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Serosal Na/Ca Exchange and H^+ and Na^+ Transport by the Turtle and Toad Bladders

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Summary. A Na/Ca exchange system has been described in the plasma membrane of several tissues and seems to regulate the concentration of calcium in cytosol. Replacement of extracellular Na by sucrose increases calcium uptake into and decreases calcium efflux from the cell, leading to an increase in cytosolic calcium. The effect of an increase in cytosolic calcium mediated by the Na/Ca exchange system on H^+ and Na transport in the turtle and toad bladder was investigated by replacing serosal Na isosmotically by sucrose or choline. Replacement of serosal by sucrose was associated with a significant inhibition of H^+ secretion or Na transport which was reversible by addition of NaCl. Replacement of mucosal Na by sucrose failed to alter H^+ secretion. Removal of serosal Na was associated with a significant increase in ^{45}Ca uptake which could be blocked by pretreatment with lanthanum chloride. Pretreatment with lanthanum chloride blunted the inhibitory effect of replacement of serosal Na by sucrose on H^+ and Na transport, thus suggesting that the increase in calcium uptake and the inhibition of transport are causally related. Under anaerobic conditions the rate of H^+ or Na transport are linked to the rate of lactate production. The inhibition of Na or H^+ transport by removal of serosal Na was accompanied by a proportional decrease in lactate production, thus suggesting that an increase in cytosolic calcium does not inhibit transport by uncoupling glycolysis from transport. Replacement of serosal Na by sucrose did not alter the force of the H^+ or Na pump but led to an increase in resistance of the active pathway of H^+ and Na transport. The inhibition of Na transport by replacement of serosal Na with sucrose could be reversed by addition of amphotericin B, an agent which increases luminal permeability to Na, thus suggesting that decreased Na entry across the apical membrane is the mechanism responsible for the inhibition of Na transport. The results of the present studies strongly suggest that an increase in cytosolic calcium through the serosal Na/Ca exchange system inhibits H^+ and Na transport in the turtle and toad bladder probably by increasing the resistance of the luminal membrane.

Key Words turtle bladder · toad bladder · calcium uptake and efflux · H^+ secretion · Na transport · lactate production · ATP · ADP

Introduction

The factors regulating calcium extrusion from the cell, although well characterized in other systems (Borle, 1973; Rasmussen & Goodman, 1977), have not been well studied in the toad or turtle bladders. One system that seems particularly important in the regulation of cytosolic calcium is Ca/Na exchange (Reeves & Sutko, 1979; Taylor & Windhager, 1979; Van Breeman, Aaronson & Loutzenhiser, 1979). This system seems to be important mainly at the blood side of epithelia as suggested by studies performed in the frog skin. It has been known for many years that removal of Na from the blood side in the frog skin or toad bladder *in vitro* is associated with inhibition of Na transport (*see* review by Macknight, DiBona & Leaf, 1980). Recently, it has been suggested that this phenomenon is the result of an increase in calcium uptake (Grinstein & Erlij, 1978).

In other systems, removal of Na increases calcium uptake and decreases calcium efflux; both events will lead to an increase in cytosolic calcium levels (Blaustein, 1974; Baker, 1978; Mullins, 1981; Reeves & Sutko, 1979; Van Breemen et al., 1979). In the absence of serosal Na the increase in calcium uptake is explained by the fact that the outward movement of Na due to the favorable concentration gradient leads to an increase in calcium uptake. When Na is present in the serosal solution the energy generated by passive entry of Na into the cell serves to extrude calcium. This system seems to be of extreme importance in maintenance of cytosolic calcium in several tissues (Blaustein, 1974; Baker, 1978; Mullins, 1981; Reeves & Sutko, 1979; Van Breemen et al., 1979). Recently, this system has been isolated from cardiac membranes and reconstituted into liposomes (Miyamoto & Racker, 1980). The reconstituted system was capable of calcium extrusion in the presence of Na.

Intracellular calcium has been shown to play an important role in the transport of Na, H^+ and water by the turtle and toad bladder (Hardy, 1977; Weisman, Sinha & Klahr, 1977, 1978; Grinstein

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& Erlij, 1978; Arruda, 1979; Arruda, Dytko & Mola, 1979; Hardy, 1979; Hardy, Balsam & Bourgoignie, 1979; Taylor & Windhager, 1979; Arruda & Sabatini, 1980*a–c*). The role of the Na/Ca exchange system on Na transport by the toad bladder has been only partially studied (Taylor & Windhager, 1979; Chase & Al-Awqati, 1981). The role of this system in the regulation of H^+ secretion by the turtle bladder has not been examined.

In the present study we evaluated the effect of serosal Na removal on H^+ and Na transport by the turtle (Schilb & Brodsky, 1966) and toad bladders and correlated the alterations in transport with alterations and calcium uptake and efflux.

Materials and Methods

Measurement of Na or H^+ Transport

The methods used to measure Na and H^+ secretion have been described in detail previously (Arruda, 1979; Arruda et al., 1979; Arruda & Sabatini, 1980*a–c*). In brief, urinary bladders of fresh water turtles (*Pseudemys scripta*) or toads (*Bufo marinus*) were removed, divided in two halves, and each hemibladder was mounted in a Lucite® chamber. The exposed area of the bladder was 8 cm^2, and the results are expressed for this area. The two sides of the turtle bladder were bathed in 10 ml of Ringer's solution of the following composition (in mmol/liter): NaCl 114.4, KCl 3.5, Na_2HPO_4 2.0, dextrose 5, $CaCl_2$ 1.8, and $MgCl_2$ 2.5. The toad bladders were bathed in 10 ml of Ringer's solution of the following composition (in mmol/liter): Na 112, Cl 113, K 5.4, Ca 1.0, HPO_4 2,4, H_2PO_4 0.6, dextrose 5, pH 7.4. In the experiments utilizing $LaCl_3$, phosphate was replaced by Tris. The mucosal solution was bubbled with compressed air or N_2 and the serosal solution was bubbled with a gas mixture containing 99% air and 1% CO_2 or 95% N_2 and 5% CO_2. The spontaneous potential difference (PD) was measured with 3 M KCl-agar bridges and calomel half-cells which were connected to a Keithley model 600B Voltmeter (Keithley Instruments, Inc., Cleveland, Ohio). An automatic voltage clamp was used to supply enough current via KCl-agar bridges and Ag-AgCl electrodes to nullify the spontaneous PD. The short-circuit current (SCC) was measured by a Simpson microammeter. All experiments were performed in the short-circuited state. Bladders that failed to maintain a spontaneous PD greater than 15 mV during the first hour were discarded. The rate of Na transport was taken to be equal to the SCC (Nakagawa, Klahr & Bricker, 1967). The rate of H^+ secretion was measured either as the reverse short-circuit current (RSCC) after Na transport was abolished by 5×10^{-4} M ouabain added to the serosal side or with the pH-stat technique as previously described (Arruda, 1979). In the present experiments we demonstrated that the RSCC equals the rate of H^+ secretion (measured with pH-stat technique) when serosal Na was replaced by sucrose. In the experiments reported here the RSCC or SCC was recorded continuously except for brief intervals when the PD was measured. In all experiments, unless otherwise stated, the pH of the bathing solutions was maintained at 7.4. In all experiments the hemibladders were allowed to stabilize for 1 to 2 hr before changing the serosal solution. In some experiments, the pH gradient necessary to nullify the rate of H^+ secretion (designated as the apparent protonmotive force) was measured before and after 1 hr of replacement of serosal Na by sucrose. In these experiments the pH of the mucosa was lowered in a stepwise fashion from 7.4 to 6.4, 5.4 and then to a level at which H^+ secretion was nullified. At each of these levels H^+ secretion was measured. The apparent protonmotive force (expressed in pH units) was calculated as the difference between the serosal pH, which was kept at 7.4, and the mucosal pH at which H^+ secretion was nullified.

In some experiments the unidirectional sodium flux (mucosa to serosa) was measured with ^{22}Na. Ten μCi of ^{22}Na was added to the mucosal solution of the turtle bladder and 60 min were allowed for equilibration. After this time 20-μl samples were obtained from the mucosal and serosal solutions at the beginning and at the end of two periods of 30-min duration. The serosal solution was then replaced by a Na-free sucrose solution and 30 min were allowed for equilibration. Samples were then obtained at the beginning and at the end of two periods of 30-min duration. The radioactivity of ^{22}Na was counted in a gamma counter.

After the RSCC or SCC was stable for at least 30-min, the serosal solution was exchanged for a solution in which NaCl was replaced isosmotically by sucrose or by choline chloride; the serosal side of the other hemibladder (control) was drained and replaced with the same solution (control wash). In the experiments in which NaCl was replaced by sucrose, Cl was present in the solution as KCl. The RSCC or SCC was measured continuously and recorded with a Fisher recorder for at least one hour after each experimental maneuver.

Estimation of the Electromotive Force (E_{Na})

The method utilized to measure E_{Na} was a modification of the method of Isaacson (1977) which utilizes amiloride. The assumption behind the use of amiloride to measure E_{Na} is that the drug only affects the resistance of the active pathway. In these experiments E_{Na} was measured in the beginning and at the end of the experiment in the same hemibladder. The resistance of the shunt pathway (R_{sh}) can be calculated from the following formula:

$$1/R_{sh} = (1/PD_A - 1/PD_B)/(1/SCC_A - 1/SCC_B)$$

where A and B refer to values obtained during and before amiloride administration (10^{-6} M), respectively (Isaacson, 1977). E_{Na} can be calculated from the following formula:

$$1/PD \times SCC = 1/R_{sh} + 1/E_{Na} \times SCC.$$

An alternative technique to measure E_{Na} using the technique of Isaacson is to use different concentrations of amiloride (10^{-7}, 5×10^{-7} and 10^{-6} M) and record PD and SCC before and after addition of each dose (Isaacson, 1977). The latter technique was used in the present study.

During amiloride administration the total conductance of the system (G) is linearly related to the SCC according to the following equation:

$$G = G_{sh} + \frac{1}{E_{Na}} \times SCC$$

where G_{sh} = conductance of the shunt pathway, E_{Na} = electromotive force and SCC = short circuit current during amiloride administration. By applying linear regression analysis to the total conductance and SCC, a linear relationship is obtained. In the experiments reported in this paper the correlation coefficient between total conductance and SCC during amiloride administration was greater than 0.98. The slope of the equation is the inverse of the electromotive force and the conductance of the shunt pathway is the intercept. The conductance across the active pathway can then be calculated for the equation

$G = G_{sh} + G_a$ (G_a = conductance of the active pathway). This method is analogous to that of Yonath and Civan (1971) which measures E_{Na} with the use of vasopressin.

Calcium Uptake and Efflux, ATP, ADP, and Lactate

The methods utilized to measure calcium uptake and calcium efflux by the stripped turtle bladder and by isolated toad bladder epithelial cells were identical to those previously described (Arruda & Sabatini, 1980*a*, *c*). The methods to measure ATP and ADP levels on scrapings of epithelial turtle bladder cells were done as previously described, using the firefly luciferase technique (Arruda, Sabatini & Westenfelder, 1981). Lactate measurements on samples of the serosal solution were done utilizing a commercially available enzymatic assay.

Data are expressed for 8 cm^2 and presented as mean ± SEM. The values presented represent the average values obtained 30 min before replacement of serosal Na by sucrose or choline and during the last 30 min of the experiment. The data were normalized by expressing the SCC or RSCC in the experimental period as the percentage of the control values. The "*t*"-test for paired data was used for statistical analysis unless otherwise indicated.

Results

Effect of Removal of Serosal Na on H^+ Secretion by the Turtle Bladder

Table 1 shows the effect of replacement of serosal Na by sucrose on H^+ secretion by the turtle bladder. Substitution of serosal Na by sucrose resulted in a significant decrease in H^+ secretion to half of control values. The inhibition of H^+ secretion was present after 2 min of substitution of serosal Na by sucrose and the inhibition of H^+ secretion remained constant throughout the 2 hr of observation. The decrease in H^+ was accompanied by a much smaller decline in PD resulting in an increase in the calculated resistance. In six additional experiments we measured the rate of H^+ secretion both with the pH-stat technique and with RSCC. Removal of serosal Na resulted in a decrease in H^+ secretion from 1.47 ± 0.06 to 0.63 ± 0.07 μmol/hr, $P < 0.001$, $n = 6$. In presence of sucrose substitution for Na the RSCC was also identical to the rate of H^+ secretion measured with the pH-stat technique [16.7 ± 2.3 vs. 17.8 ± 2.6 μA, NS) (not significant)]. In the absence of ouabain, replacement of serosal Na by sucrose decreased H^+ secretion (measured with pH-stat technique) from 1.66 ± 0.34 to 1.24 ± 0.23 μmol/hr, $n = 5$, $P < 0.025$ (paired *t*-test).

Substitution of serosal Na by choline also resulted in a significant decrease in H^+ secretion (Table 1). The magnitude and time pattern of the inhibition with choline was very similar to that observed with sucrose. The inhibitory effect of sucrose or choline was totally reversible with readdition of only 20 mM NaCl to the serosal solution and partially reversible with addition of 10 mM NaCl.

In the presence of amiloride, 10^{-4} M in the mucosal solution, substitution of serosal Na by sucrose produced the same inhibitory effect on H^+ secretion (control 96.3 ± 2.1%, NS; Na-free sucrose 57.0 ± 10.5%, $P < 0.025$, $n = 4$). Baseline H^+ secretion was not different between the two groups (43.3 ± 5.1 *vs.* 45.3 ± 10.2 μA).

The left panel of Fig. 1 shows the effect of various concentrations of serosal Na on H^+ secretion. In all these experiments, serosal Na was replaced isosmotically by sucrose. The desired Na concentration in the serosal solution was verified by mea-

Table 1. Effect of substitution of serosal or mucosal Na by sucrose or choline on H^+ secretion by the turtle bladder

Group	Baseline H^+ secretion (μA/8 cm^2)	Experimental/ baseline H^+ secretion (%)	Potential difference (mV)		
			B		*E*
Control ($n = 6$)	34.1 ± 4.1 NS	92.8 ± 5.7 < 0.001	10.3 ± 3.1	NS	9.7 ± 3.1
Serosal Na-free sucrose	39.1 ± 4.5	46.5 ± 5.5	12.5 ± 2.9	< 0.025	9.8 ± 2.7
Control ($n = 6$)	36.0 ± 7.5 NS	103.8 ± 5.5 < 0.001	9.6 ± 2.7	NS	9.8 ± 2.6
Serosal Na-free choline chloride	34.1 ± 5.0	56.8 ± 8.6	8.0 ± 2.2	< 0.01	4.8 ± 1.5
Control ($n = 5$)	34.4 ± 5.1 NS	94.4 ± 3.1 NS	8.4 ± 1.8	NS	6.4 ± 0.2
Mucosal Na-free sucrose	27.0 ± 3.1	95.8 ± 2.6	7.3 ± 2.0	NS	11.0 ± 3.3

NS = not significant. *B* and *E* refer to values obtained before and 60 min after substitution of Na by sucrose or choline in experimental hemibladders or after control wash in the control hemibladders. The data were analyzed by the paired "*t*"-test.

suring the Na concentration by flame photometry. H^+ secretion was inhibited to $48.3 \pm 6.5\%$ of baseline values, $n=11$, in presence of 0 mM Na, $65.3 \pm 3.3\%$, $n=6$, in presence of 10 mM Na, and $77.6 \pm 6.3\%$, $n=7$ in presence of 15 mM Na. All these values were significantly different from control, $P<0.01$. In the presence of 20 mM Na, H^+ secretion was not different from control (20 mM Na, $100.1 \pm 4.1\%$, $n=8$ and 110 mM, $93 \pm 5.7\%$, $n=6$, NS). The apparent half-maximal inhibition occurred at approximately 15 mM Na.

The inhibitory effect of replacement of serosal Na by sucrose was not reversible when the serosal solution was bubbled with 5% CO_2. In five such experiments replacement of serosal Na by sucrose decreased H^+ secretion significantly (control $97.8 \pm 4.7\%$; Na-free $57.0 \pm 9.3\%$ of baseline values, $P<0.01$, $n=6$). Addition of 5% CO_2 to serosal solution increased H^+ secretion from 97.8 ± 4.7 to 132.0 ± 12.3 ($P<0.02$) in control hemibladders but failed to elicit the same response in experimental hemibladders (57.0 ± 9.3 and $52.5 \pm 16.5\%$, NS).

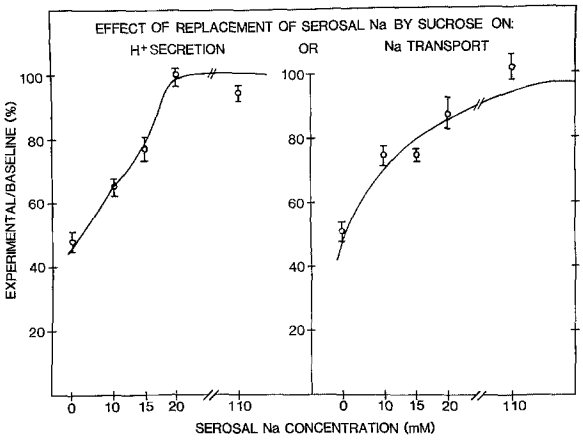

Fig. 1. Effect of different serosal Na concentration of H^+ secretion (left panel) on Na transport (right panel). Serosal NaCl was replaced isosmotically by sucrose. The curves were drawn by inspection

Table 1 shows that substitution of mucosal Na by sucrose did not result in any inhibitory effect on H^+ secretion. In six additional experiments we compared the effect of removal of serosal Na to the removal of both serosal and mucosal Na on H^+ secretion. H^+ secretion decreased to $41.3 \pm 10.5\%$ of baseline values with replacement of both serosal and mucosal Na by sucrose and to $56.5 \pm 10.0\%$ with the replacement of serosal Na by sucrose, values not significantly different.

Relationship between Calcium Uptake and Efflux by the Stripped Turtle Bladder Epithelial Layer and the Inhibition of H^+ Secretion by Replacement of Serosal Na by Sucrose or Choline

Table 2 shows ^{45}Ca uptake by the stripped turtle bladder epithelial layer under various experimental conditions. Substitution of sodium by sucrose or choline was associated with a significant increase in calcium uptake as compared to control hemibladders. The increase in calcium uptake elicited by replacement of Na with sucrose or choline could be totally prevented by pretreatment with 2 mM

Table 2. ^{45}Ca uptake by the stripped turtle bladder epithelial layer

Group		N	^{45}Ca Uptake	
			(nanomole/mg protein/5 min)	(log nanomole/mg protein/5 min)
I	Control	8	21.2 ± 5.2	1.33 ± 0.72
II	Na-free sucrose	8	82.9 ± 18.4[a]	1.92 ± 1.26[a]
III	Na-free choline	7	59.4 ± 5.7[b]	1.77 ± 0.76[b]
IV	Control + 2 mM $LaCl_3$	6	24.4 ± 4.9	1.39 ± 0.69
V	Na-free sucrose + 2 mM $LaCl_3$	6	10.3 ± 1.5	1.01 ± 0.18
VI	Na-free choline + 2 mM $LaCl_3$	7	21.8 ± 2.6	1.33 ± 0.41

[a] $P<0.025$.

[b] $P<0.001$ as compared to control. The *t*-test for unpaired data was used to analyze the data. The data were also analyzed by one way analysis of variance which showed there was a significant difference in calcium uptake among all the groups ($P<0.05$). Calcium uptakes in Na-free sucrose and in Na-free choline were greater than those of all other groups ($P<0.001$). Calcium uptake was not different among groups (I, IV, VI). Calcium uptake tended to be greater in Na-free sucrose than in Na-free choline but the difference did not achieve statistical significance. The experiments were performed at 21 °C in a turtle Ringer's solution containing 1.8 mM Ca. In the experiments utilizing $LaCl_3$, lanthanum was added before the incubation with Na-free solution.

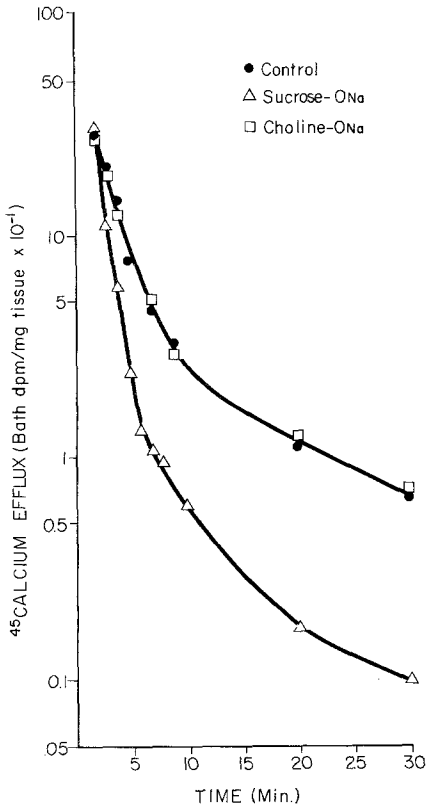

Fig. 2. ^{45}Ca efflux in hemibladders loaded with ^{45}Ca for 40 min and allowed to efflux in regular turtle Ringer's, and in turtle Ringer's in which Na was replaced isosmotically either by sucrose or by choline. ^{45}Ca efflux was significantly lower in Na-free sucrose than in controls; calcium efflux in Na-free choline Ringer's was not different from control

$LaCl_3$. Lanthanum alone did not alter calcium uptake as compared to control hemibladders.

Figure 2 shows that replacement of Na by sucrose decreased the rate of ^{45}Ca efflux from turtle bladders which had been preloaded with ^{45}Ca. Choline did not alter ^{45}Ca efflux.

To determine whether the inhibition of H^+ secretion was related to an increase in calcium uptake, one set of hemibladders was pretreated with 2 mM $LaCl_3$ in the serosal solution and 30 min later the serosal solution of both hemibladders was replaced with an identical solution in which Na was substituted by sucrose or choline (Table 3). Lanthanum by itself (2 mM added to serosal solution) failed to alter H^+ secretion (*data not shown*). Baseline H^+ secretion was not different between the groups. Pretreatment with $LaCl_3$ totally abolished the inhibitory effect of serosal Na replacement by sucrose on H^+ secretion. Replacement of Na with choline resulted in a significant inhibition of H^+ secretion which was significantly blunted by pretreatment with $LaCl_3$. It is unclear why $LaCl_3$ almost totally prevented the effect of sucrose but only partially prevented the effect of choline.

Table 3. Effect of pretreatment with 2 mM $LaCl_3$ on the inhibitory effect of removal of serosal Na on H^+ secretion by the turtle bladder

Group	Baseline H^+ secretion ($\mu A/8\ cm^2$)	Experimental/ baseline H^+ secretion (%)
Na-free sucrose ($n=6$)	28.3 ± 4.3 NS	58.5 ± 6.8 < 0.01
La + Na-free sucrose	25.9 ± 2.8	85.0 ± 6.0
Na-free choline ($n=6$)	37.3 ± 6.1 NS	36.0 ± 3.2 < 0.025
La + Na-free choline	46.1 ± 10.5	55.8 ± 4.6

The data were analyzed by the paired "t"-test.

Since a Ca/H^+ exchange has been described in other systems (Miyamoto & Racker, 1980), we investigated whether lowering of the serosal pH and addition of the protonophore, dinitrophenol, would reverse the inhibitory effect of serosal Na replacement on H^+ secretion. Replacement of serosal Na by sucrose resulted in a significant decrease in H^+ secretion as compared to controls (92.0 ± 6.5 vs. $42.3 \pm 9.1\%$, $P > 0.01$, $n=6$). Lowering of the serosal pH to 5 failed to reverse the inhibition of H^+ secretion (42.3 ± 9.1 vs. $47.8 \pm 8.0\%$, NS). Addition of H^+ ionophore dinitrophenol (5 μM) to serosal solution of these hemibladders also failed to reverse the inhibition of H^+ secretion (47.8 ± 8.0 vs. $59.0 \pm 11.3\%$, NS).

Effect of Replacement of Serosal Na by Sucrose on H^+ Secretion under Anaerobic Conditions

Table 4 shows the effect of replacement of serosal Na by sucrose on bladders bubbled with 100% N_2 in the mucosal solution and 95% N_2 and 5% CO_2 in the serosal solution. Replacement of serosal Na by sucrose caused a significant decrease in H^+ secretion and in lactate production. In control hemibladders H^+ secretion and lactate production remained unchanged. In control and in experimental hemibladders the rate of lactate production was related to the rate of H^+ secretion as recently described by Steinmetz, Husted, Mueller and Beauwens (1981) (control $y = 1.45 \pm 0.65\ x$, experimental $y = 1.47 \pm 0.45\ x$). The slopes of these two lines were not significantly different by analysis of covariance. These findings indicate that the inhibition of H^+ secretion by replacement

Table 4. Effect of replacement of serosal Na by sucrose on H^+ secretion by the turtle bladder under anaerobic conditions

Group	H^+ secretion (μmol/hr)			Lactate production (μmol/hr)			ATP (nanomol/mg/protein)	ADP
	B		*E*	*B*		*E*	*E*	*E*
Control	0.52±0.06	NS	0.57±0.11	1.69±0.27	NS	1.93±0.28	26.3±3.2	15.5±5.7
	NS		<0.01	NS		NS	NS	NS
Na-free sucrose	<0.57±0.08	0.001	0.19±0.06	1.44±0.15	<0.01	0.76±0.17	27.1±2.6	10.5±6.1
		(*n*=11)			(*n*=11)		(*n*=8)	(*n*=8)

B and *E* refer to values obtained under baseline conditions and 90 min after control wash in control hemibladders or after replacement of serosal Na by sucrose in the experimental hemibladders. H^+ secretion and lactate production are expressed for 8 cm^2 surface area. The data were analyzed by the paired "*t*"-test.

Table 5. Effect of replacement of serosal Na by sucrose on the protonmotive force (PMF) of the turtle bladder

Group	Baseline H^+ secretion (μA/8 cm^2)	Experimental/ baseline H^+ secretion (%)	PMF (pH unit)			Active conductance (μA/pH unit)		
			B		*E*	*B*		*E*
Control	47.6±8.7	95.5±2.2	3.02±0.21	NS	2.86±0.17	16.2±3.1	NS	16.1±3.1
(*n*=6)	NS	<0.001						
Na-free sucrose	43.6±9.5	38.3±6.2	2.89±0.26	NS	3.09±0.27	14.6±2.1	<0.005	5.3±1.4

NS = not significant. *B* and *E* refer to values obtained before and 60 min after changing the serosal solution to Na-free sucrose or control wash. The data were analyzed by the paired "*t*"-test.

Table 6. Effect of substitution of serosal Na by sucrose on SCC by the turtle bladder

Group	Baseline SCC (μA/8 cm^2)	Experimental/ baseline SCC (%)	Potential difference (mV)		
			B		*E*
Control	263.6±48.3	108.6±12.5	45.8±5.9	NS	45.6±17.2
n=7	NS	<0.01			
Na-free	375.7±70.5	54.7±10.4	52.7±10.3	NS	40.7±11.1
Na-free sucrose	231.5±50.2	38.2± 8.5	41.2± 8.4	<0.05	29.3± 8.6
n=6	NS	<0.025			
La + Na-free sucrose	300.0±53.4	77.3±12.0	34.2±7.2	NS	36.7±6.7

B and *E* refer to values obtained at baseline and 60 min after replacement of serosal by sucrose or control wash. The data were analyzed by the paired "*t*"-test.

of serosal Na by sucrose is accompanied by a proportional decrease in lactate production and likely cannot be explained by a metabolic effect. In support of this contention is the fact that ATP and ADP levels were not significantly different between control and experimental hemibladders.

Effect of Replacement of Serosal Na by Sucrose on the Protonmotive Force

Table 5 shows the effect of substitution of serosal Na by sucrose on the protonmotive force and the active conductance of protons. Replacement of serosal Na by sucrose resulted in a significant inhibition of H^+ secretion as compared to control hemibladders. The protonmotive force was unchanged in control of Na-free bladders. The decrease in H^+ secretion was due to a decrease in the active conductance of protons in Na-free bladders.

Effect of Replacement of Serosal Na by Sucrose on Na Transport

Table 6 shows the effect of substitution of serosal Na by sucrose on Na transport by the turtle

Table 7. Effect of replacement of serosal Na by sucrose on SCC and lactate production in turtle bladders bubbled with 100% N_2 ($n=6$)

Group	Na transport (μA/8 cm²)			Lactate production (μmol/hr/8 cm²)			ATP (nanomol/mg protein)	ADP (nanomol/mg protein)
	B		*E*	*B*		*E*	*E*	*E*
Control	141.0±40.8	NS	135.0±40	1.64±0.21	<0.05	1.25±0.22	23.8±2.9	5.0±1.1
Na-free	162.0±62.0	<0.005	118.0±60	1.82±0.37	<0.02	0.98±0.21	24.6±4.0	5.8±1.8
	($n=6$)		($n=6$)	($n=6$)		($n=6$)	($n=6$)	($n=5$)

B and *E* refer to values obtained under baseline conditions and 60 min after control wash in control hemibladders and replacement of serosal Na by sucrose in the experimental hemibladders. The data were analyzed by the paired "*t*"-test.

bladder. Replacement of serosal Na by sucrose resulted in a significant decrease in SCC. Potential difference decreased slightly but the change did not achieve statistical significance. The decline in SCC following replacement of serosal Na by sucrose was associated with a significant increase in resistance from 1.2±0.08 to 1.8±0.3 KΩ cm² ($P<0.05$). In control hemibladders SCC, PD and resistance remained unchanged. The decline in SCC following substitution of serosal Na by sucrose was fully reversible upon addition of Na to the serosal solution.

The right panel of Fig. 1 shows the effect of various concentrations of serosal Na on SCC. Serosal Na was replaced isosmotically by sucrose. SCC was inhibited to 54.5±10.4% of baseline values in the presence of 0 mM Na, $n=7$, 78.5±4.4% in presence of 10 mM Na, $n=8$, 74.6±4.0% in presence of 15 mM Na, $n=8$, and 87.1±4.7 in presence of 20 mM Na, $n=8$. All these values, except the last one, were significantly different from controls. The apparent half-maximal inhibition occurred at 10–15 mM Na concentration.

In an additional group of ten hemibladders unidirectional flux (mucosa to serosa) of ^{22}Na was measured before and after replacement of serosal Na by sucrose. Mucosal to serosal flux of ^{22}Na was not significantly different from SCC before and after replacement of serosal Na by sucrose (before, measured SCC 330.0±49.3, SCC calculated from ^{22}Na flux 313.0±16.2 μA; and after replacement of serosal Na by sucrose, 240.0±25.0 and 221.0±21.5 μA, respectively).

To investigate whether the increase in calcium uptake elicited by replacement of Na by sucrose (Table 2) was related to the inhibition of Na transport, we pretreated one side with $LaCl_3$, 2 mM, and then replaced the serosal solutions with Na-free solutions. $LaCl_3$, 2 mM, in the serosal solution alone did not alter SCC (*data not shown*). Pretreatment with $LaCl_3$ significantly decreased the inhibitory effect of serosal Na replacement by sucrose on SCC (Table 6).

Effect of Replacement of Serosal Na by Sucrose on SCC under Anaerobic Conditions

Table 7 shows the effect of replacement of serosal Na by sucrose on turtle bladders bubbled with 100% N_2 in the mucosal and in the serosal solution. Replacement of serosal Na by sucrose caused a significant decrease in SCC and in lactate production. In control hemibladders SCC remained stable, but lactate production decreased significantly. The decline in lactate production was significantly greater in experimental hemibladders than in control hemibladder (net lactate decrease 0.84±0.24 vs. 0.40±0.12 μmol/hr, $P<0.05$). ATP and ADP levels were not significantly different between the two groups.

Effect of Replacement of Serosal Na by Sucrose on Electrical Parameters

Table 8 shows the effect of replacement of serosal Na by sucrose on electrical parameters of the toad bladder and turtle bladder measured with the use of amiloride (Isaacson, 1977). In these experiments, amiloride was used before and after replacement of serosal Na by sucrose in experimental hemibladders and before and after the control wash in control hemibladders.

In the toad bladder, as in the turtle bladder, replacement of serosal Na by sucrose resulted in a significant inhibition of SCC. In the toad bladder replacement of Na by sucrose also caused a significant increase in calcium uptake (control 5.36±0.56, $n=12$, sucrose 9.62±0.56, $n=8$ nanomole/mg protein/5 min, $n=8$ ($P<0.001$). In the toad bladder, replacement of serosal Na by sucrose was associated with a significant decrease in SCC but did not alter E_{Na}. Total resistance and the resistance of the active pathway increased significantly.

Table 8. Effect of replacement of serosal Na by sucrose on the electromotive force (E_{Na}) of Na pump of the toad bladders

Group	PD (mV)			SCC (μA/8 cm²)			R_t (KΩ cm²)		
	B	*P*<	*E*	*B*	*P*<	*E*	*B*	*P*<	*E*
Toad Bladders									
Control $n=6$	63.5 ±6.4	NS	57.5 ±9.1	277.5 ±50.3	NS	252.4 ±69.6	2.0 ±0.24	NS	2.3 ±0.32
Na-free	74.1 ±5.1	0.02	58.4 ±7.7	377.9 ±69.9	0.01	170.6 ±0.7	1.9 ±0.7	0.05	2.8 ±0.09
Turtle Bladders									
Control $n=6$	56.0 ±6.7	NS	65.3 ±7.4	328.0 ±75.1	NS	380.6 ±72.6	1.7 ±0.33	NS	1.6 ±0.27
Na-free	66.8 ±9.9	NS	65.0 ±8.2	458.3 ±35.1	0.001	230.0 ±14.7	1.1 ±0.1	0.001	2.2 ±0.2

R_t=total resistance; R_a=resistance of the active pathway; R_{sh}=resistance of shunt pathway. *B* and *E* refer to values obtained under baseline and 60 min after replacement of serosal Na by sucrose in experimental and control wash in control hemibladders. The data were analyzed by the paired "*t*"-test.

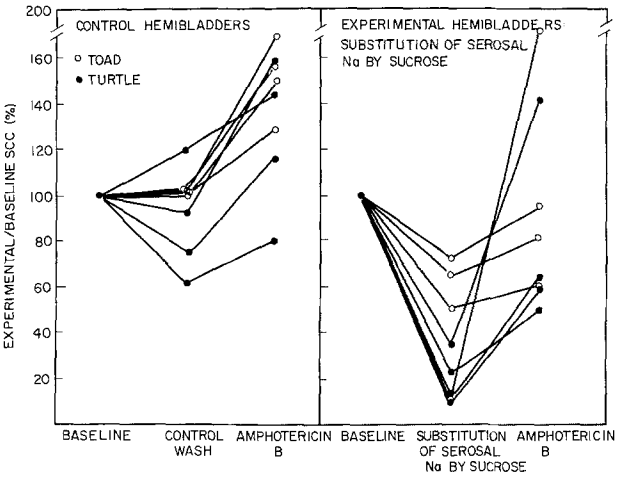

Fig. 3. Left panel SSC, expressed as percentage of baseline values, in control hemibladders and during amphotericin B addition to the mucosal solution (1.5 μg/ml). Right panel SCC during replacement of serosal Na by sucrose and during addition of amphotericin B to the mucosal solution (1.5 μg/ml)

In control hemibladders all these parameters remained unchanged.

The technique used was capable of detecting changes in the E_{Na} since dinitrophenol, 10^{-4} M, an agent thought to affect E_{Na} (Isaacson, 1977), decreased E_{Na} in the toad bladder from 125.0±6.0 to 43.2±16.3 mV, $P<0.001$, $n=7$).

In the turtle bladder replacement of serosal Na by sucrose was also associated with a decrease in SCC and unaltered E_{Na}. In the turtle bladder there was an increase in total resistance and in the resistance of the active and shunt pathway. In control turtle bladders all parameters remained unchanged.

Effect of Amphotericin B on the Inhibition of the SCC by Replacement of Serosal Na by Sucrose

Since the apical membrane probably represents the major resistance to Na transport in the toad (Macknight et al., 1980) and in turtle bladder, an increase in total resistance following replacement of serosal Na with sucrose suggests that reduced apical membrane permeability to Na is the factor responsible for the decrease in SCC. To investigate this possibility we utilized amphotericin B, an agent which increases the permeability of the mucosal membrane to Na (Lichtenstein & Leaf, 1965; Macknight et al., 1980) by creating new Na channels. If removal of serosal Na decreases Na transport by decreasing the permeability of apical membrane to Na, then amphotericin B addition to the mucosal solution should reverse the inhibition of SCC. Figure 3 shows the effect of replacement of serosal Na by sucrose on SCC expressed as percentage of baseline values. The pattern and the magnitude of inhibition of SCC with replacement of serosal Na by sucrose was very similar in the turtle and in the toad bladder; for this reason the data were combined. In control hemibladders SCC remained unchanged whereas replacement of serosal Na by sucrose was associated with a significant decrease in SCC (93.8±6.6 and 35.1±12.2%, $P<0.001$, $n=8$).

and turtle bladders measured with the use of amiloride

E_{Na} (mV)			R_{sh} (KΩ cm^2)			R_a (KΩ cm^2)		
B	*P*<	*E*	*B*	*P*<	*E*	*B*	*P*<	*E*
134.4 ±15.3	NS	134.6 ±8.2	5.4 ±0.91	NS	4.6 ±0.44	5.5 ±1.67	NS	7.4 ±2.45
114.6 ±7.8	NS	120.6 ±7.7	6.1 ±0.52	NS	4.7 ±0.59	3.3 ±0.82	0.025	7.0 ±2.3
104.3 ±6.7	NS	116.8 ±11.6	3.6 ±0.56	NS	3.7 ±0.43	3.8 ±1.25	NS	3.2 ±0.66
110.2 ±9.3	NS	112.0 ±13.8	3.1 ±0.50	0.01	6.2 ±0.98	2.0 ±0.08	0.01	3.8 ±0.4

Addition of amphotericin B to the mucosal solution, in a concentration of 1.5 μg/ml, resulted in a significant increase in SCC in controls and hemibladders in which serosal Na was replaced by sucrose. Amphotericin increased SCC from 93.8±6.6 to 135.0±10.5%, $P<0.001$, in control hemibladders and from 35.1±12.2 to 92.3±17.3%, $P<0.001$, in experimental hemibladders. The net increase in SCC elicited by amphotericin B was not significantly different between control and experimental hemibladders (control 41.0±7.7, experimental 57.0±19.6%, NS).

Discussion

The results of the present study clearly demonstrate that replacement of Na by sucrose in the serosal solution is associated with a significant inhibition of H^+ and Na transport in turtle and toad bladders. Replacement of serosal Na by choline also resulted in a significant inhibition of H^+ secretion in the turtle bladder. The effect of replacement of serosal Na by choline on Na transport was not studied since this maneuver has been shown to result in a significant stimulation of Na transport by the frog skin probably as the result of a metabolic effect (Macey & Koblick, 1963). Although this metabolic effect of choline could also play a role in the inhibition of H^+ secretion, we think that this is unlikely since one would expect H^+ secretion to increase as observed previously by us when 40 mM of choline was added to the turtle bladder (Arruda, Dytko, Mola & Kurtzman, 1980). This effect is probably secondary to metabolism of choline.

The present study was designed to determine whether an increase in cytosolic calcium through the Na/Ca exchange system would inhibit H^+ and Na transport by the turtle and toad bladder. Replacement of serosal Na by sucrose was associated with a significant increase in calcium uptake which could be blocked by pretreatment with $LaCl_3$. Lanthanum was used at 2 mM, a concentration which has been shown to be the optimal concentration to block the increase in calcium uptake elicited by Na-free solutions in other tissues (Van Breemen et al., 1979). Pretreatment with 2 mM lanthanum blunted the inhibitory effect of Na removal on H^+ secretion and on the SCC. This finding strongly suggests that the two phenomena, i.e. the increase in calcium uptake and the inhibition of transport are causally related. In addition to the increase in calcium uptake, substitution of Na by sucrose, but not by choline, was associated with a significant decrease in calcium efflux suggesting that replacement of serosal Na by sucrose increases cytosolic calcium both by promoting uptake and by decreasing efflux (Table 2 and Fig. 2). Despite repeated experiments we failed to demonstrate an effect of replacement of Na by choline on calcium efflux, although we were able to show an increase in calcium uptake by this maneuver. The reason for this discrepancy is not readily apparent.

We chose not to determine whether lowering of the extracellular calcium concentration would also blunt the inhibitory effect of Na removal on H^+ and SCC. The reason for this is the fact that it is impossible to perform experiments in the absence of extracellular calcium in the turtle and in toad bladder owing to poor viability of these membranes under these circumstances. Furthermore, in the presence of low extracellular calcium

concentration, the ^{45}Ca technique may fail to detect an increase in calcium uptake (Arruda & Sabatini, 1980*b*).

Since a Ca/H^+ exchange system has been demonstrated in other tissues we sought to investigate whether the observed inhibition of H^+ secretion could be reversed by lowering the serosal pH (Miyamoto & Racker, 1980). This maneuver should increase the influx of protons into the cells and stimulate the efflux of calcium. It should thus reverse the inhibition of H^+ secretion if a Ca/H^+ exchange system were present in the turtle bladder. If Ca/H^+ exchange were present in the turtle bladder, one would expect lowering of the serosal pH or addition of protonophore dinitrophenol to promote influx of protons and cause efflux of calcium. This would decrease cytosolic calcium and the inhibition of H^+ secretion should be reversed or blunted. We utilized the protonophore, dinitrophenol (in a concentration which does not affect H^+ secretion), and lowered the serosal pH in an attempt to reverse the inhibition of H^+ secretion. Both maneuvers, i.e. lowering of serosal pH and addition of dinitrophenol failed to reverse the inhibition of H^+ secretion. In order for dinitrophenol, however, to reverse the inhibition of transport, it would have to promote influx of H^+ through the Ca/H^+ exchange system. If dinitrophenol promotes H^+ influx through a parallel pathway to the Ca/H^+ exchange system then one would not expect this drug to reverse the inhibition of transport. The failure of low serosal pH to reverse the inhibition of transport suggests that a serosal Ca/H^+ exchange is not present in the turtle bladder serosa.

The data of the present study strongly suggest that a Na/Ca exchange system is present on the serosal side of the turtle and toad bladder. The Na/Ca exchange is present in the plasma membrane of several tissues (Reeves & Sutko, 1979; Van Breemen et al., 1979; Chase & Al-Awqati, 1981). It has been proposed that the system can operate in two ways: under normal conditions the electrochemical gradient moves Na inside the cell and the energy generated by downhill movement of Na serves to extrude Ca from the cell (Mullins, 1981). When Na is replaced by sucrose the concentration gradient of Na moves Na out and Ca into the cell. It is interesting to note that half-maximal inhibition of transport occurred at a serosal Na concentration very close to the intracellular Na concentration. The apparent K_i observed in the present study was very similar to that of the Na/Ca exchange system of the plasma membrane of the heart (Reeves & Sutko, 1979; Miyamoto & Racker, 1980). These observations suggest that the concentration gradient of Na across cell membranes is a critical determinant of cytosolic Ca. Chase and Al-Awqati (1981) have recently proposed that changes in intracellular sodium may modulate the cytosolic concentration of calcium. An increase in intracellular sodium would promote an increase in cytosolic calcium and this would then decrease the permeability of the luminal membrane to Na, thus decreasing intracellular Na. The fact, however, that removal of serosal Na inhibits Na, H^+ and water transport suggests that this system does not solely affect Na transport.

It is interesting to note, that the inhibition of H^+ secretion by replacement of serosal Na by sucrose was not altered by amiloride or ouabain, thus suggesting that the operation of the Na/Ca exchange system in the turtle bladder is still present in the absence of Na transport. This is similar to data obtained in the axon, where inhibition of (Na + K)-ATPase by ouabain does not alter Na/Ca exchange (Baker, 1978).

The mechanism whereby an increase in cytosolic calcium through manipulation of the Na/Ca exchange system inhibits H^+ and Na transport by the turtle bladder was examined under anaerobic conditions. Under anaerobic conditions the rate of H^+ or Na transport is closely coupled to the rate of lactate production (Klahr & Bricker, 1965; Steinmetz et al., 1981). The present experiments demonstrate that the inhibition of H^+ and Na transport was the same in the presence and in the absence of mitochondrial function and it is thus unlikely that alterations in mitochondrial function account for the inhibition of H^+ secretion. This is important since an increase in cytosolic calcium may lead to uncoupling of oxidative phosphorylation which then may decrease transport. Since the rate of H^+ or Na transport in the turtle bladder is closely linked to the rate of lactate production (Klahr & Bricker, 1965; Steinmetz et al., 1981), one can use this concept to analyze the mechanism of inhibition of transport. If transport is primarily inhibited then the rate of lactate production should decrease secondarily in proportion to the decline in transport. On the other hand, if glycolysis is uncoupled from transport then the rate of lactate production would rise despite a decrease in transport. The present study demonstrates that the rate of lactate production declines in parallel with the fall in the rate of Na or H^+ transport, thus strongly suggesting that the mechanism of inhibition of transport is not uncoupling of metabolism from transport. Further evidence against a metabolic effect is the fact that ATP and ADP levels were

not decreased in experimental hemibladders, suggesting that ATP availability was not rate-limiting for Na or H^+ transport

Since the above studies did not provide evidence that replacement of serosal Na by sucrose has a direct effect on metabolism, we examined the effect of this maneuver on the protonmotive force and on E_{Na}. The inhibition of H^+ secretion by removal of serosal Na was due to a decrease in active conductance of protons through the pump and not to a decrease in the force of the pump. Other maneuvers which decrease intracellular calcium also affect the conductance of protons (Arruda, 1979; Arruda & Sabatini, 1980*a, c*).

The inhibition of SCC by removal of serosal Na could arise either as the consequence of an increase in the resistance of the active or shunt pathway for Na transport or as the result of an inhibitory effect on the electromotive force. We measured the electromotive force with amiloride using a technique modified after Isaacson (1977). This technique has been utilized extensively to measure E_{Na} (Isaacson, 1977; Macchia, 1977; Aguilera, Kirk & DiBona, 1978). The assumption underlying the use of amiloride to measure E_{Na} is that the drug alters Na transport solely by increasing the resistance of the active pathway to Na transport and not by altering the resistance of the shunt pathway or the Na pump (Isaacson, 1977; Navarte & Finn, 1980). The evidence available regarding the mechanism of action of amiloride supports such an assumption (Macknight et al., 1980). The results of the present study demonstrate that removal of serosal Na does not affect the force of the Na pump but leads to a significant increase in resistance of the active pathway.

Since the apical membrane represents the major resistance to Na transport (Reuss & Finn, 1974; Macknight et al., 1980), one would expect the permeability of the apical membrane to be decreased by removal of serosal Na. Amphotericin B is an agent which increases the permeability of the apical membrane to Na by creating new channels thus leading to an increase in SCC. If the inhibition of Na transport was the consequence of decreased permeability of the apical membrane to Na, one would expect amphotericin B addition to reverse the inhibition of SCC. Figure 3 shows clearly that in the toad and turtle bladder addition of amphotericin B partially reverses the inhibition of SCC induced by removal of serosal Na. Chase and Al-Awqati (1981) have reported similar findings with amphotericin B in the toad bladder. Since amphotericin B causes an increase in K flux in the toad bladder (Gatzy, Reuss & Finn, 1979), one has to be cautious in attributing the increase in SCC with amphotericin B as being equivalent to an increase in Na transport. More direct evidence that replacement of serosal Na by sucrose inhibits apical Na transport has been provided by Chase and Al-Awqati (1981). They recently demonstrated that replacement of serosal Na by choline decreases the rate of ^{22}Na uptake by the luminal membrane, thus suggesting decreased permeability of the luminal membrane to Na. The finding of a decrease in active conductance of protons through the H^+ pump (located in the luminal membrane), suggests that an increase in cytosolic calcium decreases H^+ transport by increasing the resistance of the apical membrane to flow of H^+. In agreement with the suggestion that an increase in cytosolic calcium increases the resistance to the flow of protons through the pump is the finding that addition of 5% CO_2 to serosal solution (a maneuver which increases the conductance of protons through the pump) failed to stimulate H^+ secretion in the absence of serosal Na.

In conclusion, replacement of serosal Na by sucrose in the turtle and in the toad bladder leads to an increase in calcium uptake. This increase in cytosolic calcium inhibits H^+ and Na transport probably by increasing the resistance of the luminal membrane. The results suggest that the serosal Na/Ca exchange is an important regulator of cytosolic calcium in the turtle and toad bladder.

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