPEN-CIRCUIT SKINS

Skin No.	*ATP* ($nmoles \cdot cm^{-2}$)		*ADP* ($nmoles \cdot cm^{-2}$)	
	Open	*Shorted*	*Open*	*Shorted*
1	13.9	12.1	2.5	2.7
2	20.0	19.0	2.1	4.3
3	16.8	14.7	2.7	2.1
4	17.1	15.0	2.5	2.1
5	14.3	15.0	2.7	2.8

TABLE III

THE SUM OF THE CONCENTRATIONS OF ATP AND ADP UNDER AEROBIC AND ANAEROBIC CONDITIONS

Skin No.	*ATP* + *ADP* ($nmoles \cdot cm^{-2}$)	
	Aerobic	*Anaerobic*
1	15.5	18.2
2	13.4	18.8
3	24.0	19.0
4	19.6	21.6
5	8.4	8.2
6	18.9	17.9
7	20.7	21.2
8	14.2	11.8
9	17.3	15.9
Mean	16.9	17.0

DISCUSSION

The relation indicated in Fig. 3 between short-circuit current and ATP/ADP ratio may be explained in various ways. One possibility is that the changes in the two parameters are both effects of the lack of oxygen. The other possibility is that the ATP/ADP ratio in some way regulates the net sodium transport across the frog skin.

The first possibility cannot really be ruled out. One of the mechanisms, which ought to be discussed in this connection, is that the increase in glycolysis, which has been shown to occur under anaerobic conditions[6], decreases the intracellular pH, which then may influence the mechanism responsible for the sodium transport. The changes in sodium transport under high CO_2 tensions found by FUNDER, USSING, AND WIETH[14] were believed to be the result of a decrease in intracellular pH, but these workers found no change in the efflux of sodium. On the basis of the efflux

experiments shown in Table IV, it may be concluded that the effects of CO_2 and anaerobic conditions are at least not identical. A direct role of oxygen in the transport process seems unlikely because of the measurements of ZERAHN[4], who found the Na/O_2 ratio to be 18.

TABLE IV

EFFLUX OF SODIUM MEASURED UNDER AEROBIC AND ANAEROBIC CONDITIONS ON THE SAME SKIN WITH ^{22}Na ON THE INSIDE UNDER SHORT-CIRCUIT CONDITIONS

The skin area was 7 cm^2.

Expt. No.	*Efflux of sodium (μequiv/h)*	
	Aerobic	*Anaerobic*
1	0.41	0.57
2	0.87	1.20
3	0.36	0.66
4	0.22	0.47
5	0.10	0.19
6	0.22	0.51
7	0.19	0.93
8	0.10	0.37

The next problem, which should be discussed, is whether the results can be explained as a direct action of the ATP/ADP ratio on the transport mechanism. It then seems reasonable first to test if it is thermodynamically possible that the hydrolysis of ATP is the energy-furnishing process for the active transport. For the red blood cell it has been shown by GARRAHAN AND GLYNN[15] that the sodium/potassium exchange pump may work reversibly. If this is also true for the frog skin system, it enables us to make an estimate of the electromotive force of the frog skin pump according to the general expression for an electrochemical cell: $-\Delta G = zFE$, in which ΔG is the free energy of hydrolysis of ATP, which may be calculated according to the procedure of BURTON AND KREBS[16]. The pH and the orthophosphate concentrations are not known, but a pH of 7 and orthophosphate concentrations between 1 mM and 10 mM in the cells may be considered very likely. The ATP/ADP ratio in aerobic skins was 6.25, and using a Na/O ratio of 9 (ref. 4) and a P/O ratio of 3, the electromotive force can be calculated to be between 180 mV and 199 mV. The highest potential differences actually measured across frog skins in this laboratory have not exceeded 165 mV when the skins have been bathed in Ringer's solution containing sulfate, a non-permeating anion, instead of chloride. In the frog skin it is therefore thermodynamically possible that the free energy of hydrolysis of ATP is used for the transport of sodium.

CALDWELL *et al.*[1] found that the extrusion of sodium from squid axons was partially uncoupled from potassium influx, when the ATP/ADP ratio was low. GARRAHAN AND GLYNN[3] found evidence that a sodium–sodium exchange was substituted for the normal sodium–potassium exchange in red blood cells when the ATP/ADP ratio was low or the orthophosphate concentration high. Therefore the possibility exists that the increase in efflux of sodium in anaerobic skins shown in Table IV may be due to an exchange of sodium against sodium over the inside facing membranes of the frog skin epithelial cells.

The results of the present work are thus consistent with the view that there may be some similarity between the sodium pumps of red blood cells and frog skin. One implication is that one of the efflux paths for sodium in the frog skin is identical to the active transport path.

REFERENCES

1 P. C. CALDWELL, A. L. HODGKIN, R. D. KEYNES AND T. I. SHAW, *J. Physiol.*, 152 (1960) 561.
2 J. G. FORTE, *Federation Proc.*, 24 (1965) 1382.
3 P. J. GARRAHAN AND I. M. GLYNN, *J. Physiol.*, 192 (1967) 189.
4 K. ZERAHN, *Acta Physiol. Scand.*, 36 (1956) 300.
5 A. LEAF AND A. RENSHAW, *Biochem. J.*, 65 (1957) 90.
6 P. KRISTENSEN AND A. SCHOUSBOE, *Biochim. Biophys. Acta*, 153 (1968) 132.
7 A. WOLLENBERGER, O. RISTAU AND G. SCHOFFA, *Arch. Ges. Physiol.*, 270 (1960) 399.
8 H. RASMUSSEN AND R. NIELSEN, *Acta Chem. Scand.*, 22 (1968) 1745.
9 K. RANDERATH AND E. RANDERATH, *J. Chromatog.*, 16 (1964) 111.
10 E. RANDERATH AND K. RANDERATH, *J. Chromatog.*, 16 (1964) 126.
11 K. RANDERATH AND E. RANDERATH, *J. Chromatog.*, 22 (1966) 110.
12 H. H. USSING AND K. ZERAHN, *Acta Physiol. Scand.*, 23 (1951) 110.
13 H. H. HANSEN AND K. ZERAHN, *Acta Physiol. Scand.*, 60 (1964) 189.
14 J. FUNDER, H. H. USSING AND J. O. WIETH, *Acta Physiol. Scand.*, 71 (1967) 65.
15 P. J. GARRAHAN AND I. M. GLYNN, *J. Physiol.*, 192 (1967) 237.
16 K. BURTON AND H. A. KREBS, *Biochem. J.*, 54 (1953) 94.