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RAPID MODULATION OF Na^+/K^+-ATPase ACTIVITY IN OSMOREGULATORY TISSUES OF A SALMONID FISH

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Summary

The effects of cyclic AMP on Na^+/K^+-ATPase activity were studied in the gill and kidney of the euryhaline brown trout *Salmo trutta* using two different experimental approaches. In the first series of experiments, *in situ* Na^+/K^+-ATPase activity was analyzed by measuring the ouabain-sensitive uptake of non-radioactive rubidium (Rb^+) into gill cells and blocks of gill and kidney tissue. Rubidium uptake was linear for at least 30 min and was significantly inhibited by 1 mmol l^{-1} ouabain. Several agents presumed to increase the intracellular cyclic AMP concentration inhibited ouabain-sensitive Rb^+ uptake in both gill (0.5 and 2 mmol l^{-1} dibutyryl-cyclic AMP, 1 mmol l^{-1} theophylline, 10 μmol l^{-1} forskolin and 10 μmol l^{-1} isoproterenol) and kidney (10 μmol l^{-1} forskolin) tissue from freshwater-acclimated fish. In a separate series of experiments, ATP hydrolase activity was assayed in a permeabilised gill membrane preparation after incubation of tissue blocks with 10 μmol l^{-1} forskolin. Forskolin elevated gill cyclic AMP levels 40-fold, inhibited maximal enzymatic Na^+/K^+-ATPase activity (V_{max}) in gill tissue from both freshwater- and seawater-acclimated fish and reduced the apparent K^+ affinity in the gills of seawater-acclimated fish, demonstrating that the effects are mediated through modifications of the enzyme itself. The protein phosphatase inhibitors okadaic acid and cyclosporin A did not affect forskolin-induced inhibition of Na^+/K^+-ATPase activity, indicating that forskolin-mediated modulation was stable for the duration of assay. We suggest that cyclic-AMP-mediated phosphorylation through protein kinases may underlie the rapid modulation of Na^+/K^+-ATPase activity in the osmoregulatory tissues of euryhaline teleosts.

Key words: Na^+/K^+-ATPase, osmoregulation, gill, kidney, cyclic AMP, forskolin, brown trout, *Salmo trutta*.

Introduction

In transporting epithelia, the Na^+/K^+-ATPase (Na^+ pump) is an important transducer of chemical energy into the ion gradients that drive the uptake or excretion of essential ions. In teleosts, the gill epithelium generally serves diverse functions such as respiration, acid–base regulation and ion regulation (for a review, see Evans et al., 1999). In euryhaline teleosts, such as the brown trout *Salmo trutta*, the gill is capable of adapting to both net ion uptake and ion excretion, depending on the ionic composition of the external medium. In fresh water, the gill epithelium is 'tight' and is responsible for the active uptake of Na^+ and Cl^-. The cellular site for this process has not finally been established, but both pavement cells and mitochondria-rich cells have been implicated (see Wilson et al., 2000). According to the current model (Avella and Bornancin, 1989), Na^+ enters the epithelial cells through apical Na^+ channels energised by apically located vacuolar-type H^+-ATPase activity. Basolaterally, Na^+ leaves the cell *via* Na^+/K^+-ATPase transport. Cl^- uptake is believed to take place in exchange for HCO_3^-. In sea water, the gill epithelium changes into a leaky epithelium, excreting Na^+ and Cl^-. Interlamellar mitochondria-rich cells (chloride cells) are established as the cellular site for this transport (Foskett and Scheffey, 1982) and proliferate during acclimation to sea water (e.g. Hiroi et al., 1999). In this situation, the Na^+/K^+-ATPase maintains the basolateral transmembrane electrochemical gradient for Na^+, making Na^+/Cl^- and/or $Na^+/K^+/2Cl^-$ cotransport feasible and, thereby, energising transcellular, uphill Cl^- transport and paracellular, downhill Na^+ transport (Marshall, 1995).

The abundance of Na^+/K^+-ATPase in gill epithelia of most euryhaline fish is adaptively changed when the external salinity is changed (McCormick, 1995), a process that is controlled largely by hormonal modulation of gene and protein expression. This slow regulation of Na^+/K^+-ATPase generally takes 3–7 days and involves a number of hormones; it is particularly well documented in salmonid teleosts (Bern and Madsen, 1992). In contrast, rapid modulation of Na^+/K^+-ATPase activity in fish tissues is poorly described and has only been addressed in a few studies. Towle et al. (Towle et al.,

1977) and Mancera and McCormick (Mancera and McCormick, 2000) reported rapid modulation of gill Na^+/K^+-ATPase activity in *Fundulus heteroclitus* in response to salinity change. In addition, rapid regulation of Na^+/K^+-ATPase activity has been demonstrated to result from the activation of protein kinase C (PKC) (*Gadus morhua* gill cells; Crombie et al., 1996) and the activation of cyclic-AMP-mediated protein kinase A (PKA) (*Squalus acanthias* rectal gland; Marver et al., 1986). More extensive information about the rapid regulation of Na^+/K^+-ATPase has been obtained in other vertebrates, primarily mammalian tissues and cell lines, in which several studies have shown that a variety of chemical mediators are capable of modulating the activity of the enzyme (Ewart and Klip, 1995). Protein kinases and protein phosphatases generally play a crucial role in the modulatory event.

The aim of the present study was to evaluate the influence of cyclic AMP and PKA activation on Na^+/K^+-ATPase activity in the gill and kidney tissue of the brown trout *Salmo trutta*, a euryhaline teleost. For this purpose, a method was established for measuring *in situ* Na^+ pump activity in these fish tissues, using ouabain-sensitive non-radioactive Rb^+ uptake as a measure of its activity (in accordance with Longo et al., 1991). This was combined with measurements of maximal hydrolytic Na^+/K^+-ATPase activity (V_{max}) and of the apparent Na^+ and K^+ affinity in homogenized gill tissue.

Materials and methods

Animals

One-year-old brown trout (*Salmo trutta*, 40–60 g) were obtained from the Vork Hatchery (Denmark) and kept in an indoor fibreglass tank containing running dechlorinated tap water at the Odense University Campus (in mmol l^{-1}: 1.4 Cl^-, 1.5 SO_4^{2-}, 1.5 Na^+, 0.16 K^+, 3.0 Ca^{2+}, 0.6 Mg^{2+}, pH 8.3; 15 °C; 12 h:12 h light:dark artificial photoperiod). They were fed a maintenance diet of commercial trout pellets (1 % of body mass per day). Seawater (SW)-acclimated fish were obtained by transferring a batch of fish to natural, recirculated and filtered sea water (26 ‰; 15 °C) for 12 days prior to experiments.

Preparation 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 cord, and the gill arches were excised and rinsed in salmon Ringer's solution equilibrated with 99 % O_2/1 % CO_2 (in mmol l^{-1}:140 NaCl, 15 $NaHCO_3$, 2.5 KCl, 1.5 $CaCl_2$, 1.0 KH_2PO_4, 0.8 $MgSO_4$, 5.0 D-glucose and 5.0 *N*-2 hydroxyethyl-piperazine propanesulphonic acid, Hepps; 310 mosmol kg^{-1}, pH 7.8). Each gill arch was then cut transversely into blocks of 3–5 pairs of filaments (5–10 mg), held together proximally by the cartilage and laterally by the interfilament septum. Blocks of kidney tissue (approximately 4–8 mg) were excised from the posterior part of the trunk kidney and rinsed in Ringer. In all experiments, gill or kidney samples were pooled in a Petri dish before being transferred, without selection, into plastic baskets fitted into glass tubes. Tissues were supplied with Ringer and continuously gassed with 99 % O_2/1 % CO_2 at 15 °C. Samples were pre-equilibrated for 60–90 min in Ringer prior to all incubations and treatments.

Preparation of gill cell suspensions

After cutting the spinal cord, 2000 i.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 i.u. ml^{-1}) Ca^{2+}- and Mg^{2+}-free Ringer's solution followed by 5 min of perfusion with heparin-free Ringer. 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 was suspended in 10 ml of lysis buffer (9 parts of 0.17 mol l^{-1} NH_4Cl, 1 part of 0.17 mol l^{-1} Tris-HCl, pH 7.4; Yust et al., 1976; according to the method of Verbost et al., 1994). The remaining blood cells were lysed, and tissue fractionation was performed by incubation in lysis buffer for 10–20 min at room temperature (20 °C). The cells were suspended before and after incubation by drawing them through the 3 mm bore of a 10 ml pipette. The suspension was filtered through nylon gauze (80 μm) to remove cartilage and larger pieces of cell debris. The cells were washed three times in Ca^{2+}- and Mg^{2+}-free Ringer's solution and centrifuged at 150 ***g*** for 5 min between each wash. They were finally resuspended in 15 ml of Ca^{2+}- and Mg^{2+}-free Ringer and kept on ice until use. Viability was checked with the Trypan Blue exclusion method (Sharpe, 1988) and was typically approximately 90 %.

Measurement of ouabain-sensitive Rb^+ uptake

Tissue blocks

Non-radioactive rubidium (Rb^+) was used as a substitute for K^+ to evaluate K^+ uptake (Longo et al., 1991). To measure Rb^+ uptake, the Ringer was replaced with a Rb^+-Ringer containing 3.5 mmol l^{-1} RbCl and 1.0 mmol l^{-1} NaH_2PO_4 instead of KCl and KH_2PO_4, respectively. Ouabain-sensitive Rb^+ uptake (*in situ* Na^+/K^+-ATPase activity) was calculated as the difference between total uptake and uptake in samples pre-incubated (for 10 min in Ringer) and incubated (in Rb^+-Ringer) with 1 mmol l^{-1} ouabain. In an initial series of experiments, the time course of Rb^+ uptake was established and shown to be linear for at least 60 min in gill samples (see Fig. 1A) and 30 min in kidney samples (results not shown). The uptake was clearly inhibited by ouabain. The viability of the tissue was verified by measuring the Na^+ and K^+ content of the tissue samples incubated in standard Ringer by flame photometry (Instrumentation Laboratory 243, Lexington, MA, USA); both were constant for at least 2 h, indicating that the tissue was able to maintain ionic homeostasis during the incubation period. In all routine measurements, gill and kidney samples were incubated in Rb^+-Ringer for 30 and 20 min, respectively, which is well within the linear range of the Rb^+ uptake curve. Following incubation, the extracellular space was washed free of Rb^+, using four washes, each of 15 min at 0 °C in Tris-

sucrose buffer (in mmol l^{-1}: 2.5 KCl, 1.5 $CaCl_2$, 1.0 KH_2PO_4, 0.8 $MgSO_4$, 10 Tris, 260 sucrose, pH 7.8), equilibrated with 99 % O_2/1 % CO_2. The samples were then blotted gently on filter paper and weighed to the nearest 0.01 mg.

Ion extraction was performed overnight at 5 °C in 5 % trichloroacetic acid (TCA). KCl was added to the extracts to a final concentration of 20 mmol l^{-1}, and [Rb^+] was measured by atomic absorption spectrophotometry at 780.8 nm with a slit width of 2.0 nm and using a red filter (Perkin Elmer 2380; Mountain View, CA, USA). The standard curve was linear between 0.25 and 32 μmol l^{-1}. The rate of Rb^+ uptake was expressed as nmol mg^{-1} wet mass h^{-1}.

Cell suspensions

The cell suspension was pelleted (150 ***g***, 5 min) and resuspended in a minimal volume of Ca^{2+}- and Mg^{2+}-free Ringer. The incubation was started by transferring samples (20 μl) to Rb^+-Ringer in 24-well plates with or without 1.0 mmol l^{-1} ouabain at 15 °C. After an initial time course study (see Fig. 1B), the standard incubation time was set to 10 min. Incubation was terminated by pelleting the cells (15 000 ***g***, 30 s) and washing them three times with 0.1 mol l^{-1} $MgCl_2$. Rb^+ 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 the protein content was determined. The rate of Rb^+ uptake was expressed as nmol mg^{-1} protein h^{-1}.

Experimental series

Several series of experiments were performed to evaluate the effects of cyclic AMP on gill and kidney Na^+/K^+-ATPase activity. In series 3–5, tissues were generally pre-incubated for 10 min with the test agent in Rb^+-free Ringer prior to the incubation described below. This excludes isoproterenol in series 4, which was added without pre-incubation. Control incubations were always performed as true vehicle controls. The vehicle was Ringer solution (in series 1, 2 and 4) or dimethyl sulphoxide (DMSO; in series 3, 5 and 6). The final concentration of DMSO was 0.1 %.

Series 1

The effects of dibutyryl cyclic AMP (dbcAMP), a membrane-permeable analogue of cyclic AMP, on ouabain-sensitive Rb^+ uptake in gill blocks were studied by incubation in Rb^+-Ringer containing 0, 0.5 or 2.0 mmol l^{-1} dbcAMP (Sigma, St Louis, MO, USA).

Series 2

The effects of dbcAMP on ouabain-sensitive Rb^+ uptake in isolated gill cells were studied by incubation in Rb^+-Ringer containing 0 or 2.0 mmol l^{-1} dbcAMP.

Series 3

The effects of theophylline, a phosphodiesterase inhibitor, and forskolin, an adenylate cyclase activator, on ouabain-sensitive Rb^+ uptake in gill blocks were studied by incubation in Rb^+-Ringer containing 0 or 1 mmol l^{-1} theophylline (Sigma, St Louis, MO, USA), 10 μmol l^{-1} forskolin (Sigma) or 1 mmol l^{-1} theophylline plus 10 μmol l^{-1} forskolin.

Series 4

The effects of theophylline and isoproterenol on ouabain-sensitive Rb^+ uptake in gill blocks were studied by incubation in Rb^+-Ringer containing 0 or 1 mmol l^{-1} theophylline, 10 μmol l^{-1} isoproterenol (Sigma) or 1 mmol l^{-1} theophylline plus 10 μmol l^{-1} isoproterenol.

Series 5

The effects of forskolin on ouabain-sensitive Rb^+ uptake in kidney blocks were studied by incubation in Rb^+-Ringer containing 0 or 10 μmol l^{-1} forskolin.

Series 6

The effects of forskolin on maximal Na^+/K^+-ATPase enzymatic activity (V_{max}) were studied in gill blocks from freshwater (FW)- and SW-acclimated fish by incubating blocks in Ringer containing 0 or 10 μmol l^{-1} forskolin for 40 min. Paired gill samples from each treatment group were then immediately transferred and homogenized in ice-cold SEIDM buffer (in mmol l^{-1}: 300 sucrose, 20 Na_2-EDTA, 50 imidazole, 10 β-mercaptoethanol, 0.1 % sodium deoxycholate, pH 7.3) using a hand-held glass homogenizer, with or without the addition of two protein phosphatase inhibitors, okadaic acid (0.25 μmol l^{-1}) and cyclosporin A (25 nmol l^{-1}). Following low-speed centrifugation at 5000 ***g*** for 30 s, Na^+/K^+-ATPase activity was assayed in the supernatant at 35 °C using a microplate reader (SPECTRAmax PLUS, Molecular Devices, Sunnyvale, CA, USA; according to the protocol of McCormick, 1993).

ATP hydrolysis was enzymatically coupled to the conversion of NADH to NAD^+ by pyruvate kinase and lactic dehydrogenase and was analyzed by kinetic readings at 340 nm for 10 min. For each sample, Na^+/K^+-ATPase activity was assayed in paired solutions with or without the addition of the two protein phosphatase inhibitors. The assay conditions used for routine measurements of V_{max} were (in mmol l^{-1}): 54 NaCl, 15 KCl, 2.5 $MgCl_2$, 0.50 Na_3-phosphoenolpyruvate, 0.50 Na_2-ATP (vanadate free), 0.09 Na_2-NADH, 0.40 KCN, 2.6 units ml^{-1} pyruvate kinase, 2.0 units ml^{-1} lactic dehydrogenase, in 50 mmol l^{-1} imidazole buffer, pH 7.5. Each homogenate was analyzed in triplicate with or without the addition of 0.5 mmol l^{-1} ouabain. Protein content was measured by a microassay based on the method of Lowry et al. (Lowry et al., 1951), and enzyme activity was normalized to protein content and expressed as μmol ADP mg^{-1} protein h^{-1}.

Series 7

The effects of forskolin on maximal gill Na^+/K^+-ATPase activity (V_{max}) and apparent affinity for Na^+ and K^+ (K_m) were established after incubating gill blocks with or without 10 μmol l^{-1} forskolin for 40 min. No phosphatase inhibitors were used in this series, because series 6 showed that their presence did not influence enzymatic activities or the effects

of forskolin. The apparent K_m for K^+ was established by performing the enzyme assay (same assay conditions as in series 6) with varying concentrations of K^+ (0–30 mmol l^{-1}) while keeping Na^+ concentration constant (54 mmol l^{-1}) and *vice versa* for apparent K_m for Na^+ ([Na^+] 0–100 mmol l^{-1}; [K^+] 15 mmol l^{-1}). This was followed by hyberbolic Michaelis–Menten curve-fitting and V_{max} and K_m analyses (Microcal Origin ver. 4.10, Northampton, MA, USA).

Series 8

To ascertain that forskolin treatment induced elevated cyclic AMP levels, cyclic AMP was measured in gill tissue in a separate series. Gill tissue blocks (30–40 mg wet mass) were incubated in Ringer with or without forskolin (10 μmol l^{-1}) for 40 min. Following homogenization in 0.1 mol l^{-1} HCl using a Polytron homogenizer, the crude homogenate was centrifuged (13 000 ***g*** for 15 min), and a sample of the supernatant was used for protein determination (Lowry et al., 1951). Another sample (0.5 ml) of the supernatant was dried overnight in a Speedyvac centrifuge, resuspended in 1 ml of assay buffer and centrifuged (13 000 ***g*** for 15 min). Cyclic AMP was analyzed in the supernatant using a commercial enzyme immunoassay (EIA) (Amersham Pharmacia Biotech, Uppsala, Sweden) following the manufacturer's protocol, and [cyclic AMP] was calculated relative to tissue protein content (pmol cyclic AMP mg^{-1} protein).

Statistical analyses

In series 1, the data were analyzed by a randomized one-way block analysis of variance (ANOVA) and subsequently compared using the Tukey HSD procedure. Data from series 3, 4 and 6 were analyzed for main factor effects using a randomized two-way block ANOVA. In series 2, 5, 7 and 8, the data were analyzed using a paired Student's *t*-test. All statistical analyses were carried out using Systat (Evanston, IL, USA), and significance was accepted when $P<0.05$.

Results

The *in vitro* Rb^+ uptake was linear for at least 30 min in gill (Fig. 1A) and kidney samples (results not shown) and isolated gill cells (Fig. 1B), and was clearly inhibited by the addition of 1 mmol l^{-1} ouabain. The ouabain-sensitive component of the Rb^+ uptake amounted to 50 % of the total uptake in gill samples and 85 % in isolated gill cells and kidney samples of FW-acclimated fish. Ouabain-sensitive uptake was approximately 10 times faster in kidney than in gill tissue, matching a similar ratio between the enzymatic activities (V_{max}) routinely measured in homogenates of the two tissues in FW-acclimated trout.

The lipid-permeant cyclic AMP analogue dbcAMP induced an overall, concentration-related inhibition of the rate of ouabain-sensitive Rb^+ uptake ($P<0.01$) in gill tissue; a significant effect was seen at 2.0 mmol l^{-1} (series 1; Fig. 2). The two concentrations tested resulted in 12 % (0.5 mmol l^{-1}) and 25 % (2.0 mmol l^{-1}) inhibition of the rate of ouabain-

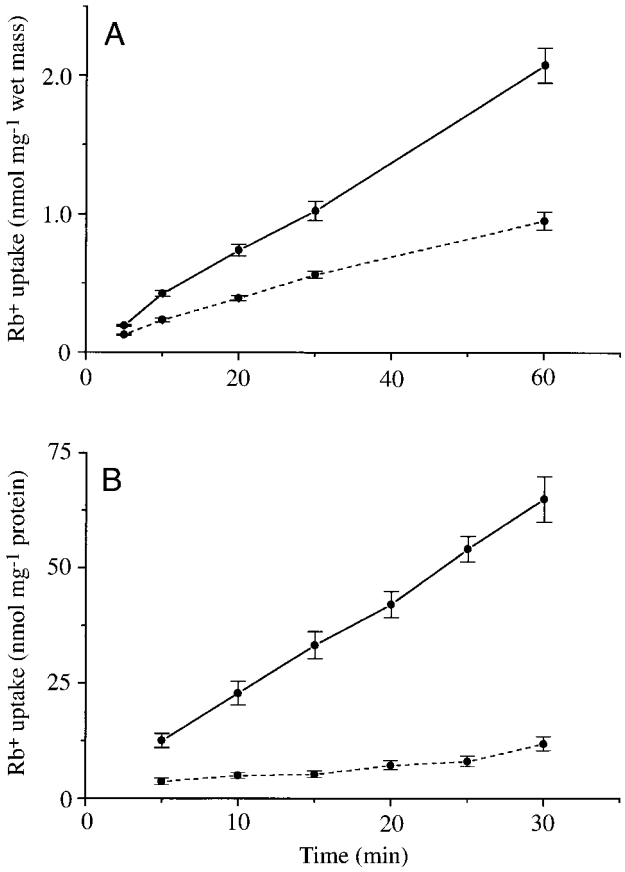

Fig. 1. Time course of Rb^+ uptake by samples of gill tissue (A) and by isolated gill cells (B) from freshwater-acclimated brown trout. The samples were incubated either without (solid line) or with (dashed line) 1 mmol l^{-1} ouabain, including a 10 min pre-incubation period for the gill samples. Values are means ± S.E.M. (N=6, except in gill tissue without ouabain, for which N=12).

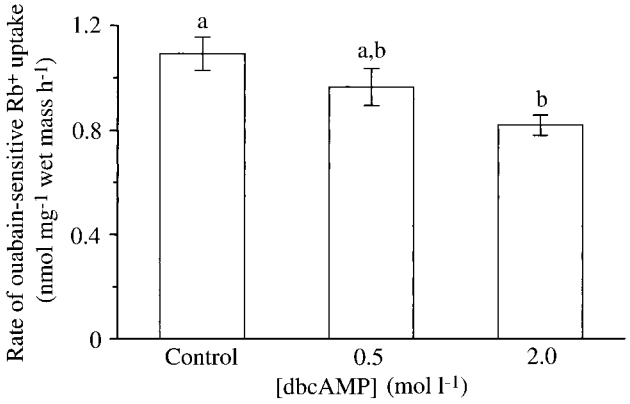

Fig. 2. Effects of 0.5 and 2.0 mmol l^{-1} dibutyryl cyclic AMP (dbcAMP) on the rate of ouabain-sensitive Rb^+ uptake by samples of gill tissue from freshwater-acclimated brown trout. Different letters indicate a significant difference ($P<0.05$). dbcAMP has an overall inhibitory effect on ouabain-sensitive Rb^+ uptake ($P<0.01$). Values are means ± S.E.M. (N=12).

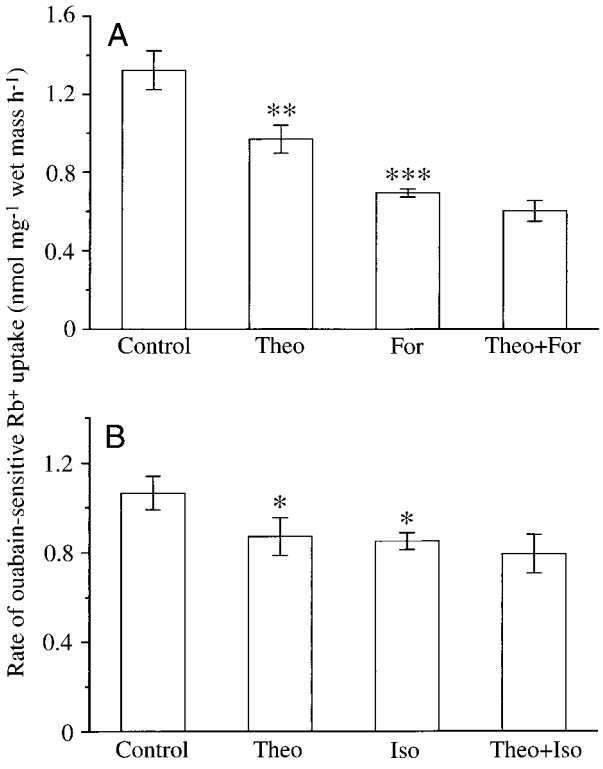

Fig. 3. Effects of 10 μmol l^{-1} forskolin (For), 1 mmol l^{-1} theophylline (Theo) (A) and 10 μmol l^{-1} isoproterenol (Iso) and 1 mmol l^{-1} theophylline (B) on the rate of ouabain-sensitive Rb^+ uptake by samples of gill tissue from freshwater-acclimated brown trout. Asterisks indicate an overall effect of the treatment (*P<0.05; **P<0.01; ***P<0.001; there was no significant interaction). All three agents inhibit ouabain-sensitive Rb^+ uptake. Values are means ± S.E.M. (N=8).

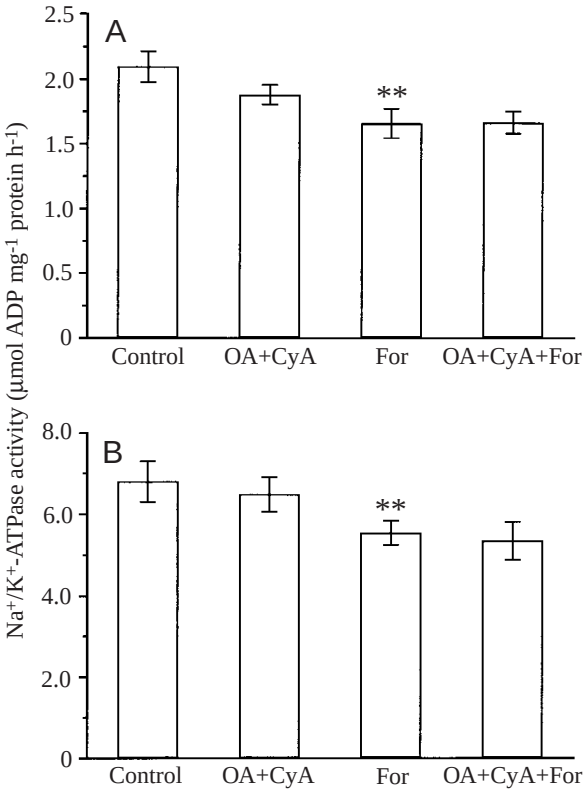

Fig. 4. Effects of 10 μmol l^{-1} forskolin (For) on the maximal Na^+/K^+-ATPase activity in gill samples of freshwater-acclimated (A) and seawater-acclimated (B) brown trout. Following incubation, gill samples were homogenized and assayed in the presence or absence of the two protein phosphatase inhibitors okadaic acid (OA) and cyclosporin A (CyA). Asterisks indicate an overall effect of the treatment (**P<0.01; there was no significant interaction). Forskolin (For) inhibits Na^+/K^+-ATPase activity in gills from freshwater- and seawater-acclimated trout; OA and CyA do not affect enzymatic activity. Values are means ± S.E.M. (N=8).

sensitive Rb^+ uptake. dbcAMP (2.0 mmol l^{-1}) induced an inhibition of ouabain-sensitive Rb^+ uptake (P<0.05) of similar magnitude in isolated gill cells (series 2; control, 136.6±16.6 nmol mg^{-1} protein h^{-1}; dbcAMP, 113.8±18.3 nmol mg^{-1} protein h^{-1}; means ± S.E.M., N=8).

To investigate further the inhibitory effects of cyclic AMP found in series 1 and 2, the adenylate cyclase activator forskolin and the phosphodiesterase inhibitor theophylline were tested in series 3. Both agents induced an overall inhibition of ouabain-sensitive Rb^+ uptake in gill tissue (Fig. 3A; theophylline P<0.01; forskolin P<0.001; no significant interaction).

To investigate the roles of different agonists involved in cyclic-AMP-mediated inhibition of *in situ* Na^+/K^+-ATPase activity, the β-adrenergic agonist isoproterenol was tested in combination with theophylline in series 4. Both agents induced an overall inhibition of ouabain-sensitive Rb^+ uptake in gill tissue (Fig. 3B; P<0.05, no significant interaction).

The inhibitory effect of cyclic AMP was not restricted to gill tissue: series 5 demonstrated that forskolin inhibited ouabain-sensitive (P<0.05) Rb^+ uptake in kidney tissue (control, 26.2±3.1 nmol mg^{-1} wet mass h^{-1}; forskolin, 18.5±1.9 nmol mg^{-1} wet mass h^{-1}; means ± S.E.M., N=8).

In all the experiments described above, the inhibitory effects of cyclic AMP on Rb^+ uptake could theoretically have been mediated by reductions in the intracellular Na^+ concentration rather than by cyclic-AMP-mediated modulation of the Na^+ pump. To discriminate between these possibilities, we carried out another series of experiments in which the effects of forskolin on the maximal Na^+/K^+-ATPase activity (V_{max}) of gill tissue were tested. Incubation with forskolin for 40 min significantly reduced V_{max} compared with control values when subsequently tested in homogenized tissue using optimal (V_{max}) assay conditions (series 6; Fig. 4; P<0.01). The effect was present in gills from both FW- and SW-acclimated fish. Enzymatic activity was unaffected by the presence of the two protein phosphatase inhibitors okadaic acid and cyclosporin A, and there was no significant interaction effect (P>0.05).

To investigate further the effects of forskolin on the Na^+/K^+-ATPase, both V_{max} and apparent K_m for K^+ and Na^+ were analyzed in gill tissue incubated for 40 min with or without

Table 1. *Effects of 10 μmol l^{-1} forskolin on enzymatic Na$^+$/K$^+$-ATPase activity in gill samples of freshwater- and seawater-acclimated brown trout*

	V_{max} (μmol ADP mg^{-1} protein h^{-1})	K_m for K$^+$ (mmol l^{-1})	K_m for Na$^+$ (mmol l^{-1})
Freshwater-acclimated			
Control	2.31±0.17	0.98±0.13	9.30±0.88
Forskolin	1.85±0.07*	1.29±0.20	8.73±0.95
Seawater-acclimated			
Control	8.07±0.44	1.13±0.09	8.31±0.29
Forskolin	6.46±0.27**	1.41±0.06*	7.94±0.29

Following incubation, gill samples were homogenized and assayed with different ion concentrations, and V_{max} and the apparent K_m for K$^+$ and Na$^+$ were determined.

An asterisk indicates a significant effect of treatment (*P<0.05, **P<0.01).

Values are means ± S.E.M. (N=8).

forskolin (series 7; Table 1). In this experiment, forskolin inhibited V_{max} in the gills of FW- and SW-acclimated fish and also increased the apparent K_m for K$^+$ (significant only for SW-acclimated fish). The apparent K_m for Na$^+$ was unaltered.

Series 8 showed the expected increase in cyclic AMP levels in gills incubated with forskolin *in vitro* (control, 13.9±1.3 pmol cyclic AMP mg^{-1} protein; forskolin, 540.2±49.8 pmol cyclic AMP mg^{-1} protein; P<0.01; N=4).

Discussion

This is the first study to identify rapid cyclic-AMP-mediated modulation of Na$^+$/K$^+$-ATPase activity in the gill and kidney of a euryhaline teleost. In the gill, cyclic AMP inhibits both ouabain-sensitive Rb$^+$ uptake (*in situ* Na$^+$/K$^+$-ATPase activity) and maximal enzymatic activity (V_{max}) and reduces the apparent affinity for K$^+$. In the kidney, a similar inhibition of *in situ* Na$^+$/K$^+$-ATPase activity is seen.

Validation of the Rb$^+$ uptake assay

A non-radioactive assay to study *in situ* Na$^+$/K$^+$-ATPase activity (Longo et al., 1991) was adapted to fish tissues and cell suspensions. In intact gill tissue, Rb$^+$ uptake is linear for 60 min and inhibited by 50 % by ouabain (Fig. 1A). The degree of inhibition is stable during the 60 min incubation, indicating that the Na$^+$ pump was maximally inhibited following pre-incubation for 10 min with ouabain. The constant levels of Na$^+$ and K$^+$ in the tissue during the incubation period (results not shown) indicate that the tissue is indeed able to maintain overall ionic homeostasis and, hence, is viable during the incubation period, even though we cannot exclude the possibility that minor ion movements between intra- and extracellular compartments may occur. In isolated gill cells, Rb$^+$ uptake is linear for at least 30 min and is approximately 85 % inhibited by ouabain (Fig. 1B). By choosing a routine assay period well within the linear uptake period (30 min for gill tissue; 10 min for isolated gill cells), we ensured that initial velocities were measured.

Effects of manipulating [cyclic AMP] on in situ *Na$^+$/K$^+$-ATPase activity*

The results of this study indicate that activation of the cyclic-AMP-protein kinase A (PKA) pathway inhibits Na$^+$/K$^+$-ATPase activity in the gill and kidney of the euryhaline brown trout *Salmo trutta*. In the gill of FW-acclimated trout, a significant inhibition of *in situ* Na$^+$/K$^+$-ATPase activity (i.e. ouabain-sensitive Rb$^+$ uptake) is induced by several agents presumed to increase intracellular [cyclic AMP]. Indeed, both the addition of exogenous dbcAMP (Fig. 2) and increasing endogenous cyclic AMP levels, by inhibiting phosphodiesterase with theophylline and activating adenylate cyclase with forskolin (Fig. 3), result in significant inhibition of the Na$^+$ pump. The same result is obtained in response to β-adrenergic stimulation with isoproterenol (Fig. 3), which generally acts through G-protein-dependent stimulation of the adenylate cyclase (Levitzki, 1988). The inhibiting effect of forskolin is not restricted to gill tissue: kidney tissue from FW-acclimated brown trout also shows a significant inhibition of pump activity when treated with forskolin.

Effects of manipulating [cyclic AMP] on Na$^+$/K$^+$-ATPase activity (V_{max}) and apparent ion affinity (K_m)

It is not evident from the Rb$^+$ uptake experiments whether the inhibition of the Na$^+$/K$^+$-ATPase depends on modulation of the intracellular Na$^+$ concentration, which is a potentially important factor in determining actual *in situ* Na$^+$ pump activity. Incubation of gill tissue with forskolin, however, significantly inhibits maximal Na$^+$/K$^+$-ATPase enzymatic activity (V_{max}) measured in homogenates from both FW- and SW-acclimated brown trout (Fig. 4; Table 1). This treatment increases cyclic AMP concentration approximately 40-fold and also lowers the apparent affinity of the enzyme for K$^+$ (significant for SW-acclimated fish only), whereas Na$^+$ affinity is unaltered (Table 1). The result of this different experimental approach with controlled ion concentrations allows us to conclude that at least part of the effect is mediated through an action on the enzyme itself. As expected, enzymatic activity

was higher in the gills of SW-acclimated than FW-acclimated fish, reflecting the qualitative difference in gill function in the two media (see McCormick, 1995). However, the inhibitory effect was present in both FW- and SW-acclimated fish, suggesting a general effect on the enzyme that is independent of its exact cellular location and function in ion uptake or ion extrusion, respectively. Okadaic acid and cyclosporin A were added to the homogenization and assay buffers in series 6 to inhibit protein phosphatase activity (Bialojan and Takai, 1988; Fruman et al., 1992). The presence of these inhibitors alone and in combination with forskolin, however, had no significant effect on enzyme activity, indicating that the forskolin-mediated modulation was stable during the assay procedure.

Mechanism, significance and perspective

There is sound evidence that the activation of PKA by cyclic AMP modulates Na^+/K^+-ATPase activity in a variety of vertebrate tissues. The mechanism has been reported to involve changes in both V_{max} and the substrate affinity of the enzyme (e.g. Fisone et al., 1994). Activation of PKA has been shown to inhibit (rat cortical collecting duct, Satoh et al., 1992; rat aortic smooth muscle, Borin, 1995; COS cells, Cheng et al., 1997; pig endothelial cells, Gruwel et al., 1998; ground squirrel skeletal muscle, MacDonald and Storey, 1999) and to stimulate (rat skeletal muscle, Clausen and Flatman, 1977; shark rectal gland, Marver et al., 1986; rat proximal convoluted tubule, Carranza et al., 1998) Na^+/K^+-ATPase activity. The observed discrepancies could relate to tissue-specific differences or to different experimental procedures. In COS cells, for instance, Cheng et al. (Cheng et al., 1999) demonstrated that intracellular $[Ca^{2+}]$ may be decisive for the observed effect. They found that inhibition by PKA of Na^+/K^+-ATPase activity at low intracellular $[Ca^{2+}]$ (120 nmol l^{-1}) was reversed into stimulation at higher intracellular $[Ca^{2+}]$ (420 nmol l^{-1}). The variability in observed responses to PKA may also result from disparate regulatory pathways in different cell systems. Thus, the inhibition by PKA of Na^+/K^+-ATPase activity in COS cells associated with phosphorylation of the α-subunit (Cheng et al., 1997) is unrelated to internalization of the enzyme (Andersson et al., 1998). Conversely, stimulation of Na^+/K^+-ATPase activity by PKA in rat proximal convoluted tubules seems to be related to recruitment of Na^+/K^+-ATPase from endosomes (Carranza et al., 1998).

Several possible mechanisms for the regulation of the Na^+/K^+-ATPase by PKA have been proposed. These include trafficking between endosomes and the basolateral membrane (Carranza et al., 1998), activation of arachidonic acid metabolism (Satoh et al., 1992) and direct phosphorylation of the enzyme, affecting its activity (Cheng et al., 1997). The functional significance of direct phosphorylation is supported by several lines of evidence. A site for PKA phosphorylation has been identified on the α-subunit at the serine-943 location (Beguin et al., 1994; Fisone et al., 1994). Direct phosphorylation of the purified α-subunit of the Na^+/K^+-ATPase by PKA *in vitro* affects enzyme activity (Bertorello et al., 1991; Cornelius and Logvinenko, 1996), and phosphorylation by PKA in intact cells is also accompanied by modulation of enzyme activity (Cheng et al., 1997).

In contrast to experiments in mammals, very few studies have reported rapid modulation of Na^+/K^+-ATPase activity in fish tissues. Because of the high permeability of the respiratory branchial epithelium, fish are constantly threatened by diffusional ion loss or ion uptake in hypo- and hyperosmotic surroundings, respectively. At the same time, the gill epithelium is the site for active, compensatory ion transport. Modification of the enzyme level has adaptive significance and is well known in situations in which euryhaline teleosts move between changing salinities (McCormick, 1995). This response normally takes several days, suggesting that transcriptional regulation is involved. Because of the central importance of Na^+/K^+-ATPase activity for both ion uptake and ion excretion, one would expect that, in addition to long-term regulation, rapid short-term regulation of the enzyme must also have a large potential for adjusting compensatory branchial ion transport. Towle et al. (Towle et al., 1977) have reported a case of rapid (<0.5 h) modulation of Na^+/K^+-ATPase activity (V_{max}) in the gills of *Fundulus heteroclitus* induced by salinity changes. This was recently confirmed by Mancera and McCormick (Mancera and McCormick, 2000), although Jacob and Taylor (Jacob and Taylor, 1983) were unable to confirm this. A few studies have reported *in vitro* modulation of fish Na^+/K^+-ATPase activity by pharmacological and hormonal agents. Activation of protein kinase C has been shown to inhibit Na^+/K^+-ATPase activity in isolated chloride cells from the gill of the marine cod *Gadus morhua* (Crombie et al., 1996). Angiotensin II modulates gill and kidney Na^+/K^+-ATPase activity in the eel *in vitro* (Marsigliante et al., 1997; Marsigliante et al., 2000). Studies on the rectal gland of the shark have demonstrated a stimulation of Na^+/K^+-ATPase activity by cyclic-AMP-mediated PKA activation (Marver et al., 1986). Phosphorylation of the α-subunit of Na^+/K^+-ATPase by PKA *in vitro*, using purified Na^+/K^+-ATPase from shark rectal gland, inhibits (Bertorello et al., 1991) or activates (Cornelius and Logvinenko, 1996) enzyme activity.

The present study reports a rapid cyclic-AMP-mediated regulation of Na^+/K^+-ATPase activity in the gill and kidney of a salmonid. Interestingly, our data agree with a recent report (Lucu and Flik, 1999) of rapid inhibitory regulation of Na^+/K^+-ATPase through the cyclic AMP/PKA pathway in the gill of the shore crab *Carcinus maenas*. Even though the exact cellular and molecular mechanisms are unknown, the present investigation suggests that regulatory phosphorylation of the Na^+/K^+-ATPase may be involved in teleosts, as in mammals. In accordance with this, the potential PKA phosphorylation site on the α-subunit is present in two teleosts (*Catostomus commersoni*, Schönrock et al., 1991; *Anguilla anguilla*, Cutler et al., 1995).

The precise physiological significance of the present effects can only be speculated upon. Even though no case of a rapid change in enzymatic activity has been reported following salinity adjustments in the brown trout or any other salmonid (Madsen et al., 1995; Mancera and McCormick, 2000), rapid

modulation of branchial and renal ion fluxes may be important in situations such as feeding, exercise, acute salinity shifts and stress. A few studies have, however, investigated rapid modulation of monovalent ion fluxes in teleost gills from a physiological perspective. In agreement with the present results, stress induces ionic imbalance in both FW- and SW-acclimated fish (Avella et al., 1991), and the impaired ionic regulation may well be related to β-adrenergic inhibition of gill Na^+/K^+-ATPase. In line with this suggestion, adrenaline causes an inhibition of Na^+ and Cl^- excretion in SW-acclimated trout (Girard, 1976). In the FW-acclimated perfused trout head, however, β-adrenergic agonists inhibit Cl^- uptake and stimulate Na^+ uptake, whereas α-adrenergic agonists have the reverse effect on Cl^- uptake (Payan et al., 1975; Perry et al., 1984).

In conclusion, cyclic-AMP-mediated inhibition of Na^+/K^+-ATPase has been demonstrated in the gill and kidney of FW- and SW-acclimated brown trout. The inhibition affects V_{max} and the apparent K^+ affinity in gill tissue, suggesting a direct, substrate-independent mechanism. Future studies should investigate how Na^+/K^+-ATPase activity is modulated in different cell types because, in the gill in particular, salinity (and developmental hormones) induces a significant rearrangement of Na^+/K^+-ATPase-rich ionocytes between the lamellar and primary filament epithelia (Seidelin et al., 1999).

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