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TEMPERATURE DEPENDENCE OF Na^+ TRANSPORT IN THE ISOLATED TOAD BLADDER

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SUMMARY

1. Na^+ transport, measured in the urinary bladder of the toad, was positively dependent on temperature over the range 18–27° and negatively dependent over the range 27–36°. At all temperatures, there was a 1:1 correspondence between radioisotopically measured net Na^+ flux and short-circuit current (s.c.c.).

2. Three possible mechanisms for the positive temperature dependence, *i.e.* (1) thermal activation of Na^+ pump, (2) thermal enhancement of metabolism, and (3) thermal facilitation of Na^+ entry across the apical membrane, were explored by a one-step rapid change in temperature from 18–25° (the temperature-jump method).

3. In the presence of ouabain, an inhibitor of ATPase, the response to the temperature jump was more impaired than the response to vasopressin, suggesting that thermal activation of the Na^+ pump may be involved. Metabolic interference with iodoacetate, rotenone or antimycin A abolished the s.c.c. response to the temperature jump, while considerable responsiveness to the stimulating actions of vasopressin and amphotericin B was preserved in the presence of these inhibitors. These results suggest a role for metabolic pathways in mediating thermal activation of Na^+ transport.

4. Reduction of the inward Na^+ gradient significantly reduced the response to the temperature jump, whereas pretreatment with vasopressin or amphotericin B, presumed activators of apical conductance, enhanced the subsequent response to the temperature jump. Thus, heat does not appear to act on the same Na^+ conductance pathway as do vasopressin or amphotericin B.

5. These studies indicate that thermal activation of Na^+ transport does not involve a single unique rate-limiting step, a conclusion consistent with the nonlinear character of the Arrhenius plot.

6. Owing to the complex character of the temperature dependence of active Na^+ transport, the response to increments in temperature does not provide useful information on the mechanism of action of mineralocorticoids as implied in an earlier study.

INTRODUCTION

Active Na^+ transport is coupled to the expenditure of metabolically derived energy, and both the transport and metabolic processes are, in principle, temperature

Abbreviation: s.c.c., short-circuit current.

dependent[1-7]. The overall effect of changes in temperature on active Na^+ transport may involve multiple effects or be dominated by an effect at a very temperature-sensitive rate-limiting step. To obtain information on the nature of the temperature dependence of transepithelial Na^+ transport, we studied these effects in the presence and absence of metabolic inhibitors.

In 1907, LESSER[3] reported that the spontaneous potential of amphibian skin was dependent on the ambient temperature. It wasn't until 1956, however, that ZERAHN[4] recorded a direct correlation between temperature, active Na^+ transport and O_2 consumption in the frog skin. These results were amplified by SNELL AND LEEMAN[5] who obtained a maximum effect on Na^+ transport at a temperature of 25° and concluded that the thermodynamic efficiency of Na^+ transport increased linearly as the ambient temperature was reduced from 20 to 2.5° (ref. 1). Somewhat differing results were reported by TAKENAKA[6] who found that the temperature dependence of transport in the frog skin was nonlinear in an Arrhenius plot. The temperature coefficient for active Na^+ transport was greater over the 6–14° than over the 14–20° range. Addition of metabolic inhibitors, NaN_3, dinitrophenol and iodoacetate, reduced the temperature coefficient of the 6–14° range to that of the 14–20° range. Based on these findings, TAKENAKA[6] suggested that active Na^+ transport in frog skin involves at least two distinct mechanisms. The effect of temperature on active Na^+ transport in the isolated toad bladder has been recently reported by DALTON AND SNART[8]. No attempt was made, however, to evaluate the metabolic component of the temperature effect. They attributed the temperature effect to an increase in the permeability of the apical plasma membrane to Na^+. In these studies, Na^+ transport was linearly dependent on temperature over the 4–28° range.

One of the purposes of the present study was to explore the validity of the inferences drawn by DALTON AND SNART[8]. The primary purpose, however, was to obtain additional information on the metabolic requirements of temperature-induced changes in active Na^+ transport with the use of metabolic inhibitors as well as hormonal and pharmacological activators of Na^+ transport. These studies were designed to add information on the fundamental characteristics of transepithelial Na^+ transport. A thorough understanding of the Na^+ transport system should include the temperature dependence of each of the key reactions in the system. The present study emphasizes the difficulties in identifying the key rate-limiting steps.

METHODS

Paired urinary hemibladders, removed after rapid double pithing of *Bufo marinus*, were mounted in glass chambers as previously described[9]. The temperature of the frog Ringer's solution (Na^+ = 114 mequiv/l, K^+ = 3.5 mequiv/l, Ca^{2+} = 5.4 mequiv/l, Cl^- = 120.4 mequiv/l, HCO_3^- = 2.5 mequiv/l, osmolarity = 0.288, pH in air = 8.4) which bathed the hemibladders was varied between 18 and 36° by means of water circulating through a heating–cooling unit and thin-walled glass coils immersed in each glass chamber. The rate of change in temperature of the media in the glass chambers was 0.5°/min following a square-wave change in the temperature of the pump-circulated water. In most experiments the hemibladders were mounted in the chambers in the evening and left open-circuited overnight at 18°. The overnight solutions contained 5 mM glucose, kanamycin (50 μg/ml) and penicillin (0.01 μg/ml).

In some of the experiments we incubated hemibladders overnight in substrate-free frog Ringer's solution in order to deplete them of endogenous substrate. The following morning the solutions were replaced with fresh frog Ringer's and a continuous short-circuit current (s.c.c.) was applied after the technique of USSING AND ZERAHN[2].

Bidirectional Na^+ fluxes were measured by adding 21 μC of $^{22}Na^+$ to the serosal chamber and 40 μC of $^{24}Na^+$ to the mucosal chamber of paired hemibladders and taking 0.5-ml aliquot samples at 0.5-h intervals. The samples were assayed in a Nuclear Chicago dual-channel γ-ray spectrometer preset for a 1% counting error. Isotope separation was achieved by re-counting all samples after 21 days had elapsed to allow complete decay of $^{24}Na^+$.

Glucose or sodium pyruvate was added to the media to give a final concentration of 5 mM unless otherwise indicated. The metabolic inhibitors that were used included: iodoacetic acid, ouabain, *p*-fluorophenylalanine, L-phenylalanine, cycloheximide, antimycin A and actinomycin D. In those experiments in which vasopressin was used, sufficient undiluted material was added to the serosal solution to achieve a final concentration of 80 munits/ml and the s.c.c. read at 30-sec intervals until a peak response was evident. In some experiments, amphotericin B was added to the mucosal solutions to give a final concentration of 13 μg/ml.

RESULTS

The effects of changes in temperature on Na^+ transport

S.c.c. studies. S.c.c. was measured during stepwise 2° increments in the temperature of the bathing media from 18 to 32° and back to 18° in freshly mounted hemibladders and following overnight incubation at 18°. After each change, 15 min were

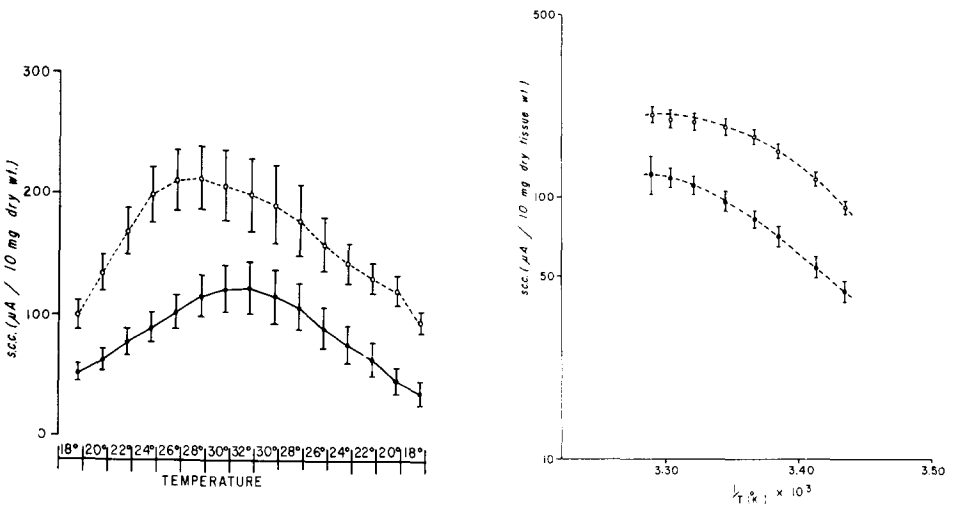

Fig. 1. The s.c.c. as a function of temperature. The solid circles represent eight pairs of hemibladders incubated at 18° for 14 h prior to stepwise changes in temperature. The open circles represent eight pairs of hemibladders incubated at 18° for 2 h prior to stepwise changes in temperature. All values are given as mean ± S.E.

Fig. 2. Arrhenius plot of s.c.c. as a function of temperature (in degrees Kelvin). The data are taken from Fig. 1. The solid and open circles are defined in the legend of Fig. 1.

allowed for stabilization of the temperature and three readings were taken at 5-min intervals. The mean s.c.c. values at each temperature were used to construct Fig. 1. The open circles are the data obtained in hemibladders 2 h after mounting: the solid dots represent data in hemibladders following 14–16 h of incubation at 18°. The general contour of the inscribed curves was similar in both groups, but the peak response was shifted from 28 to 32° following overnight incubation. Arrhenius plots[7] of these data are shown in Fig. 2. The nonlinear response between 18 and 28° is distinctly different from the linear response, over the same temperature range, reported by DALTON AND SNART[8].

Bidirectional Na^+ flux studies. Simultaneous bidirectional Na^+ fluxes were obtained in continuously short-circuited hemibladders at 18, 21, 24, 27, 30, 33 and 36°. Each flux period was separated by a 0.5-h interval to allow the rate of Na^+ transport to stabilize after each change in temperature. The results are given in Table I and Fig. 3. At all of the temperatures studied, net Na^+ flux and s.s.c. (expressed in μequiv/h) were the same. The s.c.c. technique, therefore, provides a valid measure of active Na^+ transport under these conditions. Active Na^+ transport was positively temperature dependent between 18 and 27° and negatively dependent between 30 and 36°. At the higher temperatures, the rise in the serosal to mucosal flux $\phi_{s\to m}$ is more abrupt than the rise in mucosal to serosal flux $\phi_{m\to s}$, resulting in a fall in net Na^+ transport. The linear dependence of $\phi_{m\to s}$ on temperature is apparently the result of two simultaneous effects that average out to a linear process the negative dependence in s.c.c. indicates a fall in active Na^+ transport which is masked by the steeper rise in the movement of Na^+ through passive pathways. This assumes, of course, that $\phi_{s\to m}$

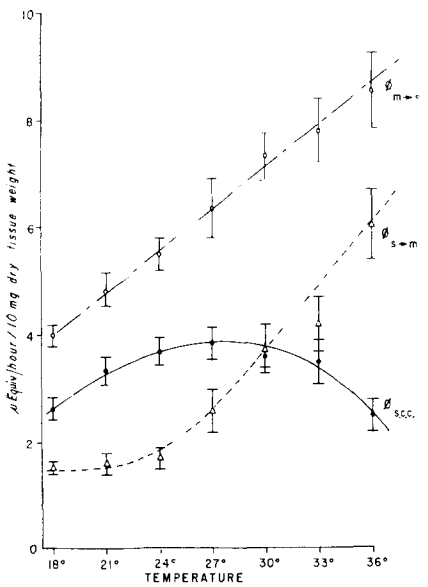

Fig. 3. Unidirectional and net flux of Na^+ as a function of temperature. $\phi_{m\to s}$ denotes the mucosal to serosal flux and is shown as open circles; $\phi_{s\to m}$ denotes the serosal to mucosal flux and is shown as triangles; $\phi_{s.c.c.}$ denotes the net flux and is shown as solid circles. All values are given as mean ± S.E. $N = 15$ pairs.

TABLE I

NET FLUX OF Na^+ AND S.C.C. AT VARIOUS TEMPERATURES

All values are expressed in μequiv/h per 10 mg dry wt. Net flux was calculated as the difference in simultaneously measured bidirectional fluxes using $^{24}Na^+$ in the mucosal media and $^{22}Na^+$ in the serosal media. $N = 15$, mean $\pm$ S.E. P = significance of the difference between net flux and s.c.c. n.s., not significant.

	18°	21°	24°	27°	30°	33°	36°
Net flux	2.68 ± 0.20	3.23 ± 0.32	3.79 ± 0.25	3.75 ± 0.43	3.54 ± 0.35	3.63 ± 0.45	2.45 ± 0.33
s.c.c.	2.66 ± 0.19	3.36 ± 0.23	3.68 ± 0.24	3.86 ± 0.32	3.58 ± 0.23	3.49 ± 0.38	2.48 ± 0.30
P	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

TABLE II

EFFECT OF OUABAIN ON THE RESPONSE TO A TEMPERATURE JUMP AND TO VASOPRESSIN

In the a.m., ouabain (24 μM, final concn.) was added to the serosal media of one of each pair of hemibladders. 1 h later, the temperature of the media of all hemibladders was raised to 25°. 30 min after adjustment of the temperature, all hemibladders were challenged with vasopressin, final concn. = 80 munits/ml in the serosal media. All results are expressed as mean ± S.E. n.s,, not significant.
$s.c.c._{18°}$ = the s.c.c. following overnight incubation at 18°; $\Delta s.c.c._I$ = the difference in s.c.c. 1 h after addition of the inhibitor, ouabain (24 μM); $\Delta s.c.c._{\Delta T}$ = the difference in s.c.c. 0.5 h after changing the temperature to 25°; $(\%\Delta s.c.c._{\Delta T})_c$ = the difference in s.c.c. corrected for the diffferences in the absolute s.c.c. at the time of the change in temperature; $s.c.c._B$ = the s.c.c. just prior to the addition of vasopressin (80 munits/ml); $\Delta s.c.c_v$. = the difference in s.c.c. between $s.c.c._B$ and the s.c.c. at the maximum (peak) response to vasopressin; $\%\Delta s.c.c._v = (\Delta s.c.c._v/s.c.c._B) \times 100$.

Conditions	*N*	$s.c.c._{18°}$ (μA)	$s.c.c._I$ (μA)	$s.c.c._{\Delta T}$ (μA)	$(\%\Delta s.c.c._{\Delta T})_c$ (%)	$s.c.c._B$ (μA)	$s.c.c._v$ (μA)	$\%\Delta s.c.c._v$ (%)
Control	8	103 ± 16	−13 ± 5	42 ± 8	61 ± 11	118 ± 16	81 ± 14	74 ± 13
Ouabain	8	92 ± 19	−39 ± 10	16 ± 3	30 ± 6	56 ± 8	36 ± 6	68 ± 11
P		n.s.	<0.05	<0.025	<0.005	<0.025	<0.05	n.s.

is a measure of passive movement of Na^+ in either direction, since the flux studies were carried out in the absence of an electrochemical gradient.

The linear region of the positive temperature-dependent response was studied in additional experiments using a one-step jump in temperature from 18 to 25°. In eight paired experiments the increase in s.c.c. was complete within 0.5 h following the change in temperature, which is in agreement with the results reported by KARGER AND KRAUSE[10] in the isolated frog skin. As shown in Fig. 4 the new steady-state s.c.c. remained fairly constant over an additional 5.5 h of observation and the pattern of the response was the same in the control (glucose-treated) and substrate-deprived hemibladders.

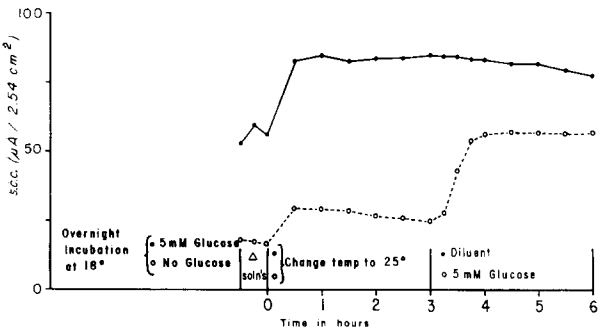

Fig. 4. Response of the s.c.c. to temperature jump (18→25°) and glucose. The solid circles represent hemibladders incubated in 5 mM glucose overnight. The open circles represent hemibladders incubated in glucose-free frog Ringer's solution. The baseline s.c.c.'s just prior to the change in temperature were 56 ± 9 μA per 2.54 cm² (solid circles) and 27 ± 2 μA (open circles). All values are the mean for eight pairs of hemibladders.

Magnitude of the temperature-dependent effect. The effect of the temperature jump from 18 to 24° on active Na^+ transport was estimated as both the absolute and relative increase in s.c.c. which occurred 0.5 h after raising the temperature to 25°. The absolute increase was denoted as Δs.c.c.$_{\Delta T}$, and the relative increase $\%\Delta$s.c.c.$_{\Delta T}$ (*i.e.* 100 × the absolute increase divided by the s.c.c. at 18°).

The possibility that overnight preincubation modified the magnitude of the response was tested in twenty-four paired experiments; the effect of the temperature jump was measured in twelve pairs of hemibladders 2 h after placement in the chambers and in twelve additional pairs the response was measured after overnight preincubation in glucose Ringer solution. In the fresh preparations, Δs.c.c.$_{\Delta T}$ averaged 66 ± 6 and 67 ± 7 μA, and in the pairs maintained overnight, 63 ± 7 and 67 ± 8 μA. Similarly, there was no significant difference when the results were expressed on a percent basis.

As many of the experiments to be performed involved the use of metabolic inhibitors which depress the baseline, the influence of spontaneous variations in baseline s.c.c. on either Δs.c.c.$_{\Delta T}$ or $\%\Delta$s.c.c.$_{\Delta T}$ was analyzed in 201 nonpaired observations. The results are shown in Fig. 5. The magnitude of the absolute response tended to be greater the higher the baseline s.c.c. and the $\%\Delta$s.c.c.$_{\Delta T}$ was linearly dependent on the baseline value with a slope of −0.31. This curve was used to assess non-specific (*i.e.* lowering of the baseline s.c.c.) effects in the experiments in which inhibitors were used. The experimentally measured value is given as ($\%\Delta$s.c.c.$_{\Delta T}$) and the corrected

value as $(\%\Delta s.c.c._{\Delta T})_c$. The corrected value was computed by adding 0.31 [$s.c.c._{18°,control} - s.c.c._{18°,exptl.}$] to the experimental value; where $s.c.c._{18°,control}$ and $s.c.c._{18°,exptl.}$ denote the baseline s.c.c. at 18° in the control and experimental hemibladders, respectively.

Role of the Na^+ pump in the temperature-dependent response

In previous experiments, we found that ouabain (24 μM) inhibited the s.c.c. by 39 ± 3 % after 1 h (J. V. BURPEE AND G. A. PORTER, unpublished observation). At the same concentration, ouabain inhibited the response to maximally effective con-

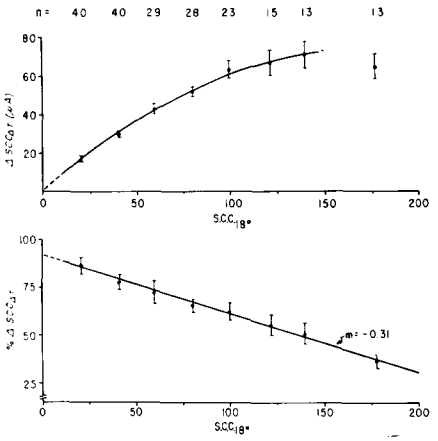

Fig. 5. Change in s.c.c. following the temperature jump (18→25 °) as a function of the baseline s.c.c. at 18°. $\Delta s.c.c._{\Delta T}$ denotes the absolute change in s.c.c. 30 min after the temperature jump. $\%\Delta s.c.c._{\Delta T}$ denotes the percent change in s.c.c. calculated from $\Delta s.c.c._{\Delta T}$ and $s.c.c._{18°}$. All values are in mean ± S.E. N denotes the number of observations averaged for the respective points.

TABLE III

EFFECT OF IODOACETATE ON THE RESPONSE TO A TEMPERATURE JUMP

In the a.m., iodoacetate (0.1 mM, final concn.) was added to the serosal media of one of each pair of hemibladders. In one series, no inhibitor was added and in another the media were fortified with sodium pyruvate (5 mM, final concn.). 1 h after the addition of the inhibitor the temperature of the media of all hemibladders was raised to 25°. The symbols are defined in the legend of Table II. All values are mean ± S.E. n.s., not significant.

Conditions	*Overnight glucose*	*N*	$s.c.c._{18°}$ (μA)	$\Delta s.c.c._I$ (μA)	$\Delta s.c.c._{\Delta T}$ (μA)	$(\%\Delta s.c.c._{\Delta T})_c$ (%)
Control	+	8	53 ± 8	3 ± 3	27 ± 4	50 ± 3
No iodoacetate	−		18 ± 2	− 1 ± 1	13 ± 3	65 ± 10
P			<0.005	n.s.	<0.025	n.s.
Control	+	8	91 ± 21	−15 ± 8	40 ± 7	59 ± 7
0.1 mM iodoacetate	−		30 ± 4	− 5 ± 3	8 ± 3	14 ± 9
P			<0.01	n.s.	<0.001	<0.005
5 mM pyruvate	−	8	29 ± 3	23 ± 4	31 ± 8	125 ± 32
5 mM pyruvate (0.1 mM iodoacetate)	−		32 ± 5	21 ± 9	24 ± 6	70 ± 9
P			n.s.	n.s.	n.s.	n.s.

centrations of aldosterone by 90 %. The s.c.c. response to changes in temperature from 18 to 25° and to vasopressin following pretreatment with ouabain (24 μM) is summarized in Table II. Ouabain inhibited the response to the jump in temperature by about 50% but did not impair the relative increase in s.c.c. produced by vasopressin. The selective impairment of the response to the temperature jump implies that part of the effect may be mediated by the temperature dependence of the Na^+ pump. This effect, however, may also reflect the influence of temperature on the availability of substrates to the pump, *i.e.* ATP or intracellular Na^+. Accordingly, the interrelation between metabolism and temperature on Na^+ transport was examined.

Role of metabolism in the temperature-dependent response

Effects of metabolic inhibitors. The results of these experiments are given in Table III. Overnight incubation in glucose-free media at 18° reduced the baseline s.c.c. presumably because of depletion of endogenous substrate. The temperature-dependent increase in s.c.c. however, was proportionate in the substrate-depleted and control hemibladders. The addition of iodoacetate (0.1 mM) to substrate-depleted hemibladders had an insignificant effect on s.c.c. but impaired the response to the temperature jump to a very considerable extent. That this effect is attributable to inhibition of glycolysis by iodoacetate is indicated by the restoration of the response to the temperature jump by pyruvate. These results imply that the temperature effect may involve or depend on an intact system for oxidative phosphorylation.

To test the possible role of oxidative metabolism in the temperature effect, three modes of inhibition were used, (1) anaerobiosis (prepurified N_2 with less than 8 ppm O_2); (2) antimycin A, an inhibitor of electron transport at the level of cytochrome *b*, and (3) rotenone, an inhibitor of NADH dehydrogenase activity[11]. The results of these experiments are summarized in Table IV and indicate that impairment of electron transport and coupled ATP synthesis reduced the baseline s.c.c. to very low levels and abolished the effect of the temperature jump on Na^+ transport. A possible explanation

TABLE IV

EFFECT OF INHIBITORS OF OXIDATIVE METABOLISM ON THE RESPONSE TO A TEMPERATURE JUMP

In the a.m., following exchange of the media for fresh frog Ringer's solution, the inhibitors were added as indicated in the table. 1 h later the temperature of the media of all hemibladders was raised to 25°. The symbols are defined in the legend to Table II. All values are mean ± S.E. n.s., not significant.

Conditions	*N*	$s.c.c._{18°}$ (μA)	$\Delta s.c.c._{1}$ (μA)	$\Delta s.c.c._{1T}$ (μA)	$(\%\Delta s.c.c._{1T})_c$ (%)
Control	4	67 ± 8	−12 ± 1	61 ± 13	91 ± 5
N_2		78 ± 10	−70 ± 9	− 1 ± 1	−33 ± 11
P		n.s.		<0.01	<0.01
Control	16	92 ± 11	− 1 ± 1	50 ± 14	68 ± 7
2μM antimycin A		100 ± 11	−74 ± 8	− 9 ± 3	−40 ± 7
P		n.s.		<0.001	<0.001
Control	4	53 ± 4	− 7 ± 1	43 ± 7	93 ± 10
4 μM rotenone		45 ± 10	−35 ± 8	2 ± 1	8 ± 4
P		n.s.		<0.01	<0.005

of these results is that the inhibitors, antimycin A and rotenone, imposed an absolute limit on active Na^+ transport and thereby eliminated the response to the temperature jump. To test the capacity of the transport system under these conditions, vasopressin and amphotericin B were chosen as activators since FANESTIL *et al.*[11] reported that the s.c.c. response to these agents was preserved under similar conditions.

TABLE V

EFFECTS OF METABOLIC INHIBITORS ON THE RESPONSE TO VASOPRESSIN OR AMPHOTERICIN B

In the a.m., following exchange of the media for fresh solutions, antimycin A (2 μM, final concn.) or rotenone (4 μM, final concn.) was added to the serosal media of one of each pair of hemibladders. 1 h later vasopressin (80 munits/ml, final concn.) was added to the serosal media or amphotericin B (13 μg/ml, final concn.) to the mucosal media of both members of the pair. All values are mean ± S.E. n.s., not significant.

Pairs	*Inhibitor*	*Activator*	$s.c.c._B$ (μA)	$\Delta s.c.c.$ (μA)	*P*	$\%\Delta s.c.c.$ (%)	*P*
10	0	Vasopressin	62 ± 11	26 ± 6		41 ± 6	
					<0.05		n.s.
	Antimycin A	Vasopressin	9 ±	8 ± 2		111 ± 38	
9	0	Amphotericin B	107 ± 21	39 ± 7			
					n.s.		
	Antimycin A	Amphotericin B	8 ± 1	36 ± 4			
4	0	Vasopressin	79 ± 7	71 ± 22		86 ± 26	
					n.s.		<0.05
	Rotenone	Vasopressin	7 ± 2	24 ± 6		406 ± 91	

Effects of metabolic inhibitors on the response to vasopressin and amphotericin B. Pretreatment with antimycin A lowered the baseline s.c.c. to very low levels and reduced the absolute increase in s.c.c. induced by vasopressin (Table V). The absolute response to amphotericin B as well as the relative response to vasopressin, however, were not impaired by this inhibitor. Rotenone may have reduced the absolute response to vasopressin (although statistical significance was not achieved owing to the small number of pairs), but the relative degree of activation was even greater in the inhibited hemibladders. These results are in contrast to the complete suppression of the response to the temperature jump produced by these inhibitors (*cf.* Tables IV and V). These results imply that the Na^+ transport response to the temperature jump is, at least in part, mediated by effects on metabolic pathways. As a means of assessing the role of metabolism in the response to the temperature jump, additional experiments on substrate depletion and repletion were carried out.

Effects of substrate depletion and repletion. The s.c.c. response to the temperature jump was tested with cross-over experiments using glucose or pyruvate after overnight incubation at 18° in substrate-free frog Ringer's solution. As shown in Fig. 6, the effects of substrates and the temperature jump were simply additive rather than synergistic. The cross-over in the design of the experiment eliminated the possibility that additivity was a result of the sequence in which the changes were made. A comparison of these results with those obtained with metabolic inhibitors suggests

that metabolic pathways play a partial or permissive role in the response to the temperature jump. Thus, additional experiments were performed to explore the possibility of an action by the temperature jump on Na^+ entry across the apical plasma membrane.

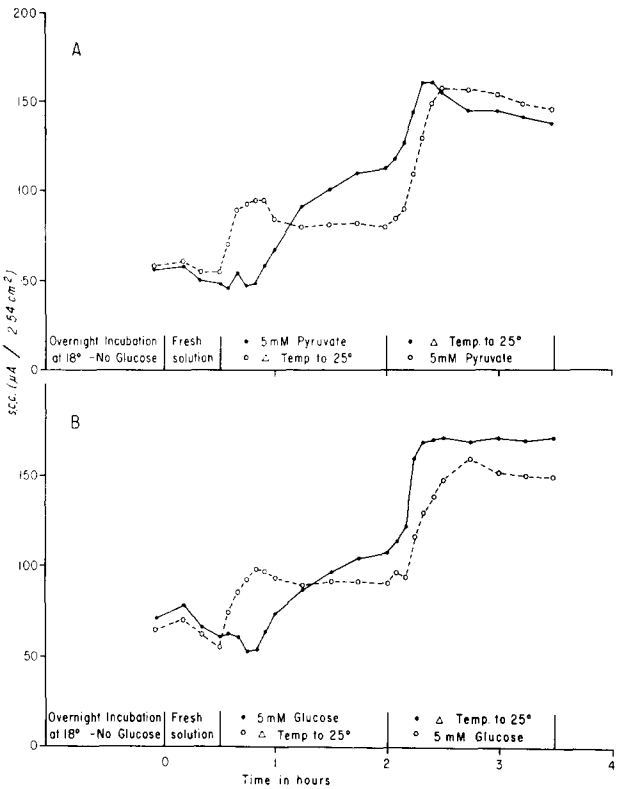

Fig. 6. The response of the s.c.c. to sequential additions of substrate and the temperature jump (18→25°). All hemibladders were preincubated overnight in substrate-free frog Ringer's solution. The order of additions is indicated in the subcompartments. N = seven pairs of hemibladders for each set of experiments. All values are given as the mean at each point.

Role of Na^+ entry in the temperature-dependent response

Effect of mucosal Na^+ concentrations. FRAZIER *et al.*[12] found that active Na^+ transport across the toad bladder is limited by the concentration of Na^+ in the mucosal medium at concentrations less than about 60 mequiv/l. At mucosal Na^+ concentrations of about 10 mequiv/l, transepithelial Na^+ transport was linearly dependent on mucosal Na^+ concentration in the presence of vasopressin and in its absence. As the entry of Na^+ across the apical boundary is believed to depend on the apical transmembrane electrochemical gradient[13], the response to the temperature jump should be attenuated when this gradient is reduced. This has been shown to be the case for vasopressin, for example[12]. As summarized in Table VI, reduction of mucosal Na^+ concentration to 11 mequiv/l reduced both the absolute and relative effects of the temperature jump on the s.c.c. by about one-third. To confirm the assumption that mucosal Na^+ was rate-limiting under these conditions, all of the mucosal media were replaced with standard

TABLE VI

EFFECT OF Na^+ GRADIENT ON THE RESPONSE TO A TEMPERATURE JUMP

In the a.m., the media were exchanged for fresh glucose frog Ringer's except that the mucosal media of one of each pair was replaced with low-Na^+ sucrose Ringer's. The s.c.c. was allowed to stabilize for 1 h at which time the temperature of all of the media was raised to 25°. The symbols are defined in the legend to Table II. All values are mean ± S.E.

Conditions	*Na^+ concn. Mucosal: serosal*	*N*	*s.c.c.$_{18°}$* (μA)	*Δs.c.c.$_{\Delta T}$* (μA)	(%Δs.c.c.$_{\Delta T}$)$_c$ (%)
Frog Ringer's	110:110	8	85 ± 13	45 ± 8	53 ± 5
Sucrose Ringer's	11:110		66 ± 10	26 ± 5	32 ± 4
P			<0.05	<0.001	<0.001

TABLE VII

EFFECT OF VASOPRESSIN OR AMPHOTERICIN B ON THE RESPONSE TO A TEMPERATURE JUMP

Vasopressin was added to the serosal solution and amphotericin B to the mucosal solution of the experimental hemibladders 30 min before the temperature of all of the media was raised to 25°. The symbols are defined in the legend to Table II. All values are mean ± S.E. n.s., not significant.

Number of pairs	*Vasopressin (80 munits/ml)*	*Amphotericin B (13 μg/ml)*	*s.c.c.$_{18°}$* (μA)	*Δs.c.c.$_{\Delta T}$* (μA)	(%Δs.c.c.$_{\Delta T}$)$_c$ (%)
	—	—	84 ± 10	40 ± 8	57 ± 10
8					
	+	—	103 ± 20	67 ± 15	81 ± 11
P			n.s.	<0.025	0.05
	—	—	24 ± 8	35 ± 4	94 ± 9
8					
	—	+	40 ± 11	57 ± 6	211 ± 38
P			n.s.	<0.05	<0.025

frog Ringer's solution at the end of each experiment. Restoration of the mucosal Na^+ concentration to 110 mequiv/l resulted invariably in a sharp increase in s.c.c. The spike lasted 5–10 min and was followed by stabilization of the current at a level that was 24 ± 7% higher than in the presence of a 1:10 Na^+ gradient. These results, therefore, are consistent with the concept that the temperature jump increases the Na^+ permeability of the apical plasma membrane. It has previously been proposed that vasopressin and amphotericin B have similar sites of action, *i.e.* on mucosal entry of Na^+ (refs. 12–14). As a further test of the apical site of action of the temperature jump, the effects of sequential pharmacological and temperature changes were assessed.

Effects of vasopressin and amphotericin B on the response to the temperature jump. If the primary site of action of two agents is shared, the expectation is that pretreatment with one of these agents, at the maximum concentration, will attenuate the response to the second agent. In these experiments, hemibladders were pretreated with either vasopressin or amphotericin B for 30 min and then challenged with the temperature jump. As shown in Table VII, these agents significantly enhanced both the relative and absolute response. Thus, if vasopressin and amphotericin B act primarily on apical entry of Na^+, at least part of the temperature-dependent effect is

attributable to either enhanced ATP production or activation of the Na^+ pump or both.

Role of RNA and protein synthesis in the temperature-dependent response

DALTON AND SNART[8] proposed that the rising phase of the dependence of s.c.c. on temperature reflects an action on mucosal entry of Na^+ and that aldosterone also acts at this site. There is now a considerable body of evidence indicating that the action of aldosterone is mediated by DNA-dependent RNA synthesis and linked *de novo* synthesis of proteins[9,15,16]. The possibility of an induction mechanism in the response to the temperature jump is also raised by the existence of temperature-sensitive bacterial mutants[17]. Actinomycin D, an inhibitor of DNA-directed RNA synthesis and cycloheximide, an inhibitor of ribosomal assembly of proteins, were added at concentrations sufficient to eliminate the mineralocorticoid action of aldosterone[16]. These agents were added 1.5 h before the temperature jump as M. LAHAV AND I. S. EDELMAN (personal communication) have recently found that the action of these inhibitors is essentially complete in this time interval. The results in Table VIII clearly show that the inhibitors of RNA and protein synthesis did not alter either the absolute or relative response to the temperature jump. Furthermore, the rapidity of the temperature-dependent response speaks against an induction mechanism (see Fig. 6).

TABLE VIII

EFFECT OF INHIBITORS OF RNA AND PROTEIN SYNTHESIS ON THE RESPONSE TO A TEMPERATURE JUMP FROM 18 TO 25°

The inhibitor was added to the media of the experimental hemibladders 1.5 h before the temperature of all of the media was raised to 25°. The symbols are defined in the legend to Table II. All values are mean ± S.E. n.s., not significant.

Conditions	*N*	$s.c.c._{18}$ (μA)	$\Delta s.c.c._{I}$ (μA)	$\Delta s.s.c._{1T}$ (μA)	$(\%\Delta s.c.c._{1T})_c$ (%)
Control	10	57 ± 15	− 6 ± 3	43 ± 11	94 ± 11
Actinomycin D (5 μg/ml)		66 ± 19	−13 ± 7	35 ± 7	80 ± 6
P		n.s.		n.s.	n.s.
Control	10	62 ± 13	− 7 ± 7	25 ± 4	68 ± 11
Cycloheximide (0.5 μg/ml)		69 ± 21	− 8 ± 4	26 ± 5	63 ± 13
P		n.s.		n.s.	n.s.

DISCUSSION

To rationalize the nature of the effect of changes in temperature on transepithelial transport of Na^+, the key rate-limiting steps in the Na^+ transport system must be defined. The most plausible model that has yet been proposed, visualizes a system of membrane boundaries in series and is based on isotopic, biochemical and microelectrode studies[14,18–20]. Na^+ entry across the apical plasma membrane is believed to be a passive process which follows saturation kinetics[12,13,18,21]. Na^+ is actively extruded from the cell across the basal-lateral surface of the plasma membrane, at the expense of ATP break-down. Evidence in favor of the electrogenic character of the Na^+ pump

has been presented by BRICKER and co-workers[19,22] and FRAZIER AND LEAF[13]. According to this model, Na^+ transport could be regulated in three different ways: (1) by modifying the passive entry of Na^+ across the apical plasma membrane; (2) by adjusting the rate of ATP synthesis and the supply of metabolic energy to the Na^+ pump; and (3) by altering the activity of the Na^+ pump (which may be expressed or measured as an effect on the $(Na^+ + K^+)$-activated ATPase of SKOU[20].

The dependence of transepithelial Na^+ transport on metabolism and available substrates has been documented by MAFFLY AND EDELMAN[23]. In analyzing the mechanism of action of aldosterone divergent views have been expressed: EDELMAN and co-workers[11,25] proposed that aldosterone-induced proteins increase the rate of synthesis of ATP and the supply of metabolic energy for Na^+ transport. SHARP AND LEAF[26] and CRABBÉ[27] contend that the aldosterone-induced protein is a "permease" which facilitates the passive entry of Na^+ across the apical plasma membrane. DALTON AND SNART[8] supported the latter contention based on the response in s.c.c. to changes in temperature in the presence and absence of aldosterone. They noted that an increase in temperature from 8 to 20° stimulated the s.c.c. and that aldosterone enhanced the response. The Arrhenius plot of their results is strikingly similar to that shown in Fig. 2. DALTON AND SNART[8] attributed the initial steep portion of the slope to changes in apical permeability to Na^+ based on the assumption that the conductance of the apical boundary is rate-limiting for Na^+ transport under these conditions. Implicit in their interpretation is the idea that the temperature jump had no significant effect on either ATP synthesis or the activity of the Na^+ pump. HEARON[28], however, has reviewed the difficulties in interpreting temperature coefficients in complex biological systems and, in particular, the difficulties in identifying or measuring master reactions.

The s.c.c. or net Na^+ transport is composed of passive and active fluxes across the basal-lateral cell boundary and the passive bidirectional fluxes across the apical boundary. There are considerable technical difficulties in measuring these four flux components directly. Thus, a straight-forward evaluation of the assumption that the positive phase of the dependence of s.c.c. on temperature is determined by an effect on apical permeability to Na^+ is not available. The transepithelial unidirectional fluxes indicate that the rising phase of the response to a rise in temperature correlates with an increase in $\phi_{m \to s}$ and not the $\phi_{s \to m}$ (Fig. 3). The former is composed of both active and passive fluxes whereas the latter is determined by the passive fluxes. These results, therefore, do not provide support for the assumption of a primary effect on passive entry of Na^+.

The results obtained in the present study support the prediction of multiple sites of action of changes in temperature including effects on the Na^+ pump, energy metabolism and apical entry of Na^+. Ouabain, a specific inhibitor of active Na^+ transport and $(Na^+ + K^+)$-activated ATPase[20,30], impaired the response to the temperature jump to a significantly greater extent than the response to vasopressin (Table II). Inasmuch as the action of vasopressin has been located at the site of apical entry of Na^+ (ref. 12), these results imply at least a partial effect of the temperature jump on the Na^+ pump or factors that modify the activity of the pump.

The participation of metabolic pathways in the temperature-dependent effect was explored with a variety of metabolic inhibitors. The response to the temperature jump was insensitive to substrate depletion and repletion with glucose or pyruvate

(Table III, Fig. 6) which is in contrast with the dependence of mineralocorticoid action on an adequate supply of intracellular substrate[9,15]. Interference with the primary metabolic pathways and especially oxidative metabolism, however, virtually abolished the response to the temperature jump. This effect was clearly evident with antimycin A, an inhibitor of electron transport at the level of cytochrome *c*, and rotenone, an inhibitor of NADH dehydrogenase activity[11]. As shown in Tables IV and V, the response to vasopressin and amphotericin B is reasonably intact in the presence of these inhibitors whereas the response to the temperature jump is nil. These results provide evidence for the participation of metabolic pathways in the positive response to a rise in temperature.

The evidence suggesting that the temperature effect involves both activation of metabolism and of the Na^+ pump does not exclude an additional effect on permeation across the apical boundary. Indeed, when the apical gradient for Na^+ was lowered by reduction of the Na^+ concentration in the mucosal media, the response to the temperature jump was significantly attenuated (Table VI). It is unlikely, however, that the determinant of the response to the change in temperature is the same as the determinants of the action of vasopressin and amphotericin B, as pretreatment with either of these stimulating agenst enhanced the response to the temperature jump (Table VII). It is possible that each of these agents, temperature, vasopressin and amphotericin B, activate independent parallel pathways across the apical boundary. Alternatively, additional effects of temperature on metabolism and the Na^+ pump would be expected to enhance the response to an increase in apical conductance of Na^+.

The weight of the available evidence, as well as the general principles governing the rate-determining effects of changes in temperature do not support the inference of a unique site of action of changes in temperature on Na^+ transport. The Arrhenius plot shown in Fig. 2 clearly indicates that multiple reaction rates are involved in the dependence of transepithelial Na^+ transport on temperature. Moreover, the effects are biphasic over a reasonable range of temperature with a critical change in passive permeability to Na^+ appearing at temperatures above 27° (Fig. 3). Almost coincident with this effect is inhibition of active Na^+ transport. Temperature dependence of Na^+ transport appears to be a multisite process that does not lend itself to simple interpretation. A corollary of this conclusion is that it is not yet possible to elucidate the mechanism of action of hormonal regulators of active Na^+ transport by an analysis of temperature dependence.

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