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Metabolic Regulation of Apical Sodium Permeability in Toad Urinary Bladder in the Presence and Absence of Aldosterone

Haim Garty,* Isidore S. Edelman and Bernd Lindemann
II. Physiologisches Institut der Universität des Saarlandes, 6650 Homburg/Saar, West Germany, and
Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York

Summary. In the present study, further evidence was adduced for energy-dependent regulation of passive apical transport of Na in toad bladder epithelium. In potassium-depolarized preparations studied by current-voltage analysis, additions of pyruvate or glucose to the media of substrate-depleted bladders evoked proportionate increases in the transepithelial Na current and in apical Na permeability. These reponses were large in aldosterone pretreated hemibladders and almost absent in the aldosterone-depleted preparations or when hormonal action was blocked by spironolactone or cycloheximide. The substrate-induced increases in apical Na permeability were fully reversed by appropriate metabolic inhibitors, i.e. 2-deoxyglucose and oxythiamine. Moreover, the inhibitory effect of 2-deoxyglucose was bypassed by the addition of pyruvate to the serosal medium. Thus apical Na permeability is clearly sensitive to the supply of cellular energy. The possibility that changes in intracellular free Na activity may mediate metabolic regulation of apical Na permeability was evaluated by prolonged exposure to Na-free mucosal and serosal media, with and without inhibition of the Na/K-pump by ouabain. The stimulatory and inhibitory effects of pyruvate, 2-deoxyglucose and oxythiamine on Na currents and Na conductances were preserved under these circumstances. Furthermore, reduction of serosal Ca to a minimal level of 3 μM, was without effect on the response to metabolic inhibition. These experiments demonstrate the existence of Na-independent metabolic regulation of apical Na transport and imply that neither basal-lateral nor mitochondrial Na/Ca exchange is required for this regulatory process under the imposed conditions. The possibility that a Na-independent, Ca transport mechanism in mitochondria or endoplasmic reticulum may be involved in metabolic regulation of apical Na transport, however, remains to be evaluated.

Key Words aldosterone · metabolic regulation · sodium permeability · toad bladder

Introduction

Transepithelial Na transport by the toad urinary bladder is considered to be a two-step process as suggested by Koefoed-Johnsen and Ussing for frog skin [21]. This model proposes that Na diffuses passively from the lumen across the apical membrane and is actively extruded into the interstitial space across the basal-lateral membrane by the Na pump.

Transepithelial Na transport by the toad bladder is stimulated by the mineralocorticoid, aldosterone [5]. Although this effect is well documented in many epithelia, the exact sites of action are under current investigation. Previous studies have proposed alternative sites of action of aldosterone, including augmentation of P_{Na}, or the supply of metabolic energy or both [4, 6, 8, 10, 20, 25, 29, 33, 34]. Stimulation of Na transport by aldosterone is dependent on the availability of specific metabolic substrates. In substrate-depleted toad bladder preparations, aldosterone has a very limited influence on Na transport and the full stimulatory effect is restored upon addition of substrates [8, 10, 32]. This observation was taken as evidence for an action of the hormone on the energy supply to the Na pump [8–10]. On the other hand, Sharp and Leaf [34] proposed that aldosterone increases P_{Na} even in substrate-depleted preparations but the effect on Na transport will be manifest only when enough energy is available for enhanced output of the Na pump. The possibility of metabolic control of P_{Na} or a bipolar effect has also been suggested [9, 11, 18, 25].

Recently, Palmer et al. [30, 31] found that in K-depolarized (basal-lateral side) bladders, 2DG decreased I_{Na} and P_{Na} proportionately while addition of pyruvate to substrate-depleted, aldosterone-treated preparations, increased I_{Na} and P_{Na} proportionately. Metabolic regulation of P_{Na} could be mediated by changes in Na_c or Ca_c or both. For example, modulation of the energy supply could limit Na pump activity and thereby modulate Na_c. Changes in Na_c in turn could regulate P_{Na} either directly, by feedback inhibition [22, 23, 36, 37] or indirectly by altering Ca_c [1, 2, 35].

This paper describes studies on the interplay between hormonal and metabolic regulation of

* *Present address:* Department of Membrane Research, The Weizmann Institute of Science, Rehovot, Israel.

P_{Na}. We found that P_{Na} is down-regulated when energy metabolism is inhibited regardless of the presence or absence of aldosterone. Stimulation of P_{Na} by addition of substrates, however, was evident only in aldosterone-pretreated bladders. Neither a change in Na_c nor uptake of Ca from the serosal medium was required for the response of I_{Na} to changes in metabolic status. Other modes of energy-dependent metabolic regulation of P_{Na}, therefore, deserve consideration.

Abbreviations: 2DG, 2-deoxyglucose; DMSO, dimethylsulfoxide; I_{sc}, short-circuit current; I_{Na}, sodium-specific (amiloride-blockable) current; V, transepithelial electrical potential; G, transepithelial slope conductance; G_{Na}, Na-specific slope conductance; P_{Na}, apical Na permeability; Na_c, cytoplasmic Na activity; Na_s, interstitial (serosal) Na activity; Ca_c, cytoplasmic Ca activity.

Materials and Methods

Male toads (*Bufo marinus*; Mexican origin, obtained from Lemberger, Wisconsin) were maintained in tanks in tap water. In one series of experiments, tap water was replaced by NaCl Ringer's 3 to 5 days before the experiment. The animals were double-pithed, and the urinary bladders were quickly removed and mounted on a Lucite® ring (free internal area 3 cm^2). The serosal side was supported by a piece of filter paper, and a ring-shaped Sylgard® gasket was placed on the mucosal side to improve sealing and minimize edge damage. The entire assembly was mounted in a plastic chamber with a serosal compartment of 18 ml open to the atmosphere and stirred by aeration. The mucosal compartment of 1 ml volume was perfused at a rate of 1 to 10 ml/min [14, 30].

The serosal solution was either Na-Ringer's containing (in mM): 110.0 NaCl, 1.0 $CaCl_2$, 0.5 $MgCl_2$, 3.5 Kphosphate (pH = 7.5), or depolarizing K-Ringer's in which NaCl was replaced by 85 mM KCl + 50 mM sucrose. This combination of KCl and sucrose was found to maintain the original cell volume [30]. The mucosal solution was either Na-free sulfate Ringer's containing (in mM): 55.0 K_2SO_4, 4.0 K_2HPO_4, 1.0 KH_2PO_4, 1.0 Ca-gluconate (pH = 7.5), or Na containing Ringer's in which 18.2 mM K_2SO_4 was replaced by the same concentration of Na_2SO_4. In all mucosal solutions, the combined Na and K activity was 65 mM. In the Na-containing mucosal solutions, the Na activity was 20 mM.

The bladders were voltage clamped to zero potential and I_{sc} was recorded continuously. The transepithelial electrical slope conductance (G) was calculated from the current changes obtained upon brief (1.0 sec) 10-mV displacements of the clamping potential. I_{Na} and G_{Na} were calculated either from the differences in the respective values measured in the presence and absence of 80 μM amiloride in the mucosal perfusate or from the differences in the respective values on incubation in Na-free and Na-containing mucosal solutions. On addition of amiloride, I_{sc} fell sharply and the residual shunt current and conductance were established within seconds. In most experiments, the shunt conductance was $\leqq 30\%$ of the total conductance. Reperfusion with amiloride-free Ringer's restored I_{sc} and G to control values within 5 min.

Near-instantaneous I-V relations were measured in K-depolarized bladders by stepwise voltage clamping and recording of the corresponding currents [13]. The voltage was changed in 5-mV steps from +20 to −100 mV (mucosal relative to serosal). Step duration was 5 msec, a period sufficient to achieve a constant current value, which was frequently verified by displaying the current transients on an oscilloscope. The voltage displacements had no long-term effects and repeating the measurement within 5 min gave the same $I-V$ data. Each measurement was repeated in the presence of 80 μM amiloride (less than 5 min after the first one). The difference between the two measurements was taken to represent the $I_{Na}-V$ curve.

The $I_{Na}-V$ curves were analyzed by closest fit with the constant field equation [13, 15]. As observed previously [13, 30], the constant field equation always fits the data between zero potential and the Na-reversal potential ($V_{I_{Na}=0}$). From the best fit in this voltage range, P_{Na} and Na_c were estimated [13]. In these calculations, as well as in calculating I_{Na} and G_{Na}, the electrical membrane capacitance was used to estimate membrane area [30], assuming a capacity of 1 μF/cm^2. Typically, membrane area inferred from capacitance was 2 to 4 times larger than the cross-sectional chamber area (3 cm^2).

Control of the voltage clamp, storage of data, subtraction of curves and curve-fitting were done with the aid of a computer (Data General, Nova 1230).

Statistics

Unless otherwise indicated, data are expressed as mean ±SE. Probabilities were calculated using the student t-test.

Materials

Aldosterone was obtained from CIBA, cycloheximide and oxythiamine from Serva, and spironolactone from Sigma. All of the conventional chemicals were reagent grade.

Results

Urinary bladders from toads kept for 3 to 5 days either in Na-Ringer's (to minimize the background aldosterone concentration) or in tap water, were mounted in chambers. After measuring the initital I_{Na} and G_{Na} (2 to 4 hr after mounting), one of each pair of hemibladders was incubated in 5×10^{-7} M aldosterone (added to the serosal side only, from an aqueous 0.5 mg/ml stock solution) while the other served as a control. The pairs were maintained overnight (12 to 16 hr) in substrate-free media with Na-free sulfate Ringer's on the mucosal side. The control hemibladders were incubated either (a) in the absence of aldosterone, or (b) with aldosterone and the specific antagonist spironolactone (added to a final concentration of 5×10^{-5} M from a stock solution of 0.1 M in DMSO), or (c) in the presence of aldosterone and the protein synthesis inhibitor, cycloheximide (added to a final concentration of 2×10^{-6} M from an aqueous solution of 2×10^{-2} M). Each addition was accompanied by adding an equal volume of diluent to the second preparation. In pilot experiments ($n=4$), this concentration of DMSO (0.05%) had no effect

Table 1. Effect of environmental NaCl on the response to aldosterone[a]

Pre-treatment *in vivo*	Pairs (n)	Initital values		Fractional change after overnight incubation			
		I_{Na_o} ($\mu A/cm^2$)	G_{Na_o} (mS/cm^2)	Aldosterone-treated		Control	
				I_{Na}/I_{Na_o}	G_{Na}/G_{Na_o}	I_{Na}/I_{Na_o}	G_{Na}/G_{Na_o}
NaCl-Ringer's	10	4.6±0.95	0.045±0.007	2.2±0.8	2.5±0.25	0.55±0.40	1.0±0.13
Tap water	9	10.2±2.1	0.039±0.014	2.6±0.2	2.9±0.27	1.0 ±0.27	1.2±0.1

[a] Toads were maintained for 3 to 5 days in either Na-Ringer's or tap water before harvesting of the urinary bladders. I_{Na_o} and G_{Na_o} were measured 2 to 4 hr after mounting of the hemibladders. The hemibladders were incubated in substrate-free media, with or without aldosterone (5×10^{-7} M).

on I_{sc} or G. At the end of overnight incubation the mucosal solution was replaced with Na-sulfate Ringer's and I_{Na} and G_{Na} were remeasured.

Table 1 summarizes the effects of *in vivo* salt loading on the initital Na transport parameters and overnight response to aldosterone in the absence of substrate and mucosal Na. Salt loading decreased the initial I_{Na} ($P<0.01$) but the two populations did not differ in their relative response to aldosterone ($P>0.10$). In both populations, the hormone elicited more than twofold increases in I_{Na} and G_{Na}. No systematic differences were noted in the three control conditions (i.e. no aldosterone, or aldosterone + spironolactone, or aldosterone + cycloheximide) after overnight incubation. Accordingly the control results were lumped. Although I_{Na} of the controls tended to fall in hemibladders from salt-loaded toads after overnight incubation, none of the fractional changes in I_{Na} or G_{Na} were statistically significant.

After measuring the Na-specific parameters, K-depolarizing-Ringer's was substituted for the NaCl-Ringer's on the serosal side. This substitution caused an immediate drop in I_{sc} and an increase in G. Most of the decrease in I_{sc}, however, reversed spontaneously. The fall in I_{sc} is attributable to the decrease in the transapical driving force associated with abolition of the electric field across both membranes. In addition, removal of serosal Na may have increased Ca_c by abolition of the Na_s/Ca_c exchange and thereby reduce P_{Na} [2]. I_{Na} 90 min after depolarization differed from the value measured immediately before depolarization by a factor of 0.75 + 0.08 ($n=20$).

$I_{Na}-V$ curves were obtained in depolarized bladders with 20 mM Na on the mucosal side to study the influences of substrates and metabolic inhibitors on P_{Na}, I_{Na} and Na_c. A typical experiment in which sequential additions of glucose, 2DG, pyruvate and oxythiamine were made in control and aldosterone-treated paired hemibladders is shown in Fig. 1. In the aldosterone-treated hemibladders both glucose and pyruvate gave rise to substantial increases in I_{Na} accompanied by equivalent increases in P_{Na}. These effects were fully reversed on addition of the metabolic inhibitors. The inhibitory effect of 2DG was bypassed by addition of pyruvate. More complex was the response of Na_c to manipulations of the metabolic status. Forty minutes after addition of pyruvate, I_{Na} had increased from 8 to 14 $\mu A/cm^2$ and Na_c had dropped from 1.95 to 1.2 mM. In the subsequent 90 min, Na_c increased from 1.2 to 1.7 mM while I_{Na} and P_{Na} continued to increase further. This behavior may reflect independent augmentation of

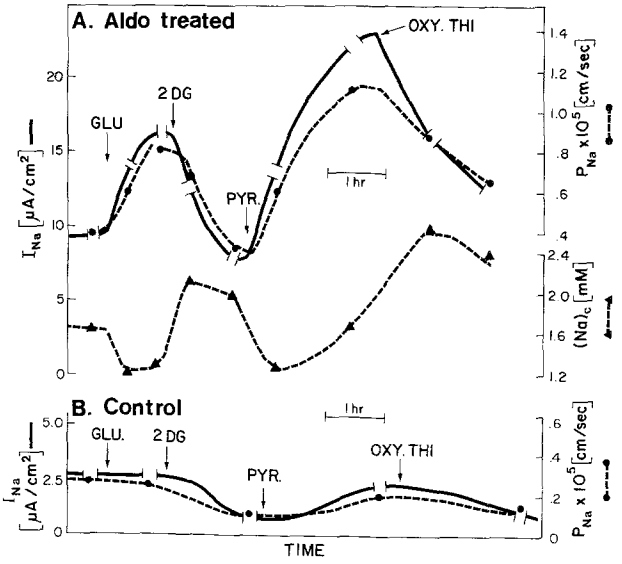

Fig. 1. Effects of substrates and inhibitors on Na-specific electrical parameters. I_{sc} was continuously recorded in aldosterone-treated (*A*) and control (*B*), K-depolarized hemibladders. At the times indicated by the gaps in the current trace, $I_{Na}-V$ measurements were performed and used to estimate P_{Na} and Na_c. Glucose (5 mM), 2-DG (5 mM), pyruvate (6.5 mM) and oxythiamine (5 mM) were added to the serosal media sequentially at the times indicated by arrows. Time-zero was at least one hour after substitution of the depolarizing K-Ringer's solution for the conventional Na-Ringer's serosal medium

Table 2. Fractional change in sodium-specific parameters induced by substrates and inhibitors[a]

	Aldosterone-treated		Control	
	I_{Na}	P_{Na}	I_{Na}	P_{Na}
Glucose	1.4±0.16 (7)[b]	1.3±0.12 (7)[b]	1.1±0.16 (7)[c]	1.1±0.08 (7)[c]
2-Deoxyglucose	0.5±0.06 (7)	0.4±0.06 (7)	0.6±0.08 (7)	0.65±0.08 (7)
Pyruvate	2.5±0.14 (7)	2.3±0.16 (7)	1.4±0.25 (5)[c]	1.4±0.25 (5)[c]
Oxythiamine	0.5±0.06 (4)	0.5±0.06 (4)	0.4±0.09 (4)	0.45±0.11 (4)[b]

[a] The experimental protocol is as shown in Fig. 1. Fractional changes are calculated as the ratio of the values measured at the maximal effect to that measured immediately before addition of the agent. The numbers in parentheses are the number of experiments.
[b] $P<0.025$.
[c] $P>0.05$.

In all other cases $P<0.005$.

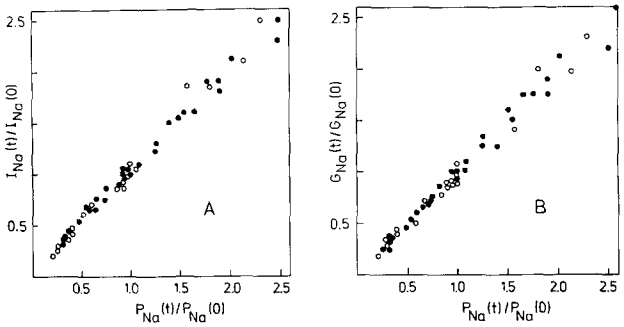

Fig. 2. Analysis of the correlations between Na-specific transport parameters. In *A*, the fractional change in Na current ($I_{Na}(t)/I_{Na}(O)$) is plotted as a function of the fractional change in apical Na permeability ($P_{Na}(t)/P_{Na}(O)$). In *B*, the fractional change in Na conductance ($G_{Na}(t)/G_{Na}(O)$) is plotted as a function of $P_{Na}(t)/P_{Na}(O)$. The reference values $I_{Na}(O)$, $P_{Na}(O)$ and $G_{Na}(O)$ were obtained 1 hr after depolarization with K-Ringer's. $I_{Na}(t)$, $P_{Na}(t)$ and $G_{Na}(t)$ are the values of these parameters at various times after the addition of substrates or inhibitors to the serosal media. The aldosterone-pretreated hemibladders are denoted by (●), ($n=31$) and the controls by (○), ($n=19$)

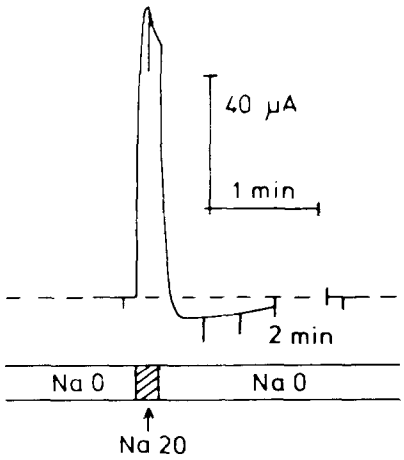

Fig. 3. Protocol used to assess I_{Na} and G_{Na} in the presence of Na-free mucosal solutions. Hemibladders were maintained in K-depolarizing serosal solutions and Na-free sulfate Ringer's (Na 0) on the mucosal side. During brief (~20 sec) intervals, sulfate Ringer's containing 20 mM Na (Na 20) was substituted for the Na-free solutions. The dashed line is the zero current ($I_{sc}=0$) and the continuous line the current response to addition and withdrawal of mucosal Na

P_{Na} and the energy supply of the Na pump. Since transepithelial sodium transport is usually limited by the rate of Na entry, an increase in P_{Na} will give rise to a similar increase in I_{Na}. In contrast, Na_c is controlled by both P_{Na} and the Na pump. A faster effect of pyruvate on the pump rate will result in the observed biphasic change in Na_c. In this experiment (Fig. 1), the control (aldosterone plus spironolactone) did not respond to added glucose but showed a clear down-regulation of apical transport parameters after addition of 2DG. This inhibition was bypassed by subsequent addition of pyruvate.

Table 2 summarizes the maximal fractional changes in I_{Na} and P_{Na} in the experiments described above ($n=14$ pairs of hemibladders). In another 4 pairs there was little response to substrates and in 5 other pairs the paracellular shunts had become too conductive to allow accurate measurements after overnight incubation. The changes in metabolic status invariably had significant effects on I_{Na} and P_{Na} in the aldosterone-treated hemibladders. In the control hemibladders, glucose and pyruvate did not increase I_{Na} and P_{Na} significantly. In contrast, the magnitude of fractional inhibition of I_{Na} and P_{Na} by either 2DG or oxythiamine was comparable in the control and aldosterone-treated tissues.

Regardless of hormonal status the fractional change in I_{Na} varied linearly with the fractional change in P_{Na}, with a slope of 0.9±0.1 (SD) and a correlation coefficient of 0.994 (Fig. 2*A*). A simi-

Table 3. Metabolic effects on I_{Na} and G_{Na} in the presence and absence of mucosal sodium[a]

Mucosal medium	Pairs (n)	I_{Na_o} ($\mu A/cm^2$)	G_{Na_o} (mS/cm^2)	Additions (mM)	I_{Na}/I_{Na_o}	G_{Na}/G_{Na_o}
Na-Ringer's	5	12.7±2.5	0.26±0.04	Pyruvate (5)	1.37±0.22	1.21±0.11
				Oxythiamine (5)	0.46±0.06	0.50±0.07
Na-free Ringer's	7	19.1±4.0	0.27±0.06	Pyruvate (5)	1.89±0.37	2.28±0.51
				Oxythiamine (5)+2DG (5)	0.34±0.05	0.38±0.08

[a] Paired hemibladders were incubated overnight in substrate-free, NaCl-Ringer's supplemented with aldosterone (5×10^{-7} M) serosally and Na-free-SO_4-Ringer's on the mucosal side. In the morning, depolarizing potassium-Ringer's (Na-free) was substituted for all of the serosal solutions and the mucosal sides were perfused with either Na-sulfate Ringer's (20 mM) or Na-free sulfate-Ringer's. I_{Na_o} and G_{Na_o} values were recorded one hr after depolarization and then challenged serially with serosal K-pyruvate and either oxythiamine alone or oxythiamine +2DG. In the Na pulse experiments, oxythiamine and 2-DG were used in combination since little effect was obtained on I_{Na} or G_{Na} with either inhibitor alone in this series.

lar correlation was established between G_{Na} and P_{Na} (Fig. 2*B*, $r=0.990$). Agreement between P_{Na} and G_{Na} is expected if changes in Na_c are small enough to keep the sodium gradient across the apical membrane nearly constant. In contrast, the fractional changes in P_{Na} and Na_c varied independently of each other; a plot of $P_{Na}(t)/P_{Na}(0)$ *vs.* $Na_c(t)/Na_c(0)$ had a correlation coefficient of 0.280. These results imply that P_{Na} is not uniquely regulated by feedback inhibition by Na_c under experimental conditions in which the changes in Na_c are limited to values below 3 mM. The imprecision in measuring Na_c by the curve-fitting method, however, made it prudent to examine this issue by another experimental design: Metabolic status was varied under completely Na-free conditions and the resulting changes in G_{Na} and I_{Na} were measured during periods of exposure to mucosal Na which were brief enough to prevent significant changes in Na_c. Accordingly, paired hemibladders were incubated in the Na-free, sulfate-Ringer's solution on the mucosal side (denoted as Na 0 in Fig. 3) and at intervals the mucosal solutions were rapidly exchanged for Na_2SO_4-Ringer's solutions containing 20 mM Na (denoted as Na 20). The total exposure time to Na 20 was 20 sec. Continuous recordings of I_{sc} and G revealed a virtual elimination of I_{sc} during exposure to Na 0. Conductance measurements, shown as the vertical spikes in Figs. 3 and 4), represent the paracellular shunt slope conductance during exposure to Na 0 and the combined conductances (cellular and paracellular) during exposure to Na 20. Introduction of Na 20 into the mucosal compartment evoked immediate increases in I_{sc} and G, which reached maximal values in 1 to 3 sec (Fig. 3). I_{Na} and G_{Na} were calculated from the differences in the values obtained at the peak of the responses and those recorded immediately before substitution of Na 20 for Na 0. The magnitude of the rise in I_{Na} on addition of Na to the mucosal medium reflects the uptake of Na when Na_c is almost nil. Fifteen to 20 sec after perfusion with Na 20, Na 0 was reintroduced into the mucosal compartment. This resulted in a rapid fall in I_{sc} followed, at the nadir, by a transient negative current (reversed I_{sc}, Fig. 3). This reversed current (serosal side negative) is abolished by addition of amiloride (80 μM) to the mucosal side. Moreover, the magnitude of the reversed I_{sc} is increased on exposure to serosal ouabain (1 mM). The sensitivity to amiloride and augmentation by ouabain indicate that some Na penetrated into the cells (presumably sub-apical) during the brief exposure to Na 20 and back diffused, via the Na channels, into the mucosal compartment on reperfusion with Na 0.

To assess the role of accumulation of intracellular Na in the responses to hormone and substrate, paired hemibladders (aldosterone-treated, substrate-depleted) were incubated in serosal Na-free, K-depolarizing Ringer's and either mucosal Na-free Ringer's or Na-sulfate Ringer's (controls). Both hemibladders were challenged with K-pyruvate (5 mM) 1 hr after depolarization. Subsequently, oxythiamine (5 mM) alone or oxythiamine (5 mM) plus 2DG (5 mM) was added to the serosal media. The changes in I_{Na} and G_{Na} were measured at the expected maxima of the responses, i.e. 1 to 2 hr after addition of the substrate and then 1 to 2 hr after addition of the inhibitors. The control hemibladder was continuously exposed to 20 mM mucosal Na except for brief intervals of exposure to the Na-free mucosal solution, during which time the shunt conductance was measured. The experimental bladder was continuously exposed to Na-free mucosal solution except for brief intervals of perfusion with 20 mM Na during which time I_{Na} and G_{Na} were measured as described above. Thus, Na_c should have been virtually nil except for the brief periods of exposure to mucosal

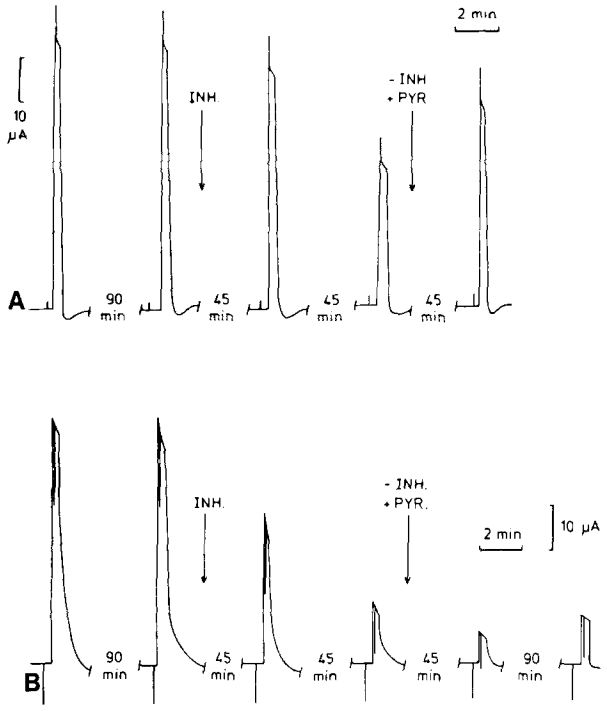

Fig. 4. Hemibladders were incubated for 2 to 4 hr in Na-free sulfate Ringer's (mucosal) and substrate-free, K-depolarizing Ringer's + 1 mM ouabain (serosal). Current (I_{sc}) and conductance (G) were measured during short exposure of the mucosal side to Na-sulfate Ringer's (20 mM). Ninety min after the peak I_{Na} values (in response to a pulse of mucosal Na) became constant, 5 mM 2DG + 5 mM oxythiamine (denoted by *INH*) were added to the serosal compartment (first arrow). The serosal solutions were replaced (90 min later) by K-Ringer's containing 10 mM pyruvate (denoted by *PYR*) instead of 2DG + oxythiamine (second arrow). The spikes are current deflections in response to 10 mV voltage displacements. (*A*) and (*B*) display two of the variations in the responses in single hemibladders

Na and the time required for subsequent elimination of Na_c (about 2 min).

In the Na-depleted preparations, the initital values (I_{Na_o} and G_{Na_o}) were comparable to those with continued exposure to mucosal Na (Table 3). With or without Na in the media, I_{Na} and G_{Na} rose proportionately on the addition of pyruvate, and fell proportionately on the addition of oxythiamine or oxythiamine plus 2DG. Moreover, in the Na-free media no attenuation was evident in the magnitude of the responses to substrate or inhibitors. These results indicate that with virtually no Na available to the intracellular pools, metabolic modulation of apical Na conductance is at least as evident (both relatively and absolutely) as in the presence of Na.

Palmer et al. [30] reported that 2DG applied to K-depolarized bladders shortly after harvesting induced proportionate falls in I_{Na} and P_{Na}. In our studies on the effects of mucosal Na on the responses to substrates and inhibitors all of the hemibladders were incubated overnight in substrate-free, aldosterone-supplemented media. To determine if freshly harvested hemibladders (i.e. without depletion of endogenous substrate stores) are similarly responsive to metabolic regulation in Na-free media we measured the effects of 2DG, oxythiamine and pyruvate without overnight incubation and without exposure to aldosterone.

In these experiments hemibladders were mounted in depolarizing K-Ringer's on the serosal side. One hour later, Na was removed from the mucosal side and the Na pump was blocked with 1 mM ouabain (serosal). I_{Na} and G_{Na} were measured from time to time during brief exposure to mucosal Na Ringer's, as described above (*cf.* Fig. 3). After stabilization of I_{Na} and G_{Na} (2 to 4 hr), the bladders were challenged with inhibitors. Oxythiamine or 2 DG alone did not significantly affect I_{Na} or G_{Na}, but their combination caused a substantial drop in these values (Fig. 4). Removal of the metabolic inhibitors and adding pyruvate partially reversed the inhibition of I_{Na} and G_{Na}, but reversibility was more evident in those cases where only a moderate inhibition of transport had taken place (*compare* Figs. 4*A* and 4*B*). In four hemibladders that exhibited responses to substitution of pyruvate for the inhibitors, the ratios were $I_{Na}/I_{Na_o} = 1.04 \pm 0.23$, and $G_{Na}/G_{Na_o} = 1.19 \pm 0.25$, 160 min after the exchanges. As discussed above, the initial rise in I_{Na}, on addition of Na to the medium, reflects the initial uptake of Na when Na_c is almost nil. It is noteworthy that impairment of metabolism significantly reduced this initial current (*see* Fig. 4). This observation shows most clearly that the inhibition develops in the absence of cellular Na. Furthermore, a second exposure to mucosal Na, immediately after I_{Na} returned to zero, produced an identical rise in the current trace *(data not shown)*. This result implies that intracellular accumulation of Na during the brief period of measurement does not influence either I_{Na} or G_{Na}. Eleven freshly mounted Na-depleted hemibladders not supplemented with exogenous substrate or aldosterone also displayed ~50% reductions in I_{Na} and G_{Na} in response to metabolic inhibition (first two columns of Table 4). Thus under these conditions, as well as after overnight incubation, metabolic regulation of apical Na transport is fully expressed even in Na-free media, i.e. not dependent on feedback inhibition by Na_c.

These results also imply that changes in Ca_c mediated by basal-lateral Na/Ca exchange [2] or by Na-dependent Ca transport across mitochondria [1] is not an obligatory step in the metabolic

Table 4. Metabolic effects on passive Na transport in Na-free media as a function of serosal Ca[a]

	1 mM serosal Ca		3 μM serosal Ca	
	I_{Na}/I_{Na_o}	G_{Na}/G_{Na_o}	I_{Na}/I_{Na_o}	G_{Na}/G_{Na_o}
90 min incubation just prior to the addition of inhibitors	1.02±0.03 (11)	1.03±0.03 (11)	1.07±0.03 (7)	1.10±0.12 (7)
120 min after adding 2DG and oxythiamine	0.43±0.06 (11)	0.50±0.06 (11)	0.26±0.03 (7)	0.38±0.03 (7)

[a] Potassium-depolarized hemibladders were incubated for 2 to 4 hr without substrates in the presence of 1 mM ouabain before the experiment. These preparations were not exposed to aldosterone. The protocol is given in Fig. 3. I_{Na_o} in 1 mM Ca; 16±2.1 μA/cm² (11); in 3 μM Ca; 21.9±4.1 μA/cm² (7). G_{Na_o} in 1 mM Ca; 0.35±0.05 mS/cm² (11); in 3 μM Ca; 0.48±0.13 mS/cm² (7).

control of P_{Na}. Nevertheless, Ca_c may be involved via other mechanisms. One possibility is that the basal-lateral membrane contains a Na-independent Ca transport system. It would permit Ca uptake which is counterbalanced by an ATP-limited Ca pump. The latter might also be contained in the basal-lateral membrane [19]. Its metabolic impairment would cause net Ca uptake from the interstitial compartment. To test the involvement of Na-independent Ca uptake, the protocol of Fig. 4 was repeated using serosal solutions which contained only 3 μM Ca. This concentration proved sufficient to maintain the conductance of the paracellular pathway at a low level. The serosal compartment was replaced 4 times with the low-Ca Ringer's in order to assure complete exchange. Although the actual Ca activity on the basal-lateral membrane surface could have been higher than 3 μM, this substitution (in a separate set of experiments) was sufficient to prevent the inhibition of P_{Na} induced by basal-lateral Na/Ca exchange (Garty & Lindemann, *unpublished observations*). The results are shown in columns 3 and 4 of Table 4. In the presence of this minimal concentration of serosal Ca the inhibitors were at least as effective as in higher Ca in reducing I_{Na} and G_{Na}. This observation suggests that *uptake* of interstitital Ca is not required for the down-regulation of apical Na transport in response to metabolic impairment.

Discussion

In a recent study Palmer et al. [31] noted that aldosterone induced equivalent increases in I_{Na} and P_{Na} in toad bladder. Current fluctuation analysis revealed that the increase in P_{Na} is a result of an increase in the density of electrically detectable Na channels with no significant change in the single-channel current. An increase in P_{Na} was also evoked by addition of pyruvate to aldosterone pretreated but substrate-depleted bladders [31].

The present study concerns metabolic regulation of transepithelial Na transport. In substrate-depleted aldosterone-treated but not in the aldosterone-depleted hemibladders we observed a substantial increase in P_{Na} on addition of pyruvate or glucose to the serosal media, although pyruvate was the more effective substrate (Table 2). In the aldosterone-depleted preparations, addition of glucose and pyruvate induced smaller increases in I_{Na} and P_{Na} (i.e. comparing glucose to glucose + aldosterone or pyruvate to pyruvate + aldosterone) and these increases failed to achieve statistical significance. Furthermore, the metabolic inhibitors 2DG and oxythiamine produced striking declines in I_{Na} and P_{Na} in both control and aldosterone-treated hemibladders. A number of features of the response patterns imply that the substrates act via effects on energy turnover rather than by direct modulation of transport components: 1. Stimulation of I_{Na} and P_{Na} by glucose addition was virtually abolished by 2DG, suggesting that glucose was being metabolized by glycolysis. 2. The 2DG block was fully reversed by pyruvate, presumably by providing substrate for the tricarboxylic acid cycle.[1] 3. The pyruvate-dependent increase in P_{Na} and I_{Na} was in turn overcome by oxythiamine, suggesting utilization of pyruvate by the tricarboxylic acid cycle. 4. These effects were obtained in Na-free serosal media, eliminating possible cotransport of Na and glucose, or Na and pyruvate as the basis of the effects.

These and previous [11, 18, 30, 31] findings indicate the existence of a metabolic control mechanism which reversibly activates Na channels. One

[1] 2-DG inhibits metabolism by two mechanisms: 1. formation of end-product, 2-DGP, with a consequent depletion of intracellular PO_4, or 2. competitive inhibition of the conversion of glucose to glucose-6-PO_4, thereby inhibiting glycolysis [17]. In the present experiments, the media contained 3.5 mM PO_4 which may have served to replenish intracellular PO_4. Reversal of inhibition with pyruvate, under these circumstances, implies that 2-DG inhibited I_{Na} primarily by depletion of glycolytic intermediates.

function of this control mechanism would be to synchronize the rates of Na entry and exit, and thus prevent large changes in Na_c which would otherwise occur when for metabolic reasons the rate of active Na extrusion cannot cope with the rate of Na entry. Indeed Na_c does appear to vary relatively little despite large changes in Na transport mediated by changes in metabolism. For example, in 10 hemibladders pretreated with aldosterone the fractional values were $P_{Na}/P_{Na_o} = 2.1 \pm 0.2$ and $Na_c/Na_{c_o} = 1.1 \pm 0.15$ in response to substrates; in 9 treated with 2DG and oxythiamine the values were $P_{Na}/P_{Na_o} = 0.45 \pm 0.05$ and $Na_c/Na_{c_o} = 1.1 \pm 0.1$. In contrast, partial inhibition of the Na pump with ouabain caused an easily detectable sixfold increase in Na_c [30]. Lipton and Edelman [25] noted no detectable change in chemical estimates of intracellular Na after stimulation by aldosterone, and postulated coordinate regulation of Na entry and exit.[2]

The above experiments were carried out in the presence of high K-sucrose solutions (basal-lateral depolarization) on the serosal side; a "nonphysiological state." This condition, and the use of Na-free mucosal solutions, were used in order to evaluate the effects of regulators on apical Na permeability and the possible dependence of P_{Na} on Na_c. The justification for the physiological validity of this preparation is based on the following: 1. The substitution of the high K-sucrose solution has no significant effect on epithelial cell volume and perturbs I_{Na} only minimally (i.e. ~10% fall) [30]. 2. In K-depolarized preparations the I_{sc} responses to aldosterone, and to metabolic substrates and inhibitors, both in magnitude and time-course are indistinguishable from those obtained with Na-Ringer's solutions on both sides [30, 31, and the present paper].

At least three possible mechanisms can account for coupling of P_{Na} to metabolism: 1. There may be activation of a pre-existing set of channels in the apical membrane by chemical modification. Such modification could be phosphorylation or dephosphorylation, which has been postulated as a possible mechanism for controlling ion permeability [7, 26]. Alternatively, channels may be activated by changes in membrane lipids (e.g. fatty acid turnover and composition [16, 24], prostaglandin metabolism [39]). 2. Energy-dependent recruitment of channels from granules or vesicles underlying the apical membrane may take place, as was postulated for vasopression [28]. The possibility of substrate dependent *de novo* synthesis of channels is not likely since the onset of the response to substrate addition is almost immediate. Furthermore, inhibition of transcription in substrate-depleted aldosterone-pretreated bladders did not affect the pyruvate-induced increase in I_{sc} for at least one hour [3]. 3. The available energy supply could control the activity of intracellular ions (e.g. Na, Ca, H), which in turn might modify the apical Na channels. Lewis et al. [23] and Frizzell and Schultz [12] noted (in rabbit bladder and rabbit colon, respectively) that aldosterone enhanced apical Na conductance. One explanation for this effect was considered by both groups: a possible primary action on active Na extrusion resulting in a fall in Na_c and a subsequent release of G_{Na} from feedback inhibition.[3] An alternative mechanism for control of G_{Na} was proposed by Taylor and Windhager [35] who suggested that Ca_c, controlled by a Na/Ca antiport in the basal-lateral membrane, regulates P_{Na}. Indeed, Wiesmann et al. [38] and Ludens [27] showed that addition of the Ca ionophore A23187 to the serosal medium in the presence of serosal Ca inhibits I_{Na} in toad bladder, presumably by increasing Ca_c. Recently, Chase and Al-Awqati [2] provided more direct evidence for the existence of a 3Na/1Ca antiport at the basal-lateral membrane.

In our studies, the possible regulation of P_{Na} by Na_c was evaluated by measuring G_{Na} in Na-free serosal and mucosal media, except for brief exposures to mucosal Na. In some experiments the Na pump was also effectively inhibited by ouabain (despite the high concentration of serosal K [30]). We found that under these conditions, where the transport pool of Na was virtually eliminated, both stimulation and inhibition of G_{Na} in response to substrates and inhibitors was entirely preserved. It is true that during the brief exposures to mucosal Na, required to measure the Na-transport parameters, a small amount of Na necessarily entered the cells. However, the establishment of transport inhibition is already apparent in the reduced amplitude of the peak Na-current observed at the onset of the Na-pulse. At the time of this peak current the

[2] Subsequent studies distinguished between chemical analysis of tissue Na and Na derived primarily from the mucosal solution (i.e. radiosodium labeling) as a means of measuring the transport pool of Na (A. Leaf & A.D.C. MacKnight, 1972, *J. Steroid Biochem.* **3**:237, and R. Rick et al. 1978, *J. Membrane Biol.* **39**:313). These studies showed some increase in the transport pool of Na in response to aldosterone, as was the case in the present study.

[3] Figure 2*B* indicates that under our experimental conditions G_{Na} and P_{Na} correlated closely. Accordingly, these parameters are taken to represent the same property of the apical membrane for the purpose of this discussion.

gain in Na_c must be vary small. Thus Na_c does not appear to mediate metabolic regulation of P_{Na}. This result also does not support the possibility of metabolic regulation of P_{Na} by Ca_c via a basal-lateral or mitochondrial Na/Ca exchange mechanism.

Consideration was also given to the possibility of regulation of P_{Na} by Ca_c under the control of an energy-dependent Ca extrusion mechanism which is Na-independent. A 300-fold reduction in serosal Ca concentration, i.e. from 1 mM to 3 μM, however, did not restrain the inhibitory response to 2DG + oxythiamine. Thus our data do not signal the participation of basal-lateral Ca *uptake* in the metabolic regulation of P_{Na}. Of course, Ca_c could be involved in these processes via other pathways (e.g. energy-dependent Ca *release* in mitochondria or other organelles).

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