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H^+-ATPase ACTIVITY IN CRUDE HOMOGENATES OF FISH GILL TISSUE: INHIBITOR SENSITIVITY AND ENVIRONMENTAL AND HORMONAL REGULATION

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Summary

N-ethymaleimide-sensitive ATPase activity was measured in crude homogenates of gill tissue from rainbow trout using a coupled-enzyme ATPase assay in the presence of EGTA, ouabain and azide. This NEM-sensitive ATPase activity, determined to be about 1.5 μmol mg^{-1} protein h^{-1} at 15°C for freshwater trout, is also inhibited by other H^+-ATPase blockers such as DCCD, DES, PCMBS and bafilomycin. It is concluded, therefore, that the NEM-sensitive ATPase activity was generated by a proton-translocating ATPase. Since this NEM-sensitive ATPase was also sensitive to the plasma membrane ATPase inhibitor vanadate, we conclude that the H^+-ATPase in fish gill is of the plasma membrane type. The major role of the H^+-ATPase in the gill epithelium is to facilitate Na^+ uptake from fresh water. Sodium concentration in the external medium was the primary regulator of the H^+-ATPase in fish gills, with low sodium levels being associated with high H^+-ATPase activity. High external calcium concentration had a marked stimulatory effect on H^+-ATPase activity in fish gills when the sodium level was low. Environmental hypercapnia induced a 70% increase in the H^+-ATPase activity in fish gills. H^+-ATPase activity was also elevated in freshwater fish after chronic cortisol infusion.

Introduction

The Na^+/H^+ exchanger in fish gill epithelium was postulated to be the major pathway for Na^+ uptake and acid excretion (Wright and Wood, 1985). The sodium concentration in fresh water, however, is usually lower than 1 mmol l^{-1}, and the intracellular sodium concentration in the gill epithelial cell, although lowered by the Na^+/K^+-ATPase in the basolateral membrane, may be much higher than 1 mmol l^{-1} (intracellular sodium ion activity in frog skin epithelium was measured by Harvey and Ehrenfeld, 1986, as 6.2 mmol l^{-1} using double-barrelled ion-sensitive microelectrodes). It seems unlikely that the sodium gradient across the apical membrane could drive the Na^+/H^+ exchange. An alternative mechanism which will account for Na^+ and H^+ transport in opposite directions is an electrogenic H^+-translocating ATPase coupled with a sodium conductive channel (Avella and Bornancin, 1989; Lin and Randall, 1991), as demonstrated in freshwater frog skin (Ehrenfeld and Garcia-Romeu, 1977; Ehrenfeld *et al.* 1985). This so-

Key words: H^+-ATPase, *N*-ethymaleimide, sodium uptake, gill, rainbow trout, *Oncorhynchus mykiss.*

called proton pump will consume ATP, actively exclude hydrogen ions across the membrane and generate a negative potential inside the apical membrane, which will then drive sodium influx *via* the sodium channel.

The existence of the H^+-ATPase is well documented not only in freshwater frog skin, which has the same Na^+ uptake function as freshwater fish gills, but also in other tight epithelia, such as turtle urinary bladder (Steinmetz and Andersen, 1982) and mammalian renal collecting tubule (Gluck and Al-Awqati, 1984; Ait-Mohamed *et al.* 1986). *N*-ethymaleimide, a covalent SH-reactive compound, is an H^+-ATPase inhibitor commonly used to identify H^+-ATPases in different organisms (Pedersen and Carafoli, 1987).

Our previous *in vivo* studies (Lin and Randall, 1991) showed that proton excretion across the gill epithelium of freshwater trout was sensitive to external pH, P_{CO_2}, vanadate (a plasma membrane ATPase inhibitor) and acetazolamide (a carbonic anhydrase inhibitor), but was not sensitive to 0.1mmol l^{-1} amiloride, which blocked the Na^+ influx across the gills of rainbow trout completely (Wright and Wood, 1985). All these characteristics are typical of H^+ transport mediated by H^+-ATPases in other tight epithelia and thus indicate the presence of a proton pump in the gill epithelium. The first objective of these studies was to measure H^+-ATPase activity directly in crude homogenates of gill tissue.

Influx of sodium in freshwater fish is affected by different environmental factors, such as hypercapnia (Goss *et al*. 1992) and water Ca^{2+} levels (Avella *et al.* 1987). If the proton pump does provide the driving force for sodium uptake in freshwater fish, these factors could act on H^+-ATPase activity and variations in Na^+ influx would be the secondary outcome. It has also been suggested that the molecular target of the mineralocorticoid hormone aldosterone on urine acidification is H^+-ATPase in the kidney collecting tubule of mammals (Mujais, 1987; Garg and Narang, 1988; Khadouri *et al.* 1989). The second objective of these studies, therefore, was to examine the effects of the environment and hormones on H^+-ATPase activity in fish gills.

Materials and methods

Experimental animals

Rainbow trout, *Oncorhynchus mykiss* (Walbaum), weighing 300–500g were kept in aerated, dechlorinated Vancouver tap water ($[Na^+]$, 0.89mmol l^{-1}; $[Ca^{2+}]$, 0.03mmol l^{-1}; $[Cl^-]$, 0.92mmol l^{-1}) at 10–15°C ambient temperature. Some animals were acclimated to sea water (34–38‰) for 8–10 weeks. All animals were fed commercial trout pellets twice a week; feeding was terminated at least 1 day before usage or treatment.

Preparation of gill tissue homogenates

A crude homogenate of rainbow trout gill tissue was prepared using a method modified from Zaugg (1982). Fish were killed with a blow to the head. The gills were perfused through the heart with heparinized saline in order to clear red blood cells. Gill filaments (approximately 1g wet mass) were trimmed from supporting arches and immersed in 2ml of a cool homogenate medium I containing 300mmol l^{-1} sucrose, 2mmol l^{-1} EGTA,

1 mmol l^{-1} dithiothreitol and 100mmol l^{-1} Tris–HCl at pH7.3. Tissue was then homogenized with a Kontes micro ultrasonic cell disrupter for 20 strokes, 2ml of distilled water was added to the homogenates and another 20 strokes were employed to ensure that all filaments were disintegrated. The diluted homogenates were centrifuged for 7min in a Janetzli laboratory table centrifuge (model T32c) at about 4000revs min^{-1} (2000 RCF). Supernatant solutions were discarded and pellets suspended in 1ml of homogenate medium II (homogenate medium I containing 6% Chaps, a zwitterionic detergent) were homogenized twice for 20 strokes each. The resulting homogenates were centrifuged as before and supernatant solutions were removed for ATPase assay. We found that the ATPase activity in the supernatant was stable for at least 1 month when stored in a freezer at −80°C. The protein concentration of the supernatant was around 6–8mg ml^{-1}.

Determination of NEM-sensitive ATPase activity

NEM-sensitive ATPase activity was measured by a modified coupled-enzyme ATPase assay used for determination of Na^+/K^+-ATPase activity (Scharschmidt *et al.* 1979). Stock reaction buffer containing 130.9mmol l^{-1} Tris (pH7.4 at 15°C), 1.05mmol l^{-1} EGTA and 13.09mmol l^{-1} KCl was prepared in advance. On the day of assay, NADH, phosphoenolpyruvate, ouabain and sodium azide were added to the reaction buffer in amounts necessary to bring their concentrations to 0.52mmol l^{-1}, 2.62mmol l^{-1}, 2.12mmol l^{-1} and 5.24mmol l^{-1}, respectively. NEM was added to half of the reaction buffer to a concentration of 1.06mmol l^{-1}. Tris–ATP was dissolved in 200mmol l^{-1} $MgCl_2$ solution to yield a 200mmol l^{-1} concentration. A lactate dehydrogenase/pyruvate kinase (LDH–PK) enzyme mixture (1000 units each per millilitre) was purchased from Sigma. All reagents were kept on ice. To perform the assay, 0.945ml of reaction buffer containing NADH and phosphoenolpyruvate, 0.025ml ATP–$MgCl_2$ solution and 0.01ml LDH–PK mixture were added to a 1.5ml cuvette. The reaction was begun by adding 0.02ml of crude homogenate and mixing the contents of the cuvette by inversion. The final 1ml reaction mixture thus contained 125mmol l^{-1} Tris buffer, 1mmol l^{-1} EGTA, 12.5mmol l^{-1} KCl, 5mmol l^{-1} NaN_3, 2mmol l^{-1} ouabain, 5mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} ATP, 2.5mmol l^{-1} phosphoenolpyruvate, 0.5mmol l^{-1} NADH and 10 units each of LDH and PK (with or without 1mmol l^{-1} NEM). The oxidation of NADH was monitored continuously at 340nm at 15°C in the temperature-controlled cuvette compartment of a continuously recording spectrophotometer (Perlin-Elmer Lambda 2). ATPase activity was calculated from the slope of the linear portion of the tracing, the NADH millimolar extinction coefficient, the volume of the reaction mixture and the milligrams of crude homogenate protein added:

$$\text{ATPase activity } (\mu\text{mol P}_\text{i}\,\text{mg}^{-1}\,\text{h}^{-1}) = \frac{\text{slope (ODunits h}^{-1})}{6.22\ (\text{ODunits ml}^{-1}\,\mu\text{mol}^{-1})} \times \frac{1\text{ml}}{\text{protein (mg)}}.$$

Protein concentrations in the crude homogenates was determined by the method of Bradford (1976). The differences between the ATPase activity with and without NEM represent the NEM-sensitive ATPase activity.

In experiments with the inhibitors DCCD, DES and PCMBS, stock solutions were

made by dissolving the drugs in 100% ethanol and then added to the reaction mixture to the required concentration. Stock solution of bafilomycin (purchased from Dr Altendorf, Fachbereich Biologie/Chemie, U. Osnabruck, FRG) were prepared in dimethylsulphoxide (Bowman *et al.* 1988). Control samples containing the proper amount of solvent were assayed simultaneously. KNO_3, acetazolamide and sodium vanadate are water-soluble and assays were performed in the same way as with NEM.

Acclimation to various external sodium and calcium concentrations

Four kinds of external media were prepared by dissolving NaCl and/or $CaCl_2$ into dechlorinated Vancouver tap water: 100mmol l^{-1} NaCl; 100mmol l^{-1} NaCl plus 1 mmol l^{-1} $CaCl_2$; 1mmol l^{-1} $CaCl_2$; and 10mmol l^{-1} $CaCl_2$. Fish were placed into 100 l opaque fibreglass tanks (density less than 25 g l^{-1}) filled with different external media for an acclimation period of 10–14 days. The tanks were well-aerated and temperature was maintained at ambient levels with cooling cords. The external medium was changed daily to prevent ammonia accumulation.

Chronic cortisol treatment

Plasma cortisol levels were elevated in both freshwater- and seawater-adapted trout by means of implanted Alzet mini osmotic pumps containing cortisol (Reid and Perry, 1991). Mini osmotic pumps were loaded with cortisol (hydrocortisone 21-hemisuccinate, Sigma) or saline (in sham treatments) and surgically implanted into the peritoneal cavity of anaesthetized fish. The nominal calculated plasma cortisol concentration was 200ng ml^{-1}, and fish were sampled for gill tissue and blood (by caudal puncture) 7 days after the implantation. Plasma cortisol level was measured using a Gammacoat [^{125}I]cortisol radioimmunoassay kit (Incstar Corp.).

Hypercapnia treatment

Rainbow trout were placed into a 100 l opaque fibreglass tank supplied with flowing aerated dechlorinated Vancouver tap water (pH5.8–6.2) and allowed to acclimate for 24h. Control fish were sampled immediately before the 48h hypercapnia treatment (2 % CO_2 in air, mixed using a Wösthoff gas-mixing pump, water pH5.0–5.5) and experimental fish were sampled at 6, 24 and 48h of hypercapnia. They were then allowed to recover for 24h and samples were taken at 6 and 24h of recovery.

NH_4Cl injection

Rainbow trout were fitted with dorsal aortic catheters under MS-222 anaesthesia (1:10000 in $NaHCO_3$-buffered fresh water). Fish were then allowed to recover for 24h in a sectioned Plexiglas box. 2ml kg^{-1} bodymass of saline (control group) or 1mol l^{-1} NH_4Cl in saline was injected daily for 2 days into the dorsal aorta of the fish over a period of approximately 5min. 48h after the first injection, blood samples were taken for pH measurement using a microcapillary pH electrode (Radiometer G279/G2) coupled to a PHM84 pH meter, and fish were killed for gill tissue sampling.

Statistical analysis

All ATPase assays were performed in triplicate and data are presented as mean ± standard error. Student's two-tailed *t*-test was used to test for significant ($P<0.05$) differences.

Results

A substantial amount of the ATPase activity in the crude homogenates of gill tissue, in the presence of azide, ouabain and EGTA, was sensitive to NEM. Fig. 1A shows the ATPase activity of gill tissue in response to various concentration of NEM. NEM causes a dose-dependent inhibition of ATPase activity. Maximal inhibition is observed in $1\,mmol\,l^{-1}$ NEM, which accounted for more than 70% of the total ATPase activity (Table 1). The difference between ATPase activity with and without $1mmol\,l^{-1}$ NEM is referred to as the NEM-sensitive ATPase activity; at 15°C, it was determined to be about $1.5\pm0.09\,\mu mol\,mg^{-1}\,protein\,h^{-1}$ for freshwater-adapted trout (Fig. 2).

Vanadate ($0.1mmol\,l^{-1}$) suppressed 60% of the ATPase activity in gill tissue (Table 1). When $1mmol\,l^{-1}$ NEM and $0.1mmol\,l^{-1}$ vanadate were applied together, the percentage of ATPase affected was only increased slightly. The combination of $1\,mmol\,l^{-1}$ of NEM and $0.1mmol\,l^{-1}$ vanadate suppressed 80% of the ATPase in crude homogenates, which indicates that, of the 60% of the ATPase that was sensitive to vanadate, 50% is from the NEM-sensitive ATPase.

The effect of DCCD was also examined. A maximum of 52% of the total ATPase activity was suppressed by $1mmol\,l^{-1}$ of DCCD (Table 1). A similar dose–response curve to that produced by NEM was observed with DCCD (Fig. 1B). The sensitivity profiles of fish gill ATPase towards both NEM and DCCD are similar to those found for the inhibition of H^+-ATPase of rat kidney (Ait-Mohamed *et al.* 1986).

DES and PCMBS had maximal inhibitory effects at a much lower concentration and accounted for inhibition of 63% and 45% of the total ATPase activity, respectively (Table 1). Bafilomycin, a very specific and potent inhibitor of vacuolar H^+-ATPase (Bowman *et al.* 1988), significantly inhibited ATPase activity of fish gills only at concentration above $25\,\mu mol\,l^{-1}$ (Table 1), while vacuolar H^+-ATPases are completely blocked at concentrations as low as $0.1\,\mu mol\,l^{-1}$ (Bowman *et al.* 1988). Potassium nitrate, another inhibitor used to distinguish vacuolar H^+-ATPase from plasma membrane H^+-ATPase (Bowman, 1983), caused a less than 30% reduction in fish gill ATPase activity (Table 1) at a concentration of $100mmol\,l^{-1}$, a dosage that is sufficient to inhibit 80% of the vacuolar H^+-ATPase. $0.1mmol\,l^{-1}$ acetazolamide, however, had no effect on the ATPase activity of fish gills.

In rainbow trout acclimated to different external level of Na^+ and/or Ca^{2+}, gill NEM-sensitive ATPase activity decreased as Na^+ acclimation level increased (Fig. 2). NEM-sensitive ATPase activity was significantly lower in fish acclimated to $100mmol\,l^{-1}$ Na^+ (with or without Ca^{2+}) than in control fish. Seawater-adapted rainbow trout had only one-third of the NEM-sensitive ATPase activity of their freshwater-adapted counterparts. The addition of Ca^{2+} to high-Na^+ water made no difference to NEM-sensitive ATPase

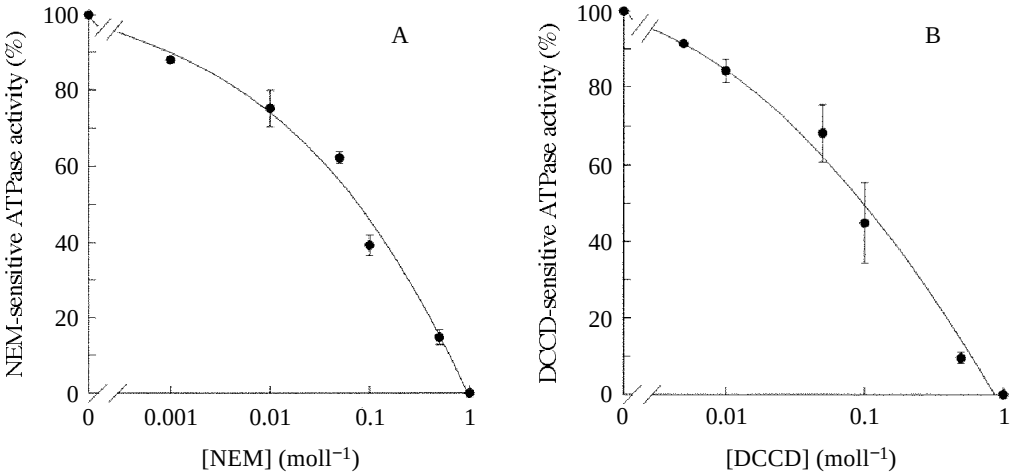

Fig. 1. NEM-sensitive (A) and DCCD-sensitive (B) ATPase activity in crude homogenates of trout gill tissue in response to various concentrations of NEM and DCCD, respectively. Activity is expressed as percentage assuming that the ATPase activity is 0 with 1mmol l^{-1} of NEM/DCCD and 100% without NEM/DCCD. Each point is the mean ± S.E.M. of four replicates.

Table 1. *Effects of inhibitors on ATPase activity in crude homogenate of trout gill tissue*

Inhibitor	Concentration (mmol l^{-1})	Relative activity (%)	*N*
None		100	
NEM	1	27.08±2.36	6
Vanadate	0.1	38.18±3.01	6
NEM+vanadate	1+0.1	20.87±2.37	6
DCCD	1	47.43±0.65	4
DES	0.025	36.88±0.82	3
PCMBS	0.010	55.35±4.36	3
Bafilomycin	0.025	58.02±2.25	3
KNO_3	100	72.25±2.61	3
Acetazolamide	0.1	97.19±5.66	3

NEM, *N*-ethylmaleimide; DCCD, dicyclo hexylcarbodiimide; DES, diethylstilbestrol; PCMBS, *p*-chloromercuribenzenesulfonate

Values are mean ± S.E.M.

activity. Increasing the Ca^{2+} level in low-Na^+ media, however, had a marked stimulatory effect and resulted in a twofold increase in NEM-sensitive ATPase activity in fish gills (Fig. 2).

Chronic cortisol infusion into freshwater rainbow trout caused a 170% increase in plasma cortisol level (Fig. 3B) and a 30% increase in NEM-sensitive ATPase activity in gill tissue (Fig. 3A). Seawater-adapted animals, in contrast, showed no increase in NEM-sensitive ATPase activity in response to a similar cortisol treatment, although their plasma cortisol level increased fourfold.

Hypercapnia treatment induced an immediate increase in the NEM-sensitive ATPase activity, which stabilized at a level 70% higher than that during normocapnia (Fig. 4). The elevated NEM-sensitive ATPase activity returned to control levels after 24h of recovery.

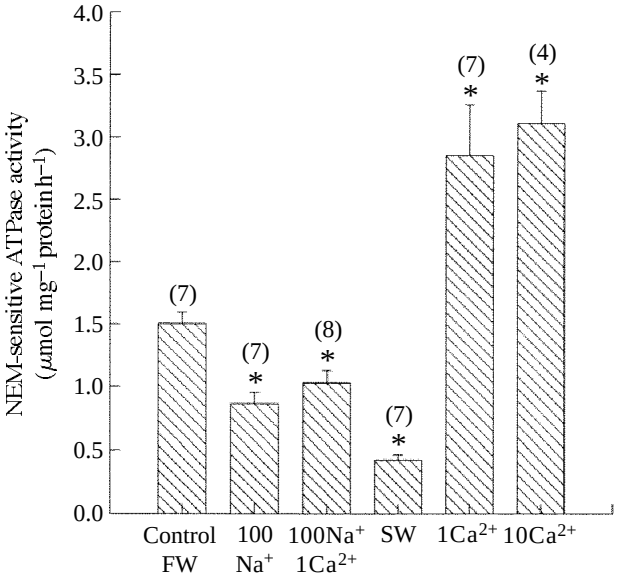

Fig. 2. NEM-sensitive ATPase activity in the gill tissue of rainbow trout acclimated to various Na^+ and Ca^{2+} levels (in mmol l^{-1}) in the external medium for 10–14 days. Mean ± S.E.M. * indicates a significant difference from the control value (P<0.05). Numbers in parentheses indicate the sample size. FW, fresh water; SW, sea water.

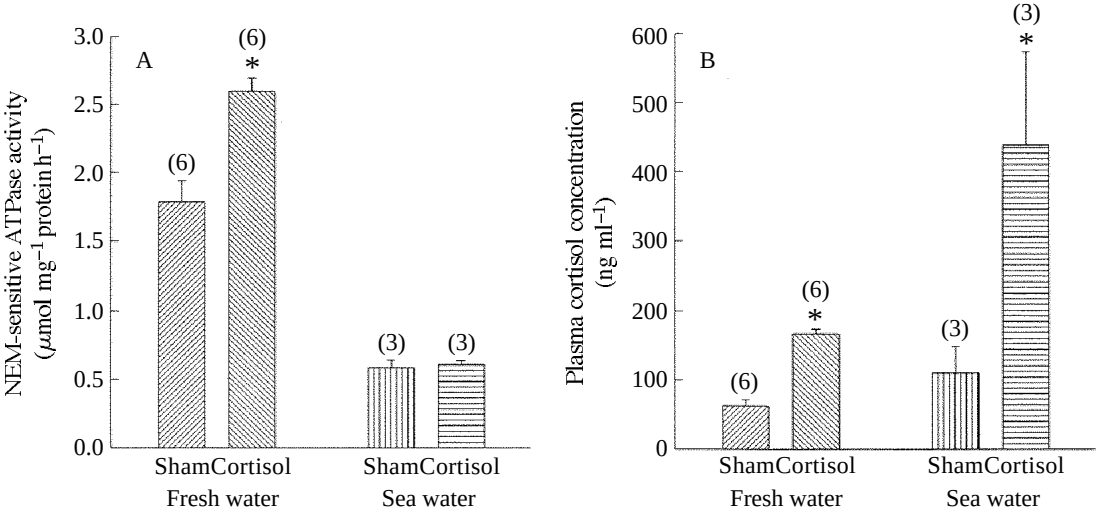

Fig. 3. NEM-sensitive ATPase activity in the gill tissue (A) and plasma cortisol concentration (B) of freshwater- and seawater-adapted rainbow trout after 7 days of chronic cortisol treatment. Mean ± S.E.M. * indicates a significant difference from the sham treatment value (P<0.05). Numbers in parentheses indicate the sample size.

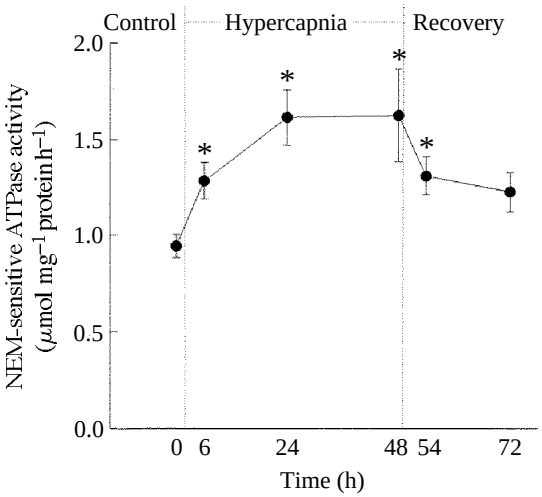

Fig. 4. NEM-sensitive ATPase activity in the gill tissue of freshwater-adapted rainbow trout during 48h of hypercapnia treatment and 24h of recovery. Mean ± S.E.M. (*N*=6). * indicates a significant difference from the control value ($P<0.05$).

Table 2. *Plasma pH and NEM-sensitive ATPase activity in gill tissue of NH_4Cl-injected rainbow trout*

	Plasma pH	NEM-sensitive ATPase activity ($\mu mol\,mg^{-1}\,protein\,h^{-1}$)
Saline-injected	7.70±0.12	0.985±0.123
NH_4Cl-injected	7.37±0.22*	1.020±0.224

*Indicates a significant difference between saline and NH_4Cl injection values ($P<0.05$).
Values are mean ± S.E.M., *N*=6.

A blood acidosis was associated with NH_4Cl injection (Table 2). NEM-sensitive ATPase activity, however, was not altered by NH_4Cl injection. Daily injections of NH_4Cl were given to two fish for 5 days and no change in NEM-sensitive ATPase activity was observed (data not shown).

Discussion

These studies demonstrate for the first time the existence of an NEM-sensitive ATPase in crude homogenates of fish gill tissue. H^+-ATPase has been reported to be either bound to membranes or packaged in cytoplasmic vesicles (Arruda *et al.* 1990). The crude homogenates prepared with the current method contain mainly the membrane fraction of gill cells, and the NEM-sensitive ATPase we detected in this study was released by protein solubilizer from the membrane fraction. Soluble cell material, mitochondria, cytoplasmic vesicles and other organelles would have been discarded in the supernatant of the first centrifugation (2000 ***g***) because much higher relative centrifugal forces are

required to spin down this material. Little ATPase activity was found in the discarded supernatant, but protein solubilizer was never applied. If there was H^+-ATPase packaged in cytoplasmic vesicles, perhaps protein solubilizer was required to release it for subsequent detection.

NEM is an alkylating agent that is relatively selective for sulphydryl groups (SH-). It inhibits vacuolar H^+-ATPase in an ATP-protectable manner at concentrations under $10\,\mu mol\,l^{-1}$ (Forgac, 1989; Pedersen and Carafoli, 1987). Phosphorylated ATPases (including Na^+/K^+-ATPase, Ca^{2+}-ATPase and plasma membrane H^+-ATPase) are sensitive to higher concentrations ($100\,\mu mol\,l^{-1}$ to $1 mmol\,l^{-1}$) of NEM (Forgac, 1989). Since the assay was carried out in the presence of EGTA, a Ca^{2+} chelator that should abolish Ca^{2+}-ATPase activity, azide, a mitochondrial H^+-ATPase inhibitor, and ouabain, a Na^+/K^+-ATPase inhibitor, the contribution of unrelated ATPase activity was minimized. Thus, the ATPase activity in the crude homogenate of gill tissue that was sensitive to $1 mmol\,l^{-1}$ NEM probably originated from plasma membrane H^+-ATPase.

30% of the ATPase activity was NEM-insensitive and is of unknown origin. Unidentified NEM-insensitive ATPase has also been detected in mammalian kidney (Ait-Mohamed *et al.* 1986; Garg and Narang, 1988). Bornancin *et al.* (1980) presented evidence of Cl^-/HCO_3^--ATPase in gill plasma membrane of rainbow trout. This might account for the NEM-insensitive ATPase activity in the gill tissue crude homogenates.

The gill ATPase sensitive to NEM was also sensitive to vanadate. Orthovanadate, VO_4^{3-}, acting as a phosphate transition analogue, blocks the formation of phosphorylated intermediates in all P-type ATPases. $0.1 mmol\,l^{-1}$ vanadate has been reported to inhibit branchial proton excretion in freshwater trout (Lin and Randall, 1991). Urinary acidification by turtle bladder (Arruda *et al.* 1981) and proton transport across freshwater frog skin (Ehrenfeld *et al.* 1985), both mediated by H^+-ATPase, are also vanadate-sensitive. However, vanadate fails to inhibit NEM-sensitive ATPase activity and proton transport in mammalian kidney (Gluck and Al-Awqati, 1984; Ait-Mohamed *et al.* 1986), which were believed to be mediated by vacuolar H^+-ATPase (Forgac, 1989).

DCCD inhibits mitochondrial, vacuolar and plasma membrane H^+-ATPase by binding to the *c* subunit of the hydrophobic channel portion (Pedersen and Carafoli, 1987). Mitochondrial H^+-ATPase has the highest sensitivity to DCCD ($0.1–0.5\,\mu mol\,l^{-1}$), followed by vacuolar H^+-ATPase ($1–10\,\mu mol\,l^{-1}$) and then plasma membrane H^+-ATPase ($10–100\,\mu mol\,l^{-1}$). The dose–response curves of the ATPase activity in gill tissue for NEM and DCCD are very similar to those reported for rat kidney H^+-ATPase (Ait-Mohamed *et al.* 1986). DES and PCMBS were also reported to inactivate the F_o moiety of H^+-ATPase (Pedersen and Carafoli, 1987). Proton transport mediated by H^+-ATPase in bovine kidney medulla was completely blocked by $10\,\mu mol\,l^{-1}$ PCMBS (Gluck and Al-Awqati, 1984), which partially inhibited H^+-ATPase in fish gills.

Bafilomycins are macrolide antibiotics that have a specific and potent inhibitory effect on vacuolar H^+-ATPase (Bowman *et al.* 1988). Mitochondrial H^+-ATPase is resistant to up to $1 mmol\,l^{-1}$ bafilomycin, whereas vacuolar H^+-ATPase is completely inhibited by less than $0.1\,\mu mol\,l^{-1}$ of the antibiotic. Phosphorylated ATPase exhibits intermediate sensitivities, with I_{50} values between 10 and $100\,\mu mol\,l^{-1}$. The sensitivity of the ATPase in gill tissue to bafilomycin is within this intermediate range.

Vacuolar H^+-ATPase also demonstrates a unique sensitivity to KNO_3 with an I_{50} value of about 50mmol l^{-1}. The resistance of fish gill ATPase to nitrate indicates that the H^+-ATPase we measured is not of the vacuolar type. Taken together, these pharmacological properties indicate that H^+-ATPase in fish gills is of the plasma membrane type and not the vacuolar type.

Acetazolamide has been demonstrated to inhibit luminal acidification in turtle bladder by stimulating the endocytosis of apical membrane (Dixon *et al.* 1988; Graber *et al.* 1989). The inhibition appeared to be independent of cell pH, which ruled out the possibility of a secondary effect due to inhibition of carbonic anhydrase. The inhibitory effect of acetazolamide on *in vivo* proton excretion (Lin and Randall, 1991) cannot be reproduced in the *in vitro* ATPase assay, indicating that acetazolamide has no direct effect on H^+-ATPase itself.

The observation that fish acclimated to water with low Na^+ level have higher H^+-ATPase activity than fish acclimated to water with a high Na^+ level indicates that the functional significance of the H^+-ATPase in fish gills is to generate an electrochemical gradient for Na^+ uptake from a dilute medium. Ehrenfeld *et al.* (1985) demonstrated that sodium absorption across freshwater frog skin was mediated by an active proton pump indirectly coupled with a sodium channel, instead of Na^+/H^+ exchange. The proton pump in frog skin was inhibited by DCCD and vanadate. Since the gill epithelium in freshwater fish is a tight epithelium similar to that of freshwater frog skin, it is reasonable to suppose that they have the same mechanism to solve the same osmoregulatory problem. When external Na^+ level is high, H^+-ATPase is down-regulated, possibly by endocytosis of membrane protein into intracellular vesicles (Schwartz and Al-Awqati, 1986). The residual H^+-ATPase in fish gills may play a role in acid–base regulation, although it is not required for sodium absorption or sodium excretion in fish that live in high-sodium environments.

The stimulatory effect of Ca^{2+} on H^+-ATPase could be explained using the cellular model of Wendelaar Bonga *et al.* (1992), who proposed the existence of an apical Ca^{2+} channel. When the external medium has a high Ca^{2+} but a low Na^+ level, Ca^{2+} might compete with Na^+ by entering the cell *via* the Ca^{2+} channel, reducing the potential gradient generated by the H^+-ATPase and therefore Na^+ influx. The resulting high Ca^{2+} concentration in the cell might stimulate the insertion of proton pumps from intracellular vesicles into the apical membrane in order to maintain Na^+ influx. Exocytosis of vesicles containing proton pumps into the cell membrane is Ca^{2+}-dependent in turtle bladder epithelium (Adelsberg and Al-Awqati, 1986).

Variations in Ca^{2+} level in freshwater environments have been reported to affect gill morphology and sodium influx in rainbow trout (Avella *et al.* 1987). Na^+ influx increased 2.5-fold in fish acclimated to fresh water+10mmol l^{-1} $CaCl_2$ for 15 days and new globular chloride cells appeared and proliferated in the secondary lamellae. Fish acclimated to fresh water+5mmol l^{-1} $CaCl_2$ for 5 days showed no change in gill morphology or sodium flux, perhaps because 5 days was too short for morphological modification.

We have also investigated the effects of chronic infusion of cortisol on H^+-ATPase activity in gill tissue in freshwater and seawater rainbow trout. Aldosterone treatments,

either long-term (7 days) or short-term, have been reported to stimulate H^+ secretion mediated by H^+-ATPase in the collecting duct of mammalian kidney (Garg and Narang, 1988; Mujais, 1987). The functionally parallel steroid hormone in fish is cortisol. Perry and Laurent (1989) have shown that plasma cortisol level rose transiently in fish exposed to deionized water. Daily intramuscular injections of cortisol for 10 days caused an increase in Na^+ uptake. The 30% increase in H^+-ATPase activity observed in freshwater trout following chronic cortisol treatment is probably responsible for this increased Na^+ uptake. Similar cortisol treatment has no effect on seawater-acclimated rainbow trout, indicating that Na^+ concentration is the predominant regulator of the H^+-ATPase in fish gills.

Another possible function of the H^+-ATPase in fish gill epithelium is acid–base regulation. We induced respiratory acidosis through hypercapnia treatment and observed an increase in H^+-ATPase activity in fish gills. Similar hypercapnia treatment in freshwater catfish was reported to cause a marked increase in Na^+ influx, which might be correlated with the increased H^+-ATPase activity. The elevated H^+-ATPase activity could be induced by the CO_2-mediated insertion of proton pumps *via* exocytosis, as demonstrated in turtle bladder epithelium (Cannon *et al.* 1985; Arruda *et al.* 1990). High CO_2 levels reduced the intracellular pH of the proton-secreting cells, which increased the intracellular Ca^{2+} concentration and, in turn, stimulated the fusion of cytoplasmic vesicles containing proton pumps into the apical membrane. This would then correct the intracellular acidosis.

A chronic metabolic acidosis was induced in the fish by NH_4Cl injection; it resulted in no significant change in H^+-ATPase activity. This indicates that high plasma hydrogen ion levels alone do not stimulate H^+-ATPase activity in gill tissue. We conclude that elevated CO_2 levels increase H^+-ATPase activity *via* a depression of epithelial pH. A metabolic acidosis, however, will only activate H^+-ATPase activity if the acidosis is transferred into the gill intracellular compartment. If we follow this argument, then presumably NH_4Cl infusion has little or no effect on epithelium pH because H^+-ATPase activity was unchanged. Unfortunately, we were not be able to measure the effects of NH_4Cl injection on gill epithelial pH.

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