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Interrelationship of H^+ Excretion and Na^+ Reabsorption in the Toad Urinary Bladder*

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Summary. Recent results from this laboratory have shown that in the absence of mucosal Na^+ and exogenous CO_2, H^+ excretion is reduced in the toad urinary bladder. This study was done to determine if H^+ excretion is linked to Na^+ reabsorption in the toad bladder. Na^+ transport was inhibited by ouabain or by substitution of the sodium with choline chloride. Both of these agents produced an inhibition of H^+ excretion. However, when dinitrophenol was added to stimulate CO_2 production by the tissue, H^+ excretion returned to control levels even though Na^+ transport was still inhibited. Vasopressin, which stimulates Na^+ transport in toad urinary bladder, had no effect on H^+ excretion. In addition, simultaneous energies of activation were determined for H^+ excretion and Na^+ reabsorption in the toad bladder. The activation energies for the two processes were significantly different. These results suggest that Na^+ and H^+ are not coupled in an exchange mechanism at the mucosal surface of the cell. H^+ excretion, however, does appear to be limited by the endogenous CO_2 production of the bladder in the absence of exogenous CO_2.

The toad urinary bladder is known to be capable of excreting H^+ (Frazier & Vanatta, 1971; Ludens & Fanestil, 1972). In a later study, Frazier and Vanatta (1973) demonstrated that in the absence of exogenous CO_2 the H^+ excretion was decreased by the removal of mucosal Na^+. This finding could be interpreted in one of two ways: (1) a direct coupling could exist at the mucosal surface by a $Na^+ - H^+$ exchange pump; or (2) H^+ excretion could be limited by CO_2 production of the bladder since their study was done in the absence of exogenous CO_2.

The purpose of the present study was to elucidate the relationship between Na^+ reabsorption and H^+ excretion in the toad bladder. Our first experiments utilized two different methods of decreasing the Na^+ pumping

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activity in the bladder. Since it is known that a large portion of CO_2 production by the bladder arises from the activity of the sodium pump (Leaf, Page & Anderson, 1959), dinitrophenol (DNP) was added to stimulate CO_2 production during the period of reduced Na^+ pumping activity. In addition, the energy of activation (E_a) for Na^+ reabsorption and H^+ excretion was measured simultaneously in paired hemibladders. Our data indicate that H^+ excretion and Na^+ reabsorption are not coupled to an obligatory exchange mechanism at the mucosal surface. It does appear, however, that in the absence of exogenous CO_2, the endogenous CO_2 supplied by the Na^+ pump may be necessary for maximum H^+ excretion. Under these circumstances, when the action of the Na^+ pump is reduced, the H^+ excretion is limited due to the reduced CO_2 available for formation of carbonic acid.

Materials and Methods

The toads used in these experiments were *Bufo marinus* of Colombian origin and were supplied by Charles P. Chase of Miami, Florida. The routine care of toads, solutions, the procedure of inducing acidosis and the method of measuring H^+ excretion were as previously described (Frazier & Vanatta, 1973). In all experiments, the H^+ excretion was calculated from change in pH and the concentration of buffer in the mucosal solution. A Corning Model 110 digital pH-meter was used for all pH determinations. All Na^+ analyses were performed on an IL flame photometer with an internal lithium standard. Calomel electrodes immersed in Ringer's solution were used in measuring the potential difference (p.d.) across the bladder with agar bridges between the electrode bath and incubation chambers. The asymmetry of the electrodes and bridges was checked before and after each experiment and was less than 1.0 mV. One hundred per cent humidified O_2 was bubbled into the serosal medium throughout each experiment. The Ringer's solution used contained (in mM): NaCl, 114.5; KCl, 3.0; $CaCl_2$, 0.9; and sodium phosphate, 0.5; the final pH was 6.80. Pitressin (Parke, Davis and Co.) was used as vasopressin. Bladders from toads in metabolic acidosis were used in all experiments. All statistics were performed as the mean difference on paired hemibladders or between two consecutive time periods on the same bladder.

In the experiments in which Na^+ reabsorption was inhibited, one hemibladder was used to measure H^+ excretion and the paired hemibladder was used to monitor the p.d. The hemibladder for measuring p.d. was mounted between two glass chambers, each of which held 10 ml of Ringer's solution. The p.d. was read every 5 min during the experiment and the readings averaged for each control or experimental period. H^+ excretion was measured in bladder sac preparations. The bladder sac was tied around the outlet of a 1-ml plastic chamber as reported elsewhere (Frazier & Vanatta, 1972). The chamber contained a fitting to hold the pH electrode as well as an additional outlet to accommodate a 5-ml syringe. Five ml of the Ringer's solution was placed in the bladder sac. The bladder was then immersed in a beaker containing 50 ml of the same Ringer's solution. The bladder contents were mixed throughout the experiment by aspirating with the syringe and reinjecting into the bladder sac every 5 min. The total flux period was 90 min. After a 15-min equilibration period the first 30-min control excretion period was obtained. At the end of this time either ouabain (10^{-4} M) (strophanthin-G) was added to the serosal medium or Na^+-free choline Ringer's was placed

on both the serosal and mucosal surfaces and a 30-min experimental flux obtained. In the final 30-min period, DNP (10^{-4} M) was added to the serosal bath. DNP is a known uncoupler of oxidative phosphorylation and has been shown to stimulate CO_2 production in the turtle bladder (Schwartz & Steinmetz, 1971). In experiments in which Na^+ was removed from both media, it was replaced on a mole-for-mole basis by choline chloride.

A third series of experiments was performed in which vasopressin (40 mU/ml) was added to the serosal medium during the second flux period. At the end of this period the serosal surface was washed twice with Ringer's solution and the final 30-min control flux period was obtained.

The E_a for both sodium reabsorption and H^+ excretion was determined by mounting paired hemibladders between Lucite chambers each of which held 2 ml. The bladders were allowed to equilibrate for 15 min and then simultaneous H^+ excretion and Na^+ flux were determined. The flux period was for 60 min. The bidirectional flux of Na^+ was determined using ^{22}Na and ^{24}Na supplied by New England Nuclear of Boston, Mass. Gamma counting was done on a Baird Atomic scaler attached to a well-type scintillation detector. Standard counting techniques and calculations were used in calculating the Na^+ flux. Counts were to a statistical accuracy of 1%. One hemibladder was done at room temperature (23 °C), while the paired hemibladder was done in the cold (2 to 4 °C). From this data the E_a for each process was then calculated using the Arrhenius equation (Knight, 1970).

Results

Effect of Na^+ Inhibition on H^+ Excretion

Since it is known that ouabain inhibits active Na^+ reabsorption in the toad bladder, the effects of this agent were determined on H^+ excretion. A control excretion period was performed first in which the Ringer's solution bathed both surfaces. This was followed by a period of ouabain (10^{-4} M) application in the serosal bath. In Table 1 is shown the average H^+ excretion and p.d. for eight bladders. As can be seen, ouabain did inhibit H^+ excretion. However, during the final period when DNP (10^{-4} M) was added to the serosal bath there was not a significant difference from the initial control period ($p > 0.10$). The p.d. was decreased by ouabain application and continued to decrease throughout the period of DNP application.

In the next series of experiments the Ringer's solution was replaced on both surfaces with a Na^+-free choline Ringer's solution during the second period. This was followed by application of DNP (10^{-4} M) in the third period. It can be seen in Table 2 that H^+ excretion was drastically reduced by replacing the Na^+ in both media with choline. When DNP was placed in the serosal medium during the final period, the H^+ excretion was again increased. There was essentially no difference in the H^+ excretion between the control and final period with DNP ($p > 0.30$). The p.d.'s across all bladders again continued to decrease throughout the experimental periods.

Table 1. Effect of ouabain and DNP on H^+ excretion in the toad bladder

	Control	Ouabain, 10^{-4} M	Ouabain, 10^{-4} M + DNP, 10^{-4} M
H^+ Excretion [a] (nmoles/100-mg bladder × min)	20.2 ± 3.01	12.1 ± 1.75	17.8 ± 2.50
Mean difference [b] with (p value) [c]		8.14 ± 1.85 ($p < 0.005$)	−5.70 ± 1.78 ($p < 0.025$)
Potential difference [d] (mV)	21.5 ± 4.51	10.0 ± 2.66	4.50 ± 1.19

[a] Average of eight experiments ± SEM.
[b] ± SEM.
[c] Calculated from the mean difference.
[d] Average of eight bladders ± SEM.

Table 2. Effect of choline and DNP on H^+ excretion in the toad bladder

	Control	Na-Free choline	Na-Free choline + DNP, 10^{-4} M
H^+ Excretion [a] (nmoles/100-mg bladder × min)	18.4 ± 2.90	9.50 ± 1.35	17.0 ± 2.98
Mean difference [b] with (p value) [c]		8.90 ± 4.56 ($p < 0.005$)	−7.46 ± 2.95 ($p < 0.025$)
Potential difference [d] (mV)	29.4 ± 5.33	6.19 ± 1.69	3.86 ± 0.90

[a] Average of nine experiments ± SEM.
[b] ± SEM.
[c] Calculated from the mean difference.
[d] Average of nine bladders ± SEM.

Both the serosal and mucosal baths were collected and analyzed for Na^+ at the end of each experimental period. The serosal baths in the nine experiments contained an average Na^+ concentration of 0.34 ± 0.11 μM/ml. The mucosal baths contained an average Na^+ concentration of 1.77 ± 0.17 μM/ml.

Effect of Na^+ Stimulation on H^+ Excretion

A third series of experiments was performed in which Na^+ reabsorption was stimulated by the addition of vasopressin to the serosal medium during

Table 3. Effect of ADH on H^+ excretion by the acidotic toad urinary bladder

	Control	ADH in serosal bath	Control
H^+ Excretion[a] (nmoles/100-mg bladder × min)	25.0 ± 2.60	23.8 ± 6.40	23.6 ± 2.39
Mean difference[b] with (p value)[c]		1.18 ± 1.46 ($p < 0.20$)	0.26 ± 1.36 ($p < 0.40$)
Potential difference[d] (mV)	47.7 ± 7.68	66.6 ± 7.28	53.0 ± 7.50

[a] Average of nine experiments ± SEM.
[b] ± SEM.
[c] Calculated from the mean difference.
[d] Average of nine bladders ± SEM.

the experimental period. At the end of this period the serosal surface was washed twice with Ringer's solution and another 30-min post-treatment control excretion period was obtained. Table 3 shows that there was no significant change in the H^+ excretion during any of the periods. It can also be seen that in the experimental period the average p.d. was increased across all bladders in response to vasopressin.

In order to confirm that DNP and vasopressin stimulate CO_2 production in the toad bladder a separate series of experiments was done. The experiments were performed in the same manner as the previous experiments except a P_{CO_2} electrode was substituted for the pH electrode in the chamber. P_{CO_2} accumululation was measured in the mucosal medium of bladder sacs for a 30-min control period according to the method of Severinghaus and Bradley (1958). At the end of this period DNP (10^{-4} M) or vasopressin (40 mU/ml) was added to the serosal medium and the CO_2 accumulation was measured for a second 30-min period. There was an approximately linear increase in P_{CO_2} in all experiments, so the P_{CO_2} at the end of the 30-min period is given as an index of CO_2 production. P_{CO_2} in mm Hg ± SEM for each 30-min period was as follows: control = 0.23 ± 0.01 (n = 12); DNP in serosal bath = 0.28 ± 0.01 (n = 6); vasopressin in serosal bath = 0.27 ± 0.01 (n = 6). It is obvious that DNP and vasopressin stimulate CO_2 production in the toad bladder ($p < 0.005$ in both cases).

Energy of Activation (E_a) for Na^+ Reabsorption and H^+ Excretion

A further argument may be made with respect to a $Na^+ - H^+$ exchange pump at the mucosal border of the cell based on the E_a of the two processes.

Table 4. Activation energy for H^+ excretion and Na^+ reabsorption in the toad urinary bladder

	Flux (nmoles/100-mg bladder × min)		E_a (kcal mole^{-1})
	Room temp.	Cold	
H^+ (Cell → M) (7)[a]	49.3 ± 13.3[b]	20.4 ± 4.37	6.88 ± 1.07
Na^+ (M → S) (7)	250.0 ± 30.5	52.2 ± 14.10	20.10 ± 5.01 ($p < 0.025$)[c]
Na^+ (Net M → S) (3)	138.9 ± 17.0	30.5 ± 10.70	16.50 ± 5.55 ($p < 0.10$)

[a] Numbers in parentheses indicate number of experiments.
[b] ± SEM.
[c] ± Calculated from the mean difference.

If the two processes are coupled then one would expect the activation energy for Na^+ reabsorption and H^+ excretion to be of similar magnitude as determined from the Arrhenius equation. The E_a was determined for both Na^+ reabsorption and H^+ excretion as described above.

In Table 4 are shown the results of this experiment. The average H^+ excretion at room temperature and in the cold is given along with the calculated E_a. Also shown are the mucosal-to-serosal fluxes of Na^+ at room temperature and in the cold along with its calculated E_a. The E_a for the Na^+ mucosal-to-serosal flux was found to be significantly greater than that for H^+ excretion ($p < 0.025$). There was a net mucosal-to-serosal flux of Na^+ in only three hemibladders performed in the cold; therefore, the E_a for the net mucosal-to-serosal movement, while considerably higher than for H^+ excretion, was not significant. However, the fact that the net reabsorptive flux of Na^+ was zero in four hemibladders in the cold, while the H^+ excretion still continued argues against a mucosal $Na^+ - H^+$ exchange mechanism.

Discussion

Preliminary evidence in an earlier paper (Frazier & Vanatta, 1973) suggested that H^+ excretion in the toad bladder may be coupled to the reabsorption of Na^+. A $Na^+ - H^+$ exchange mechanism at the luminal surface has also been postulated for the distal nephron of the mammalian kidney (Pitts, 1968). This study was undertaken to further elucidate the relationship of Na^+ reabsorption and H^+ excretion in the toad urinary bladder.

If Na^+ mucosal-to-serosal flux is coupled in an obligatory manner to H^+ excretion then the H^+ excretion should be reduced in the presence of ouabain and/or the absence of Na^+ from the bathing medium. Our experi-

ments show that, in the presence of ouabain or removal of Na^+ from the bathing media, H^+ excretion is drastically reduced. However, if these same experimental conditions were maintained and DNP was added to the system, H^+ excretion returned to essentially pretreatment control levels. These data show that H^+ excretion can proceed normally even in the presence of reduced or negligible Na^+ reabsorption. As mentioned previously, DNP has been shown to stimulate CO_2 production in the turtle urinary bladder (Schwartz & Steinmetz, 1971). In addition, we have performed experiments showing that DNP also stimulates CO_2 production in the toad bladder.

In the experiments using vasopressin a similar argument may be made. If H^+ excretion is linked to Na^+ reabsorption then one would expect to see an increase in H^+ excretion when Na^+ reabsorption is stimulated with vasopressin. Our experiments did not support this hypothesis. H^+ excretion was not affected by the stimulation of Na^+ reabsorption.

Since DNP and vasopressin each stimulate CO_2 production in the toad bladder it seems paradoxical that DNP would return the H^+ excretion to normal while vasopressin has no effect on H^+ excretion. However, it should be pointed out that in the experiments using DNP, the DNP was added to a system in which H^+ excretion was already reduced; whereas, in the vasopressin experiments the vasopressin was added to an uninhibited H^+ excretory system. It is possible that this latter system was already secreting H^+ at near maximal level and therefore the effect of increasing CO_2 production by vasopressin would not have contributed further to H^+ excretion. Studies are currently underway in which we hope to answer this question by measuring simultaneously H^+ excretion and CO_2 production in the presence of DNP and vasopressin.

In considering a coupled transport between Na^+ and H^+, the possibility of an electrical coupling must be considered. Such a coupling has been suggested for Na^+ and K^+ in rat distal nephron (Malnic, Klose & Giebisch, 1964). Since Na^+ reabsorption in the toad bladder is responsible for the development of an electrical gradient (mucosa negative with respect to serosa), this could be the driving force for H^+ excretion if the two ions are electrically coupled. Our experiments demonstrate that the spontaneous p.d. could be decreased by 80 to 85% in the presence of DNP and ouabain without significant effect on the H^+ excretion. The p.d. was also increased by 50% in the vasopressin experiments without an effect on H^+ excretion. In addition, Frazier and Vanatta (1971) have shown that when the spontaneous p.d. is nullified by short-circuit current, the H^+ excretion is significantly increased. This evidence argues against the possibility of an electrical coupling between Na^+ and H^+ in the toad bladder.

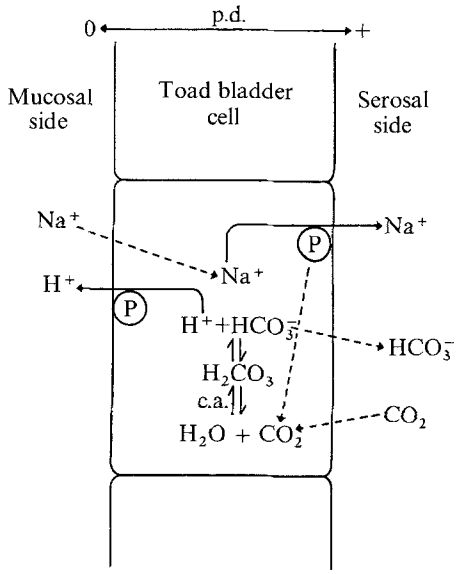

Fig. 1. Schematic view of Na^+ reabsorption and H^+ excretion in the toad bladder epithelial cell. Na^+ reabsorption is shown as an electrogenic pump on the serosal surface, which gives rise to the transepithelial p.d. H^+ excretion is via an active pump on the mucosal border of the cell and the rate of H^+ formation is dependent on the presence of carbonic anhydrase (c.a.) (Frazier & Vanatta, 1972; Ludens & Fanestil, 1972). In the absence of exogenous CO_2 the H^+ formation is dependent on the CO_2 arising from the Na^+ pump

An additional argument may be made regarding a $Na^+ - H^+$ exchange pump at the mucosal border of the cell. If the two ions are obligatorily exchanged at the mucosal surface, then one would expect the E_a for Na^+ reabsorption and H^+ excretion to be of similar magnitude. It is obvious from our experiments that the E_a for the two systems are drastically different. This is additional evidence against the presence of a $Na^+ - H^+$ exchange mechanism at the mucosal border of the cell.

It is interesting to note that the E_a of H^+ excretion and net Na^+ reabsorption of 6.88 and 16.5 kcal/mole, respectively, are well above that usually considered the upper limit (5 kcal/mole) for a purely passive process, such as diffusion (Teorell, 1953). This is additional support for the hypothesis that H^+ excretion is via an active transport process in the toad urinary bladder. The E_a for Na^+ reabsorption in the dog kidney has been reported to be 8.4 kcal/mole (Isaacson, 1962). This is about 50% of the value we obtained in the toad bladder. However, the measurement in the dog kidney was a mean activation energy for the entire nephron. This

large difference in the E_a is not surprising since it is between two different species.

In summary, three lines of evidence have been presented which argue against a $Na^+ - H^+$ coupled exchange mechanism. First, if Na^+ mucosal-to-serosal flux is coupled to H^+ excretion then the H^+ excretion should be reduced in the presence of ouabain and absence of Na^+ in the bathing media. This was not the case as shown when DNP was added to stimulate CO_2 production by the bladder during these two states. Second, it should also hold that H^+ excretion would increase in the presence of increased Na^+ reabsorption. The experiments using vasopressin did not support this hypothesis. Third, the E_a for two systems which are coupled by sharing a common carrier or reaction site would be expected to be similar. Again, the E_a for the two systems were distinctly and significantly different.

In Fig. 1 is shown the current concept of Na^+ transport in the toad bladder along with a possible mechanism for H^+ excretion. The results of the present experiments indicate that H^+ excretion and Na^+ reabsorption are not coupled to an exchange mechanism at the mucosal surface. It does appear, however, that in the absence of exogenous CO_2 the endogenous CO_2 supplied by the Na^+ pump is necessary for maximum H^+ excretion in the toad urinary bladder.

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