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Regulation of Na^+/K^+-ATPase activity by nitric oxide in the kidney and gill of the brown trout (*Salmo trutta*)

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Summary

In teleost fish, successful osmoregulation involves controlled ion transport mechanisms in kidney and gill epithelia. In this study, the effect of nitric oxide (NO) on Na^+/K^+-ATPase was investigated *in vitro* in these two tissues in brown trout (*Salmo trutta*) acclimated to freshwater. The NO donor sodium nitroprusside (SNP) inhibited *in situ* Na^+/K^+-ATPase activity, measured as ouabain-sensitive Rb^+ uptake, in both samples of kidney and gill tissue and in isolated gill cells. The effect was dose-dependent in both tissues, with a maximal observed inhibition of approximately 40–50% (1 mmol l^{-1} SNP). The time-course of inhibition revealed a maximum effect with 10 min pre-incubation. The effect of SNP was reproduced with another NO donor, papa-nonoate (NOC-15; 200 μmol l^{-1}), and was prevented by the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO; 1 mmol l^{-1}). To further investigate the mechanism of the NO effect, whole-tissue Na^+ and K^+ levels were analysed. In kidney, SNP (1 mmol l^{-1}) led to an increase in tissue Na^+ levels and a decrease in K^+ levels in a 3:2 ratio. In gill tissue, no change in either ion was observed. These observations indicate that the effect on Na^+/K^+-ATPase is direct rather than due to a decrease in intracellular Na^+, its rate-limiting substrate. SNP elevated the level of cyclic GMP (cGMP) in both kidney and gill tissue. Dibutyryl cyclic GMP (db-cGMP; 1 mmol l^{-1}) also inhibited Na^+/K^+-ATPase activity in both tissues. Hence, a possible mechanism may involve the cGMP-activated kinase, even though other mechanisms cannot be excluded.

Key words: brown trout, *Salmo trutta*, kidney, gill, Na^+/K^+-ATPase, nitric oxide, SNP, sodium nitroprusside, cyclic GMP.

Introduction

Na^+/K^+-ATPase is an important energizer for ion transport in epithelial tissue. In the teleost kidney, reabsorption of most solutes and water is secondary to reabsorption of Na^+, which is energized by Na^+/K^+-ATPase localized in the basolateral membrane. Teleosts in freshwater (FW) have a high glomerular filtration rate (GFR), which offsets the substantial osmotic uptake of water, and NaCl is conserved by reabsorption. In seawater (SW), teleosts maintain only 10% of the GFR attained in FW, the main role of the kidney being excretion of divalent ions (Brown et al., 1980; Evans, 1993). No change is seen in the kidney Na^+/K^+-ATPase activity in salmonids in response to salinity changes (rainbow trout, Jürss et al., 1985; Atlantic salmon, McCormick et al., 1989; brown trout, Madsen et al., 1995), and the decline in GFR is produced by a reduction in the number of filtering glomeruli rather than a fall in single nephron filtration (Brown et al., 1980).

Compensatory ion transport is accomplished by the gill epithelium in both FW and SW, countering NaCl loss and influx, respectively. In FW, the combined action of basolateral Na^+/K^+-ATPase and apical H^+-ATPase constitutes the driving force for uptake of NaCl *via* apical Na^+ channels and Cl^-/HCO_3^- exchange (Avella and Bornancin, 1989; Marshall, 2002). Both chloride cells and pavement cells may be involved in the uptake of NaCl (Wilson et al., 2000). In SW, Cl^- excretion involves apical Cl^- channels and basolateral Na^+/K^+-ATPase and $Na^+/K^+/2Cl^-$ cotransporter, Na^+ excretion being paracellular (see Marshall, 2002). The cellular site for this salt secretion is known to be chloride cells (Foskett and Scheffey, 1982).

Hence, the Na^+/K^+-ATPase is of major importance in the salmonid kidney and gill. When the external salinity changes, expression of this and several other ion transporters in the gill is changed, a process that is mediated by several slow-acting hormones (McCormick, 2001; Evans, 2002). Hormonal long-term regulation of Na^+/K^+-ATPase seems less prominent in the kidney (Madsen et al., 1995), where regulatory adjustments may rely more on rapid alterations of ion transport protein activity. Whereas expressional regulation of the Na^+/K^+-ATPase is well documented in teleosts, short-term regulatory events are less well investigated. This is despite the fact that such regulation of pump activity has a great potential, as seen in various mammalian tissues (Therein and Blostein, 2000). A

few studies have, however, documented short-term hormonal regulation in teleost tissue. For instance, in eel kidney and gill, angiotensin II modulates Na^+/K^+-ATPase activity within minutes (Marsigliante et al., 1997, 2000). The second messenger systems involving protein kinase C (PKC; Crombie et al., 1996) and protein kinase A (PKA; Tipsmark and Madsen, 2001) have been shown to modulate Na^+/K^+-ATPase activity in the cod and trout gill, respectively. From the mammalian field, it is known that the nitric oxide–cyclic GMP (NO–cGMP) messenger system is involved in regulation of the Na^+/K^+-ATPase in the kidney and many other tissues (Therein and Blostein, 2000; Ortiz and Garvin, 2002). Nitric oxide synthase (NOS) catalyzes the production of NO, working as an autocrine and paracrine messenger. NO itself can activate the soluble guanylate cyclase (sGC) and can therefore work through the action of cGMP and the cGMP-activated kinase (PKG; Lincoln and Komalavilas, 2000).

It is not known whether NO plays a part in the regulation of Na^+/K^+-ATPase in lower vertebrates. The aim of the present study was to investigate the possible effect of NO and cGMP on Na^+/K^+-ATPase activity of the kidney and gill of the brown trout *Salmo trutta*. We used an *in vitro* system to analyse the effect of NO and cGMP on *in situ* Na^+/K^+-ATPase activity, measured as ouabain-sensitive Rb^+ uptake. To further investigate potential mechanisms, the Na^+, K^+ and cGMP concentrations in the tissues were analysed.

Materials and methods

Animals

Brown trout (*Salmo trutta* L.; mass, 40–60 g; >1 year old; mixed sex) were obtained from the Vork Hatchery (Egtved, Denmark) and kept in an indoor fibreglass tank with running tapwater (1.4 mmol l^{-1} Cl^-, 1.5 mmol l^{-1} SO_4^{2-}, 1.5 mmol l^{-1} Na^+, 0.16 mmol l^{-1} K^+, 3.0 mmol l^{-1} Ca^{2+}, 0.6 mmol l^{-1} Mg^{2+}, pH 8.3) at the Odense University Campus (15°C, 12 h:12 h light:dark artificial photoperiod). Experiments were performed from August to December and all fish used were classified as morphological parr. They were fed a maintenance diet of commercial trout pellets (2% body mass every second day).

Preparations of tissue blocks

After stunning the fish with a blow to the head, blood was drawn from the caudal blood vessels. The fish was then killed by cutting the spinal chord and pithing of the brain. Blocks of kidney tissue (approximately 4–8 mg) were excised from the posterior part of the trunk kidney and rinsed in chilled salmon Ringer's solution equilibrated with 99% O_2/1% CO_2 (140 mmol l^{-1} NaCl, 15 mmol l^{-1} $NaHCO_3$, 3.5 mmol l^{-1} KCl, 1.5 mmol l^{-1} $CaCl_2$, 1.0 mmol l^{-1} NaH_2PO_4, 0.8 mmol l^{-1} $MgSO_4$, 5.0 mmol l^{-1} D-glucose and 5.0 mmol l^{-1} *N*-2-hydroxyethyl-piperazine propanesulfonic acid; osmolality, 310 mosmol kg^{-1}; pH 7.8). The gill filaments were excised free of the cartilage and rinsed in Ringer's. Each gill arch was then cut transversally into blocks of 3–5 pairs of filaments (5–10 mg), held together by the interfilament septum. Samples were equilibrated for 30–60 min in Ringer's at 15°C prior to all incubations and treatments.

Preparations of cell suspensions

After cutting the spinal chord and pithing of the brain, 2000 U of heparin (in Ringer's solution) was injected into the heart. The bulbus arteriosus was cannulated and perfused over 10 min with 10–15 ml of heparinized (20 U ml^{-1}) Ca^{2+}- and Mg^{2+}-free Ringer's solution followed by 5 min of perfusion with heparin-free Ringer's. All gill arches were excised and rinsed in ice-cold Ca^{2+}- and Mg^{2+}-free Ringer's solution. The arches were scraped with a micro slide and the soft tissue suspended in 10 ml lysis buffer (9 parts 0.17 mol l^{-1} NH_4Cl, 1 part 0.17 mol l^{-1} Tris-HCl, pH 7.4: Yust et al., 1976) according to Verbost et al. (1994). Lysis of remaining blood cells and tissue fractionation was obtained by incubation in lysis buffer for 10–20 min at room temperature. The cells were suspended at the beginning and re-suspended at the end of this incubation by drawing them through a 10 ml pipette (3 mm bore diameter). The suspension was filtered through nylon gauze (80 μm) to remove cartilage and major cell debris. The cells were washed three times in Ca^{2+}- and Mg^{2+}-free Ringer's solution and spun down at 150 ***g*** for 5 min between each wash. They were finally re-suspended in 15 ml Ca^{2+}- and Mg^{2+}-free Ringer's and kept on ice until use. Viability was checked with the tryphan blue exclusion method (Sharpe, 1988) and was typically around 90%.

Incubations and experiments

In situ Na^+/K^+-ATPase activity

The method employed followed Tipsmark and Madsen (2001) with minor modifications. In order to measure Rb^+ uptake in tissue blocks, the Ringer's was replaced with Rb^+-Ringer's solution containing 3.5 mmol l^{-1} RbCl instead of KCl. In all routine measurements, kidney and gill blocks were incubated in Rb^+-Ringer's for 10 min at 15°C. Ouabain-sensitive Rb^+ uptake (representing *in situ* Na^+/K^+-ATPase activity) was calculated as the difference between total uptake and uptake in samples both pre-incubated (10 min in Ringer's) and incubated (10 min in Rb^+-Ringer's) with 1 mmol l^{-1} ouabain. Following incubation, the extracellular space was washed free of Rb^+ for 4×15 min at 0°C in Tris-sucrose buffer (10 mmol l^{-1} Tris, 260 mmol l^{-1} sucrose, pH 7.8). The tissue blocks were then blotted on filter paper and extraction of ions was performed overnight at 4°C in 5% trichloroacetic acid (TCA).

Rubidium uptake in cell suspensions was measured as described previously (Tipsmark and Madsen, 2001). The cell suspension was pelleted (150 ***g***, 5 min) and re-suspended in a minimal volume of Ca^{2+}- and Mg^{2+}-free Ringer's. The incubation was started by transferring aliquots (20 μl) to Rb^+-Ringer's in 24-wells with or without 1.0 mmol l^{-1} ouabain at 15°C. The standard incubation time was 10 min. Incubation was terminated by pelleting the cells (15 000 ***g***, 30 s) and washing three times with 0.1 mol l^{-1} $MgCl_2$. Rubidium was determined after extraction in 5% TCA at 4°C for 1 h. The

pellet was solubilized overnight in 0.2 mol l^{-1} NaOH and protein content was determined according to Lowry et al. (1951).

For Rb^+ measurements, KCl was added to the extracts to a final concentration of 20 mmol l^{-1}, and Rb^+ was determined by atomic absorption spectrophotometry at 780.8 nm with a slit of 2.0 nm and a red filter (Perkin Elmer 2380; Mountain View, CA, USA). Rubidium uptake in tissue blocks was expressed as nmol mg^{-1} wet mass h^{-1}. Rubidium uptake in cell suspensions was expressed as nmol mg^{-1} protein h^{-1}.

Experiments

In all experiments where Na^+/K^+-ATPase activity was measured, test agents were present both during incubation in Rb^+-Ringer's and during a 10 min pre-incubation, unless otherwise indicated. Donors of NO were always dissolved in Ringer's immediately before the experiment.

The dose–response relationship of the NO donor sodium nitroprusside (SNP; Sigma, St Louis, MO, USA; 0.001 mmol l^{-1}, 0.01 mmol l^{-1}, 0.1 mmol l^{-1}, 1 mmol l^{-1}; 10 min pre-incubation) on Na^+/K^+-ATPase activity in kidney and gill tissue blocks was investigated in one experiment.

A time-course experiment of the effect of 1 mmol l^{-1} SNP on Na^+/K^+-ATPase activity in kidney and gill tissue was done by pre-incubation for 0 min, 10 min and 60 min with SNP.

In three separate experiments the effect of (1) the NO donor papa-nonoate (NOC-15; Sigma; 0.2 mmol l^{-1}; concentration selected in accordance with Maragos et al., 1993), (2) lipid-soluble dibutyryl cyclic GMP (db-cGMP; Sigma; 1 mmol l^{-1}) and (3) the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO; Sigma; 1 mmol l^{-1}) in combination with SNP (0.5 mmol l^{-1}) on Na^+/K^+-ATPase activity in kidney and gill tissue blocks was examined. In each experiment, a parallel incubation with SNP (1.0 mmol l^{-1}, 1.0 mmol l^{-1} and 0.5 mmol l^{-1}, respectively) was used for direct comparison. The effect of SNP (1 mmol l^{-1}) on Na^+/K^+-ATPase activity of gill cells in suspension was examined without pre-incubation with SNP.

The effect of SNP (1 mmol l^{-1}) on tissue Na^+ and K^+ levels was examined by incubating kidney and gill tissue in triplicate in Ringer's solution with or without SNP for 2×30 min followed by blotting of the tissue, weighing and extraction overnight at 4°C in 5% TCA. The Na^+ and K^+ content of the supernatant was determined using a flame photometer (Instrumentation Laboratory 243; Lexington, MA, USA) with lithium as an internal standard. The values were expressed in nmol mg^{-1} wet mass.

The [^{14}C]inulin space was determined after 2×30 min and 3×30 min incubation in Ringer's solution containing [^{14}C]inulin (3700 Bq ml^{-1}). The tissues were incubated with or without SNP (1 mmol l^{-1}).

The effect of SNP on the cGMP concentration in kidney and gill tissue was examined. Kidney and gill blocks were incubated in Ringer's with or without SNP (1 mmol l^{-1}) for 10 min. Following homogenization in 0.1 mol l^{-1} HCl (1 ml) with a polytron homogenizer (30–40 mg tissue wet mass), the crude homogenate was centrifuged (13 000 ***g***, 15 min) and an aliquot of the supernatant was used for protein determination (Lowry et al., 1951). Another aliquot (0.5 ml) was dried overnight in a Speedyvac centrifuge, re-suspended in 1 ml of assay buffer and centrifuged (13 000 ***g***, 15 min). Cyclic GMP was analyzed in the supernatant after acetylation of the samples using a commercial enzyme immunoassay (Amersham Pharmacia Biotech, Uppsala, Sweden) following the manufacturer's protocol, and cGMP concentration was calculated relative to tissue protein content (fmol cGMP mg^{-1} protein).

Statistical analyses

Dataset with more than two groups were analysed by a randomized one-way block analysis of variance (ANOVA) and subsequently compared by the Tukey honest significant difference (HSD) procedure. Dataset with two groups were analysed by a paired Student's *t*-test. All statistical analyses were done using Systat (Evanston, IL, USA) and significant differences were accepted when $P<0.05$. Indicated *N* values signify the number of fish represented in each group.

Results

We examined the dose dependence of inhibition of SNP on *in situ* Na^+/K^+-ATPase activity in intact tissue, using a 10 min pre-incubation period. As shown in Fig. 1, a dose-dependent inhibition of the enzyme activity by SNP was found in both

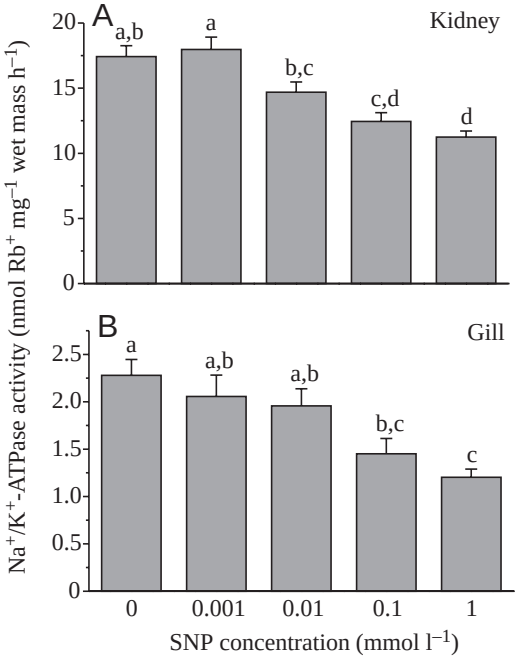

Fig. 1. The effect of sodium nitroprusside (SNP) concentration on *in situ* Na^+/K^+-ATPase activity (measured as ouabain-sensitive Rb^+ uptake) in kidney (A) and gill (B) tissue. Samples were pre-incubated for 10 min with the dose indicated before the Rb^+ uptake assay. Different letters indicate significant difference ($P<0.05$). Values are means ± S.E.M. ($N=8$).

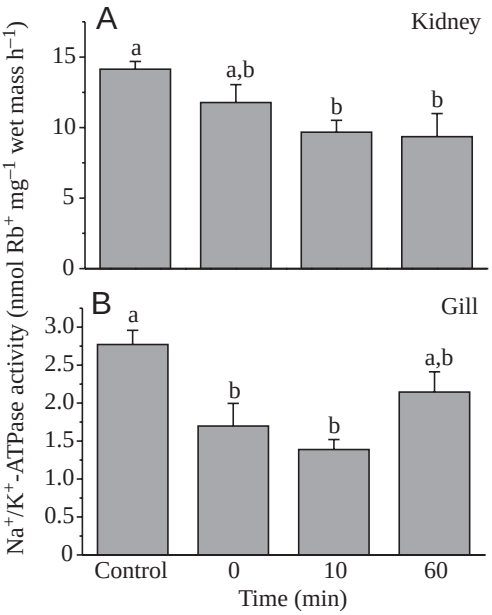

Fig. 2. The effect of pre-incubation time with sodium nitroprusside (SNP) on *in situ* Na^+/K^+-ATPase activity (measured as ouabain-sensitive Rb^+ uptake) in kidney (A) and gill (B) tissue. The samples were pre-incubated with SNP (1 mmol l^{-1}) for the time indicated before the Rb^+ uptake assay. Different letters indicate significant difference (P<0.05). Values are means ± S.E.M. (N=8).

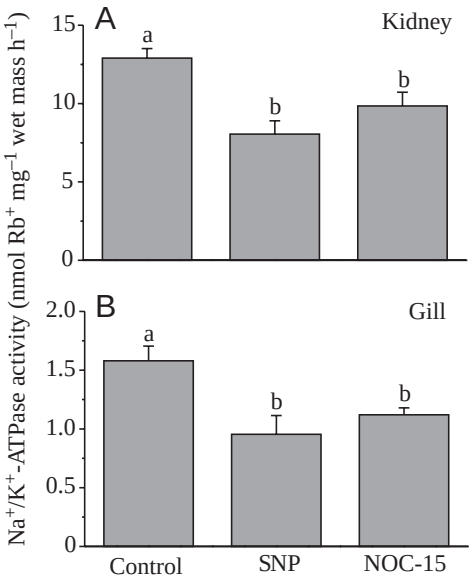

Fig. 3. The effect of the nitric oxide (NO) donors sodium nitroprusside (SNP; 1 mmol l^{-1}) and papa-nonoate (NOC-15; 0.2 mmol l^{-1}) on *in situ* Na^+/K^+-ATPase activity (measured as ouabain-sensitive Rb^+ uptake) in kidney (A) and gill (B) tissue. Samples were pre-incubated for 10 min with the NO donors before the Rb^+ uptake assay. Different letters indicate significant difference (P<0.05). Values are means ± S.E.M. (N=8).

the kidney and gill. A significant effect of SNP was observed at the two highest concentrations (0.1 mmol l^{-1} and 1 mmol l^{-1}). The maximal observed inhibition of pump activity was approximately 40% and 50% (1 mmol l^{-1} SNP) in kidney and gill, respectively.

The time-course dependence of the SNP effect on Na^+/K^+-ATPase activity was investigated using 1 mmol l^{-1} SNP. As shown in Fig. 2, Na^+/K^+-ATPase activity was inhibited after 10 min and 60 min pre-incubation in kidney tissue and after 0 min and 10 min in the gill. In a separate experiment, the effect of SNP on isolated gill cells was examined, and Na^+/K^+-ATPase activity was also inhibited by approximately 50% (control, 85.5±4.7 nmol Rb^+ mg^{-1} protein h^{-1}; 1 mmol l^{-1} SNP, 43.6±9.2 nmol Rb^+ mg^{-1} protein h^{-1}).

To confirm that the effect of SNP was indeed associated with NO release, we examined the effect of another NO donor (NOC-15) and the effect of SNP in combination with the NO scavenger PTIO on Na^+/K^+-ATPase in intact tissue. As shown in Fig. 3, NOC-15 (0.2 mmol l^{-1}), like SNP, significantly inhibited Na^+/K^+-ATPase activity in both the kidney and gill. When employing the NO scavenger PTIO (1 mmol l^{-1}) in combination with SNP (0.5 mmol l^{-1}), the effect of SNP on the Na^+/K^+-ATPase was abolished in both tissues (Fig. 4).

Because intracellular Na^+ concentration is a primary substrate regulator of Na^+/K^+-ATPase activity, we examined whether NO-associated effects on intracellular ion levels could possibly explain the inhibitory effect. Accordingly, Na^+ and K^+ content and inulin space in the tissue following incubation with SNP (1 mmol l^{-1}; 2×30 min) were analysed (Table 1). In

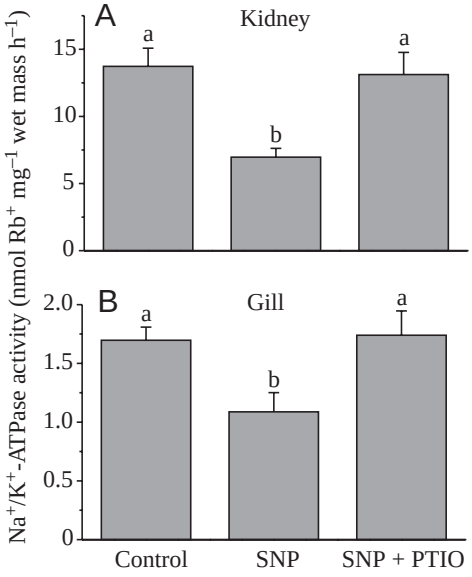

Fig. 4. The effect of sodium nitroprusside (SNP; 0.5 mmol l^{-1}) on *in situ* Na^+/K^+-ATPase activity (measured as ouabain-sensitive Rb^+ uptake) in kidney (A) and gill (B) tissue incubated in the presence or absence of the nitric oxide scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO; 1 mmol l^{-1}). Samples were pre-incubated for 10 min before the Rb^+ uptake assay. Different letters indicate significant difference (P<0.05). Values are means ± S.E.M. (N=8).

Table 1. *The effect of 1 mmol l^{-1} sodium nitroprusside (SNP) on Na^+ and K^+ content and inulin space in kidney and gill tissue from brown trout*

	Na^+ content (nmol mg^{-1} wet mass)	K^+ content (nmol mg^{-1} wet mass)	Inulin space (μl mg^{-1} wet mass)	cGMP concentration (fmol mg^{-1} protein)
Kidney tissue				
Control	65.8±1.1	78.6±0.6	0.142±0.009	53±4
SNP	73.3±1.1*	73.6±0.8*	0.139±0.004	469±62*
Gill tissue				
Control	89.1±1.1	69.9±0.8	0.124±0.004	230±27
SNP	89.8±1.3	68.9±1.2	0.120±0.005	365±31*

*Significant effect of treatment (P<0.05). Values are means ± S.E.M. (N=6).

The tissues were incubated under standard conditions for 2×30 min. Cyclic GMP (cGMP) concentrations were determined in both tissues following 10 min incubation with or without 1 mmol l^{-1} SNP.

the kidney, Na^+ content increased and K^+ content decreased in a 3:2 ratio. In the gill, there was no effect of SNP on the level of either ion. To ensure that the effect of SNP on Na^+ and K^+ content were indeed due to changes in intracellular concentrations and not caused by changes in the extracellular compartment, tissue inulin space was determined under similar conditions. The inulin space was unaffected by SNP (1 mmol l^{-1}) during 2×30 min incubation with SNP (Table 1). To validate that 2×30 min incubation was sufficient to estimate the inulin space, a parallel incubation for 3×30 min with or without SNP (1 mmol l^{-1}) was performed. Since this led to no significant change in inulin space, 2×30 min incubation was sufficient to ensure a correct estimation.

To further address the possible mechanism of NO, cGMP concentration in the tissues was analysed following 10 min incubation with SNP (1 mmol l^{-1}). NO significantly increased the cGMP level in both kidney and gill tissue (Table 1; P<0.05, N=6). To further evaluate the significance of these results, the effect of the lipid-soluble cGMP analogue db-cGMP on *in situ* Na^+/K^+-ATPase activity was examined. This was done on intact kidney and gill tissue in parallel with SNP. As shown in Fig. 5, Na^+/K^+-ATPase activity in both kidney and gill was significantly inhibited by db-cGMP (1 mmol l^{-1}) as well as by the NO donor (1 mmol l^{-1} SNP).

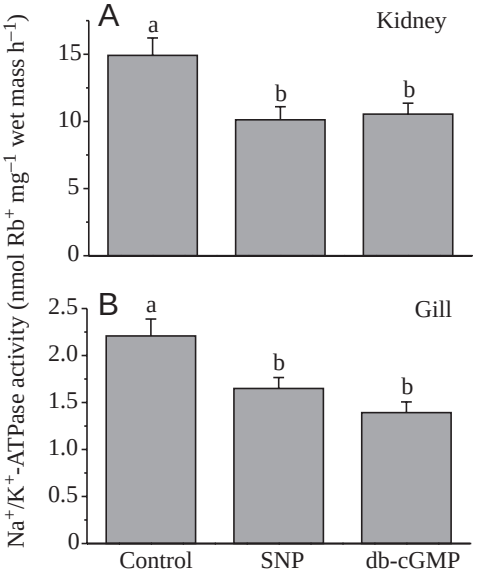

Fig. 5. Effect of sodium nitroprusside (SNP; 1 mmol l^{-1}) and dibutyryl cyclic GMP (db-cGMP; 1 mmol l^{-1}) on *in situ* Na^+/K^+-ATPase activity (measured as ouabain-sensitive Rb^+ uptake) in kidney (A) and gill (B) tissue. Samples were pre-incubated for 10 min before the Rb^+ uptake assay. Different letters indicate significant difference (P<0.05). Values are means ± S.E.M. (N=8).

Discussion

In the euryhaline brown trout, ion transport in the gill and kidney epithelia is essential when osmoregulating in both FW and SW. This study investigated the effects of the potential autocrine and paracrine modulator NO on Na^+/K^+-ATPase activity in these epithelia. We found that (1) NO inhibited Na^+/K^+-ATPase in both tissues, (2) the effect was not related to reduction in the intracellular level of the important substrate Na^+ and (3) the effect of NO could be mediated by a cGMP-dependent mechanism.

The present study is the first to demonstrate effects of NO on Na^+/K^+-ATPase in fish tissues. In both kidney and gill, the NO donor SNP inhibited *in situ* Na^+/K^+-ATPase activity in a dose-dependent manner. Since another NO donor (NOC-15) had a similar effect, and an NO scavenger (PTIO) abolished the inhibition of the pump, the effect is indeed related to NO itself. The present study was performed on FW-acclimated fish. Acclimation salinity is of potential importance for the observed effect, as Na^+/K^+-ATPase subunit isoform expression may be salinity dependent (Lee et al., 1998) and isoforms may be differentially regulated by NO (Blanco et al., 1998; Pontiggia et al., 1998). However, since a similar inhibitory effect of NO on gill Na^+/K^+-ATPase has been observed recently in SW-acclimated Atlantic salmon (L. O. E. Ebbesson and C. K. Tipsmark, unpublished), the results also apply to SW-acclimated salmonids.

Nitric oxide has been shown to modulate ion transport in several mammalian tissues, the effect apparently being tissue dependent. Inhibitory effects on the Na^+/K^+-ATPase have been found in the kidney (opossum, Liang and Knox, 1999; rat, McKee et al., 1994), the choroid plexus (bovine, Ellis et al., 2000), alveolar cells (rat, Guo et al., 1998), aortic endothelial cells (porcine, Gruwel and Williams, 1998), liver (rat, Muriel and Sandoval, 2000) and brain (porcine, Sato et al., 1997). Stimulatory effects have been found in rat trachea (de Oliveira Elias et al., 1999) and rabbit aorta (Gupta et al., 1994). The effect of NO has also been shown to differ among different kidney segments (Ortiz and Garvin, 2002). Isoform-specific effects on Na^+/K^+-ATPase of PKG, which is often found to mediate NO effects, have also been observed. Thus, PKG inhibits the α_1- and α_3- but not the α_2-isoform in infected SF-9 cells (Blanco et al., 1998) and α_1- but not the α_2- and α_3-isoform in brain endothelial cells (Pontiggia et al., 1998).

In addition to effects on the Na^+/K^+-ATPase, NO has been shown to inhibit the $Na^+/K^+/2Cl^-$ cotransporter (kidney, Ortiz et al., 2001), the Na^+/H^+-exchanger (kidney, Garvin and Hong, 1999) and apical Na^+ channels (kidney, Stoos et al., 1994). Since these ion transport proteins are also present in fish osmoregulatory tissues, the observed action of NO on Na^+/K^+-ATPase activity could be indirect and possibly mediated by reduced access to Na^+, the rate-limiting substrate. Assuming that changes in intracellular Na^+ content are reflected in measurements of whole-tissue Na^+ content, the present data indicate that the inhibition by NO was not caused by reduced Na^+ access in either tissue. Hence, the effect seems to be directly on the Na^+/K^+-ATPase. The above assumption requires that the extracellular space is unaffected by treatment and that there is a diffusion equilibrium between the extracellular space and incubation medium. Inulin-space estimates validated the former assumption. The latter assumption was validated through optimisation of the assay procedure (Tipsmark and Madsen, 2001), where it was shown that Rb^+ uptake was linear over time and lacked an initial lag phase. Thus, the present changes in whole-tissue ion levels most likely reflect changes at the intracellular level. A minor problem with interpretation of the gill ion data, however, is that the Na^+/K^+-ATPase is specifically concentrated in a minor fraction of the epithelial cells, the chloride cells. These cells constitute 10% or less of the total cell numbers (Wilson and Laurent, 2002), and any change in the ion content of these cells may be masked by a different or lack of change in the majority of other cells. So, the gill ion data should be interpreted cautiously, and future studies should focus on changes in intracellular ion concentrations within specific cell types of the gill.

Activation of the sGC leads to increased intracellular cGMP concentrations and is believed to mediate many of the physiological effects of NO, even though cGMP-independent effects have also been described (Gupta et al., 1994; Sato et al., 1997). The present study demonstrated increased whole-tissue cGMP concentration in response to SNP, apparently by activating sGC in both tissues. The lipid-soluble cGMP analogue (db-cGMP) had a similar inhibitory effect as NO on Na^+/K^+-ATPase activity. Since NO elevates the cGMP concentration in both tissues, the NO effect on the Na^+/K^+-ATPase may be a cGMP-dependent effect, possibly related to activation of PKG. Phosphorylation of a PKG substrate could underlie the regulatory event, and, in fact, the α-subunit of the Na^+/K^+-ATPase from the mammalian kidney has itself been shown to be a substrate for PKG (Fotis et al., 1999). The present effect may thus be related to cGMP-dependent phophorylation of either the α-subunit or, alternatively, a regulatory protein component, as seen for PKC modulation of the shark rectal gland enzyme (Mahmmoud et al., 2000). The α-subunit has been shown to be a substrate for PKA and PKC in several species, sometimes associated with modulation of Na^+/K^+-ATPase activity (Therein and Blostein, 2000). PKA and PKC can also phosphorylate the brown trout α-subunit *in vitro* (C. K. Tipsmark and Y. A. Mahmmoud, unpublished results), and cAMP modulates activity of the trout enzyme (Tipsmark and Madsen, 2001). Alternatively, more-complex pathways involving secondary modulators could be involved in the present effect. For example, Liang and Knox (1999) found inhibition of Na^+/K^+-ATPase in opossum kidney cells by NO to be associated with activation of the PKC α-isoform.

Several hormones and cytokines are known to influence tissue NO and cGMP concentrations. Atrial natriuretic peptide (ANP) inhibits Na^+/K^+-ATPase *via* activation of PKG in the rat kidney (Scavone et al., 1995). Angiotensin II activates NOS and sGC in the rat kidney (Zhang and Mayeux, 2001). In the mammalian kidney, bradykinin, acetylcholine and oxytocin also activate NOS and sGC and inhibit Na^+/K^+-ATPase (McKee et al., 1994). Hence, a number of chemical modulators of ion transport may work *via* NO/cGMP. Recent findings by Evans et al. (2002) indicate that the endothelin agonist sarafotoxin and NO itself inhibit short-circuit current (I_{sc}) across the killifish operculum membrane. The mechanism behind these observations may be an effect on the Na^+/K^+-ATPase, as found in the present study.

Salinity changes have been found to evoke rapid (in the order of a few hours) modulation of the Na^+/K^+-ATPase in the gill of the killifish (Towle et al., 1977; Mancera and McCormick, 2000) but not in salmonids, where only long-term expressional changes (in the order of several days) have been reported so far (Madsen et al., 1995; Mancera and McCormick, 2000). Even so, short-term adjustment of ion transport in the gill and kidney should be anticipated during feeding and stress. Short-term modulation of the Na^+/K^+-ATPase as seen in mammalian tissues (Therein and Blostein, 2000), involving hormones and second messengers, has been found in several teleosts in response to angiotensin II (eel gill, Marsigliante et al., 1997; eel kidney, Marsigliante et al., 2000), PKC activation (cod gill, Crombie et al., 1996), PKA activation (trout gill and kidney, Tipsmark and Madsen, 2001) and NO (present study).

Juxta-localization of the neuronal isoform of NOS (nNOS) and Na^+/K^+-ATPase has recently been reported using immunocytochemistry in the killifish opercular membrane (Evans, 2002). As mentioned before, the physiological

significance of this observation was supported by data showing the inhibition by NO of the I_{sc} across the opercular skin (Evans et al., 2002). In this study, the NOS inhibitor L-NAME stimulated the I_{sc}. Thus, NaCl transport over the opercular skin appears to be under tonic control by endogenously produced NO. In the Atlantic salmon gill, nNOS co-localizes with Na^+/K^+-ATPase in chloride cells and/or adjacent accessory cells, and gill Na^+/K^+-ATPase is also inhibited by NO in this species (L. O. E. Ebbesson and C. K. Tipsmark, unpublished). In the rainbow trout, nNOS has recently been localized in the kidney (Jimenez et al., 2001). These observations support the present study well and indicate that NO is an important autocrine/paracrine modulator of ion transport in the salmonid gill and kidney, adjusting it to the current need. Future studies employing NOS inhibitors and stimulators should focus on the role of endogenously produced NO in the regulation of Na^+/K^+-ATPase.

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References

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

Blanco, G., Sanchez, G. and Mercer, R. W. (1998). Differential regulation of Na,K-ATPase isozymes by protein kinases and arachidonic acid. *Arch. Biochem. Biophys.* **359**, 139-150.

Brown, J. A., Oliver, J. A., Henderson, I. W. and Jackson, B. A. (1980). Angiotensin and single nephron glomerular function in the trout *Salmo Gairdneri*. *Am. J. Physiol*. **239**, R509-R514.

Crombie, H. J., Bell, M. V. and Tytler, P. (1996). Inhibition of sodium-plus-potassium-stimulated adenosine triphosphatase (Na^+-K^+-ATPase) by protein kinase C activators in the gills of Atlantic cod (*Gadus morhua*). *Comp. Biochem. Physiol. B* **113**, 765-772.

de Oliveira Elias, M., de Lima, W. T., Vannuchi Y. B., Marcourakis, T., da Silva, Z. L., Trezena, A. G. and Scavone, C. (1999). Nitric oxide modulates Na^+-K^+-ATPase activity through cyclic GMP pathway in proximal rat trachea. *Eur. J. Pharmacol.* **367**, 307-314.

Ebbesson, L. O. E., Tipsmark, C. K., Holmqvist, B., Nilsen, T., Andersson, E., Stefansson, S. and Madsen, S. S. Nitric oxide synthase in the gill of Atlantic salmon: co-localization with and inhibition of the Na^+-K^+-ATPase. (submitted).

Ellis, D. Z., Nathanson, J. A. and Sweadner, K. J. (2000). Carbachol inhibits Na^+-K^+-ATPase activity in choroids plexus via stimulation of the NO/cGMP pathway. *Am. J. Physiol*. **279**, C1685-C1693.

Evans, D. H. (1993). Osmotic and ionic regulation. In *The Physiology of Fishes* (ed. D. H. Evans), pp. 315-341. Boca Raton, FL: CRC Press.

Evans, D. H. (2002). Cell signaling and ion transport across the fish gill epithelium. *J. Exp. Zool*. **293**, 336-347.

Evans, D. H., Roeser, J. M. and Stridham, J. D. (2002). Endothelin and nitric oxide interact to inhibit NaCl transport across the opercular skin of the killifish, *Fundulus hetroclitus*. *Bull. Mt. Desert. Isl. Biol. Lab.* **41**, 8.

Foskett, J. K. and Scheffey, C. (1982). The chloride cell: definitive identification as the salt-secretory cell in teleosts. *Science* **215**, 164-166.

Fotis, H., Tatjanenko, L. V. and Vasilets, L. A. (1999). Phosphorylation of the α-subunit of the Na^+/K^+-ATPase from mammalian kidneys and *Xenopus* oocytes by cGMP-dependent protein kinase results in stimulation of ATPase activity. *Eur. J. Biochem.* **260**, 904-910.

Garvin, J. L. and Hong, N. J. (1999). Nitric oxide inhibits sodium/hydrogen exchange in the thick ascending limb. *Am. J. Physiol.* **277**, F377-F382.

Gruwel, M. L. H. and Williams, J. P. (1998). Short term regulation of endothelial Na^+-K^+-pump activity by cGMP: a ^{133}Cs magnetic resonance study. *Mol. Membr. Biol.* **15**, 189-192.

Guo, Y., Duvall, D., Crow, J. P. and Matalon, S. (1998). Nitric oxide inhibits Na^+ absorption across cultured alveolar type II monolayers. *Am. J. Physiol.* **274**, L369-L377.

Gupta, S., McArthur, C., Grady, C. and Ruderman, N. B. (1994). Stimulation of vascular Na^+-K^+-ATPase activity by nitric oxide: a cGMP-independent effect. *Am. J. Physiol.* **266**, H2146-H2151.

Jimenez, A., Esteban, F. J., Sanchez-Lopez, A. M., Pedrosa, J. A., Del Moral, M. L., Hernandez, R., Blanco, S., Barroso, J. B., Rodrigo, J. and Peinado, M. A. (2001). Immunohistochemical localisation of neuronal nitric oxide synthase in the rainbow trout kidney. *J. Chem. Neuroanat.* **21**, 289-294.

Jürss, K., Bittorf, T. and Volker, T. (1985). Influence of salinity and ratio of lipid to protein in diets on certain enzyme activities in rainbow trout (*Salmo gairdneri* Richardson). *Comp. Biochem. Physiol. B* **81**, 73-79.

Lee, T. H., Tsai, J. C., Fang, M. J., Yu, M. J. and Hwang, P. P. (1998). Isoform expression of Na^+-K^+-ATPase α-subunit in the gills of the teleost *Oreochromis mossambicus*. *Am. J. Physiol*. **275**, R926-R932.

Liang, M. and Knox, F. G. (1999). Nitric oxide activates PKCα and inhibits Na^+-K^+-ATPase in opossum kidney cells. *Am. J. Physiol.* **277**, F859-F865.

Lincoln, T. M. and Komalavilas, P. (2000). Cyclic GMP-mediated signaling mechanisms in smooth muscle. In *Nitic Oxide; Biology and Pathobiology* (ed. L. J. Ignarro), pp. 401-425. San Diego, CA: Academic Press.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem*. **193**, 265-275.

Madsen, S. S., Jensen, M. K., Nøhr, J. and Kristiansen, K. (1995). Expression of Na^+-K^+-ATPase in the brown trout, *Salmo trutta*: in vivo modulation by hormones and seawater. *Am. J. Physiol.* **269**, R1339-R1345.

Mahmmoud, Y. A., Vorum, H. and Cornelius, F. (2000). Identification of a phospholemman-like protein from shark rectal glands. *J. Biol. Chem.* **275**, 35969-35977.

Mancera, J. M. and McCormick, S. D. (2000). Rapid activation of gill Na^+,K^+-ATPase in the euryhaline teleost *Fundulus heteroclitus*. *J. Exp. Zool.* **287**, 263-274.

Maragos, C. M., Wang, J. M., Hrabie, J. A., Oppenheim, J. J. and Keefer, L. K. (1993). Nitric oxide/nucleophile complexes inhibit the *in vitro* proliferation of A375 melanoma cells via nitric oxide release. *Cancer Res.* **53**, 564-568.

Marshall, W. S. (2002). Na^+, Cl^-, Ca^{2+} and Zn^{2+} transport by fish gills: retroperspective review and prospective synthesis. *J. Exp. Zool.* **293**, 264-283.

Marsigliante, S., Muscella, A., Barker, S. and Storelli, C. (2000). Angiotensin II modulates the activity of the Na^+/K^+-ATPase in eel kidney. *J. Endocrinol.* **165**, 147-156.

Marsigliante, S., Muscella, A., Vinson, G. P. and Storelli, C. (1997). Angiotensin II receptors in the gill of seawater- and freshwater-adapted eel. *J. Mol. Endocrinol.* **18**, 67-76.

McCormick, S. D. (2001). Endocrine control of osmoregulation in teleost fish. *Am. Zool.* **41**, 781-794.

McCormick, S. D., Moyes, C. D. and Ballantyne, J. S. (1989). Influence of salinity on the energetics of gill and kidney of Atlantic salmon (*Salmo salar*). *Fish Physiol. Biochem.* **6**, 243-254.

McKee, M., Scavone, C. and Nathanson, J. A. (1994). Nitric oxide, cGMP, and hormone regulation of active sodium transport. *Proc. Natl. Acad. Sci. USA* **91**, 12056-12060.

Muriel, P. and Sandoval, G. (2000). Nitric oxide and peroxynitrite anion modulate liver plasma membrane fluidity and Na^+/K^+-ATPase activity. *Nitric Oxide* **4**, 333-342.

Ortiz, P. A. and Garvin, J. L. (2002). Role of nitric oxide in the regulation of nephron transport. *Am. J. Physiol.* **282**, F777-F784.

Ortiz, P. A., Hong, N. J. and Garvin, J. L. (2001). NO decreases thick ascending limb chloride adsorption by reducing Na^+-K^+-$2Cl^-$ cotransporter activity. *Am. J. Physiol.* **281**, F819-F825.

Pontiggia, L., Winterhalter, K. and Gloor, S. M. (1998). Inhibition of Na,K-ATPase activity by cGMP is isoform-specific in brain endothelial cells. *FEBS Lett.* **436**, 466-470.

Sato, T., Kamata, Y., Irifune, M. and Nishikawa, T. (1997). Inhibitory effects of several nitric oxide-generating compounds on purified Na^+,K^+-ATPase activity from porcine cerebral cortex. *J. Neurochem.* **68**, 1312-1318.

Scavone, C., Scanlon, C., McKee, M. and Nathanson, J. A. (1995). Atrial natriuretic peptide modulates sodium and potassium activated adenosine triphosphatase through a mechanism involving cyclic GMP and cyclic GMP-dependent protein kinase. *J. Pharmacol. Exp. Ther.* **272**, 1036-1043.

Sharpe, P. T. (1988). *Methods of Cell Separation*. Amsterdam, New York, Oxford: Elsevier Science.

Stoos, A. S., Carretero, O. A. and Garvin, L. G. (1994). Endothelial-derived nitric oxide inhibits sodium transport by affecting apical membrane channels in cultures collecting duct cells. *J. Am. Soc. Nephrol.* **4**, 1855-1860.

Therein, A. G. and Blostein, R. (2000). Mechanisms of sodium pump regulation. *Am J. Physiol.* **279**, C541-C566.

Tipsmark, C. K. and Madsen, S. S. (2001). Rapid modulation of Na^+/K^+-ATPase activity in osmoregulatory tissues of a salmonid fish. *J. Exp. Biol.* **204**, 701-709.

Towle, D. W., Gilman, M. E. and Hempel, J. D. (1977). Rapid modulation of gill Na^++K^+-dependent ATPase activity during acclimation of the killifish *Fundulus heteroclitus* to salinity change. *J. Exp. Zool*. **202**, 179-186.

Verbost, P. M., Flik, G. and Cook, H. (1994). Isolation of gill cells. In *Analytical Techniques: Biochemistry and Molecular Biology of Fishes* (ed. P. W. Hochachka and T. P. Mommsen), pp. 239-247. Oxford: Elsevier Science.

Wilson, J. M. and Laurent, P. (2002). Fish gill morphology: inside out. *J. Exp. Zool.* **293**, 192-213.

Wilson, J. M., Laurent, P., Tufts, B. L., Benos, D. J., Donowitz, M., Vogl, A. W. and Randall, D. J. (2000). NaCl uptake by the branchial epithelium in freshwater teleost fish: an immunological approach to ion-transport protein localization. *J. Exp. Biol.* **203**, 2279-2296.

Yust, I., Smith, R. W., Wunderlich, J. R. and Mann, D. L. (1976). Temporary inhibition of antibody-dependent, cell-mediated cytotoxicity by pretreatment of human attacking cells with ammonium chloride. *J. Immunol.* **116**, 1170-1172.

Zhang, C. and Mayeux, P. R. (2001). NO/cGMP signaling modulates regulation of Na^+,K^+-ATPase activity by angiotensin II in rat proximal tubules. *Am. J. Physiol.* **280**, F474-F479.