IN VIVO BAFILOMYCIN-SENSITIVE Na$^+$ UPTAKE IN YOUNG FRESHWATER FISH

J. C. FENWICK[1,*], S. E. WENDELAAR BONGA[2] AND G. FLIK[2]

[1]*Department of Biology, University of Ottawa, PO Box 450 STN A, Ottawa, Ontario, Canada K1N 6N5* and
[2]*Department of Animal Physiology, Faculty of Science, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands*

*e-mail: james@science.uottawa.ca

Summary

In vivo treatment with external bafilomycin A$_1$, a selective inhibitor of V-ATPase H$^+$ pumps, reduced whole-body Na$^+$ influx by up to 90 % in young tilapia and 70 % in young carp. The inhibition was rapidly reversible, with whole-body Na$^+$ influx rebounding to 280 % of pre-treatment values within 20 min of removal from the bafilomycin. This rebound effect is consistent with the prior accumulation of protons during the period when the cells were exposed to bafilomycin. Bafilomycin also inhibited Cl$^-$ uptake, an effect that was still apparent 30 min after the removal of bafilomycin. These data provide circumstantial evidence for previous suggestions that Na$^+$ uptake in freshwater fish is associated with a proton-motive force created by a proton pump and indirect evidence for the major significance of this mechanism in the branchial uptake of Na$^+$ by freshwater fish.

Key words: H$^+$-ATPase, V-type ATPase, proton pump, Na$^+$, Cl$^-$, teleost, bafilomycin A$_1$, gill, tilapia, *Oreochromis mossambicus*, carp, *Cyprinus carpio*.

Introduction

Freshwater teleosts absorb Na$^+$ from the ambient medium against steep concentration gradients (Maetz and Garcia-Romeu, 1964; for a review, see Perry, 1997). This uptake was originally attributed to an electroneutral Na$^+$/H$^+$ (or NH$_4^+$) antiporter (Krogh, 1938) located in the apical membrane of the branchial epithelium (Wright and Wood, 1985; Shuttleworth, 1989; for a review, see Marshall, 1995). But several substantive observations led to this paradigm being abandoned. Wilson et al. (1994) were unable to locate an apical Na$^+$/NH$_4^+$ transporter in rainbow trout. Avella and Bornancin (1989) reported that NH$_3$ and Na$^+$ transport were not coupled and that neither the Na$^+$ electrochemical gradient nor the intracellular pH is consistent with the necessary drive for a Na$^+$/H$^+$ exchange. Further, Heisler (1990) indicated that the vast majority of ammonia excretion was by non-ionic diffusion, and Wilson et al. (1994) concluded that NH$_3$ diffusion could be responsible for all NH$_3$ excretion. In addition, the K_m of the Na$^+$/K$^+$ exchange in cultured freshwater rainbow trout gills is 8.3 mmol l^{-1} (Pärt and Wood, 1996) whereas the K_m for Na$^+$ uptake *in vivo* in the same species is approximately 0.1–0.3 mmol l^{-1} (Goss and Wood, 1991). Given this large discrepancy, it is highly unlikely that Na$^+$ uptake from, and H$^+$ excretion into, fresh water occurs *via* a Na$^+$/H$^+$ exchange mechanism, given that the Na$^+$ concentration in fresh water is normally below 1 mmol l^{-1} (Lin and Randall, 1993). As a result of such studies, the concept of an important role for Na$^+$/NH$_4^+$ exchange has been rejected. A new model proposes that branchial epithelial Na$^+$ absorption occurs passively *via* apical membrane Na$^+$-conductive channels, the uptake being driven by a proton-motive force generated by the active electrogenic extrusion of protons (H$^+$) through a V-type ATPase located in the apical branchial epithelium (Lin and Randall, 1991, 1993, 1995b; Lin et al., 1994; Laurent et al., 1994; Potts, 1994; Sullivan et al., 1995, 1996).

There is compelling evidence for this model. Na$^+$ uptake (for a review, see Perry, 1997) and H$^+$ secretion (Lin and Randall, 1991) occur at the gills, and their rates show a 1:1 stoichiometry (Wright and Wood, 1985; Pärt and Wood, 1996). In trout gills, a V-type ATPase (H$^+$-ATPase) has been demonstrated both biochemically (Lin and Randall, 1993) and immunocytochemically (Sullivan et al., 1995); moreover, mRNA for an H$^+$-ATPase has been demonstrated by *in situ* hybridisation (Sullivan et al., 1996). Although the exact location (i.e. gill cell type) of this H$^+$-ATPase remains the centre of an interesting polemic, this subject is not germane to the present paper. However, there are two very important gaps in our knowledge concerning this recent model. First, there is absolutely no direct evidence in the literature for an apical Na$^+$ conductance channel in the fish gill. And second, although there is ample evidence for the existence of H$^+$-ATPase-driven uptake of Na$^+$ through what must be separate Na$^+$ conductance uniports (Harvey, 1992; Zare and Greenaway, 1998), there is no indication of the relative importance of this mechanism in Na$^+$ uptake in fish. Thus our hypothesis is that a significant

proportion of Na^+ uptake is coupled to proton movements *via* V-ATPase. To test this hypothesis, we tested the prediction that inhibition of the V-ATPase *in vivo* would significantly reduce the overall rate of Na^+ uptake.

Bafilomycin is a naturally occurring macrolide antibiotic that is a very specific inhibitor of vacuolar proton-translocating ATPases (H^+- or V-type ATPase) and is effective *in vitro* at nanomolar concentrations (Bowman et al., 1988). Bafilomycin can be used *in vivo* to inhibit V-ATPase without killing cells (Yoshimori et al., 1991) and it does not appear to have any major effect on either F-type or typical P-type ATPases (Hanada et al., 1990). Because the ATPase studied here is purported to be located in the plasma membrane, it could be defined as a P-type ATPase, but it should be thought of as a V-type ATPase located in the plasma membrane.

An *in vivo* system was chosen for these studies because we were interested primarily in measuring the importance of the epithelial V-ATPase in Na^+ influx. Further, because Lin and Randall (1993) showed that the I_{50} of gill V-ATPase to bafilomycin in crude gill extracts was within the range $10–100 \mu mol \, l^{-1}$, because our dose–response study (see Fig. 1) indicated a K_i of $1.6 \times 10^{-7} \, mol \, l^{-1}$, and because G. Flik (unpublished results) has shown that a bafilomycin concentration of $10 \mu mol \, l^{-1}$ did not affect Na^+/K^+-ATPase, K^+-ATPase, Ca^{2+}-ATPase or Na^+/K^+ exchange in isolated membrane preparations, a bafilomycin concentration of $10^{-5} \, mol \, l^{-1}$ was used. This higher dose, relative to the K_i, was chosen because Bowman et al. (1988) reported that the I_{50} values for bafilomycin were strictly related to the total protein present in their incubation system, and we had no way of predicting what the effective equivalent protein concentration would be in our animals. Although this higher dose of bafilomycin increased the likelihood that internal V-ATPase would also be inhibited, such an occurrence would not materially affect our interpretations. Further, as we planned to use the same bafilomycin solution a number of times, we wanted to ensure that the concentration of bafilomycin would not be reduced below the effective concentration during sequential uses. Finally, we measured the effect of an NH_4Cl prepulse to determine whether we could mimic the proposed effect of bafilomycin on pHi. This test was chosen because Boron and De Weer (1976) showed in the perfused trout head preparation that addition of NH_3 to the external medium resulted in rapid intracellular alkalization as a result of the influx of NH_3 which would rapidly soak up protons. These same authors also suggested that the subsequent removal of the external ammonium would result in a rapid efflux of NH_3, resulting in an intracellular acidification.

Materials and methods
Animals

Young Mozambique tilapia, *Oreochromis mossambicus*, approximately 9 days old (out of mouth) and weighing 32–64 mg, were taken from laboratory stock reared in Nijmegen tap water ($[Na^+]$, $3.8 \, mmol \, l^{-1}$; $[Cl^-]$, $4.56 \, mmol \, l^{-1}$;

$[Ca^{2+}]$, $0.7 \, mmol \, l^{-1}$; $26 °C$; pH 7.4) under a 12 h:12 h light:dark photoperiod. Carp, *Cyprinus carpio*, obtained through *in vitro* fertilisation, were raised for 45 days (Stouthart et al., 1994) in artificial fresh water $[([Na^+], 0.4 \, mmol \, l^{-1}$; $[Cl^-]$, $3.1 \, mmol \, l^{-1}$; $[Ca^{2+}]$, $0.8 \, mmol \, l^{-1}$; $26 °C$; pH 7.4) under a 12 h:12 h light:dark photoperiod. At the time of the experiments, they weighed between 37 and 74 mg.

Fish were removed from their holding tank with a small-mesh net and immediately placed in a beaker filled with the same water. From this point on, the fish were always transferred in water. This was accomplished by cutting the end off a 5 ml pipette tip and then catching the fish in the pipette by sucking them up with a volume of water appropriate to the size of the fish. Most commonly, a 1 ml setting was used. The fish were transferred to individual wells of a 24-well (four rows of six wells each) cell culture tray (InterMed, Kamstrup, Denmark). All the initial water transferred with the fish was removed, and 1.6 ml of fresh aquarium water was added. This, and all subsequent water replacements described, was accomplished quickly so that the fish was out of water for less than 0.5 s. Fish were allowed to adjust to these conditions for 30 min prior to further experimental manipulation.

Ion influxes

The influxes of Na^+ and Cl^- were determined on young tilapia and carp as follows.

At the start of each influx measurement, a 20 μl sample of the original aquarium water labelled with sufficient ^{22}Na or ^{36}Cl to produce a radioactive concentration of approximately $1 \, kBq \, ml^{-1}$ was added to the wells. To obtain an accurate measure of the ions taken up through the body surface, tracer that was bound to the surface of the animals or retained within the buccal and branchial cavities had to be eliminated from the count. To achieve this, the fish underwent a rigorous rinsing treatment. First, the 2 ml of radioactive medium were removed and replaced with 1 ml of a rinsing solution containing an excess of the ion being measured to facilitate the displacement of surface-bound tracer. This was repeated twice more at 30 s intervals. The wells then had 2 ml of the same rinsing solution added, and each fish was observed to ensure that it was ventilating so that the buccal and branchial cavities were cleared of tracer. After this, the rinsing solution was removed, and the fish was picked up with curved forceps and killed by spinal section. The animals were blotted dry on moist tissue paper and then weighed to the nearest milligram. By testing this rinsing technique on fish held in the labelled medium for 1 min (too short a time for any significant influx), we determined that the rinsing technique was more than 99 % efficient. After weighing, the fish were placed individually into 6 ml plastic scintillation vials; 0.1 ml of water was added together with 0.7 ml of NCS-II Tissue Solubilizer (Amersham). The vials were capped and left at room temperature overnight. When the fish had fully dissolved, glacial acetic acid ($30 \mu l \, ml^{-1}$ of NCS) was added to neutralise the digest. The vial was shaken, allowed to cool, and then 4 ml of scintillant (OptiPhase 'HiSafe', Wallac, UK) was added to each vial,

which was subsequently counted in a scintillation counter (Wallac). Because of the homogeneity of the samples, disints min⁻¹ corrections were not necessary and only counts min⁻¹ were recorded. Fish were also tested to determine whether they were drinking water by immersing them for 30 min in aquarium water labelled with [^{14}C]polyethylene glycol (PEG) (5 kBq ml⁻¹).

Ion influx J was calculated using the formula:

$$J_{in}^{ion} = \frac{C_{WB}}{S_E \times M \times t} ,$$

where C_{WB} is whole-body counts min⁻¹ measured from the whole digested fish, S_E is the mean external specific activity of the water throughout the flux period, M is the mass of the fish (mg), and t is flux duration (h). The rates of influx of the ions were measured independently and for only 10–30 min to ensure that backflux was minimised.

Bafilomycin experiments

Effect of bafilomycin concentration on Na⁺ influx

Because of the prohibitive cost of bafilomycin, six young tilapia were consecutively tested in the same 1.6 ml of 10⁻⁵ mol l⁻¹ ^{22}Na-labelled bafilomycin solution. This was accomplished by placing one fish each in 1.6 ml of aquarium water in six different wells. The water in one well was then replaced with 1.6 ml of the ^{22}Na-labelled bafilomycin solution. After 20 min, this same ^{22}Na-labelled bafilomycin solution was used to replace the water in a second well. This was continued until all six fish had been treated. The solution was then diluted to reduce the bafilomycin concentration to 10⁻⁶ mol l⁻¹, additional ^{22}Na was added to bring the radioactivity back to the original activity of 1 kBq ml⁻¹, and the experiment was repeated. This process was repeated twice more to provide values for bafilomycin concentrations of 10⁻⁷ mol l⁻¹ and 10⁻⁸ mol l⁻¹. A zero bafilomycin group was also tested. The effects of the different concentrations of bafilomycin were then expressed as a percentage inhibition of the Na⁺ uptake measured in the control fish. The water in the wells was not aerated because we had previously determined that the fish could easily survive for more than 48 h in the wells used.

Effect of bafilomycin on Na⁺ influx

One fish, together with 1.6 ml of aquarium water, was placed in each well of a 24-well cell culture tray (four rows of six wells each), and the fish were left to recover from the effects of the transfer for 1 h. Na⁺ uptake was measured over 20 min in normal aquarium water, in aquarium water containing 0.05 % dimethylsulphoxide (DMSO) and during exposure to 10⁻⁵ mol l⁻¹ bafilomycin and for 20 min following 20 min of exposure to 10⁻⁵ mol l⁻¹ bafilomycin. The same 1.6 ml of 10⁻⁵ mol l⁻¹ bafilomycin was used for every fish (N=6). Each experimental group was manipulated in the same way with regard to transfer of fluids. That is, the experiment was started by replacing the water in the first well of a row with an equivalent volume of the appropriate test solution. For

example, for the DMSO-treated group, the aquarium water was replaced with aquarium water containing the same concentration of DMSO as in the bafilomycin solution. This well was then spiked with ^{22}Na, and the fish was left in this for 20 min. After this time, the water in the second well was replaced with the ^{22}Na-labelled DMSO-spiked water from the first well. The fish remaining in the first well was then treated in the manner described above. Each of the next four fish in the same row was treated in the same fashion until all six fish in a row had been tested. For the untreated group, the normal, ^{22}Na-spiked aquarium water from the first well was passed along the row. The bafilomycin solution was used to test the post-bafilomycin effects before it was used to test the immediate effects of bafilomycin exposure, to avoid the addition of ^{22}Na to the bafilomycin solution until absolutely necessary. For the post-bafilomycin group, the water in the first well of the row was replaced with aquarium water containing 10⁻⁵ mol l⁻¹ bafilomycin. The fish was left in this solution for 20 min. After 20 min, the water in the second well in the row was removed, and the bafilomycin-containing water from the first well was moved into the second well. The first well was then filled with ^{22}Na-spiked aquarium water, and Na⁺ flux was measured over the next 20 min as described above. Each fish in the row was treated identically. Finally, for the bafilomycin-treated group, the water in the first cell in the appropriate row was replaced with the water containing 10⁻⁵ mol l⁻¹ bafilomycin from the last well in the post-bafilomycin row. ^{22}Na was immediately added, and the fish was left for 20 min. This water in the first well was then passed along the row as just described. After the 20 min exposure to ^{22}Na, the fish were treated in the fashion described above to measure Na⁺ influx. In other words, the same 1.6 ml volume of bafilomycin-containing aquarium water was used for a total of 12 fish, and the same bafilomycin-plus ^{22}Na-spiked water was used six times. Because of the sequential sampling of the water from each well (10 μl) to measure the specific activity of the water, the volume of the water decreased by 10 μl with each transfer, but this was not considered to be of major significance because the procedure did not influence the specific activity of the ^{22}Na. In all experiments, the radioactive concentration of the initial sample was sufficiently high to prevent a significant decline during its five subsequent uses. For the carp experiment, the post-bafilomycin-exposed group was omitted.

Effect of bafilomycin on Cl⁻ influx

This experiment was performed in an identical manner to that described for Na⁺ with the exceptions that ^{36}Cl rather than ^{22}Na was used and that an additional group was included. That is, ^{36}Cl influx was also measured during the first 10 min following exposure to bafilomycin.

Effect of a NH₄Cl prepulse on Na⁺ and Cl⁻ uptake

This technique was developed by Boron and De Weer (1976), and its rationale for use is clearly described by Pärt and Wood (1996). In essence, NH₃ in the external medium enters cells rapidly where it takes up protons, forming NH₄⁺ and

decreasing $[H^+]_i$. As the availability of protons decreases, so should the activity of any proton pumps. And if the uptake of Na^+ is linked to proton pumping, then there should be a concomitant decrease in the rate of Na^+ uptake when the availability of H^+ is reduced. Conversely, when the NH_3 is removed from the external medium, intracellular NH_3 should leave the cell rapidly and the remaining NH_4^+ should dissociate, producing an excess of intracellular protons. If this occurs, there will a surfeit of protons available to any proton pump, which should become more active. Again, if proton pumping is associated with Na^+ uptake, the rate of Na^+ uptake should be increased following an NH_3 prepulse. Unfortunately, it is not yet possible to measure the intracellular pH of the gill cells under the experimental conditions (*in vivo*). However, Avella and Bornancin (1989) exposed the trout perfused head preparation to $20\,mmol\,l^{-1}$ NH_4Cl and found a significant alkalization of the efferent arterial fluid within 30 s; they concluded that a similar alkalization of the intracellular compartment probably also occurred. They also deduced that removal of the NH_3 would result in intracellular acidosis and stimulation of Na^+ absorption. These same data, however, suggest that exposure to NH_4Cl might effect changes in blood pH, and this could affect branchial transport mechanisms.

In these experiments, fish were exposed to $25\,mmol\,l^{-1}$ NH_4Cl for 10 min, following which the NH_4Cl-containing water was replaced with normal aquarium water. The NH_4Cl-containing aquarium water contained $6\,mmol\,l^{-1}$ Hepes and was titrated to pH 7.6 using a $10\,mmol\,l^{-1}$ Tris solution. The pH of 7.6 was chosen to ensure an adequate concentration of NH_3. Rates of Na^+ and Cl^- uptake were measured as described during the exposure to NH_4Cl and for the first, second and third 10 min intervals following removal of the NH_4Cl. As a control, rates of Na^+ and Cl^- uptake were also measured in fish handled in the same way, but the NH_4Cl exposure was excluded. $N=8$ in all cases.

Chemicals

Bafilomycin A_1 from *Streptomyces griseus* was purchased from Sigma Chemical Co. (Cat. B-8261) and was dissolved in DMSO and then diluted to the required concentration with normal aquarium water. The concentration of bafilomycin at the various nominal concentrations prepared was measured using an extinction coefficient (in methanol) of 25×10^3 at 245 nm (Werner et al., 1984). The concentration of DMSO in the experimental solutions was always below 0.1 %, and DMSO was consistently incorporated into control solutions. DMSO alone at this concentration had no effect on any of the variables tested. Sixteen fish were observed for up to 6 h following a 20 min immersion in $10^{-5}\,mol\,l^{-1}$ bafilomycin. None of these fish died or showed any overt signs of distress.

Statistical analyses

Data sets were subjected to one-way repeated-measures analyses of variance (ANOVAs) followed by Fisher's least significant difference (LSD) multiple means comparison test. In all cases, a fiducial limit of $P<0.01$ was set.

Results

Fig. 1 shows that the inhibitory effect of bafilomycin on the rate of Na^+ uptake in young tilapia is dose-dependent with a K_i of $1.6\times10^{-7}\,mol\,l^{-1}$. The inhibitory effect (20 %) was seen at a concentration as low as $10^{-8}\,mol\,l^{-1}$ and increased linearly up to a concentration of $10^{-6}\,mol\,l^{-1}$, after which it plateaued at approximately 90 % inhibition.

During exposure to bafilomycin A_1 ($10^{-5}\,mol\,l^{-1}$), whole-body Na^+ influx was inhibited by 90 % (Fig. 2). Fig. 2 also shows that the effect was quickly reversible in that, during the first 20 min after the bafilomycin was removed (Post-Baf A_1 in Fig. 2), the measured whole-body Na^+ influx increased to a value 282 % greater than in the untreated group.

To assess whether the effect of bafilomycin on Na^+ influx is pHi-mediated by inhibiting a H^+ pump, the effects of NH_4Cl and of a NH_4Cl prepulse ($25\,mmol\,l^{-1}$ NH_4Cl) on Na^+ influx were studied *in vivo*. The results of these experiments are summarised in Fig. 3. Whole-animal Na^+ influx was significantly reduced during a 10 min exposure to NH_4Cl. When the NH_4Cl was removed following this 10 min NH_4Cl prepulse, the measured rate of Na^+ influx during the first 10 min (Post 0–10) was significantly higher than in the control group. The rate of the Na^+ influx measured during the second 10 min following the NH_4Cl prepulse (Post 10–20) was even higher than during the first 10 min. This indicates that the rate of Na^+ influx increased rapidly following removal of the NH_4Cl and continued to increase after 10 min. Na^+ influx during the third 10 min following the prepulse (Post 20–30) was not significantly different from the control. Since the control values measured during each treatment did not differ from each other, only the initial control values are shown for the sake of clarity.

Whole-body Cl^- influx was inhibited by 35 % during exposure to bafilomycin A_1 (Fig. 4), and the inhibition was even greater
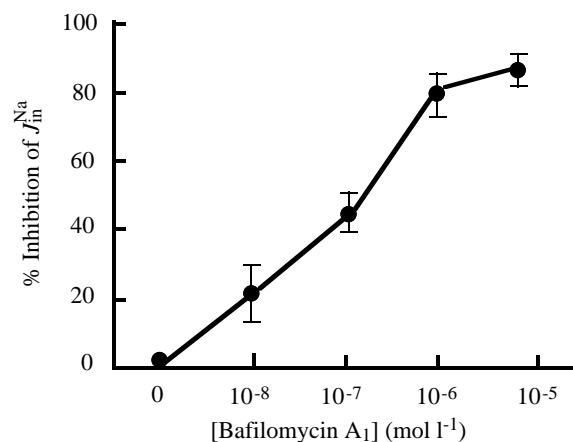

Fig. 1. *In vivo* dose–response effects of bafilomycin A_1 on relative net Na^+ uptake J_{in}^{Na} in young freshwater tilapia *Oreochromis mossambicus*. Na^+ influx was measured during a 30 min exposure to various concentrations of bafilomycin A_1. Values are means ± S.E.M., $N=6$ in all cases. The middle three values were used to construct a Hill plot, which yielded a K_i of $1.6\times10^{-7}\,mol\,l^{-1}$.
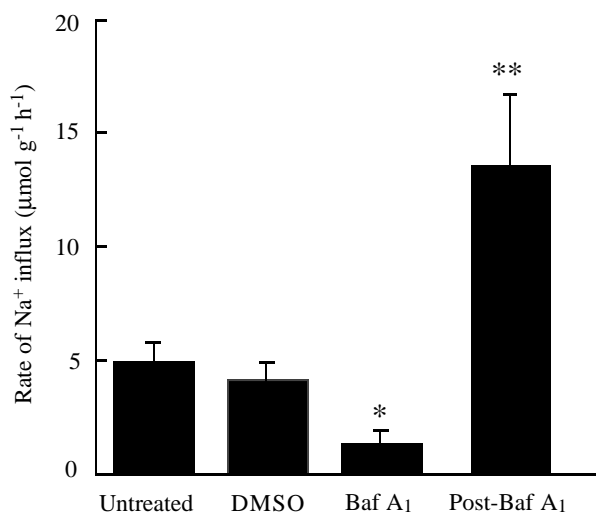
Fig. 2. *In vivo* effects of 10^{-5} mol l^{-1} bafilomycin A$_1$ on the rate of Na$^+$ influx in young freshwater tilapia *Oreochromis mossambicus*. Na$^+$ influx was measured during treatment with 0.05 % DMSO, during a 30 min exposure to 10 μmol l^{-1} bafilomycin A$_1$ (Baf A$_1$) and for 20 min following its removal (Post-Baf A$_1$). Values are means + S.E.M., *N*=6 in all cases. Asterisks indicate that the value is significantly lower (*) or higher (**) (*P*<0.01) than all other values.

during the first 10 and first 30 min after the bafilomycin was removed. The DMSO-containing control medium did not affect the rate of Cl$^-$ influx over the same time period.
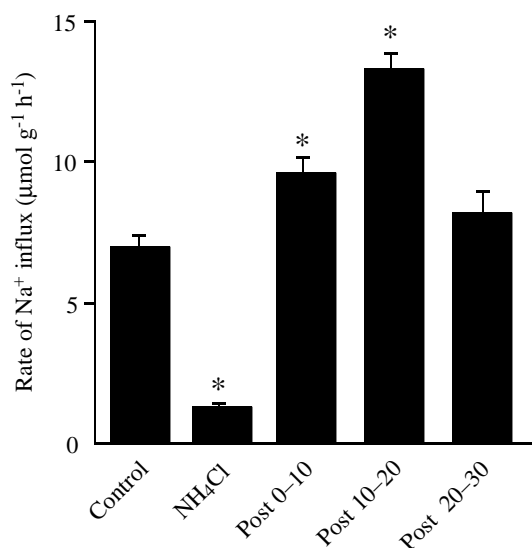

Fig. 3. *In vivo* Na$^+$ influx in young freshwater tilapia *Oreochromis mossambicus* during and after exposure to water at pH 7.6 containing 25 mmol l^{-1} NH$_4$Cl. Na$^+$ influx was measured during a 10 min NH$_4$Cl prepulse (NH$_4$Cl) and during the first (Post 0–10), second (Post 10–20) and third (Post 20–30) 10 min periods following removal of the NH$_4$Cl. Only the initial control value is shown because the control values measured during the various intervals did not differ from this. Values are means + s.e.m., *N*=8 in all cases. An asterisk indicates that the value is significantly different (*P*<0.01) from the control.
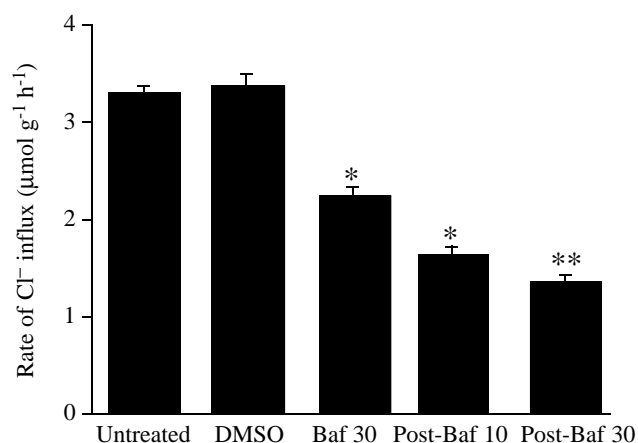

Fig. 4. *In vivo* effects of bafilomycin A$_1$ on the rate of Cl$^-$ influx in young freshwater tilapia *Oreochromis mossambicus*. Cl$^-$ influx was measured during treatment with 0.05 % DMSO, during a 30 min exposure to 10 μmol l^{-1} bafilomycin A$_1$ (Baf 30) and for 10 min (Post-Baf 10) and 30 min (Post-Baf 30) following its removal. Values are means + S.E.M., *N*=6 in all cases. An asterisk indicates that the value is significantly (*P*<0.01) lower than all values to its left. A double asterisk indicates that the value is significantly (*P*<0.01) lower than the first three columns only.

The immediate effect of NH$_4$Cl and the effect of a NH$_4$Cl prepulse (25 mmol l^{-1} NH$_4$Cl) on whole-animal Cl$^-$ influx were also measured *in vivo*. The results of these experiments are summarised in Fig. 5. Whole-animal Cl$^-$ influx was significantly increased during a 10 min exposure to NH$_4$Cl. During the first 10 min after the NH$_4$Cl had been removed, the measured Cl$^-$ influx remained higher than that of the controls. The value of the Cl$^-$ influx measured during the second 10 min following the NH$_4$Cl prepulse was lower than the Cl$^-$ influx measured during the first 10 min, but remained higher than that of the controls. These data indicate that Cl$^-$ influx increased rapidly during exposure to NH$_4$Cl and remained high during the first 20 min interval following removal of the NH$_4$Cl. Cl$^-$ influx during the third 10 min following the prepulse was not significantly different from the control. Since the control values measured during each treatment did not differ from each other, only the initial control values are shown for the sake of clarity.

During exposure to bafilomycin A$_1$ (10 μmol l^{-1}), whole-body Na$^+$ influx in young carp was inhibited by 70 % (Fig. 6). The DMSO-containing control medium did not affect the rate of Na$^+$ influx over the same time period.

Discussion

This study measured the effect of bafilomycin on fish *in vivo*, and the data clearly show that at least one of its effects is to reduce substantially the rate of Na$^+$ uptake from the environment. We contend that this effect is mediated *via* the inhibition of an H$^+$-ATPase. In the normal situation, this proton pump translocates protons vectorially from within the
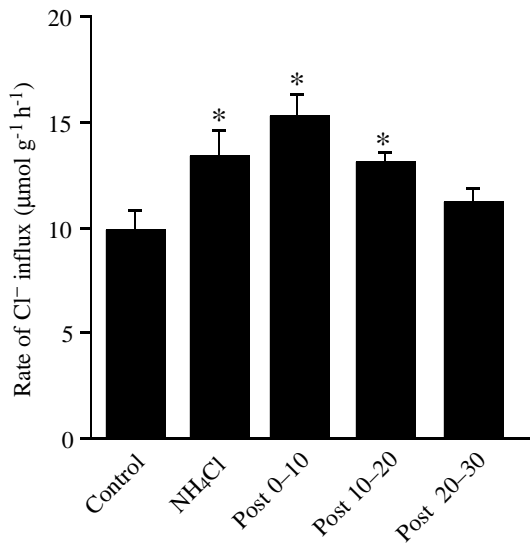
Fig. 5. *In vivo* Cl$^-$ influx in young freshwater tilapia *Oreochromis mossambicus* during and after exposure to water (pH 7.6) containing 25 mmol l^{-1} NH$_4$Cl. Cl$^-$ influx was measured during a 10 min NH$_4$Cl prepulse (NH$_4$Cl) and during the first (Post 0–10), second (Post 10–20) and third (Post 20–30) 10 min periods following removal of the NH$_4$Cl. Only the initial control value is shown because the control values measured during the various intervals did not differ from this. Values are means + s.e.m., N=8 in all cases. An asterisk indicates that the value is significantly different ($P<0.01$) from the control.

intracellular space of branchial epithelial cells into the external medium. As a result of this process, the intracellular concentration of protons decreases, providing a more favourable inwardly directed electrochemical gradient for the passive inward movement of Na$^+$ across the apical membrane. This gradient is further maintained by the removal of Na$^+$ from the intracellular compartment by the activity of a Na$^+$/K$^+$-ATPase located on the basolateral membrane. In bafilomycin-treated fish, intracellular protons accumulate and, therefore, Na$^+$ transport is inhibited.

It is clear from the data presented here that Na$^+$ uptake is very sensitive to bafilomycin. The K_i of bafilomycin on Na$^+$ uptake in the present study was 0.16 μmol l^{-1} (Fig. 1), and this is within the range of the concentrations (0.1–1.0 μmol l^{-1}) required to inhibit the acidification of lysosomes in cultured cells (Yoshimori et al., 1991). However, Pärt and Wood (1996) reported that bafilomycin had no effect on the intracellular pH of primary cultured gill pavement cells. This discrepancy might be interpreted as evidence that the V-type ATPase on the fish gill epithelium is present on the mitochondrial-rich cells rather than the pavement cells, as suggested by Sullivan et al. (1995). Lin and Randall (1993) found that total gill ATPase activity was significantly inhibited only when the bafilomycin concentration exceeded 25 μmol l^{-1}. But their study was performed *in vitro* using ATPase activity in crude gill homogenates rather than Na$^+$ uptake *in vivo* as in the present study.
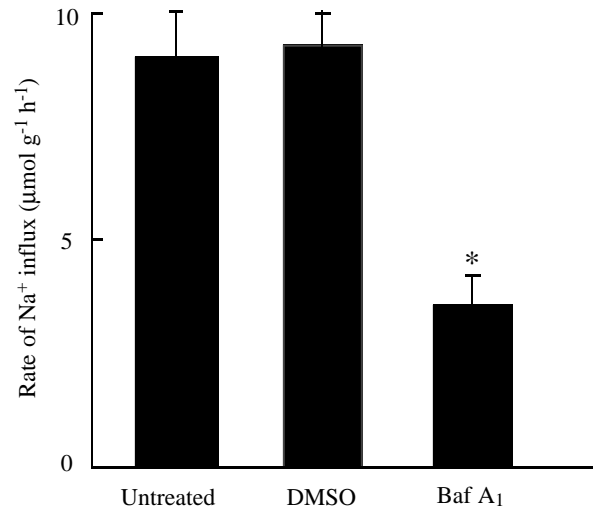

Fig. 6. *In vivo* effects of 10^{-5} mol l^{-1} bafilomycin A$_1$ on Na$^+$ influx in 45-day-old freshwater carp *Cyprinus carpio*. The rate of Na$^+$ influx was measured during treatment with 0.05 % DMSO and during a 30 min exposure to 10 μmol l^{-1} bafilomycin A$_1$ (Baf A$_1$). Values are means + s.e.m., N=6 in all cases. An asterisk indicates that the value is significantly lower ($P<0.01$) than both other values.

The mean rate of Na$^+$ uptake was reduced by approximately 80 % in young tilapia (Fig. 2) and by 70 % in young carp (Fig. 6) during a 30 min exposure to bafilomycin. In the tilapia, the mean rate of Na$^+$ uptake during the first 20 min after the bafilomycin was removed was increased almost 10-fold compared with the inhibited rate. All these observations are consistent with the hypothesis that a substantial proportion of Na$^+$ uptake is driven by a proton-motive force that favours Na$^+$ uptake through associated Na$^+$-conductive channels. Unfortunately, there are no published data that unambiguously establish the presence or localisation of Na$^+$-conductive channels in fish gills. But such channels, all sensitive to amiloride, are common in a wide variety of tight epithelia, including frog skin (Van Driessche and Lindemann, 1979), chicken copropodum (Christensen and Bindslev, 1982) and rabbit urinary bladder (Lewis et al., 1984). And since Na$^+$ uptake by the teleost fish gill is also highly sensitive to amiloride (Perry and Randall, 1981; Wright and Wood, 1985), it is likely that this tight epithelium also possesses Na$^+$-conductive channels. Thus, in the presence of bafilomycin, the V-ATPase is inhibited and [H$^+$]$_i$ increases, reducing the driving force for Na$^+$ entry so that the rate of Na$^+$ entry declines. The effect of bafilomycin was readily reversible (Yoshimori et al., 1991): after the bafilomycin had been removed, there was a rapid reactivation of the V-ATPase and, because there was an accumulated excess of protons, possibly present as NH$_4$$^+$, the rate of proton secretion was increased, resulting in an electrical gradient that strongly favoured increased Na$^+$ entry.

An inhibitory effect of bafilomycin on a H$^+$ pump is also consistent with its inhibitory effect on Cl$^-$ uptake (Fig. 4). By inhibiting the H$^+$ pump during the exposure to bafilomycin,

there will be an accumulation of H$^+$ within the intracellular compartment which will tend to decrease pHi. To compensate for this decrease in pHi, the cell could reduce the rate of secretion of HCO$_3^-$ and, because HCO$_3^-$ secretion is linked to Cl$^-$ uptake (Laurent and Perry, 1995), the rate of uptake of Cl$^-$ would be reduced. This concept is supported by the fact that the inhibitory effect of bafilomycin on Cl$^-$ uptake lasts longer than that on Na$^+$ uptake following the removal of bafilomycin. That is, as soon as bafilomycin is removed and the proton pump is reactivated, the cell would begin to excrete protons and the rate of Na$^+$ uptake would increase. However, because there would be an excess of protons, it would take some time to lower the [H$^+$] and raise pH to the point where HCO$_3^-$ secretion, and hence Cl$^-$ uptake, would resume. In the present experiments, however, Cl$^-$ uptake was still depressed 30 min after the removal of bafilomycin, and this recovery rate is slow compared with that described by Pärt and Wood (1996) in acidified primary cultured gill epithelial cells. Perhaps this suggests that the acidification in the present study extended beyond the epithelium and into the blood. If this were the case, it would certainly explain the prolonged period of enhanced Na$^+$ and decreased Cl$^-$ uptake following the removal of bafilomycin.

The effects of NH$_4$Cl exposure and removal on both Na$^+$ and Cl$^-$ uptake are entirely consistent with the effects of bafilomycin if bafilomycin inhibits a proton-translocating system. During the time when the fish were exposed to NH$_4$Cl, when NH$_3$ would be entering the cell and associating with intracellular protons to lower the availability of free [H$^+$], Na$^+$ uptake was significantly reduced (Fig. 3). Conversely, during this period of elevated pHi, there would be a significant benefit attached to increased secretion of HCO$_3^-$ in order to lower intracellular pH and, because this HCO$_3^-$ secretion is linked to Cl$^-$ uptake, the latter would increase. And, indeed, this was observed (Fig. 5). Further, the increased Na$^+$ uptake seen following the removal of the external NH$_4$Cl (Fig. 3), when there would be an increase in [H$^+$]$_i$, is in complete agreement with what was observed following bafilomycin exposure. That is, the rate of Na$^+$ uptake increased and remained elevated for up to 20 min. Similar arguments can be made from the data in Fig. 3.

There are five areas of concern in the interpretation of these experiments: (1) that the data are also consistent with a hypothesis which states that Na$^+$ is taken up by a Na$^+$/NH$_4^+$ exchanger; (2) that bafilomycin is not sufficiently specific to draw any major conclusions concerning the importance of a V-type ATPase; (3) that bafilomycin may have important extraneous effects when used in whole animals; (4) that the inhibition of Na$^+$ uptake during NH$_4$Cl exposure may have resulted from the known blocking effect of NH$_4$ on Na$^+$-conductive channels. But it is unlikely that bafilomycin exerted its effect by inhibiting a Na$^+$/NH$_4^+$ exchange because proton and ammonia efflux occur *via* separate pathways and because the rate of proton excretion can be more than twice that of NH$_3$ (Lin and Randall, 1995a). (5) The rates of Na$^+$ and Cl$^-$ influx in this study (approximately 6 and 8 μmol g^{-1} h^{-1}, respectively)

are substantially higher than the monovalent ion flux of 0.002–1.4 μmol g^{-1} h^{-1} normally cited for freshwater fish (Mayer-Gostan et al., 1987). But this latter flux is for juvenile and adult fish. In the present study, very small young fish were used, and we could find no Na$^+$ or Cl$^-$ flux studies on comparable fish in the literature. However, because the Na$^+$ fluxes reported here for the young tilapia are similar to those for the young carp, we do have an added measure of confidence in the values. Further, given the high density of branchial chloride cells reported in tilapia larvae (Li et al., 1995), it seems quite reasonable that these young fish have relatively high rates of influx of monovalent ions.

As for the specificity of bafilomycin at the concentrations we used, there is the possibility of effects on other transport enzymes. But, because bafilomycin affected both Na$^+$ and Cl$^-$ transport, and because these are separate systems, it makes more sense that both were affected by an interruption of the normal function of a proton pump. Certainly, we cannot rule out extraneous effects caused by bafilomycin in the whole animals; however, the fish did survive exposure to bafilomycin and appeared to remain healthy. Finally, although NH$_4^+$ may have blocked the Na$^+$-conductive channels during exposure to the NH$_4$Cl, the fact that the rate of Na$^+$ uptake continued to increase for as long as 10 min after the removal of the NH$_4$Cl suggests that this increased uptake was the result of some longer-term homeostatic response to a change in intracellular composition, for example a reduced pH (i.e. increased [H$^+$]).

References

Avella, M. and Bornancin, M. (1989). A new analysis of ammonia and sodium transport through the gills of the freshwater rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.* **142**, 155–175.

Boron, W. F. and De Weer, P. (1976). Intracellular pH transients in squid giant axon caused by CO$_2$, NH$_3$ and metabolic inhibitors. *J. Gen. Physiol.* **142**, 155–175.

Bowman, F. J., Siebers, A. and Altendorf, K. (1988). Bafilomycins: A class of inhibitors of membrane ATPases from microorganisms, animal cells and plant cells. *Proc. Natl. Acad. Sci. USA* **85**, 7972–7976.

Christensen, D. and Bindslev, N. (1982). Fluctuation analysis of short-circuited current in a warm-blooded sodium-retaining epithelium: site current, density and interaction with triamterene. *J. Membr. Biol.* B **65**, 19–30.

Goss, G. G. and Wood, C. M. (1991). Two-substrate kinetic analysis: a novel approach linking ion and acid–base transport at the gills of freshwater trout, *Oncorhynchus mykiss*. *J. Comp. Physiol.* B **161**, 635–646.

Hanada, H., Moriyama, Y., Maede, M. and Futai, M. (1990). Kinetic studies of chromaffin granule H$^+$-ATPase and effects of bafilomycin A$_1$. *Biochem. Biophys. Res. Commun.* **170**, 873–878.

Harvey, B. J. (1992). Energization of sodium absorption by the H$^+$-ATPase pump in mitochondria-rich cells of frog skin. *J. Exp. Biol.* **172**, 289–309.

Heisler, N. (1990). Mechanisms of ammonia elimination in fishes. In *Animal Nutrition and Transport Processes*, vol. 2, *Transport, Respiration and Excretion: Comparative and Environmental Aspects* (ed. J. P. Truchot and B. Lahlou). *Comp. Physiol.* **6**, 137–151. Basel: Karger.

Krogh, A. (1938). The active absorption of ions in some freshwater animals. *Z. Vergl. Physiol.* **25**, 335–350.

Laurent, P., Goss, G. G. and Perry, S. F. (1994). Proton pumps in fish gill pavement cells? *Arch. Int. Physiol. Biochem. Biophys.* **102**, 77–79.

Laurent, P. and Perry, S. F. (1995). Morphological basis of acid–base and ionic regulation in fish. *Adv. Comp. Env. Physiol.* **22**, 91–118.

Lewis, S. A., Ifshin, M., Loo, D. and Diamond, J. (1984). Studies of sodium channels in rabbit urinary bladder by noise analysis. *J. Membr. Biol.* **80**, 135–151.

Li, J., Eygensteyn, J, Lock, R. A. C., Verbost, P. M., van der Heijden, A. J. H., Wendelaar Bonga, S. E. and Flik, G. (1995). Branchial chloride cells in larvae and juveniles of freshwater tilapia *Oreochromis mossambicus. J. Exp. Biol.* **198**, 2177–2184.

Lin, H., Pfeiffer, D. C., Vogl, A. W., Pan, J. and Randall, D. J. (1994). Immunolocalization of H^+-ATPase in the gill epithelia of rainbow trout. *J. Exp. Biol.* **195**, 169–183.

Lin, H. and Randall, D. J. (1991). Evidence for the presence of an electrogenic proton pump on the trout gill epithelium. *J. Exp. Biol.* **161**, 119–134.

Lin, H. and Randall, D. J. (1993). H^+-ATPase activity in crude homogenates of fish gill tissue – inhibitor sensitivity and environmental and hormonal regulation. *J. Exp. Biol.* **180**, 163–174.

Lin, H. and Randall, D. J. (1995a). Proton pumps in fish gills. In *Fish Physiology*, vol. 14, *Cellular and Molecular Approaches to Fish Ionic Regulation* (ed. C. M. Wood and T. J. Shuttleworth), pp. 229–256. New York: Academic Press.

Lin, H. and Randall, D. J. (1995b). H^+-ATPase activity in crude homogenates of fish gill tissue: inhibitor sensitivity and environmental regulation. *J. Exp. Biol.* **180**, 163–174.

Maetz, J. and Garcia-Romeu, F. (1964). The mechanisms of sodium and chloride uptake by the gills of freshwater fish, *Carassius auratus*. II. Evidence for NH_4^+/Na^+ and HCO_3^-/Cl^- exchanges. *J. Gen. Physiol.* **47**, 1209–1227.

Marshall, W. S. (1995). Transport processes in isolated teleost epithelia: opercular epithelium and urinary bladder. In *Fish Physiology*, vol. 14, *Cellular and Molecular Approaches to Fish Ionic Regulation* (ed. C. M. Wood and T. J. Shuttleworth), pp. 1–23. New York: Academic Press.

Mayer-Gostan, N., Wendelaar Bonga, S. E. and Balm, P. H. M. (1987). Mechanisms of hormone actions on gill transport. In *Vertebrate Endocrinology: Fundamentals and Biomedical Implications*. vol. 2, *Regulation of Water and Electrolytes* (ed. P.

K. T. Pang, M. P. Schreibman and W. H. Sawyer), pp. 211–238. New York: Academic Press.

Pärt, P. and Wood, C. M. (1996). Na/H exchange in cultured epithelial cells from fish gills. *J. Comp. Physiol.* B **166**, 37–45.

Perry, S. F. (1997). The chloride cell: structure and function in the gills of freshwater fishes. *Annu. Rev. Physiol.* **59**, 325–347.

Perry, S. F. and Randall, D. J. (1981). Effects of amiloride and SITS on branchial ion fluxes in rainbow trout (*Salmo gairdneri*). *J. Exp. Zool.* **215**, 225–228.

Potts, W. T. W. (1994). Kinetics of sodium uptake in freshwater animals – a comparison of ion-exchange and proton pump hypotheses. *Am. J. Physiol.* **266**, R315–R320.

Shuttleworth, T. J. (1989). Overview of epithelial ion transport mechanisms. *Can. J. Zool.* **67**, 3032–3038.

Stouthart, A. J. H. X., Spanings, F. A. T., Lock, R. A. C. and Wendelaar Bonga, S. E. (1994). Effects of low water pH on lead toxicity to early life stages of the common carp. *Aquat. Toxicol.* **30**, 137–151.

Sullivan, G. V., Fryer, J. N. and Perry, S. F. (1995). Immunolocalization of proton pumps (H^+-ATPase) in pavement cells of rainbow trout. *J. Exp. Biol.* **198**, 2619–2629.

Sullivan, G. V., Fryer, J. N. and Perry, S. F. (1996). Localization of mRNA for the proton pump (H^+-ATPase) and Cl^-/HCO_3^- exchanger in the rainbow trout gill. *Can. J. Zool.* **74**, 2095–2103.

Van Driessche, W. and Lindemann, B. (1979). Concentration-dependence of currents through single sodium-selective pores in frog skin. *Nature* **282**, 519–520.

Werner, G., Hagenmaier, H., Drautz, H., Baumgartner, A. and Zähner, H. (1984). Metabolic products of microorganisms. 224 bafilomycins, a new group of macrolide antibiotics. Production, isolation, chemical structure and biological activity. *J. Antibiotics* **37**, 110–117.

Wilson, R. W., Wright, P. M., Munger, S. and Wood, C. M. (1994). Ammonia excretion in freshwater rainbow trout (*Oncorhynchus mykiss*) and the importance of gill boundary layer acidification: lack of evidence for Na^+/NH_4^+ exchange. *J. Exp. Biol.* **191**, 37–58.

Wright, P. A. and Wood, C. W. (1985). An analysis of branchial ammonia excretion in the freshwater rainbow trout: effects of environmental pH change and sodium uptake blockage. *J. Exp. Biol.* **126**, 329–353.

Yoshimori, T., Yamamoto, A., Moriyama, Y., Futai, M. and Tashiro, Y. (1991). Bafilomycin A_1, a specific inhibitor of vacuolar-type H^+-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. *J. Biol. Chem.* **266**, 17707–17712.

Zare, S. and Greenaway, P. (1998). The effect of moulting and sodium depletion on sodium transport and the activities of $Na^+ K^+$-ATPase and V-ATPase in the freshwater crayfish *Cherax destructor* (Crustacea: Parastacidae). *Comp. Biochem. Physiol.* **119**A, 739–745.