Defect in Urinary Acidification Induced In Vitro by Amphotericin B

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ABSTRACT An experimental defect in urinary acidification was induced in the isolated turtle bladder by amphotericin B and the nature of the defect was examined. Net hydrogen ion secretion was little affected by amphotericin when passive electrochemical forces across the epithelium were held at a minimum in the short-circuited state under isohydric conditions. Hydrogen ion secretion against a gradient, however, was markedly reduced by amphotericin and abolished at gradients of more than 2 pH units.

The results suggest that impaired acidification is caused by increased passive permeability of the luminal membrane and increased back diffusion of hydrogen ion rather than by failure of active transport. This interpretation is supported by evidence that amphotericin causes a large increase in the permeability to potassium and smaller increases in the sodium and chloride permeabilities. This mechanism of impaired acidification in vitro may have bearing on the renal tubular defect observed in patients treated with amphotericin B.

INTRODUCTION

One of the determinants of the excretion of acid by the kidney is the maximal hydrogen ion gradient that can be established across the epithelium of the distal segment of the nephron. This concentrating capacity for hydrogen ion, normally about 1000-fold, is impaired in a number of acquired and inherited defects of tubular function. What determines the inability to generate a maximal hydrogen ion gradient in these conditions of "renal tubular acidosis" remains unknown due to the many experimental limitations imposed by the complexity of the kidney. Among the hypotheses that have been considered are increased leakiness of the tubules, an abnormality in the membrane carrier for hydrogen, a failure in the supply of energy to the pump, and a defect in the carbonic anhydrase system (1–3).

Recently McCurdy, Frederic, and Elkinton (3), and Douglas and Healy (4) observed that patients receiving the polyene antibiotic, amphotericin B, develop impaired urinary acidification and urinary potassium wasting, tubular dysfunctions characteristic of the distal form of renal tubular acidosis. Stimulated by these observations, we studied the effects of amphotericin B in vitro in a simple urinary epithelium capable of acidification and induced an acidifying defect the nature of which could be examined. The preparation selected was the urinary bladder of the water turtle, *Pseudemys scripta elegans,* an epithelium resembling the distal nephron in its ability to secrete hydrogen ion and to reabsorb sodium against concentration gradients.

METHODS

Turtle bladders were mounted in an Ussing chamber and bathed with Ringer's solutions that were buffered with 0.3 mM sodium phosphate at pH 7.4 and free of exogenous bicarbonate and carbon dioxide as previously described (5). The net rate of addition of hydrogen ion to the luminal solution was measured by the pH stat method, and the electrical potential difference (PD) was nullified in all studies by means of an external voltage clamp. Amphotericin B (Fungizone)[1] was added to the luminal solution yielding a final concentration of 15 μg/ml and the luminal pH was kept adjusted to the control value. The small quantity of sodium desoxycholate, 12 μg/ml, contained in Fungizone was tested separately and had none of the actions of amphotericin.

For permeability measurements ^{22}Na and ^{42}K were used as the Cl salts and were counted in a Nuclear-Chicago Model 4218 well counter.[2] ^{36}Cl was prepared as the Na salt and counted in a Nuclear-Chicago Model 6801-S liquid scintillation counter. All isotopes were obtained from Cambridge Nuclear Corporation.[3]

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[1] E. R. Squibb and Sons, New York.

[2] Nuclear-Chicago Corporation, Des Plaines, Ill.

[3] Cambridge Nuclear Corporation, Cambridge, Mass.

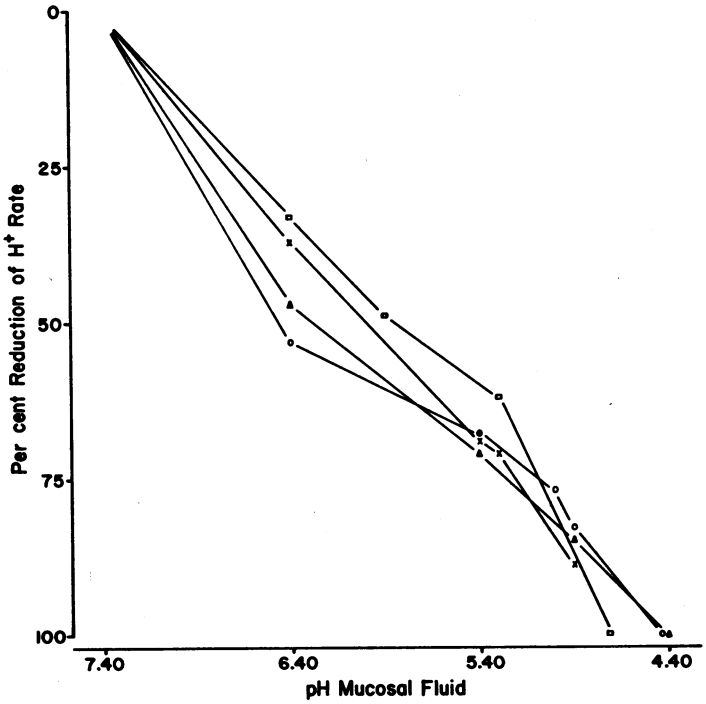

FIGURE 1 Per cent reduction in the net rate of hydrogen ion secretion with increasing pH gradient. The pH of the serosal fluid was kept at 7.40. Symbols represent four experiments.

The measurements of H^+ secretion and of the permeability of the bladder to ^{22}Na, ^{42}K, and ^{36}Cl were made during the first 60 min of exposure to amphotericin, since the amphotericin effects proved to be present in a matter of minutes and to be relatively constant during that period.

Morphological examination of bladders fixed in vitro by electron microscopy revealed that amphotericin caused rarefaction and swelling of the cytoplasm in many of the surface epithelial cells by 2 hr. In occasional surface cells cytoplasmic changes could be detected within the first 30 min of exposure. The luminal membrane itself, however, revealed no visible changes in any of the micrographs. No changes were observed, either early or late, in the basal cells (6).

RESULTS

Acidification against a gradient. To define the effects of opposing concentration gradients on acidification the net rate of hydrogen ion secretion into the mucosal (luminal) solution was measured at gradients ranging from 0 to 3 pH units. In Fig. 1 the per cent reduction in net secretion is shown for four bladders in which the mucosal pH was lowered from 7.4 to 4.4 while the serosal pH was kept at 7.4. Net secretion of hydrogen ion decreased as the pH gradient was increased and approached zero at a mucosal pH of about 4.4.

When both sides of the bladder are isohydric at 7.4 and short-circuited, passive forces for the movement of protons are reduced to a minimum and the net rate of secretion approaches the active component of flux:

$$J_{net} \approx J_{act}$$

In the presence of an opposing concentration driving force, the back flux of protons will equal the net passive flux and the net rate of secretion that is directly measurable by the pH stat technique may be described as:

$$J_{net} \approx J_{act} - J_{pass}$$

At a pH gradient of about 3 U:

$$J_{net} = 0 \text{ and } J_{act} \approx J_{pass}$$

Previous studies have shown that the intracellular pH is close to the pH of the serosal solution. The intracellular pH is about 7.5 when the serosal solution is 7.4 and this relationship holds over the range of mucosal acidification studied (7). The entire pH gradient across the bladder, therefore, is maintained at the luminal membrane of the epithelium. If the active component of flux remains constant during acidification of the luminal solution, the back flux of protons would be expected to increase in direct proportion to the increase in the concentration force, the change in the electrical

force being negligible in the short-circuited state.[4] The observed reduction in net secretion, however, was proportional to the logarithm of the concentration gradient rather than to the gradient itself. The back flux predicted on the basis of the concentration driving force would be $J_{pass} = K \cdot \Delta C$, in which K is the permeability coefficient for hydrogen ion and ΔC the difference between the mucosal and serosal hydrogen ion concentrations. If J_{pass} is calculated from the observed reductions in net secretion and related to ΔC at different values for mucosal pH in Fig. 1, the permeability coefficient is found to vary with mucosal pH; it is lowest at pH 4.4 and increases as pH increases to 7.4. According to this analysis, based on the assumption that J_{act} is constant, the coefficient decreases while back flux increases with increasing acidity of the mucosal solution. Alternatively, J_{act} or both J_{act} and K may vary with mucosal pH and account for the observed relationship. Our analysis does not permit a choice between these possibilities. Since ion permeability has been shown to be dependent on pH in many biological membranes (9) and since there is some evidence that lowering the mucosal pH decreases the permeability of the turtle bladder to another cation, namely sodium (10),[5] the assumption that hydrogen ion permeability is pH dependent appears to provide at least a working hypothesis.

For the purposes of the amphotericin study it is sufficient to define the effect of an opposing concentration gradient on acidification and to select experimental conditions in which either J_{act} or J_{pass} is the principal determinant of the net rate of hydrogen ion secretion.

Effect of amphotericin B on acidification. The effects of amphotericin B were studied, first, in the short-circuited bladder with both sides isohydric at pH 7.4 (passive forces at a minimum), and second, in the presence of a submaximal pH gradient causing back flux of hydrogen ion.

As shown in Table I, the net rate of H^+ secretion was affected little by addition of amphotericin to the luminal solution. During the period 10–40 min after amphotericin, the mean secretion rate was 1.24 ±0.15 μM/hr (SEM), a value comparable to the control rate of 1.44 ± 0.14 μM/hr (SEM). Decreases in the secretion rate did occur after this period but were relatively small.

TABLE I
Effect of Amphotericin B on Net H^+ Secretion at pH 7.4

Turtle	Net H^+ secretion	
	Control	Amphotericin B*
	μM/hr	μM/hr
a	1.11	0.84
b	0.95	0.85
c	1.10	1.08
d	2.18	2.35
e	2.20	1.58
f	1.58	1.40
g	1.47	0.87
h	1.47	1.52
i	1.30	0.83
j	1.06	1.04
Mean ±SEM	1.44 ±0.14	1.24 ±0.15

* Amphotericin B was added to the mucosal solution to a final concentration of 15 μg/ml. Net H^+ transport was measured from 10 to 40 min after addition of amphotericin B.

In contrast, net H^+ secretion was markedly reduced during acidification against a gradient. In Table II is shown that net secretion was abolished at mucosal pH values below 5.4 when the transbladder gradient was 2 or more pH U. At these mucosal pH values there was usually a slight loss of H^+ from the mucosal compartment after exposure to amphotericin. In each experiment the pH stat was engaged three times at the initial pH during the first 40 min after addition of amphotericin. Net secretion could not be recorded in any of the experiments. After failure of secretion at the control pH, the mucosal pH was increased until net secretion could again be recorded in the presence of amphotericin. As shown in the two columns on the right of Table II, some net secretion was restored after the gradient was reduced by 0.4 to 1.0 pH U.

These results indicate that amphotericin has little effect on acidification when electrochemical forces across the bladder are close to zero and, therefore, suggest that the active transport system per se or the energy supplying reactions are not affected directly by amphotericin. When acidification is opposed by a concentration driving force, however, net H^+ secretion is markedly reduced and may reach a negative value, a result consistent with increased back diffusion of H^+ from the mucosal fluid across the luminal membrane.

Effect of amphotericin B on the electrical activity and the permeability of the bladder. Exposure of the luminal membrane to amphotericin in our standard concentration of 15 μg/ml caused a decrease in the electrical

[4] The term back flux in this paper is employed to indicate net passive flux. The concentration driving force was in the direction from mucosal solution (M) to cell in all gradient experiments; the passive flux from cell to M was considered to be vanishingly small, as were the passive fluxes in both directions under isohydric conditions. Hirschhorn and Frazier (personal communication) have shown that the electrical profile of the turtle bladder is comparable to that of the toad bladder (8). During short-circuiting the electrical potential across the mucosal membrane is very small compared to the H^+ gradient that can be generated by the hydrogen ion pump.

[5] L. R. Lawson and P. R. Steinmetz, unpublished observations.

potential difference (PD) and the electrical resistance in a matter of minutes. The short circuit current (SCC) usually increased slightly during the first 20 min. This increase, however, was transient. After these initial changes, the PD and SCC remained relatively steady, declining very slowly with time.

Since the observations on acidification against a gradient were most consistent with an effect of amphotericin on the passive permeability of the mucosal membrane, a series of permeability measurements were carried out for ^{42}K, ^{22}Na, and ^{36}Cl.

Although in the steady state the transepithelial permeability may be measured either from mucosa (M) to serosa (S) or from S to M, an acute change in the permeability of one of the two cell membranes may cause asymmetric changes in the measured permeabilities in the two directions; alterations in both the concentration and the specific activity of the isotope as well as poor mixing on the serosal side of the epithelium may contribute to this asymmetry after an acute change in the permeability of the mucosal membrane. Finn (11) showed that the effects of amphotericin on the mucosal membrane of the toad bladder were best demonstrated in S to M flux measurements. In Table III, S to M permeabilities for ^{42}K are given before and after addition of amphotericin to M. 10- to 50-fold increases in the apparent S to M permeability were observed. The increases were largest during the first 30 min of exposure to amphotericin. Although the values decreased somewhat during the second 30 min, the order of magnitude remained the same. M to S flux measurements revealed only small and rather inconsistent increases in the potassium permeability. Since it was likely that this result might be attributed to a decrease in the intracellular K^+ below its steady-state control value, the M to S permeability was also measured in the presence of a high potassium Ringer's solution in the mucosal compartment to reduce the magnitude of change in intracellular K^+. In these experiments the M to S permeability was increased. In one typical experiment, for example, the control permeability values were 3.93 and 4.58×10^{-6} cm sec^{-1} and the permeability during three

TABLE II

*Effect of Amphotericin B on Net H^+ Secretion against a pH Gradient**

	Control		Amphotericin B		
Turtle	Initial M	H^+ rate	H^+ rate	New M	H^+ rate
	pH	*μM/hr*	*μM/hr*	*pH*	*μM/hr*
a	5.2	0.6	<0.1	6.2	0.6
b	5.2	0.6	<0.1	6.0	0.6
c	5.2	0.9	<0.1	5.7	0.4
d	5.2	0.6	<0.1	5.6	0.5
e	5.4	0.4	<0.1	6.0	0.6
f	5.4	0.5	<0.1	6.0	0.3

* Amphotericin B was added to the mucosal solution (M) in a final concentration of 15 μg/ml. The serosal pH was maintained at 7.4.

TABLE III

*Effect of Amphotericin B on Potassium Permeability**

		Permeability S → M ($\times 10^{-6}$ cm sec^{-1})			
		Control		Amphotericin B	
Turtle	Period:	1	2	3	4
a		5.46	3.01	276	159
b		17.2	19.3	217	193
c		5.54	4.34	174	118
d		3.69	4.44	198	108

* ^{42}K was added to the serosal solution (S) and allowed to equilibrate for at least 50 min. The permeability to ^{42}K was measured during four consecutive 30-min periods. Amphotericin B was added to a final concentration of 15 μg/ml mucosal medium (M) at the beginning of period 3. For turtles a and b both sides of the bladder were maintained at pH 7.4 and for turtles c and d the M pH was 5.0 and S pH 7.4.

TABLE IV

*Effect of Amphotericin B on Sodium and Chloride Permeability**

		Permeability S → M ($\times 10^{-6}$ cm sec^{-1})			
		Control		Amphotericin B	
Turtle	Period:	1	2	3	4
Sodium					
a		3.10	2.85	5.59	7.30
b		3.55	1.85	5.56	8.56
c		2.37	0.70	1.98	4.19
d		2.71	3.83	6.58	29.6
Chloride					
e		2.23	2.63	3.17	3.37
f		5.08	5.38	6.17	8.90
g		2.16	2.83	14.3	35.3
h		1.49	1.74	9.92	17.0

* Either ^{22}Na or ^{36}Cl was added to the serosal solution (S) and allowed to equilibrate for at least 50 min. The permeability to the isotopes were measured during four consecutive 30-min periods. Amphotericin B was added to a final concentration of 15 μg/ml mucosal medium (M) at the beginning of period 3. For turtles a, b, e, and f both sides of the bladder were maintained at pH 7.4 and for turtles c, d, g, and h the M pH was 5.0 and S pH 7.4.

consecutive half hours of mucosal amphotericin exposure were 14.1, 30.0, and 29.5 $\times 10^{-6}$ cm sec^{-1}.

In Table IV, the effects of mucosal amphotericin on the permeability of the bladder to Na^+ and Cl^- are shown. S to M fluxes for both isotopes were increased in all experiments. The results for M to S fluxes, not shown, were inconsistent.

The permeability measurements were made at two mucosal pH values, i.e., pH 7.4 and 5.0 corresponding to the conditions under which the effect of amphotericin on acidification had been studied. In Tables III and IV, the first two experiments of each series were carried out at mucosal pH 7.4 and the last two at mucosal pH 5.0, the serosal pH being 7.4 in all experiments. Similar increases in permeability were observed in the two conditions demonstrating that amphotericin increased membrane permeability not only at low pH where acidification was abolished, but also at pH 7.4 where increased back diffusion of H^+ was not apparent in the absence of a concentration driving force.

DISCUSSION

Previous studies have shown that the epithelial cells of the turtle bladder are relatively alkaline, the cellular pH of the in vitro preparation being close to 7.5 at a serosal pH of 7.4 (7). This alkalinity of the cells was preserved when the mucosal solution was acidified over the range used in the present study. The transcellular pH gradients of the present experiments, therefore, provide an approximation of the gradients across the mucosal membrane alone. In the short-circuited preparation, furthermore, the electrical gradient across this membrane is small compared to the concentration gradient that can be maintained by the H^+ pump. It is therefore possible to vary and monitor this major passive driving force by varying the pH of the mucosal solution and by measuring the net rate of acidification at these different pH levels. To study the effect of amphotericin on acidification advantage was taken of this possibility by measuring the net rate of H^+ secretion under conditions in which there was at least a 100-fold gradient favoring back diffusion of H^+ as well as under conditions in which passive forces on H^+ were minimized.

The virtually unaltered rates of acidification that were observed after amphotericin when electrochemical gradients were minimal suggest that the active transport system for H^+ and the metabolic coupling reactions were not affected directly. Since acetazolamide (Diamox)[6] had its usual inhibitory effect on acidification in the presence of amphotericin in the mucosal solution, an effect of amphotericin on the hydroxylation reaction of CO_2 may also be excluded (7).

At mucosal pH values of 5.4 or less net secretion was abolished by amphotericin. In most experiments back diffusion exceeded forward flux as judged from the slight upward drift of mucosal pH. Increased back diffusion for a given concentration gradient must represent increased passive permeability to H^+. This indirect evidence for increased H^+ permeability of the luminal membrane is supported by the results of the experiments with isotopic K^+, Na^+, and Cl^-. The increased permeabilities and especially the large increase in the K^+ permeability in turtle bladder are in accord with the effects of amphotericin on other biological membranes (11–14).

The effects of amphotericin on K^+, Na^+, and Cl^- were similar at a mucosal pH of 5.0 and 7.4. It is likely therefore that the passive permeability to H^+ was increased also at pH 7.4 when increased back diffusion was not apparent because of the absence of a significant driving force.

Alternatively it is possible that the H^+ pump has separate capacities for its maximal rate and gradient and that only the gradient capacity is affected by amphotericin. Such an action of amphotericin, however, would be unusual in itself and furthermore leaves the increased permeability of the luminal membrane to other electrolytes unexplained.

To which extent this experimental defect in vitro is comparable to the defect in the distal nephron of patients receiving amphotericin B remains to be determined. The amphotericin concentration of 15 μg/ml in the luminal solution of our preparation compares with peak plasma concentrations in patients of up to 3.5 μg/ml. Little is known about the way amphotericin is excreted by the kidney. From cumulative excretion rates given by Louria (15) for two patients it appears likely that peak urinary concentrations exceed the plasma concentrations. The former, therefore, may be of greater importance in the pathogenesis of the tubular defect than the latter. An increase in the passive permeability of the luminal surface of the tubular epithelium would increase the passive fluxes of potassium and hydrogen ions in the directions of their respective electrochemical gradients. Since recent studies by Malnic, Klose, and Giebisch (16) indicate that in the distal tubule of the rat the secretion of potassium into the urine is a passive process, a defect in the barrier function of the luminal membrane might provide an explanation for increased potassium secretion. A single alteration of the luminal membrane, therefore, might account for both the potassium wasting and the failure to elaborate an acid urine.

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