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EFFECTS OF ANTIDIURETIC HORMONE ON KINETIC AND ENERGETIC DETERMINANTS OF ACTIVE SODIUM TRANSPORT IN FROG SKIN

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The effects of antidiuretic hormone (ADH) on the rate of transepithelial active Na transport J^a_{Na} and the rate of suprabasal O_2 consumption J^{sb}_r were studied in paired hemiskins of frog. Within some 30 min following administration of ADH both J^a_{Na} and J^{sb}_r increased to near-maximal levels and then remained stable for at least an hour. On symmetric perturbation of the transepithelial electrical potential $\Delta\psi$ at 6-min intervals, the dependence of J^a_{Na} and J^{sb}_r on $\Delta\psi$ was near-linear, both in control and experimental hemi-skins. The stability and near-linearity of the system permitted systematic analysis of the parameters of linear non-equilibrium thermodynamic (NET) and electrical equivalent circuit (EC) formulations. ADH (100 mU/ml) stimulated two of the three NET phenomenological L coefficients, as well as A, the affinity (negative Gibbs free energy) of a metabolic reaction driving transport. Observations at partially depressed levels of transport indicated that the effects on kinetic and energetic factors are to some extent discrete. EC analysis showed stimulation of the amiloride-sensitive conductance κ^a, but not of the apparent electromitive force of Na transport 'E_{Na}'. Similar effects were produced by 10 mU/ml of ADH or by 10 mM dibutyryl cyclic AMP, although less marked effects on the L coefficients were noted with the lower concentration of hormone. It is suggested that, in contrast to EC analysis, the NET formulation distinguishes between kinetic and energetic determinants of transport, supporting a dual mechanism of action of ADH.

Introduction

Despite extensive study, disagreement persists as to whether antidiuretic hormone (ADH) stimulates active Na^+ transport across anuran epithelia solely by facilitating passive apical entry, or also by stimulating active basolateral extrusion [1]. We have found that, in contrast to the case in toad urinary bladder, where the effect of ADH is usually transient, in frog skins the administration of ADH results in well maintained stimulation of the rate of transepithelial active Na transport J^a_{Na} and the rate of suprabasal O_2 consumption J^{sb}_r. On symmetric perturbation of the transepithelial potential $\Delta\psi$ the response of J^a_{Na} and of J^{sb}_r is near-linear in both control and experimental tissues. This stability and near-linearity permitted systematic analysis of kinetic and energetic parameters modulating transport, as evaluated by means of non-equilibrium thermodynamic (NET) and electrical equivalent circuit (EC) formulations.

Abbreviations: ADH, antidiuretic hormone; EC formulation, equivalent circuit formulation; NET formulation, non-equilibrium thermodynamic formulation.

Methods

I. General

Frogs (*Rana pipiens*), obtained from the Carolina Biological Supply Co., Burlington, NC, were kept at room temperature in tanks which contained commercial aquarium gravel and tap water [2]. Pieces of abdominal skin were mounted in modified Ussing-Zerahn Lucite chambers of 7.1 cm^2 cross-sectional

area and bathed in glucose-Ringer solution (113.5 mM NaCl, 1.6 mM KCl, 2.4 mM $KHCO_3$, 0.9 mM $CaCl_2$, 10 mM glucose, pH 8.0, osmolality 220 mosmol/kg H_2O [3] containing 40 mg/l gentamicin sulfate to retard bacterial growth. The electrical potential difference $\Delta\psi$ across the tissues was regulated automatically by use of a voltage clamp, and the current I was recorded continuously. Oxygen tension was measured with Clark electrodes, permitting calculation of the steady state rate of oxygen consumption J_r. Details of the apparatus and procedures are presented in Lang et al. [4]. Tissues were rejected if following equilibration the short circuit current $I_0 \equiv I_{\Delta\psi=0}$ was less than ~20 $\mu A \cdot cm^{-2}$, or if at the end of a study it was found that the ratio of the amiloride-sensitive to total conductance (see below) was less than 0.45. Initial values of parameters in 18 representative tissues are given in Table I.

TABLE I

INITIAL VALUES OF PARAMETERS OF TRANSPORT AND METABOLISM

These observations were made in 18 paired hemi-skins used for the studies of the effects of 100 mU/ml ADH shown in Fig. 1 and Table II. Initial mean values in the tissues employed in other studies did not differ appreciably. See Methods for definitions.

(a) Standard observations

I_0	= 32.9 ± 1.9		$\mu A \cdot cm^{-2}$
J^a_{Na0}	= 344.6 ± 16.0		$pmol \cdot cm^{-2} \cdot s^{-1}$
J_{r0}	= 61.0 ± 4.5		$pmol \cdot cm^{-2} \cdot s^{-1}$
J^b_r	= 39.7 ± 2.2		$pmol \cdot cm^{-2} \cdot s^{-1}$
J^{sb}_{r0}	= 20.7 ± 1.7		$pmol \cdot cm^{-2} \cdot s^{-1}$
κ	= 0.68 ± 0.08		$mS \cdot cm^{-2}$ [a]
κ^p	= 0.32 ± 0.03		$mS \cdot cm^{-2}$ [a]

(b) Thermodynamic parameters

L_{Na}	= 231.6 ± 19.4	$\mu mol^2 \cdot cm^{-2} \cdot s^{-1} \cdot kcal^{-1}$
$L_{Na,r}$	= 7.83 ± 0.66	$\mu mol^2 \cdot cm^{-2} \cdot s^{-1} \cdot kcal^{-1}$
L_r	= 0.50 ± 0.04	$\mu mol^2 \cdot cm^{-2} \cdot s^{-1} \cdot kcal^{-1}$
A	= 43.0 ± 2.6	$kcal \cdot mol^{-1}$
J^a_{Na0}/J^{sb}_{r0}	= 16.1 ± 1.0	
$\Delta\psi^{sh}$	= 62.8 ± 4.0	mV

(c) Equivalent circuit parameters

κ^a	= 0.35 ± 0.03	$mS \cdot cm^{-2}$ [a]
'E_{Na}'	= 95.0 ± 4.4	mV

[a] $1\ S = 1\ \Omega^{-1}$.

II. Protocols

Most studies employed paired hemi-skins from the same animal, one being employed as a control, the other as an experimental tissue. Following equilibration at open-circuit for 1 h and at short-circuit for 1 to 1.5 h, $\Delta\psi$ was clamped sequentially at 0 and ±60 mV for 6-min periods. Measurements were made during the final 2 min at each potential, when the tissues were in a steady state, as judged by near-constancy of I and J_r. Following two such series, test agents were added to the serosal medium and after I_0 increased to a new steady state value an additional series of potential perturbations was carried out as above. Following stabilization at short-circuit $\Delta\psi$ was perturbed by ±10 mV for 5-s periods, giving from Ohm's law the '5 s' conductance $\kappa = -\delta I/\delta(\Delta\psi)$. (Similar (1–10 s) perturbations are commonly employed in the evaluation of equivalent circuit parameters. Slight deviation from square wave responses demonstrable at rapid chart speed are of minor significance as compared with the effects under study.) On completion of the above series of procedures, $2.5 \cdot 10^{-5}$ M amiloride (Merck, Sharp & Dohme, NJ) was used to depress I_0 to near zero within less than 5 min, and measurements of J_r and κ were promptly repeated [5].

The above protocol was employed to examine the effects of 100 mU/ml antidiuretic hormone (ADH, Vasopressin: Parke-Davis), 10 mU/ml ADH, and 10 mM $N^6,O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphoric acid (Sigma), and to compare the effects of 100 and 10 mU/ml ADH.

Studies of the effects of ADH in the presence of partially inhibitory concentrations of amiloride were carried out with a similar protocol, but in unpaired tissues.

III. Quantities derived by the use of amiloride

Since $2.5 \cdot 10^{-5}$ M amiloride reduced the short-circuit current to a small percentage of the control value (4.8 ± 0.0%; $n = 60$), we have considered that measurements made in the presence of this concentration of amiloride reflect tissue functions unassociated with active transepithelial sodium transport [5,6]. Thus the conductance was taken as the passive conductance,

$$\kappa^p = (\kappa)_{I_0 \simeq 0} \quad (1)$$

permitting calculation of the amiloride-sensitive conductance

$$\kappa^a = \kappa - \kappa^p \tag{2}$$

and the amiloride-sensitive current

$$I^a = I + \kappa^p \Delta\psi \tag{3}$$

Correspondingly, J_r in the presence of $2.5 \cdot 10^{-5}$ M amiloride was taken as the rate of basal oxygen consumption

$$J_r^b = (J_r)_{I_0=0} \tag{4}$$

permitting calculation of the rate of suprabasal oxygen consumption [7,8]

$$J_r^{sb} = J_r - J_r^b \tag{5}$$

Representative initial values of the above quantities are summarized in Table I(a).

Since measurements were carried out only once at the end of the experiment in the presence of $2.5 \cdot 10^{-5}$ M amiloride, an implicit assumption is that estimates of κ^p and J_r^b would remain constant during the whole period of study. Several pieces of evidence suggest the validity of this assumption under the conditions employed by us: (1) When transport is abolished by the use of amiloride or removal of mucosal Na (in the presence or absence of ouabain) the basal parameters remain constant for 2 h or more [8]. (2) In the presence of $\geqslant 10^{-5}$ M amiloride in the toad urinary bladder basal O_2 consumption and CO_2 production and κ^p are unaffected by perturbations of $\Delta\psi$ of the magnitude and duration employed here [5,6,9]. (Candia [10] has reported an influence of amiloride on the partial ionic conductance of Cl^- in frog skins exposed to 10^{-4} M amiloride. Although effects were demonstrable within 30 min they were maximal only thereafter. In the present study κ^p and J_r^b were determined within 6 min after exposure to $2.5 \cdot 10^{-5}$ M amiloride.) (3) For our purposes it was important also to evaluate any possible influence of 100 mU/ml ADH on κ^p and J_r^b. For four tissues studied at time $t = 0$ in the control state and at $t \simeq$ 60–90 min following the administration of ADH, $\kappa_t^p / \kappa_{t=0}^p = 0.98 \pm 0.07$ (S.E.) and $J_{r,t}^b / J_{r,t=0}^b = 1.07 \pm 0.04$ (S.E.). For nine pairs of control (c) and experimental (e) tissues studied simultaneously in the absence and presence of ADH, respectively, $\kappa_e^p / \kappa_c^p = 1.03 \pm 0.07$ (S.E.) and $J_{r,e}^b / J_{r,c}^b = 1.07 \pm 0.10$ (S.E.). (It must be noted that although the above findings indicate that our various observations will be related systematically they do not speak against Gordon's suggestion that amiloride may reduce the paracellular conductance [11]. The significance of this consideration will be analyzed below.)

IV. Thermodynamic interpretation

For a two-flow coupled process, with identical solutions bathing the two sides of the tissue, the linear nonequilibrium thermodynamic representation takes the form [13]:

$$J_{Na}^a = L_{Na}(-F\Delta\psi) + L_{Na,r}A \tag{6}$$
$$J_r^{sb} = L_{Na,r}(-F\Delta\psi) + L_r A \tag{7}$$

Here A is the thermodynamic affinity of the metabolic reaction driving transport, and the L's are phenomenological coefficients representing kinetic factors. The thermodynamic parameters were evaluated as previously [7,9]. Briefly, presuming that following symmetric 6-min perturbations of $\Delta\psi$, as employed here, transepithelial active Na^+ transport is conservative and κ^p, J_r^b, and A are constant, J_{Na}^a is given by I^a/F (Eqn. 3), and J_r^{sb} is given by Eqn. 5,

$$L_{Na} = -(1/F)(dJ_{Na}^a/d\Delta\psi), \tag{8}$$
$$L_{Na,r} = -(1/F)(dJ_r/d\Delta\psi), \tag{9}$$

and

$$A = J_{Na0}^a / L_{Na,r} = -I_0/(dJ_r/d\Delta\psi) \tag{10}$$

Introducing the value of J_r^{sb} at short-circuit gives

$$L_r = J_{r0}^{sb}/A \tag{11}$$

(Constancy of the L's and A is considered the simplest and most plausible basis for the observed linear responses of J_{Na}^a and J_r on variation of $\Delta\psi$. An alternative possibility, constancy of J_r^{sb}, is ruled out by the strong voltage-dependence of J_r in the face of complete voltage-insensitivity of J_r^b [6,9].)

Also of interest is the magnitude of 'static head',

given by

$$\Delta\psi^{sh} \equiv (\Delta\psi)_{J^a_{Na}=0} = (1/F)(L_{Na,r}/L_{Na})A$$
$$= I_0/FL_{Na} \quad (12)$$

Initial values of thermodynamic parameters in representative tissues are listed in Table I(b).

V. Equivalent circuit interpretation

In terms of the classical electrical equivalent circuit model [13], active Na^+ transport is the consequence of an electromotive force E_{Na} acting on the conductance of the sodium active transport pathway (which we shall call κ^a_{Na}). Thus, with identical solutions at each surface, in a steady state at short circuit [14].

$$I_0 \equiv FJ^a_{Na0} = \kappa^a_{Na}E_{Na} \quad (13)$$

We and others [5,14,15] have attempted to evaluate the equivalent circuit parameters by perturbing $\Delta\psi$ briefly (here 5 s) in order to measure the amiloride-sensitive conductance $\kappa^a = \kappa - \kappa^p$. The estimate of E_{Na} obtained by this means is then given by

$$I_0 = \kappa^a \cdot E_{Na}' \quad (14)$$

The ambiquity associated with this method will be discussed below [14].

Initial values of quivalent circuit parameters in representative tissues are listed in Tabel I(c).

VI. Analysis of data

Standard statistical procedures were used throughout [16]. Results are expressed as mean values ± standard errors of the mean (S.E.).

In studies of unpaired tissues effects were evaluated by relating the value of a parameter x at time t to that at time $t = 0$, giving the normalized quantity $r = (x_t/x_{t=0})$. In studies of paired tissues an attempt was made to correct for spontaneous variation with time by relating observations in experimental (e) and control (c) tissues, giving the doubly normalized quantity $R = (x_t/x_{t=0})_e/(x_t/x_{t=0})_c$. The significance of the difference of normalized values from 1.00 is expressed by superscripts: $^1(P(\Delta)<0.05)$; $^2(P(\Delta)<0.025)$; $^3(P(\Delta)<0.01)$; $^4(P(\Delta)<0.001)$.

Results

Effects of ADH (100 mU/ml)

Current I_0 and rate of oxygen consumption J_{r0} in the short-circuited state. In a first series of studies, in order to demonstrate maximal effects on transport and metabolism, following adequate control periods a high comcentration of ADH (100 mU/ml) was instilled into the solution bathing the serosal surface of the test skins. In almost all cases this resulted in well maintained effects on I_0 (considered equivalent to FJ_{Na0}) and on J_{r0}. With over 50 animals employed, in only four was the response to ADH transient, necessitating rejection from the study. The mean results in nine paired hemi-skins are shown in Fig. 1. As is seen, following a few minutes delay both I_0 and J_{r0} increased to near-maximal values over a period of some 30 min and then remained stable for at least an hour.

Dependence of J^a_{Na} and J_r on $\Delta\psi$. Previous studies in untreated frog skins have demonstrated that when the transepithelial electrical potential $\Delta\psi$ is perturbed symmetrically at 6-min intervals, as discussed under Methods, the response of both the rate of active sodium transport J^a_{Na} (estimated according to the amiloride techniques of Methods, Section II, III) and the rate of oxygen consumption J_r is near-linear [12,

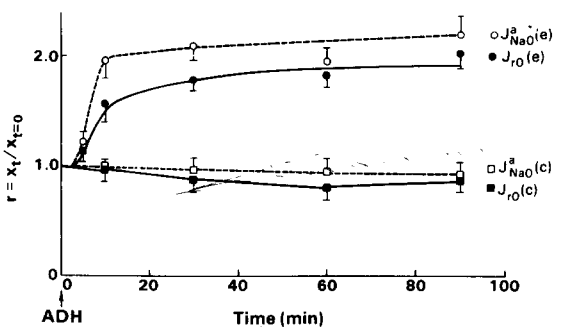

Fig. 1. Effects of 100 mU/ml ADH on $I_0(\equiv FJ^a_{Na0})$ and J_{r0}. Following equilibration, at time $t = 0$ (↑), 100 mU/ml of ADH in a small volume of Na-Ringer's solution was applied to the inside surface of the experimental (e) tissues and an equal volume of Na-Ringer's solution was applied to the inner surface of the control (c) tissues. Values of parameters x at time t were related to those at time $t = 0$ to give the reduced parameter r and are expressed as mean r ± S.E. ($n = 9$). Initial values of the parameters for the nine pairs of hemi-skins are given in Table I; initial values in (c) and (e) tissues did not differ significantly.

17]. This was the case also in the present study, both in the control (c) hemi-skins and in the experimental (e) hemi-skins exposed to ADH. Correlation coefficients for J^a_{Na} vs. $\Delta\psi$ were $r_c = 0.990 \pm 0.008$ (18), $r_e = 0.994 \pm 0.007$ (27) and for J_r vs. $\Delta\psi$ were $r_c = 0.973 \pm 0.009$ (18), $r_e = 0.981 \pm 0.009$ (27).

Non-equilibrium thermodynamic (NET) parameters. The above-noted linear response of both J^a_{Na} and J_r to perturbation of $\Delta\psi$ suggests near-constancy of the phenomenological coefficients and affinity of the thermodynamic formulation discussed above over the range and duration of perturbations employed see Methods, Section IV). This permits a systematic analysis of the effects of ADH in an attempt to discriminate between kinetic factors (influencing permeabilities and or rate coefficients) and energetic factors (influencing the free energy of the driving reaction). Such an analysis was performed 30, 60, and 90 min following exposure to ADH (Table II). In order to describe the effects concisely it is convenient to apply a double normalization procedure so that observations in paired control tissues correct those in the experimental tissues for spontaneous variation with time (Methods, Section VI). Thus a value of $R > 1$ indicates a stimulatory effect of ADH. (Initial values of the various parameters are given in Table I(b).)

TABLE II

EFFECTS OF 100 mU/ml ADH ON TRANSPORT AND METABOLISM AND NET PARAMETERS AT t = 30, 60, AND 90 MIN (EXPERIMENTS OF FIG. 1)

Effects are evaluated as $R = (x_t/x_{t=0})_e/(x_t/x_{t=0})_c$ and are expressed as mean $R \pm$ S.E. (n = 9). Superscripts indicate significance of difference of R values from 1.00 (see Methods, Section VI). Initial mean values of the parameters (Table I) did not differ significantly in (c) and (e) tissues.

	R		
	t = 30 min	t = 60 min	t = 90 min
J^a_{Na0}	2.17 ± 0.20^4	2.95 ± 0.21^4	2.52 ± 0.25^4
J^{sb}_{r0}	2.02 ± 0.17^4	2.24 ± 0.20^4	2.19 ± 0.18^4
L_{Na}	1.69 ± 0.08^4	1.75 ± 0.09^4	1.65 ± 0.10^4
$L_{Na,r}$	1.41 ± 0.11^3	1.56 ± 0.13^3	1.43 ± 0.10^3
L_r	1.06 ± 0.05	1.17 ± 0.10	1.01 ± 0.08
A	1.83 ± 0.24^3	1.64 ± 0.17^3	2.03 ± 0.24^3
J^a_{Na0}/J^{sb}_{r0}	1.27 ± 0.11	1.06 ± 0.10	1.29 ± 0.15
$\Delta\psi^{sh}$	1.14 ± 0.07^3	1.34 ± 0.06^4	1.26 ± 0.10^4

The NET analysis is consistent with the interpretation that ADH stimulates both kinetic and energetic factors modulating transport. As with the observations of Fig. 1, control values of the L's and A's varied insignificantly with the passage of time. The Phenomenological coefficients L_{Na} and $L_{Na,r}$, reflecting the dependence of J_{Na} and J_r on $\Delta\psi$, were stimulated significantly by ADH, as was the metabolic factor A. These effects were well maintained. L_r, however, was unaffected.

Also of interest is the quantity Na^+/O_2, the equivalents of sodium ions transported per mol of suprabasal oxygen consumed. Our results suggest that the ratio at short-circuit, J_{Na0}/J^{sb}_{ro}, may have increased 30 min following ADH but not at 60 or 90 min. There was also an increase of $\Delta\psi^{sh}$.

Effects of ADH (10 mU/ml)

Since 100 mU/ml is well above the concentration of ADH required to produce its maximal natriferic action, it was necessary to consider the possibility that the above results might represent unphysiological effects of the hormone. Accordingly, we tested the effect of 10 mU/ml, a concentration below that necessary to produce a maximal rate of transport. Table III shows that in 4 tissues the qualitative effects of this concentration, evaluated 60 minutes following administration of the hormone, were similar to those seen earlier with 100 mU/ml.

In order to determine the influence of the concentration of ADH more precisely, studies were carried out in six pairs of hemi-skins, one of each pair (control) being exposed to 10 mU/ml of ADH, the other

TABLE III

EFFECTS OF 10 mU/ml ADH ON PARAMETERS OF TRANSPORT AND METABOLISM AT t = 60 MIN

Effects are evaluated as $R = (x_{t=60}/x_{t=0})_e/(x_{t=60}/x_{t=0})_c$ and are expressed as mean $R \pm$ S.E. (n = 4). Superscripts indicate significance of the difference of R values from 1.00 (see Methods, Section VI).

	R		R
J^a_{Na0}	2.15 ± 0.29^3	L_r	1.01 ± 0.07
J^{sb}_{r0}	2.05 ± 0.31^2	A	1.78 ± 0.23^2
L_{Na}	1.59 ± 0.21^1	J^a_{Na0}/J^{sb}_{r0}	1.25 ± 0.14
$L_{Na,r}$	1.39 ± 0.19^1	$\Delta\psi^{sh}$	1.42 ± 0.11^3

(experimental) being exposed to 100 mU/ml. Measurements of function and thermodynamic parameters were performed 30, 60, 90, and 120 min following the administration of ADH. Since the values at these different times were found to be closely similar, they are combined in Table IV. It is seen that the higher concentration of ADH stimulates transport and metabolism to a greater extent than the lower concentration. The difference is entirely accounted for by effects on phenomenological coefficients, there being no difference with respect to the affinity. There was no difference also with respect to the Na^+/O_2 ratio at short-circuit as $\Delta\psi^{sh}$.

Effects of cyclic AMP

In order to test further the specificity of the above effects, the response to cyclic AMP was studied in 6 tissues. For this purpose, following appropriate control observations, dibutyryl cyclic AMP was added to the serosal bath to give a concentration of 10 mM. In these studies the onset of action and maximum level of activity occurred later than with ADH. After some 60 min, however, the response was stable and, as shown in Table V, quite similar to that seen with ADH, although without a demonstrable effect on $\Delta\psi^{sh}$.

Effect of level of transport on response of A to ADH

The above findings are consistent with the point of view that ADH enhances transport and metabolism as a result of effects on both kinetic and energetic factors [12]. In interpreting these effects, however,

TABLE IV

COMPARATIVE EFFECTS OF 100 mU/ml AND 10 mU/ml ADH

Effects are expressed as mean ± S.E. of $R = (x_t/x_{t=0})_{100mU/ml}/(x_t/x_{t=0})_{10mU/ml}$, pooling 20 observations made in five animals at t = 30, 60, 90, and 120 min. Superscripts indicate significance of the difference of R values from 1.00, as above.

	R		R
J^a_{Nao}	1.31 ± 0.05^4	L_r	1.09 ± 0.05
J^{sb}_{r0}	1.35 ± 0.04^4	A	0.98 ± 0.08
L_{Na}	1.13 ± 0.05^2	J^a_{Na0}/J^{sb}_r	0.98 ± 0.09
$L_{Na,r}$	1.30 ± 0.05^4	$\Delta\psi^{sh}$	1.16 ± 0.14

TABLE V

EFFECTS OF 10 mM CYCLIC AMP ON PARAMETERS OF TRANSPORT AND METABOLISM AT t = 60 AND 90 MIN

Effects are here evaluated in unpaired tissues as $r = x_t/x_{t=0}$, and expressed at each t as mean r ± S.E. (n = 6). Superscripts indicate significance of the difference of r values from 1.00, as above.

	r	
	t = 60 min	t = 90 min
J^a_{Na0}	2.23 ± 0.19^4	2.06 ± 0.16^4
J^{sb}_{r0}	1.92 ± 0.14^4	1.86 ± 0.20^3
L_{Na}	2.07 ± 0.21^4	1.86 ± 0.15^4
$L_{Na,r}$	1.40 ± 0.16^1	1.28 ± 0.15
L_r	1.22 ± 0.18	1.13 ± 0.13
A	1.66 ± 0.15^3	1.66 ± 0.10^4
J^a_{Na0}/J^{2b}_{r0}	1.09 ± 0.10	1.04 ± 0.10
$\Delta\psi^{sh}$	1.08 ± 0.05	1.11 ± 0.06

it is important to consider the possibility that the increase in affinity following the administration of ADH might not be a result of a direct action of ADH on metabolic pools, but rather a secondary effect of the enhancement of kinetic factors leading to increased transport. In order to test this possibility, the response to ADH was examined under circumstances such that the rates of transport and metabolism in the presence of hormone did not exceed significantly those in the control state. For this purpose, following initial observations, amiloride was added to the mucosal medium in a concentration (1–5 μM) adequate to reduce the short-circuit current to about 35–40% of control level. Thirty and 60 minutes later the transport and metabolic parameters were again measured. The addition of ADH to the serosal medium (10 mU/ml) then brought I_0 and J_{r0} back to near control levels; 30 and 60 min later the parameters were evaluated again. A representative experiment is depicted in Fig. 2. Detailed mean results of nine studies are given in Table VI. The depression of J^a_{Na0} and J^{sb}_{r0} by amiloride was associated with highly significant depression of all L coefficients and (at 30 min) a slight depression of A. The subsequent addition of ADH increased the values of all parameters. (In two cases the addition of ADH elevated I_0 to about 50% above control level, necessitating the use of additional amiloride; subsequent observations dif-

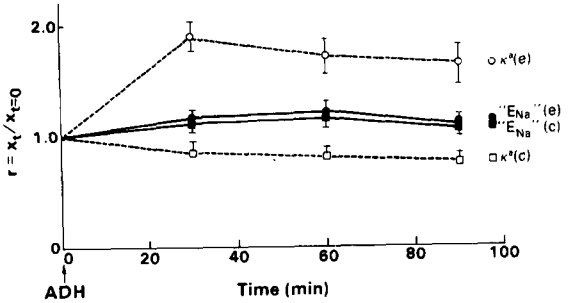

Fig. 2. Experiment depicting the effect of ADH on A in face of levels of transport and metabolism previously depressed by the administration of amiloride. In this tissue A was not depressed in association with depression of N_{Na0} and J_{r0}^{sb} by amiloride.

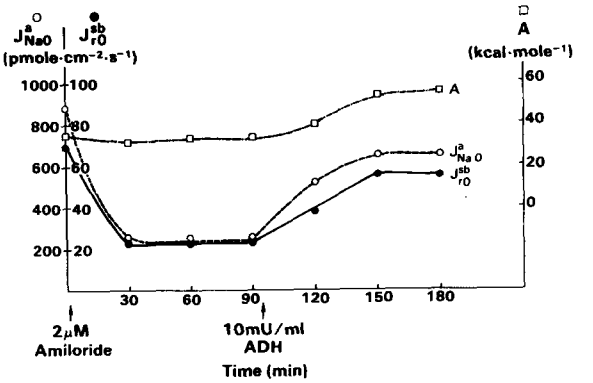

Fig. 3. Effects of 100 mU/ml ADH on equivalent circuit parameters. Effects are expressed as mean $r \pm$ S.E. ($n = 9$) as in Fig. 1. Initial values of the parameters for the nine pairs of hemi-skins are given in Table I; initial values in (c) and (e) tissues did not differ significantly.

TABLE VI

EFFECTS OF ADH ON PARAMETERS AT REDUCED LEVELS OF TRANSPORT AND METABOLISM

Effects are evaluated from r values in unpaired tissues, as in Table V.

a. 1–5 μM amiloride

	r		
	t = 30 min (n = 9)	t = 60 min (n = 9)	t = 90 min (n = 4)
J_{Na0}	0.35 ± 0.03^4	0.39 ± 0.03^4	0.36 ± 0.04^4
J_{r0}^{sb}	0.40 ± 0.05^4	0.49 ± 0.05^4	0.42 ± 0.02^4
L_{Na}	0.46 ± 0.06^4	0.38 ± 0.06^4	0.35 ± 0.08^4
$L_{Na,r}$	0.52 ± 0.06^4	0.52 ± 0.07^4	0.49 ± 0.06^4
L_r	0.57 ± 0.11^3	0.63 ± 0.13^2	0.53 ± 0.08^3
A	0.74 ± 0.06^4	0.86 ± 0.09	0.96 ± 0.12
J_{Na0}/J_{r0}^{sb}	0.88 ± 0.16	0.80 ± 0.06^3	0.86 ± 0.11
$\Delta\psi^{sh}$	0.80 ± 0.08^1	1.10 ± 0.08	1.09 ± 0.06

b. 10 mU/ml ADH

	r		
	t = 120 min (n = 9)	t = 150 min (n = 9)	t = 180 min (n = 4)
J_{Na0}	1.25 ± 0.29	1.05 ± 0.15	0.92 ± 0.09
J_{r0}^{sb}	1.09 ± 0.19	1.02 ± 0.13	0.96 ± 0.09
L_{Na}	0.84 ± 0.18	0.74 ± 0.08^3	0.69 ± 0.09^3
$L_{Na,r}$	0.76 ± 0.10^1	0.71 ± 0.05^4	0.60 ± 0.05^4
L_r	0.71 ± 0.07^4	0.70 ± 0.07^4	0.62 ± 0.05^4
A	1.56 ± 0.16^3	1.46 ± 0.10^4	1.55 ± 0.13^4
J_{Na0}/J_{r0}^{sb}	1.15 ± 0.06^1	1.03 ± 0.07	0.96 ± 0.07
$\Delta\psi^{sh}$	1.44 ± 0.11^3	1.42 ± 0.09^4	1.36 ± 0.12^2

fered insignificantly from those in the other seven animals). Of note is the fact that despite levels of transport and metabolism not in excess of control level (and with values of phenomenological coefficients below control level) 10 mU/ml of ADH induced increases in A to levels not very different from those in the absence of amiloride (Table III).

Equivalent circuit analysis

It is also of interest to analyze our system by means of the equivalent electrical circuits so widely employed in the study of transepithelial active transport processes [13]. In terms of a simple lumped circuit the rate of active transport is determined by the conductance of the cellular ('active') pathway κ^a (Eqn. 2), and the apparent electromotive force of Na^+ transport 'E_{Na}' (Eqn. 14) [14]. While it is appreciated that E_{Na} is not a purely energetic parameter, but incorporates also kinetic components [5,12], the concept that E_{Na} provides a driving force for transport provides a systematic basis for the analysis of transport under diverse conditions. As is shown in Fig. 3, 100 mU/ml caused a marked increase in κ^a, but had no effect on 'E_{Na}'.

Discussion

Although a variety of evidence has been adduced in support of a dual effect of ADH on transepithelial active Na^+ transport in anuran epithelia, with

enhancement of both apical entry and basolateral efflux, controversy remains as to the relative impor- of these effects and their interrelation [1].

Following the initial observations of Ussing and Zerahn [13] and Leaf and colleagues [19], many studies have indicated that ADH stimulates transepithelial active Na^+ transport by increasing apical permeability, with the resultant increase in cell Na^+ concentration and/or the mean electrical potential difference across the apical plasma membrane. Either effect could stimulate increased activity of the basolateral Na^+ pump. Although it has been objected that tissue heterogeneity makes for uncertainty in localization of transport pool Na^+, chemical analysis of isolated toad baldder epithelial cells [20–22] and electronmicroprobe analysis of frog skins [23] have now provided strong evidence that ADH can indeed increase the Na^+ content of transporting cells, as would be consistent with facilitation of apical entry. Furthermore, recent microelectrode studies, employing more reliable techniques than available earlier, have demonstrated that ADH increases the intracellular electrical potential in the frog skin [24].

The nature of effects of ADH at the basolateral surface is less clear. Evidence for a direct effect on the active process was the finding of Morel and Bastide [25] that in frog skins in which exposure to mucosal solutions containing less than 1 mM Na^+ was associated with net Na^+ flux from serosa to mucosa, application of ADH reversed the direction of Na^+ transport. Finn [26,27] later described a stimulatory effect of ADH on the rate coefficient for efflux across the serosal boundary of the toad bladder. Lipton and Edelman [28] found, in contrast to earlier findings of others, that ADH had no effect on the water or ion content of scraped toad bladder epithelial cells, which in view of previous evidence for enhanced apical entry was considered to indicate a basolateral effect as well. On the other hand, it has been claimed that the above-cited kinetic studies overestimated the size of the Na^+ transport pool [29], and questions have been raised as to the physiological state of scraped cell preparations [1]. Also pertinent are the microelectrode studies in frog skins of Nägel [24], mentioned above, in which it was found that ADH had no effect on intracellular electrical potentitial following the depression of apical Na^+ entry by amiloride, and that the normal effect of ADH was reversed by the subsequent administration of amiloride. While these results may indicate a lack of a direct effect of ADH on the electromotive forces at the inner cell surface, as was suggested, lacking precise knowledge of the influence of ADH on cell Na^+ activity and the response of the Na^+ pump to both chemical and electrical potential gradients, stimulation of the pump mechanism by ADH cannot be unequivocally excluded. In this regard it is pertinent that Aceves [20] has described stimulation of Na^+ extrusion from preloaded frog skin epithelial cells by oxytocin in the presence of 10^{-5} M amiloride.

Our linear NET analysis is consistent with an effect of ADH on both kinetic and energetic factors which influence transport. Thus an increase in L_{Na}, proportional to the integral conductance, is compatible with increased permeability at either the apical and/or basolateral surface; increase in $L_{Na,r}$ indicates facilitation of the mechanism coupling transport to metabolism; and increase in A suggests an increase in the negative free energy of a metabolic reaction driving the transport process. Of special interest was the observation that the kinetic and energetic effects were to some extent dicrete, since it was possible to demonstrate elevated values of A following ADH even in the presence of amiloride, with maintenance of L values and transport well below control level. Also pertinent in this regard was the observation that while 100 mU/ml of ADH increased the values of the phenomenological coefficients above those achieved with 10 mU/ml, it had no additional effect on A.

Effects on the apparent stoichiometric ratio at short-circuit, $Na^+/O_2 \equiv J_{Na0}/J_{r0}^{sb}$, were usually small or of borderline significance. Although in some studies the magnitude of steady-state $\Delta\psi$ at static head, $\Delta\psi^{sh} \equiv (\Delta\psi)_{J^a_{Na}=0}$, appeared to have increased following ADH, this is uncertain, since the value of 62.8 ± 4.0 mV calculated in the control state (Table I) is implausibly small. (Values of the static head electrochemical potential difference across the basolateral membrane in frog skin, estimated recently from measurements employing Na^+-specific electrodes, averaged about 154 mV (Nagel, W., Garcia-Diaz, J.F., Armstrong, W.McD., personal communication).) We are unable at present to explain this discrepancy, but it may be usefully analyzed by refer-

ence to Eqn. 12. Although it is possible that perturbation of $\Delta\psi$ may alter either L coefficient or A, this has seemed to us unlikely with the protocol of the present study, given the impressively linear responses of both J_{Na} and J_r on variation of $\Delta\psi$. Another possibility is that our method for evaluation of κ^p may be faulty, leading to error in the evaluation of I^a, and thus of L_{Na} (see Eqns. 3 and 6). In Materials and Methods, Section III, we have given our reasons for feeling that κ^p remained constant throughout the course of our studies, both in the absence and presence of ADH, and irrespective of the value of $\Delta\psi$. None of these considerations, however, argues conclusively against the possibility that our amiloride technique may misevaluate the conductance of the active pathway, as suggested by Gordon [11]. Gordon has concluded that the steady-state conductance of the toad bladder is associated only with the paracellular pathway. For various reasons we do not believe that this can be quite the case in the frog skin. (For one reason, the effect of ADH on total tissue conductance κ is associated with a dramatic lowering of the apical: basolateral voltage divider ratio (see Ref. 24, Fig. 6).) Nevertheless, it remains possible that, as suggested by Gordon, amiloride may depress tissue conductance by lowering the concentration of ions in the lateral intercellular space and/or the junctional complex. If so, the passive conductance in the absence of amiloride might appreciably exceed the value of κ^p measured in the presence of amiloride, even though our measurements were carried out promptly, well before secondary tissue changes could depress tracer ion fluxes between the external bathing solutions, as observed by Candia [10]. Our values for L_{Na} would then be too large and our values of $\Delta\psi^{sh}$ too low. (It should be noted, however, that erroneous absolute values of L_{Na} would not necessarily invalidate our inference that L_{Na} increases following ADH; as mentioned above, microelectrode studies support this view. Nor would erroneous values of κ^p affect our estimates of $L_{Na,r}$, A, or J_{Nao}/J_{ro}^{sb}.)

On the face of it, the equivalent circuit analysis might seem in conflict with the NET analysis, in that although again there was evidence for a kinetic effect (with increase in κ^a), there was no effect on the apparent electromotive force of Na^+ transport, E_{Na}. (Earlier studies in the toad bladder also suggested either no effect or a very slight (depressant) effect of ADH on E_{Na} [5,31]. On consideration, however, it appears that the equivalent circuit analysis is not in fact inconsistent with a dual action of ADH. As discussed recently in a study of the equivalent circuit model in the toad bladder [14], estimation of E_{Na} is based on the assumption that κ^a, the amiloride-sensitive conductance determined from a short-term perturbation of $\Delta\psi$, represents κ^a_{Na}, the conductance of the sodium transport pathway (compare Eqns. 13 and 14). To the extent that short-term perturbations result in brief flows of ions other than Na^+ across the basolateral membrane, however, κ^a will not equal κ^a_{Na} and thus 'E_{Na}' cannot reliably evaluate E_{Na}. In addition, there is the consideration that in principle E_{Na}, like $\Delta\psi^{sh}$, is not a pure energetic parameter, but reflects kinetic factors as well [12]. Further study will be required to define the dynamic responses to brief (5 s) and prolonged (6 min) perturbations of $\Delta\psi$ underlying the discrepancies between 'E_{Na}', E_{Na}, and $\Delta\psi^{sh}$.

Although the applicability of linear NET to biological systems remains to be fully tested, its use in conjunction with precise protocols allows a systematic analysis of active transport and the associated metabolism [12]. Studies to date have led to plausible values of A. (In the present study, presuming P/O ratios of about 6, values of A ranged from approx. 7 to 13 kcal per mol of ATP.) Recent theoretical analyses of kinetic models of the sodium active transport system support the utility of the formalism under appropriate conditions (Refs. 32, 33, and Caplan, S.R., submitted for publication). Studies of mitocondrial oxidative phosphorylation have demonstrated linearity and reciprocity [34,35] and the accuracy of calculation of the affinity of the oxidative driving reaction by means of a relationship analogous to our Eqn. 10 [35].

Assuming that the NET formalism is indeed appropriate, it is of interest to enquire how ADH and cyclic AMP can bring about an increase in A, presuming that this reflects the Gibbs free energy of ATP hydrolysis. Such an effect appears in fact quite consistent with the known actions of these agents. Thus, assuming the same mechanisms, as in the toad urinary bladder [36], the administration or formation of cyclic AMP results in the stimulation of phosphorylase, increased levels of fructose 6-phosphate, stimulation of phosphofructokinase, increased levels of fructose diphos-

phate, and stimulation of pyruvate kinase. Increased concentrations of pyruvate could then stimulate oxidative metabolism, ATP formation, and thus A. Since glycolysis itself results in ATP formation it might seem that ADH and cyclic AMP could stimulate active Na^+ transport significantly even without an effect on oxidative metabolism; the resultant enhancement in I_0, without a commensurate effect on the potential-dependence of O_2 consumption $dJ_r/d(\Delta\psi)$, would then lead to an over-estimate of A, as calculated from Eqn. 10. However, such an effect would be small, since the metabolism of a molecule of glucose provides only two molecules of ATP in the course of glycolysis, as against some 32 molecules of ATP on complete oxidation, and our results demonstrate marked stimulation of suprabasal O_2 consumption by both ADH and cyclic AMP [37].

Thus it appears that classical views of intermediary metabolism are consistent with primary effects of ADH and cyclic AMP on energetic factors, rather than secondary metabolic changes associated with stimulation of transport. It should be pointed out that this possibility is not contradicted by the observations that both glycogenolysis and O_2 consumption of intact toad urinary bladders are stimulated by ADH and cyclic AMP only in the presence of Na^+ ions in the medium, since in the absence of Na^+ transport, with resultant accumulation of ATP and diminution of ADP and P_i, A might be expected to rise, depressing both glycogenolysis and oxidative phosphorylation.

It is possible, of course, that mechanisms other than those considered above may be responsible for enhancement of A by ADH. For one example, ADH might activate cells of high affinity which are relatively quiescent in the absence of the hormone. This seems unlikely, given the syncytial character of the tissue [23] and the prominent electrophysiological effects of ADH noted in cells punctured at random [24]. In view of the recent suggestion that ADH and cyclic AMP transiently lower the Ca^{2+} level in epithelial cell cytoplasm, it might seem that this effect could enhance mitochondrial ATP synthesis, over and above effects on the Na-K ATPase and apical permeability which could influence L_{Na} and $L_{Na,r}$; however, given the high K_m of mitochondrial Ca^{2+} transport (~10 μM), it has been considered unlikely that cytoplasmic Ca^{2+} is sufficiently high to influence ATP synthesis significantly [38–40].

Whatever the mechanism of ADH's actions, it should be noted that a dual effect on apical entry and basolateral efflux would result in enhanced transepithelial transport with minimal alteration of cell Na activity.

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