

## BAFILOMYCIN A<sub>1</sub> AT NANOMOLAR CONCENTRATIONS SATURABLY INHIBITS A PORTION OF TURTLE BLADDER ACIDIFICATION CURRENT

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### Summary

An earlier report indicated that acid secretion in turtle urinary bladder is driven by an unusual vacuolar H<sup>+</sup>-ATPase and that the ATPase accounts for essentially all acid secreted. These results, however, are difficult to reconcile with the acid transporters currently ascribed to the renal collecting duct. Here, we re-examine the effect of bafilomycin A<sub>1</sub>, an inhibitor of vacuolar (V-type) H<sup>+</sup>-ATPases, on acid secretion by intact isolated bladders from *Pseudemys scripta* turtles. Serosal-side bafilomycin had no effect on the transepithelial acidification current (AC). In the mucosal solution, bafilomycin inhibited the AC, with inhibition developing over the range 0.1–10 nmol l<sup>-1</sup>, with a sigmoidal dose–response curve, and an IC<sub>50</sub> of 0.47 nmol l<sup>-1</sup>. At saturation, approximately 70 % of H<sup>+</sup> secretion was inhibited. The remaining 30 %

could be abolished by 30 μmol l<sup>-1</sup> Sch-28080, which is a level that in other systems is known to inhibit H<sup>+</sup>/K<sup>+</sup>-ATPase transport activity specifically and essentially completely. When the order of addition was reversed (Sch-28080 first), there was no change in the magnitude of the effect produced by either inhibitor, and the two together again eliminated the AC. The data indicate that baseline acid secretion in intact bladders is due (i) in part to a highly bafilomycin-sensitive process, with sensitivity typical of vacuolar H<sup>+</sup> ATPases; and (ii) in part to a more bafilomycin-resistant process that is sensitive to Sch-28080.

Key words: turtle, *Pseudemys scripta*, urinary bladder, acid secretion, bafilomycin A<sub>1</sub>, vacuolar H<sup>+</sup> ATPase, Sch-28080.

### Introduction

The urinary bladder of the turtle has been widely used as a model of acid secretion by the collecting duct of the mammalian kidney. It is thought that acid secretion in the turtle bladder originates predominantly from a population of the so-called carbonic anhydrase-rich cells, the counterpart of the renal intercalated cell (Stetson and Steinmetz, 1985; Scheffey et al., 1991; Kohn et al., 1997), and that a vacuolar-type H<sup>+</sup>-ATPase is involved in or contributes to the active secretion of acid (Youmans and Barry, 1989; Youmans and Barry, 1991b; Kohn et al., 1993; Kohn et al., 1997). Vacuolar (or V-type) H<sup>+</sup>-ATPases are widely distributed in nature. In eucaryotic animal cells they occur in a variety of intracellular organelles and in the plasma membranes of specialized acid-secreting cells including renal intercalated cells, bone osteoclasts, and certain cells of the male epididymis and vas deferens, as well as a growing list of other acid-transporting cells (for reviews, see Finbow and Harrison, 1997; Stevens and Forgac, 1997; Nelson and Harvey, 1999; Wiczeorek et al., 1999; Forgac, 2000). Vacuolar H<sup>+</sup>-ATPases also exist in the vacuolar membranes of yeast and other fungi (Bowman et al., 1988; Nelson and Harvey, 1999; Bowman and Bowman, 2000; Forgac, 2000) and the tonoplast of higher plants (White, 1994).

In every case tested to date, the antibiotic bafilomycin A<sub>1</sub>

acts as a potent and, at suitable concentrations, specific inhibitor of eucaryotic vacuolar H<sup>+</sup>-ATPases (Bowman et al., 1988; Dröse et al., 1993; White, 1994; Dröse and Altendorf, 1997; Finbow and Harrison, 1997; Stevens and Forgac, 1997). The potency of the inhibitor is such that inhibition seen at concentrations of 1 to a few nmol l<sup>-1</sup>, or less, is often taken as indicative of the presence of a vacuolar H<sup>+</sup>-ATPase (Dröse and Altendorf, 1997). In various intact or fractionated eucaryotic systems in which molar units were reported, and the criterion evaluated was inhibition of H<sup>+</sup> transport or H<sup>+</sup>-ATPase activity, 50 %-inhibitory concentrations (IC<sub>50</sub>) have ranged from a low of 0.2 nmol l<sup>-1</sup> (Crider et al., 1994) to a high of 3 nmol l<sup>-1</sup> (Sundquist et al., 1990; Nanda et al., 1992), with essentially complete inhibition occurring between 1 (Crider et al., 1994) and no more than 20 nmol l<sup>-1</sup> (Sundquist et al., 1990; Nanda et al., 1992).

In contrast, it has been reported that acid secretion by intact turtle bladders is inhibited by bafilomycin, but a concentration of more than 100 nmol l<sup>-1</sup> is needed to cause 50 % inhibition and 500 nmol l<sup>-1</sup> or more for essentially complete inhibition, which are levels some 25- to 500-fold higher (Kohn et al., 1993). The results have been interpreted to mean that in turtle bladders, acid secretion is driven by a single active transporter,

a V-type H<sup>+</sup>-ATPase with unusually low sensitivity to bafilomycin A<sub>1</sub> (Kohn et al., 1993). However, the concentration profile is important, for three reasons. First, such relative insensitivity to bafilomycin, if confirmed, would be an unusual finding, and might well define a new class or subclass of vacuolar ATPase. Second, it is well-known that bafilomycin A<sub>1</sub> at these higher concentrations is an effective inhibitor of P-type ion-transporting ATPases, a group that includes Na<sup>+</sup>/K<sup>+</sup>-ATPases, H<sup>+</sup>/K<sup>+</sup>-ATPases, the H<sup>+</sup>-ATPase of *Neurospora* plasma membrane, and the CaATPase of sarcoplasmic reticulum (Bowman et al., 1988; Dröse et al., 1993; Dröse and Altendorf, 1997). Bafilomycin only shows specificity for vacuolar ATPases at suitably low concentrations (Bowman et al., 1988; Dröse et al., 1993; Dröse and Altendorf, 1997), so an action observed at higher concentrations must be evaluated with care. Consequently, the possibility cannot be excluded that the inhibition seen with high bafilomycin concentrations in the turtle bladder could have been due, at least in part, to effects on other transport processes. Third, these findings, if true, would seem to indicate that the turtle bladder has different active acid-secretory mechanisms than the renal collecting duct, which is currently thought to utilize a typical vacuolar H<sup>+</sup>-ATPase and one or more H<sup>+</sup>/K<sup>+</sup> exchange ATPases (Brown et al., 1988; Wingo and Smolka, 1995; Caviston et al., 1999; DuBose et al., 1999; Jaisser and Beggah, 1999).

In preliminary studies with turtle bladders, however, we noted substantial inhibition at low, subnanomolar concentrations. In view of the discrepancy of this observation with the published literature, and considering the importance of bafilomycin A<sub>1</sub> as a characteristic inhibitor of V-type H<sup>+</sup>-ATPases, we decided to reinvestigate its action on intact turtle bladders. The purpose of the present study then was to determine: (i) the concentration dependence of bafilomycin's inhibitory effect on acid secretion by turtle bladders over a range known to be specific for other V-type H<sup>+</sup>-ATPases; and (ii) whether this range of concentrations inhibits none, a portion, or essentially all of such acid secretion.

## Materials and methods

### Measurement of acid secretion

Bladders from turtles (*Pseudemys scripta elegans*) 4–8 days after feeding (post-absorptive) were removed and mounted as flat sheets in physiological (Ussing-type) chambers as described in the literature (Durham et al., 1987; Kniaz and Arruda, 1991; Graber and Devine, 1993; Kohn et al., 1993). The animals were effectively anaesthetized before sacrifice by cooling them in steps to approx. 8 °C in baths of chilled water. The spontaneous electrical potential across isolated bladders was held to zero by an automatic voltage clamp and the resulting short-circuit current (SCC) was measured throughout the experiment. The measurements were made relative to a serosal-side ground, and so acid secretion corresponds to negative SCC values (net serosal-to-luminal movement of positive charge). Ouabain (final concentration 2 × 10<sup>-4</sup> mol l<sup>-1</sup>) was added to the serosal fluid bath to eliminate sodium

transport. In turtle bladders, the net transepithelial ion transport remaining under these conditions closely approximates the acidification current,  $J_H$  (Steinmetz et al., 1967; Durham et al., 1987; Kniaz and Arruda, 1991; Graber and Devine, 1993; Kohn et al., 1993), the rate at which net acid enters (or, occasionally, is removed from) the luminal solution. In experiments in which Sch-28080 was added alone to the mucosal solution (i.e. preceding bafilomycin), it was noted that in most (not all) experiments a transient but slowly developing 'overshoot' in the SCC ensued, before it settled down to a new, reduced, steady state in 60–90 min. In the remaining experiments there was no such overshoot and Sch-28080 addition yielded a monophasic decline to a new steady state in 30–60 min. In either case, only steady state values were tabulated. In all experiments, the Ussing chambers had a cross-sectional area of 1.43 cm<sup>2</sup> and short-circuit current values are given throughout for that area. All experiments were done at room temperature, approx. 23 °C.

### Solutions

The luminal and serosal tissue surfaces *in vitro* were bathed in identical reptilian Ringer's solution, which contained, in mmol l<sup>-1</sup>: NaCl, 80; NaHCO<sub>3</sub>, 20; KCl, 3.5; MgSO<sub>4</sub>, 1.0; CaCl<sub>2</sub>, 1.8; Na<sub>2</sub>HPO<sub>4</sub>, 1.25; and glucose, 11. The final pH after equilibrating with 95 % O<sub>2</sub>:5 % CO<sub>2</sub> was 7.3–7.4. Inhibitor stock solutions were prepared as follows: bafilomycin A<sub>1</sub>, 2 µg per 100 µl (32.1 µmol l<sup>-1</sup>) in dimethyl sulphoxide (DMSO); Sch-28080, 100 mmol l<sup>-1</sup> in DMSO. Bafilomycin A<sub>1</sub> concentrations were determined photometrically by the method of Bowman et al. (Bowman et al., 1988). The stocks were prepared ahead of time and stored in portions at -20 °C (bafilomycin) or +4 °C (Sch-28080). We found that stocks of either compound had to be used within 6–8 weeks. It was also found necessary to minimize the time during which working samples of bafilomycin A<sub>1</sub> were kept at temperatures of 0 °C or higher during an experiment. The inhibitory potency deteriorated markedly beyond a period of 4–5 h at ice temperature. The final DMSO vehicle concentration in experiments never exceeded 0.3 %. In pilot studies, no vehicle effects were seen up to at least that concentration.

### Data analysis

In general, data are presented as the mean ± S.E.M. for the number of experiments indicated. Figs 1 and 3 were prepared by scanning and digitizing the appropriate chart recorder tracing and importing the files into Sigma Plot. In the experiments in which the half-maximal inhibitory concentration (IC<sub>50</sub>) was determined, IC<sub>50</sub> was determined for each individual experiment and is expressed here as the mean ± S.E.M. The curve in Fig. 2 was calculated from the mean ± S.E.M. for the response to bafilomycin A<sub>1</sub> at each indicated concentration, using Sigma Plot. Statistical significance was determined, as appropriate, with a paired Student's *t*-test.

### Materials

Turtles were obtained from Kons Scientific, Germantown,

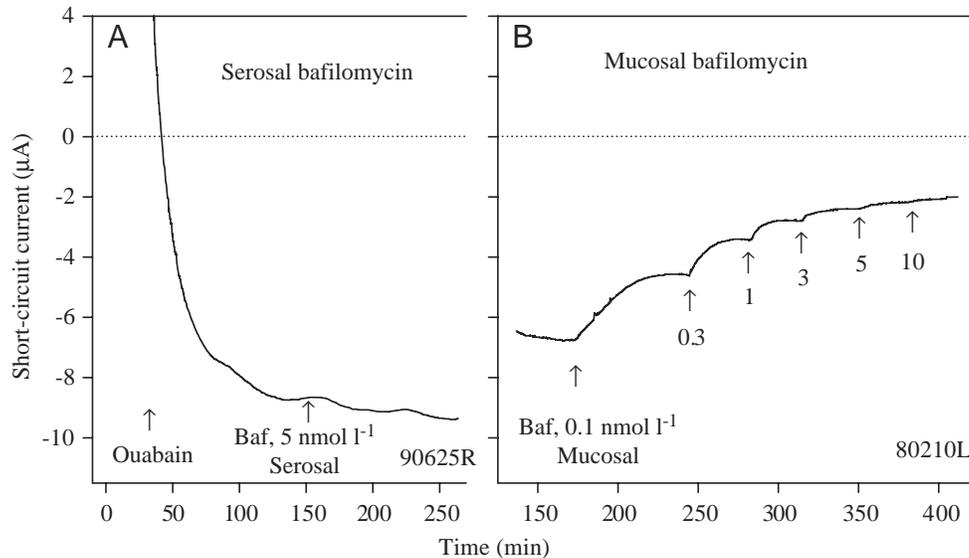


Fig. 1. Effect of bafilomycin A<sub>1</sub> exposure on acidification current. Separate experiments are shown in A and B. In both experiments, turtle bladders were mounted in Ussing chambers as described in Materials and methods and exposed to 0.2 mmol l<sup>-1</sup> ouabain in the serosal solution (arrow) to eliminate sodium transport. When the short circuit current (acidification current) subsequently reached a new steady state, the bladder was exposed to either 5 nmol l<sup>-1</sup> bafilomycin A<sub>1</sub> in the serosal bathing solution (A) or to a graded sequence of bafilomycin A<sub>1</sub> concentrations in the mucosal solution (B; final concentrations are shown in nmol l<sup>-1</sup>). Baf, bafilomycin A<sub>1</sub>. Short circuit current shown is in µA (per area of chamber, 1.43 cm<sup>2</sup>). The unlabeled number in the lower right of each panel is an index number identifying the experiment.

WI, USA and Carolina Biological, Burlington, NC, USA. Sch-28080 was a gift from Dr James Kaminski of Schering-Plough. All other reagents were obtained from Sigma.

## Results

### Effect of bafilomycin A<sub>1</sub> on acid secretion

#### Serosal addition

Bladders were mounted *in vitro* in Ussing-type chambers as described in Materials and methods and exposed to ouabain on the serosal side to eliminate transepithelial sodium transport. After ouabain was added, the short-circuit current (acidification current under these conditions) was allowed to reach a steady state, which typically was oriented serosal side-negative, indicative of net acid (H<sup>+</sup>) secretion. Bafilomycin added to the serosal bathing solution (final concentration, 5 nmol l<sup>-1</sup>) caused no discernable change in the acidification current in 30–60 min, as shown for a representative experiment in Fig. 1A. In six such experiments, after 30 min, acid secretion was 99.7±1.5% (mean ± S.E.M.) of baseline,  $P=0.85$  versus null effect, NS (baseline, -6.8±2.0 µA (per 1.43 cm<sup>2</sup>); and at 60 min ( $N=5$ ), 98.2±3.8% of baseline,  $P=0.67$  versus null, NS (baseline, -6.6±2.45 µA (per 1.43 cm<sup>2</sup>).

#### Mucosal addition

In contrast, bafilomycin A<sub>1</sub> added to the mucosal solution alone did inhibit the acidification current. The results of one experiment are depicted in Fig. 1B, which shows the effects of a graded sequence of concentrations of bafilomycin. Inhibition was pronounced at the lowest concentration tested, 0.1 nmol l<sup>-1</sup>, and saturation was reached by 5–10 nmol l<sup>-1</sup>. The

concentration dependence observed in four such experiments is summarized by the dose–response curve in Fig. 2. When the half-maximal inhibition was determined for each experiment individually, the average was 0.47±0.17 nmol l<sup>-1</sup>. The inhibition again reached a plateau at 5–10 nmol l<sup>-1</sup>, with

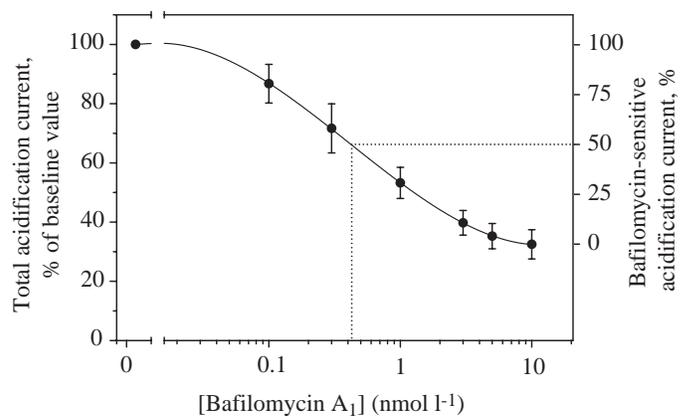


Fig. 2. Effect of bafilomycin A<sub>1</sub> on acid secretion by intact turtle bladders. Dose–response inhibition curve. Turtle urinary bladders were mounted in Ussing-type chambers and bathed in Na/Cl/HCO<sub>3</sub> Ringer's solution. The serosal solution contained 0.2 mmol l<sup>-1</sup> ouabain. Under these conditions the short-circuit current across the tissue approximates the rate of net acid–base transport and is typically oriented serosal-side negative (acid secretion; see Materials and methods). Baseline acid secretion (100%) was -12.0±4.3 µA (per area of chamber, 1.43 cm<sup>2</sup>) ( $N=4$ ). Bafilomycin A<sub>1</sub> in DMSO vehicle was added in the luminal solution to the concentrations indicated and the short-circuit current allowed to stabilize after each addition. The final concentration of DMSO never exceeded 0.3%.

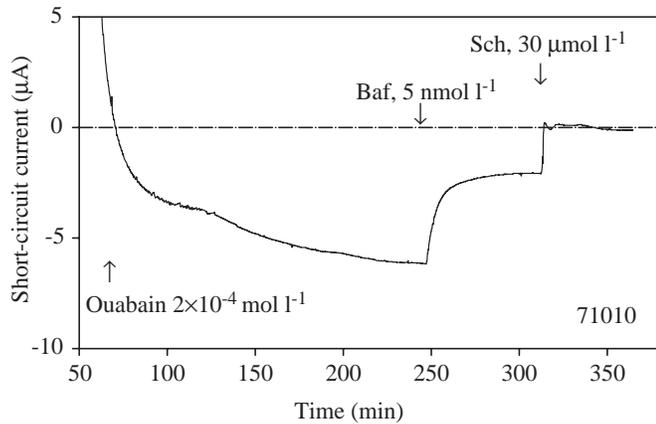


Fig. 3. Effects of bafilomycin A<sub>1</sub> and Sch-28080 on acidification current in a representative experiment. The bladder was maintained in an Ussing-type chamber and conditions were as described for Fig. 1. Baf, bafilomycin A<sub>1</sub> ( $5 \times 10^{-9}$  mol l<sup>-1</sup>, mucosal); Sch, Sch-28080 ( $3 \times 10^{-5}$  mol l<sup>-1</sup>, mucosal) were introduced where indicated (arrows).

$65 \pm 4\%$  and  $67 \pm 5\%$  of the original acid secretion inhibited and  $35 \pm 4$  and  $33 \pm 5\%$  remaining, respectively. The H<sup>+</sup> secretion remaining was significantly different from zero ( $P < 0.03$  at both 5 and 10 nmol l<sup>-1</sup>). No significant difference was found between these two highest doses ( $P > 0.1$ ). In subsequent experiments therefore, a concentration of 5 nmol l<sup>-1</sup> bafilomycin A<sub>1</sub> was used, 10 times the IC<sub>50</sub>, a multiple used commonly in transport or enzyme studies.

#### Effect of Sch-28080 on bafilomycin A<sub>1</sub>-insensitive acid secretion

Bafilomycin A<sub>1</sub> inhibited the majority of the acidification current, but there was a portion that clearly was not affected, at concentrations that are specific for V-type H<sup>+</sup>-ATPases in other preparations. Fig. 3 shows an experiment in which the bafilomycin-resistant component was tested for sensitivity to a different inhibitor of transport-ATPases, Sch-28080. In this representative experiment, acidification that was probably due to vacuolar H<sup>+</sup>-ATPase activity was first inhibited by 5 nmol l<sup>-1</sup> bafilomycin in the mucosal medium. The bolus addition of bafilomycin decreased the magnitude of the short-circuit current in this experiment from  $-6.1 \mu\text{A}$  to  $-2.1 \mu\text{A}$ , with a half-time ( $t_{1/2}$ ) of 4.1 min. A stable plateau was reached within 50 min. At this point,  $30 \mu\text{mol l}^{-1}$  Sch-28080 was added to the mucosal solution. Sch-28080 inhibited the bafilomycin A<sub>1</sub>-resistant acidification current, reducing it from  $-2.1 \mu\text{A}$  to  $+0.1 \mu\text{A}$  with a rapid onset,  $t_{1/2} = 1.5$  min. The current stabilized near zero within 15 min.

Fig. 4 summarizes the results of seven such experiments, here expressed as the percentage of total H<sup>+</sup> transport (acidification current) that was inhibited by each agent. 5 nmol l<sup>-1</sup> bafilomycin caused acid secretion to decline by  $71 \pm 4\%$  ( $P < 0.01$ ), a decrement similar to that seen in the experiments in which the concentration dependence (dose-response) was determined. The acid secretion that

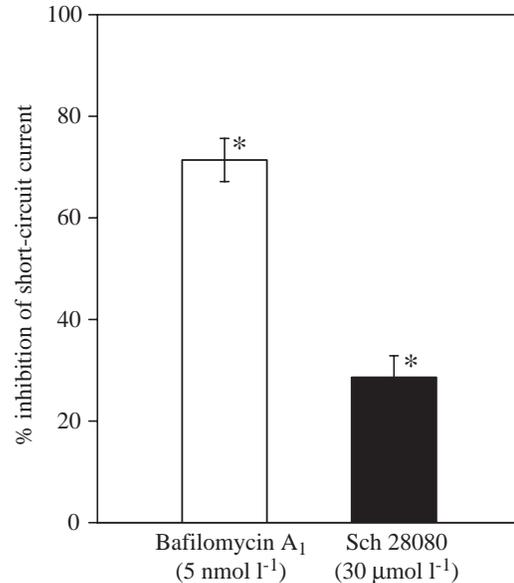


Fig. 4. Inhibition of acid secretion by bafilomycin A<sub>1</sub> and Sch-28080. Urinary bladders were mounted in vitro in chambers as described in Materials and methods and the legend to Fig. 1. When the short-circuit current was stable, bafilomycin A<sub>1</sub> was introduced into the mucosal solution to the indicated final concentration. When the short-circuit current again stabilized, Sch-28080 was then added to the mucosal solution to the concentration indicated. Bars show inhibition by each compound as a percentage of baseline acid secretion ( $-4.7 \pm 1.25 \mu\text{A}$  (per area of chamber, 1.43 cm<sup>2</sup>),  $N = 7$ ). \*Significant effect of treatment ( $P < 0.01$ ).

remained at this point, in the presence of bafilomycin only, while reduced remained significantly different from zero ( $P < 0.03$ ), as before. This residual bafilomycin-resistant acid transport was inhibited completely by  $30 \mu\text{mol l}^{-1}$  Sch-28080, which caused a decline of  $29 \pm 4\%$  of the original acid secretion ( $P < 0.01$  versus null). In the presence of both 5 nmol l<sup>-1</sup> bafilomycin and  $30 \mu\text{mol l}^{-1}$  Sch-28080, the acidification current was not statistically distinguishable from zero ( $+0.21 \pm 0.11 \mu\text{A}$ ,  $P > 0.1$ ). Thus, the results indicate that the two inhibitors, used at concentrations at which each is known in other systems to act specifically, together inhibit essentially all the acid secretion in these bladders. Further, the results are consistent with the concentrations used, 5 nmol l<sup>-1</sup> bafilomycin and  $30 \mu\text{mol l}^{-1}$  Sch-28080, being the maximally inhibiting level of each inhibitor for its respective transport process.

#### Reversal of the sequence of inhibitor addition

If bafilomycin A<sub>1</sub> and Sch-28080 exert independent effects on two different transport systems, then the order in which they are added should have little or no effect on the degree of inhibition produced by each. To test this, we ran a group of eight experiments, in each of which one hemibladder was exposed on the mucosal side first to 5 nmol l<sup>-1</sup> bafilomycin A<sub>1</sub> and subsequently to  $30 \mu\text{mol l}^{-1}$  Sch-28080, while the mated hemibladder (from the same animal) received the reverse sequence of inhibitors. The results are summarized in Table 1.

Table 1. *Effects of bafilomycin A<sub>1</sub> and Sch-28080 on short circuit current across isolated turtle urinary bladders*

Sequence	Initial SCC (μA)	Baf Δ (μA)	Sch Δ (μA)	Final SCC (μA)	Total Δ (μA)	Baf Δ as % of total Δ	Sch Δ as % of total Δ
Baf, Sch	-3.19±1.31	+2.64±0.85	+1.29±0.55	+0.74±0.94	+3.93±1.06	69.4±8.6	30.6±8.6
<i>P</i>		<0.02	<0.05	>0.45*	<0.01	<0.001	<0.01
Sch, Baf	-6.10±2.86	+2.73±0.70	+1.15±0.45	-2.22±2.08	+3.87±0.90	72.0±7.8	28.0±7.8
<i>P</i>		<0.01	<0.04	>0.3*	<0.005	<0.01	<0.001

The effects of bafilomycin A<sub>1</sub> (5×10<sup>-9</sup> mol l<sup>-1</sup>) and Sch-28080 (3×10<sup>-5</sup> mol l<sup>-1</sup>) additions, to the luminal bath, on steady state short-circuit current (SCC) values were assessed in the presence of serosal ouabain. The order of addition was bafilomycin followed by Sch-28080 (Baf, Sch sequence) or the reverse (Sch, Baf sequence). In each of eight experiments, one hemibladder was given one sequence while the mated hemibladder from the same animal was given the other.

Baf Δ, SCC change induced by bafilomycin; Sch Δ, SCC change induced by Sch-28080; Total Δ, change in SCC with both inhibitors present. Values are means ± s.e.m. (N=8); *P* values were determined using a paired Student's *t*-test.

\**Versus* zero; *P* values in other columns are *versus* null effect of treatment.

When bafilomycin was given first, it produced a decrement in the acidification current of 2.64±0.85 μA (initial baseline, -3.19±1.31 μA (per 1.43 cm<sup>2</sup>), which amounted to 69±9% of the total effect that would ultimately be produced by both inhibitors. Sch-28080 subsequently caused a further decrement of 1.29±0.55 μA, the remaining 31±9% of the combined inhibitor effect. With both inhibitors present, the SCC (acidification current) did not differ statistically from zero (+0.74±0.94 μA, *P*>0.45; *N*=8), as before. In the paired hemibladders, where Sch-28080 was given first, the SCC was decreased by 1.15±0.45 μA (baseline -6.10±2.86 μA (per 1.43 cm<sup>2</sup>), which was 28±8% of the ultimate combined inhibitor effects on the acidification current in this group of hemibladders. Bafilomycin subsequently caused a further decrement of 2.73±0.70 μA, the remaining 72±8% of the combined inhibitor effect. Again, when both inhibitors were present the acidification current did not differ significantly from zero (-2.22±2.08 μA per 1.43 cm), *P*>0.3). Table 2 shows that the order of addition had no influence on the effect produced by bafilomycin, either in terms of absolute decrement of the short circuit current (*P*>0.8, bafilomycin added first *versus* added second) or the percentage decrement (*P*>0.65 for addition first *versus* second). Likewise, the order of addition had no effect on the decrement produced by Sch-28080, either in terms of absolute current (*P*>0.7; Sch-28080 added first *versus* added second) or percentage decrement (*P*>0.65 for addition first *versus* second). These results indicate that the order of addition had no effect on the magnitude of inhibition produced by either inhibitor. The results are consistent with the hypothesis that bafilomycin A<sub>1</sub> and Sch-28080 exert independent effects on independent transport processes in this tissue.

## Discussion

### *Action of bafilomycin on different classes of ion-transporting-ATPase*

The effects of bafilomycin A<sub>1</sub>, a macrolide antibiotic, on three classes of ion-transporting ATPase were first reported by

Bowman et al. (Bowman et al., 1988). They found that the compound was a potent inhibitor of vacuolar ATPases, with IC<sub>50</sub> values typically <0.5 nmol bafilomycin mg<sup>-1</sup> protein (approx. 2–5 nmol l<sup>-1</sup> under the conditions of their experiments), while the F<sub>1</sub>F<sub>0</sub>-ATPases from bacteria and mitochondria were unaffected even at extremely high doses (up to 150 μmol bafilomycin mg<sup>-1</sup> protein; approx. 1 mmol l<sup>-1</sup>). The action on P-type (phosphorylating) ATPases was intermediate, with no inhibition in the low nanomolar range but effective inhibition at concentrations in the micromolar range (IC<sub>50</sub> values of 1–4 μmol mg<sup>-1</sup>; approx. 50 μmol l<sup>-1</sup>). These findings on potency and specificity have since been substantiated in other studies (Dröse et al., 1993; Dröse and Altendorf, 1997; Stevens and Forgac, 1997).

### *Results of the present experiments*

We made use of this specificity in the present experiments. We find that bafilomycin A<sub>1</sub> inhibits with high sensitivity

Table 2. *The effect of the order in which bafilomycin A<sub>1</sub> and Sch-28080 were added*

	Data evaluated	
	Absolute SCC Δ	SCC Δ as % of total (baf+Sch) Δ
Bafilomycin added first <i>versus</i> second		
<i>t</i>	0.14	0.44
<i>P</i>	>0.8	>0.65
Sch-28080 added first <i>versus</i> second		
<i>t</i>	0.34	0.44
<i>P</i>	>0.7	>0.65

The experiments tabulated in Table 1 were evaluated statistically for effects of the order of addition of bafilomycin A<sub>1</sub> (baf) and Sch-28080 (Sch) on the inhibition by each of the short-circuit currents (SCC).

Δ, change.

*t* and *P* values are *versus* the null hypothesis; *P* was determined using a Student's *t*-test for paired variates (*N*=8).

approximately 70% of the baseline acid secretion of intact turtle bladders, with inhibition developing over the range of 0.1 to 10 nmol l<sup>-1</sup>, giving a sigmoidal dose–response curve, and exhibiting an IC<sub>50</sub> of 0.47 nmol l<sup>-1</sup> (Fig. 2). This is the first demonstration that acid secretion in intact turtle bladders is inhibited by bafilomycin A<sub>1</sub> at concentrations known to be specific for V-type H<sup>+</sup>-ATPases in other tissues. The remaining 30% of acid secretion is more resistant to bafilomycin.

The apparent sigmoidal shape of the bafilomycin dose–response curve is not necessarily an expected result. The simplest hypothetical model would be one in which the inhibitory ligand binds reversibly to a uniform population of unhindered binding sites located, one each, on a uniform population of protein molecules. If the binding is one to one, does not induce oligomerization of the binding sites or protein molecules, and does not induce secondary conformational states of the binding site with different affinities for the receptor, then a hyperbolic dose–response relationship would be expected (Segel, 1975). The fact that this is not seen in the present case appears to indicate that at least one of these assumptions is violated. A sigmoidal curve clearly raises the possibility of cooperativity in the binding of bafilomycin A<sub>1</sub>, and hence the presence of multiple binding sites (Segel, 1975). However, there are other possible interpretations and with the information available it is not feasible to distinguish between them.

#### *Previous results with the turtle bladder*

Our results differ from those of an earlier study, which reported that turtle bladder acid secretion is inhibited by bafilomycin A<sub>1</sub>, but only at far higher concentrations than we report here (Kohn et al., 1993). The results led the authors to conclude that acid secretion in the turtle bladder is driven by a single active transport process, involving a vacuolar H<sup>+</sup>-ATPase with unusually low sensitivity to bafilomycin A<sub>1</sub>. This is, to date, a unique finding among eucaryotic cells, and the known features of bafilomycin interaction with V-ATPases in other eucaryotic systems, discussed below, argue for caution in interpreting the claim. It is not entirely clear why lower bafilomycin sensitivity was found in the earlier study, but potential sources of discrepancy can be identified. The concentrations of bafilomycin used in that study, 50 nmol l<sup>-1</sup> to >600 nmol l<sup>-1</sup>, were much greater than those presently employed. The reported IC<sub>50</sub> (approx. 100 nmol l<sup>-1</sup>) was 200-fold greater and the lowest concentration used in the study, 50 nmol l<sup>-1</sup>, was tenfold higher than required to produce saturation in our experiments. Hence, the earlier study used concentrations considerably above the range where we observed a sigmoidal dose–response to bafilomycin. Since we find that the turtle bladder displays both a highly bafilomycin-sensitive component of acid secretion and a second, less-sensitive or insensitive, component, the lower overall tissue sensitivity reported previously appears to be, at least in part, a composite effect resulting from determining a single IC<sub>50</sub> in the presence of both high- and low-sensitivity transport systems. In addition, bafilomycin is a somewhat labile

compound and storage conditions can impinge on its biological activity (Dröse et al., 1993; Farina and Gagliardi, 1999; see also Materials and methods). Finally, the possibility of unappreciated differences in the metabolic states of the animals cannot be entirely ruled out as a contributing factor.

#### *Comparison to other systems*

It is of interest to compare these two sets of findings in the turtle bladder with what is known of bafilomycin's action in other systems. The discovery by Bowman et al. (Bowman et al., 1988) that bafilomycin A<sub>1</sub> inhibited vacuolar H<sup>+</sup>-ATPases at markedly low concentrations suggested a specific interaction. However, they also reported that the IC<sub>50</sub> in molar units was strictly dependent on the amount of protein present in homogenized preparations. This raised the possibility that bafilomycin interacted nonspecifically with one or more proteins or that, as a lipophilic compound, it partitioned extensively into the lipid phase of membranes. Hanada et al. (Hanada et al., 1990) confirmed the dependence on endogenous protein concentration but also reported that neither exogenously added protein (albumin) nor lipid (phospholipids) had a detectable effect on the IC<sub>50</sub>. Hanada et al. further determined, by kinetic analysis, that the V-ATPase of chromaffin granules contained a single type of bafilomycin interaction site, that it had high affinity for the inhibitor, and that 50% inhibition occurred at a ratio of 1 mole bafilomycin per mole of enzyme (Hanada et al., 1990). Essentially 100% inhibition was reached at 7–8 moles bafilomycin. From these considerations, it can be concluded that bafilomycin interaction in these studies occurred at a distinct binding site, and that the apparent dependence on protein quantity was in fact a dependence on the number or density of that binding site in the preparation.

In coated vesicles and in osteoclasts, the binding site has been localized to V<sub>0</sub>, the membrane-bound portion of the V-ATPase assembly, although there is controversy as to which peptide subunit or subunits of V<sub>0</sub> contain the site (Hanada et al., 1990; Crider et al., 1994; Zhang et al., 1994; Mattsson and Keeling, 1996; Forgac, 2000). While the structure of the binding site is not yet known, its specificity for bafilomycin has been characterized in some detail. High-affinity binding has been shown to depend on specific structural elements contained in a 16- to 18-member macrolactone ring, which characterizes bafilomycin (16-member) and related macrolide antibiotics, including the concanamycins (Dröse et al., 1993; Farina and Gagliardi, 1999; Gagliardi et al., 1999). Artificial constructs have been prepared that contain these structures, but are otherwise structurally unrelated to bafilomycin, and a number of these also inhibit V-ATPases with high affinity (Farina and Gagliardi, 1999; Gagliardi et al., 1999). Thus, the available evidence indicates that bafilomycin interacts with eucaryotic vacuolar-ATPases through a binding site that is characterized by a high affinity and high specificity for the inhibitor.

Perhaps not surprisingly, there is a striking consistency in the threshold concentrations that have been reported to produce

inhibition. In a variety of experimental preparations (intact tissue, suspended cells, subcellular fractions or isolated molecules) in which a molar dose–response to bafilomycin was determined using inhibition of  $H^+$ -ATPase activity or  $H^+$  transport as the criterion,  $IC_{50}$  values ranging only from 0.2 to  $3\text{ nmol l}^{-1}$ , with maximal inhibition achieved by 1 to no more than  $20\text{ nmol l}^{-1}$ , have been reported, (Bowman et al., 1988; Moriyama and Nelson, 1989; Moriyama and Futai, 1990; Sundquist et al., 1990; Mattsson et al., 1991; Mattsson et al., 1993; Nanda et al., 1992; Armitage and Wingo, 1994; Crider et al., 1994; Keeling et al., 1997). Exceptions to this pattern of concentration dependence have been demonstrated only in prokaryotes (Chen and Konisky, 1993; Yokoyama et al., 1994). Thus, our present findings in the turtle bladder are consistent with results reported for a variety of eucaryotic cells, in which the structural requirements for bafilomycin's inhibitory effect on V-ATPases appear to be highly conserved.

#### *Comparison to results in the mammalian kidney*

In the mammalian kidney, acid secretion in the cortical and outer medullary collecting duct segments (CCD and OMCD, respectively) has also been shown to be driven in part, but not wholly, by V-ATPase activity (Khadouri et al., 1991; Armitage and Wingo, 1994; Wingo and Smolka, 1995; Tsuruoka and Schwartz, 1997). Immunological and biochemical evidence has demonstrated a vacuolar-ATPase in the intercalated cells of the CCD and OMCD (Brown et al., 1988; Kim et al., 1999). Furthermore, a vacuolar-ATPase has been shown to contribute to  $H^+$  secretion in intact tubule segments from the OMCD and to be maximally inhibited by  $5\text{ nmol l}^{-1}$  luminal bafilomycin (Armitage and Wingo, 1994; Tsuruoka and Schwartz, 1997). Extensive evidence also indicates that at least two  $H^+/K^+$ -ATPase isoforms are present in the CCD and OMCD, that these contribute to acid secretion, and that their activity is unaffected by nanomolar concentrations of bafilomycin (Wingo et al., 1990; Armitage and Wingo, 1994; Zhou and Wingo, 1994; Tsuruoka and Schwartz, 1997; Caviston et al., 1999; DuBose et al., 1999; Jaisser and Beggah, 1999). The activity of  $H^+/K^+$ -ATPase in the renal tubule is however inhibited by Sch-28080, with apparently full inhibition reached at  $10\text{ }\mu\text{mol l}^{-1}$  (Armitage and Wingo, 1994; Tsuruoka and Schwartz, 1997). Furthermore, in intact CCD and OMCD tubule segments, it has been reported that  $H^+$  secretion attributable to a V-ATPase and to an  $H^+/K^+$ -ATPase or ATPases together account for all acid secreted (Armitage and Wingo, 1994; Tsuruoka and Schwartz, 1997). The portion attributable to bafilomycin-sensitive  $H^+$  transport has been reported variously to be 30–65% of the total in the OMCD with the remainder being inhibitable by Sch-28080 (Armitage and Wingo, 1994; Tsuruoka and Schwartz, 1997). Thus, our findings indicate that the turtle bladder resembles the CCD and OMCD of the mammalian tubule in that all three tissues utilize two distinct active transport mechanisms to secrete acid, one of which is very likely driven by a vacuolar  $H^+$ -ATPase. In the present experiments we find that bafilomycin-sensitive  $H^+$  secretion

amounts to approximately 70% of the baseline acidification current.

#### *Bafilomycin-resistant $H^+$ secretion in turtle bladder*

The question arises as to what the remaining 30%, the bafilomycin-resistant component, is in turtle bladders. In principle it could be due either to residual  $H^+$  transport by a V-ATPase or it could arise from a different transport process. However, such a bafilomycin-resistant 'residual' is not a characteristic of V-type  $H^+$ -ATPases in other systems (Bowman et al., 1988; Moriyama and Nelson, 1989; Moriyama and Futai, 1990; Sundquist et al., 1990; Mattsson et al., 1991; Mattsson et al., 1993; Nanda et al., 1992; Armitage and Wingo, 1994; Crider et al., 1994; Keeling et al., 1997). It seems more likely then that the bafilomycin-resistant component seen in the turtle bladder represents a different transport process.

Work with isolated membranes also suggests this. Cell membranes isolated from the epithelium of the turtle bladder display ATP-dependent  $H^+$  transport, and we have shown elsewhere that part of the acid transport is highly sensitive to inhibition by vanadate ions, and is probably due to a P-type ATPase (Youmans and Barry, 1991a; Youmans and Barry, 1991b). The activity of this transporter was found to be eliminated when the donor animals were alkali-loaded, suggesting strongly that it is involved in or linked to urinary acidification (Youmans and Barry, 1991b). Furthermore, in a sodium-free medium, the vanadate-sensitive transport of  $H^+$  was found to depend absolutely on the presence of potassium and valinomycin. These findings, taken together with the direction of  $H^+$  transport, suggested that the P-type ATPase was in fact a  $K^+/H^+$  exchange ATPase (Youmans and Barry, 1991a).

Results obtained with isolated membranes thus raise the possibility that the bafilomycin  $A_1$ -resistant portion of acid secretion seen presently with intact tissues could be due to an  $H^+/K^+$ -exchange ATPase, as is the case in the renal tubule. Hence, we chose to test the susceptibility of this transport to an inhibitor of the gastric isoform of  $H^+/K^+$ -ATPase, Sch-28080 (Scott et al., 1987; Wallmark et al., 1987; Briving et al., 1988). We found that the residual  $H^+$  secretion remaining in the presence of  $5\text{ nmol l}^{-1}$  bafilomycin  $A_1$  was completely inhibited by Sch-28080 at a concentration of  $30\text{ }\mu\text{mol l}^{-1}$  (Fig. 3 and Table 1). In gastric preparations, the  $IC_{50}$  for Sch-28080 ranges from 0.1 to  $3.0\text{ }\mu\text{mol l}^{-1}$  and the full inhibitory concentration from 3.0 to  $30\text{ }\mu\text{mol l}^{-1}$ , depending on the preparation, pH and ambient  $K^+$  concentration (Scott et al., 1987; Wallmark et al., 1987; Briving et al., 1988). Sch-28080 at  $30\text{ }\mu\text{mol l}^{-1}$  thus is sufficient to maximally inhibit the gastric  $H^+/K^+$ -ATPase under a variety of experimental conditions. On the other hand, it is known that Sch-28080 does not inhibit vacuolar  $H^+$ -ATPases, including that of the renal collecting duct, at concentrations up to at least  $50\text{--}100\text{ }\mu\text{mol l}^{-1}$  (Cheval et al., 1991; Sabolic et al., 1994; Wingo and Smolka, 1995). Hence, the effect that we see at the lower concentration,  $30\text{ }\mu\text{mol l}^{-1}$ , is consistent with essentially full inhibition of an  $H^+/K^+$ -ATPase. When the sequence of inhibitor addition was

reversed (i.e. Sch-28080 given first), there was no change in either the absolute or percentage inhibition caused by either inhibitor and the two together again reduced the acidification current to zero (Table 1, Table 2). This is consistent with the two inhibitors acting independently on separate transport processes. We must point out that our findings with Sch-28080, while consistent with, do not in themselves establish the presence, of an H<sup>+</sup>/K<sup>+</sup>-ATPase in the intact turtle bladder. They do, however, seem to argue strongly against a vacuolar H<sup>+</sup>-ATPase being the source of bafilomycin-resistant acid secretion.

In summary, this is the first study to show: (i) that acid secretion by intact turtle bladders is highly sensitive to bafilomycin A<sub>1</sub>, at concentrations specific for vacuolar H<sup>+</sup>-ATPases in other systems; (ii) that a portion of the acid transport is unaffected by these low concentrations of bafilomycin; and (iii) that the bafilomycin A<sub>1</sub>-resistant portion is eliminated by Sch-28080 at a concentration similar to those which inhibit the gastric H<sup>+</sup>/K<sup>+</sup>-ATPase. Our results are consistent with the presence of two distinct acid-secretory processes in the intact turtle urinary bladder, one of which appears to be due to a 'typical' vacuolar H<sup>+</sup>-ATPase. The nature of the second process, its presence previously unrecognized in this tissue, awaits further definition.

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