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EVALUATION OF THE RATE OF BASAL OXYGEN CONSUMPTION IN THE ISOLATED FROG SKIN AND TOAD BLADDER

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Summary

In the study of active transport it is important to distinguish between oxygen consumption sustaining transepithelial transport and that responsible for other tissue functions (basal metabolism). Since amiloride blocks transepithelial active sodium transport and the associated oxygen consumption in the frog skin and toad bladder, we and others have employed this agent to evaluate the rate of basal metabolism. This technique has recently been criticized in a report that amiloride (and ouabain) increased oxygen consumption when no sodium was available for transport. We have been unable to corroborate these observations.

With magnesium-Ringer as external bathing solutions, amiloride and ouabain failed to stimulate oxygen consumption. With sodium-Ringer as external bathing solution amiloride reduced oxygen consumption about 30%, to a level indistinguishable from that found on external substitution of magnesium-Ringer for sodium-Ringer. We conclude that the use of amiloride permits evaluation of the rate of basal metabolism with acceptable accuracy; a possible slight depressant effect of ouabain on basal metabolism remains to be investigated.

Introduction

In the analysis of the relationship between active sodium transport and oxidative metabolism it is necessary to distinguish between oxygen consumption specifically associated with transepithelial active transport and that associated with other tissue functions. In previous studies of the frog skin (*Rana pipiens*) [1] and toad urinary bladder (*Bufo marinus*, Dominican Republic) [2—5] we and others have found it convenient to employ the diuretic amiloride for this

purpose, since its addition to the outer bathing solution in appropriate concentrations results in rapid near-complete suppression of active sodium transport and the simultaneous partial suppression of oxidative metabolism.

Noé et al. [6] have recently assessed the metabolic cost of transcellular sodium transport in the frog skin and its isolated epithelial layers (*Rana esculenta*) by measuring the drop in oxygen consumption upon transient withdrawal of sodium from the outer bathing solution. They concluded that the metabolic cost of sodium transport could not be estimated properly when transport was blocked by amiloride or ouabain, as these drugs were found to bring about an increase in oxygen consumption even when no sodium was available for transport. Since these observations raise questions concerning earlier interpretations of ourselves and others, we felt it important to reinvestigate the issue in the species customarily employed in our laboratories.

Materials and Methods

Frogs (*R. pipiens*) were obtained from Carolina Biological Supply Co., Burlington, North Carolina ('X-jumbo' variety, 10—12 cm in length). Large female toads (*Bufo marinus*, originating in the Dominican Republic) were obtained from National Reagents, Bridgeport, Conn. Animals were maintained and tissues were prepared as described previously [1,4,5,7]. Paired tissues (frog abdominal skin or toad urinary bladder) were mounted in carefully cleansed Ussing-Zerahn Lucite chambers of 7.1 cm^2 cross-sectional area and exposed initially at both surfaces to sodium-Ringer's solutions. After initial equilibration at open-circuit for between 1 and 2 h, the tissues were 'short-circuited' with a voltage clamp and maintained in this state throughout the remainder of the experiment. The short-circuit current I_O and the total rate of oxygen consumption J_{rO}, evaluated as described previously with Clark oxygen electrodes, were monitored continuously. At all times the O_2 tension was between 87.5 and 100% that of air, and solution temperature was maintained at 25 ± 0.03°C. Since apparent tissue O_2 consumption attributable to electrode O_2 consumption and other factors was less than 3% of basal metabolism, no correction was made for this effect. (This value corresponds to <1.2 pM $\cdot$ cm^{-2} $\cdot$ s^{-1}, as compared with Noé et al.'s 11.4 pM $\cdot$ cm^{-2} $\cdot$ s^{-1}, for which they corrected [6]).

Following equilibration for an hour or more at short-circuit the tissues were treated according to one of two protocols, involving the substitution of magnesium-Ringer for sodium-Ringer bathing solutions, exposure to 10^{-5} M amiloride (Merck, Sharp and Dohme) in the external bath, and/or 10^{-5} M ouabain (Calbiochem) in the internal bath. The Na-Ringer solution contained NaCl (113.5 mM), KCl (1.6 mM), $KHCO_3$ (2.4 mM), and $CaCl_2$ (0.9 mM). For the Mg-Ringer solution the NaCl was replaced by $MgCl_2$ (57.5 mM) plus sucrose (57.5 mM), as in the solution of Noé et al. [6]. Prior to use the solutions were filtered through a 0.45 μm bacteriological filter and the antibiotic gentamicin (Schering) was added in a concentration of 40 mg/l. The Na-Ringer and Mg-Ringer solutions were similar in pH and osmolality (8.0 and 220 mosM/kg H_2O, and 7.9 and 215 mosM/kg H_2O respectively).

The composition of the bathing solutions was changed at 30-min intervals. Measurements were made during the final 15 min of each period. The values

reported represent the mean of 3 successive 5-min measurements during this interval. Where Mg-Ringer was substituted for Na-Ringer, the change was made by gently flushing the appropriate hemi-chamber with 300 ml Mg-Ringer. In two instances in which I_O failed to fall adequately after this treatment a second 300 ml of Mg-Ringer was employed.

Studies in frog skin were performed in paired tissues, hemi-skin A being treated according to protocol A, hemi-skin B according to protocol B, as described in Table I. Studies in toad bladders utilized only protocol A.

Standard statistical procedures were used throughout [8].

Results

I. Effects on I_O and J_{rO}

When the tissues were clamped at short-circuit, both I_O and J_{rO} showed transient variability, reaching quasi-steady state values after some 30—40 min. Accordingly, all tissues were equilibrated at short-circuit for 1 h prior to the experimental perturbations. Perturbations of bathing solutions resulted in transient changes in I_O and J_{rO} for some 5—10 min, but steady states were achieved prior to the 15 min interval during which the reported measurements were carried out.

a. Frog skins. The results for 6 pairs of hemi-skins A and B are summarized in Table I. Initially, rates of both active sodium transport (short-circuit current I_O) and oxygen consumption (J_{rO}) differed insignificantly between paired tissues. Nevertheless, in order to facilitate comparisons between simultaneous observations in paired hemi-skins, J_{rO} is expressed as a percentage of the initial control value, as well as in absolute terms. It is seen that in the presence of 10^{-5} M amiloride J_{rO} is more than 30% below control level, with either Na-Ringer (B2) or Mg-Ringer (A3) as the external bathing solution.

Examination of Table I(A) shows that, unlike Noé et al. [6], we were unable to demonstrate a stimulatory effect of amiloride on O_2 consumption with Mg-Ringer as the external medium. Indeed, on comparing the mean values of periods 2 and 4 ($\overline{2,4}$) (in the absence of amiloride) with that of period 3 (in the presence of amiloride) it might appear that J_{rO} is slightly, but significantly, less in the presence of amiloride than in its absence. However, the comparison of simultaneous values in paired hemi-skins A and B (period 3) showed no significant differences. The addition of ouabain to the internal bathing solution was associated with a further slight decline in J_{rO}. In this case it was not possible to control for temporal factors, since the influence of ouabain was effectively irreversible. Under all conditions examined in Table I(A) in the absence of external sodium (periods 2—5) the short-circuit current differed insignificantly from zero.

Examination of Table I(B) shows that the depression of J_{rO} which resulted from the addition of amiloride to external Na-Ringer solution (column 2) was indistinguishable from that later associated with the substitution of external Na by Mg (column 3); similarly, there was no significant difference between J_{rO} in paired hemi-skins A and B exposed simultaneously to external Mg-Ringer or Na-Ringer-amiloride respectively (columns 2). Thus temporal factors appear here to be insignificant.

TABLE I

EFFECT OF BATHING MEDIA, AMILORIDE, AND OUABAIN ON THE RATE OF O_2 CONSUMPTION J_{rO} AND CURRENT I_O IN PAIRED SHORT-CIRCUITED FROG SKINS (*R. PIPIENS*)

Following equilibration for 60 min in standard Na-Ringer bathing solutions, paired hemi-skins were exposed sequentially to various isoosmotic test solutions and agents for 30 min periods, as indicated. Amiloride (A) and ouabain (O) were each employed at a concentration of 10^{-5} M. Values represent the arithmetic mean ± S.E. Effects of various treatments were compared by paired difference analysis. All P values indicate the statistical significance of the difference of a value from zero; n.s. represents non-significance. Since previous observations suggest near-constancy of basal metabolism over the course of a 30 min period, no correction was made for possible decline of function with time [1—5, 9]. ($\overline{2,4}$) indicates the average value of periods 2 and 4. MgR, magnesium-Ringer; NaR, sodium-Ringer.

	Period						Paired difference	
	1	2	3	4	5	6	($\overline{2,4}$)-(3)	(3)-(5)
Hemi-skin A (n = 6)								
External bath	NaR	MgR	MgR-A	MgR	MgR			
Internal bath	NaR	NaR	NaR	NaR	NaR-O			
J_{rO} (pmol · cm^{-2} · s^{-1})	55.8 ± 4.4	39.8 ± 2.9	36.5 ± 2.6	37.9 ± 1.7	32.7 ± 2.8		2.4 ± 0.9 $P < 0.025$	3.8 ± 1.4 $P < 0.05$
J_{rO} (%)	100.0	71.3 ± 3.1	65.4 ± 5.8	67.9 ± 2.6	58.6 ± 3.3		4.2 ± 1.7 $P < 0.05$	6.8 ± 2.8 $P < 0.05$
I_O (μA · cm^{-2})	28.3 ± 3.8	—0.7 ± 0.6	—1.2 ± 0.5	—1.1 ± 0.4	—1.1 ± 0.5		0.4 ± 0.2 n.s.	—0.2 ± 0.1 n.s.

							Paired difference		
Hemi-skin B (n = 6)							(2)-(3)	(3)-(4)	(5)-(6)
External bath	NaR	NaR-A	MgR	MgR	MgR-A	MgR			
Internal bath	NaR	NaR	NaR	MgR	MgR	MgR-O			
J_{rO} (pmol · cm^{-2} · s^{-1})	63.4 ± 5.7	44.1 ± 4.6	45.4 ± 4.5	57.4 ± 5.1	49.0 ± 4.0	41.9 ± 4.7	—1.4 ± 2.1 n.s.	—12.0 ± 4.6 $P < 0.025$	7.2 ± 0.7 $P < 0.001$
J_{rO} (%)	100.0	69.6 ± 4.7	71.6 ± 5.4	90.5 ± 6.3	77.3 ± 4.7	66.1 ± 5.0	—2.0 ± 1.8 n.s.	—18.9 ± 6.3 $P < 0.025$	11.2 ± 3.9 $P < 0.025$
I_O (μA · cm^{-2})	25.4 ± 3.9	0.3 ± 0.4	0.7 ± 0.5	4.4 ± 0.3	0.2 ± 0.6	—0.9 ± 0.5	1.0 ± 0.9 n.s.	5.1 ± 1.0 $P < 0.001$	1.0 ± 0.7 n.s.

TABLE II

EFFECT OF BATHING MEDIA, AMILORIDE, AND OUABAIN ON THE RATE OF O_2 CONSUMPTION J_{rO} AND CURRENT I_O IN SHORT-CIRCUITED TOAD BLADDERS (*B. MARINUS*) (n = 8)

Protocols were exactly as in Table I, hemi-skins A.

	Period					Paired difference	
	1	2	3	4	5	$\overline{(2,4)}$-(3)	(3)-(5)
External bath	NaR	MgR	MgR-A	MgR	MgR		
Internal bath	NaR	NaR	NaR	NaR	NaR-O		
J_{rO} (pmol · cm^{-2} · s^{-1})	46.6 ± 4.3	32.3 ± 3.1	28.7 ± 3.3	29.3 ± 3.5	26.6 ± 3.4	2.8 ± 0.8 $P < 0.01$	2.0 ± 0.5 $P < 0.01$
J_{rO} (%)	100.0	69.3 ± 2.0	61.5 ± 3.0	62.6 ± 4.1	56.9 ± 3.4	4.9 ± 2.0 $P < 0.05$	4.7 ± 1.7 $P < 0.05$
I_O (μA · cm^{-2})	31.2 ± 3.6	1.7 ± 0.3	1.0 ± 0.4	0.9 ± 0.4	0.7 ± 0.3	0.3 ± 0.2 n.s.	0.3 ± 0.2 n.s.

A different pattern was observed when Mg-Ringer was substituted for internal Na-Ringer in both bathing solutions. This substitution resulted in significant enhancement of both J_{rO} ($P < 0.01$) and I_O ($P < 0.001$) (Table I(B), column 4). The subsequent addition of amiloride to the external Mg-Ringer solution depressed both J_{rO} and I_O to levels indistinguishable from those measured in the presence of internal Na-Ringer (cf. columns 3 and 5). The addition of ouabain then resulted in a significant further deline in J_{rO} without significant effect on I_O, but here there is no control for temporal factors (column 6).

b. Toad bladders. The results of studies in 8 hemi-bladders, employing Protocol A, are summarized in Table II. The effects on J_{rO} were very similar to those described for frog skins in Table I. Again, the substitution of external Mg-Ringer for Na-Ringer resulted in a decline of J_{rO} by some 30%, and again we were unable to confirm a stimulatory effect of amiloride with Mg-Ringer as the external medium; as previously, serial changes suggested a slight depressant effect (columns 2—4), but in this case no paired tissues untreated with amiloride were available for simultaneous controls. As with frog skins, the addition of ouabain to tissues bathed externally with Mg-Ringer resulted in slight further depression of J_{rO}, again without significant effect on I_O. Unlike the case with frog skin, none of the maneuvers employed to depress the short-circuit current resulted in its complete inhibition. However, in each experimental period I_O was less than 6% of control level, and there was no significant variation from one period to another.

Discussion

In the analysis of the relationship between transepithelial active sodium transport and oxidative metabolism it is necessary to distinguish between metabolism specifically associated with the transport process, and that providing energy utilized in other tissue functions (basal metabolism). Accepting the view that under circumstances these processes are discrete [4,5,7,9,10], we and others have evaluated suprabasal metabolism by measuring the rate of oxygen consumption first in the absence and then in the presence of amiloride. Several considerations suggested the convenience and validity of this approach. First of all, the avoidance of replacement of Na-Ringer by Na-free media simplifies technique and avoids mechanical trauma to tissues. When amiloride is introduced in adequate concentrations into Na-Ringer's solution bathing the external surface of the frog skin or toad bladder, it results promptly in near-abolition of transepithelial active Na transport and the associated oxidative metabolism [3]. Despite its prolonged effect, the action of amiloride is promptly reversible, permitting its intermittent use where desired; in contrast, recovery from the effect of ouabain is slow and often incomplete. Of major importance is the belief that the action of amiloride is specific, resulting in the inhibition of movement of sodium across the external plasma membrane into the 'active transport' pool, without affecting the flux of other ions via either cellular or paracellular pathways [11,12]. The apparent specificity and effectiveness of the inhibition of sodium entry permitted the use of amiloride in Na-Ringer solutions, thus avoiding the alteration of the transepithelial electrochemical potential difference of sodium, with possible non-specific side

effects. In contrast, the use of external solutions free of sodium might possibly promote the leakage of Na into unstirred layers adjacent to the tissue. The subsequent active transepithelial transport of this sodium might contribute to the residual short-circuit current often associated with the use of external Mg- or choline-Ringer's solutions.

Finally, the apparent specificity of the amiloride effect for the sodium channels in the external plasma membrane suggests its superiority to ouabain for the evaluation of basal metabolism, for although the glycoside is considered to act specifically to inhibit the $(Na^+ + K^+)$-ATPase associated with the sodium pump, it might be expected to interfere thereby not only with transepithelial active Na transport but also with the pumping of Na out of muscle, connective tissue and/or other cells in the contiguous serosal adventitial layer. If so, the rate of metabolism measured in the presence of concentrations of ouabain adequate to depress the short-circuit current to near-zero might well underevaluate the rate of basal metabolism as defined for our purposes, namely that metabolism unassociated with transepithelial active sodium transport.

In their recent study of the ventral skin and isolated epithelial layer of *R. esculenta*, Noé et al. [6] conluded that the metabolic cost of sodium transport could not be estimated properly when transport was blocked either by amiloride or by ouabain, since both agents were found to result in an increase in oxygen consumption when no sodium was available for transport. They suggested that a more appropriate means for evaluating the metabolic cost of sodium transport was temporary removal of sodium from the external bathing solution. Using very similar protocols, we were unable to confirm their findings in either preparation tested by us. Both the substitution of external Mg for Na and the addition of amiloride to external Na-Ringer solution resulted in a decrease of J_r of some 30%. It was not possible to demonstrate stimulation of J_r by amiloride in the absence of external Na; if anything, there may have been slight further depression of J_r, but this effect was of border-line significance. Similarly, the subsequent addition of ouabain to the internal solution (in the absence of external Na) appeared to result in a slight further decrease in J_r, but although this effect was statistically significant, precise interpretation is precluded by lack of control for possible spontaneous decline of basal metabolism with the passage of time.

In 6 hemi-skins we examined the effects of amiloride and ouabain in the complete absence of Na from both bathing solutions, but these observations are difficult to interpret, since the substitution of internal Mg for Na resulted in an increase in both J_{rO} and I_O. Possibly these effects may have been the consequence of leakage of intracellular Na into the Na-free bathing media; in the absence of an adverse electrochemical potential difference of Na across the tissue the pump may have been stimulated by low concentrations of Na in the external medium. On the other hand, it is likely that removal of Na from the internal medium causes important and poorly defined effects on epithelial cell composition and transport characteristics, and so we cannot rule out the possibility that leakage of intracellular electrolyte (e.g. K^+ at the internal surface, Cl^- at the external surface) may contribute to the enhanced short-circuit current. Be these facts as they may, sequential exposure to Mg-Ringer + amiloride/Mg-Ringer and then to Mg-Ringer/Mg-Ringer + ouabain was asso-

ciated in each case with a significant decrement in J_t. Since there was no suitable control study of the time course of function in the presence of bilateral Mg-Ringer solutions it is not possible to conclude whether this decline in metabolism was attributable to either agent. It seems, however, that neither substance stimulated metabolism.

We are not able to account for the significant differences between our results and those of Noé et al. [6], since our experiments were similar, employing the same bathing solutions and the same concentrations of both amiloride and ouabain. We continue to feel, however, that under the conditions of our experiments the use of amiloride permits the evaluation of the rate of basal metabolism with an accuracy adequate for our purposes.

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