# Effect of osmotic shrinkage and hormones on the expression of Na⁺/H⁺ exchanger-1, Na⁺/K⁺/2Cl⁻ cotransporter and Na⁺/K⁺-ATPase in gill pavement cells of freshwater adapted Japanese eel, *Anguilla japonica*

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## Summary

It is well-known that gill epithelial cells are important in fish osmoregulation. However, studies on the effect of osmotic stress on the direct cellular responses of the gill epithelial cells are limited. In this paper, we aimed to determine the effects of osmotic hypertonicity, hormones and cellular signaling molecules on the expression of ion transporters in the cultured primary freshwater pavement cells (PVCs), prepared from freshwater-adapted eels (*Anguilla japonica*). Our data demonstrated that the hypertonic (500 mOsmol l⁻¹) treatment of the isolated PVCs induced cell shrinkage, followed by regulatory volume increase (RVI). Application of blockers (i.e. ouabain, bumetanide and EIPA) demonstrated that Na⁺/K⁺-ATPase, Na⁺/K⁺/2Cl⁻ cotransporter (NKCC) and Na⁺/H⁺ exchanger-1 (NHE-1) were involved in RVI. Western blot analysis of the hypertonic-treated cells revealed a significant induction of NHE-1, NKCC and, α and β subunits of Na⁺/K⁺-ATPase. In nonshrunken cultured PVCs, we found that dexamethasone and dibutyryl cAMP treatments significantly stimulated the expression levels of the three ion transporters. Both prolactin and insulin-like growth factor-1, can only induce the expression of NKCC. The effect of thyroid hormone (T₃) and dibutyryl cGMP was negligible. In this study, the induction of ion transporter expression was found to be post-transcriptionally regulated as no significant change in mRNA levels was detected. This observation implies that the regulation is rapid and is probably induced *via* nongenomic actions.

Key words: dexamethasone, hypertonic stress, dibutyryl cAMP, regulatory volume increase.

## Introduction

Eel (*Anguilla japonica*) is a euryhaline fish. To acclimate to the wide range of salinities during migration, the cellular and molecular reorganization of gill epithelia is essential for osmoregulation (Evans et al., 2005; Wong and Chan, 1999a). It had been reported in trout that during seawater transfer, transient increase in plasma osmolarity imposes hypertonic stress, which causes the shrinkage of red blood cells (Brauner et al., 2002). In addition, the drinking response in seawater-acclimating fish induced cell shrinkage in intestinal epithelia (Ando et al., 2003; Lionetto et al., 2001). Under the same circumstances, it is anticipated that the gill epithelium, which is in direct contact with water, would be exposed to the large osmotic fluctuations in the surrounding water. Surprisingly, there has been no study to address the effect of the hypertonic stress on the disturbance of gill cell volume.

Cell volume regulation is a fundamental and highly conserved cellular process found in most cells (Chamberlin and Strange, 1989; Lang et al., 1998b; Lang et al., 1998a). It is particularly important in euryhaline fish as they may require a rapid and effective mechanism to respond to the changing water salinity. The capability of gill epithelial cells to maintain a constant intracellular osmotic conditions is critical for normal cell functioning. As the gill is one of the major osmoregulatory organs in fish, the ability of the gill epithelial cells to recover from osmotic shrinkage/swelling, may relate to fish euryhalinity.

In vertebrates, hormones and growth factors play a critical role in cell volume regulation (Hoffmann and Dunham, 1995; Pedersen et al., 2006). In teleost fish, a number of key hormones have been identified as essential for euryhalinity and osmotic adaptability. The osmoregulatory hormones can be classified into two groups: (i) fast-acting hormones (i.e. natriuretic peptides and epinephrine, which are mediated by the action of cGMP and cAMP, respectively), and (ii) slow-acting hormones (i.e. cortisol, growth hormone/insulin-like growth factors and prolactin) (McCormick and Bradshaw, 2006). A considerable number of studies has demonstrated the biological effects of these hormones on gill functions, including the expression/distribution/activities of ion

transporters and the proliferation/differentiation of chloride cells (Evans, 2002; Evans et al., 2005; Perry, 1997; Tse et al., 2006; Wong and Chan, 1999a; Wong and Chan, 1999b). Despite this body of evidence, however, because of the anatomical complexities of the gill epithelium, it is difficult to elucidate the direct hormonal effect as well as the immediate biological responses of the gill epithelial cells, using an *in vivo* approach.

Gill epithelial culture has been established in freshwater trout for the study of unidirectional ion fluxes and for the characterization of ion transporter properties (Kelly et al., 2000; Kelly and Wood, 2001c; Kelly and Wood, 2001a; Kelly and Wood, 2001b; Kelly and Wood, 2002a; Kelly and Wood, 2002b; Kelly and Wood, 2003; O'Donnell et al., 2001; Wood et al., 2002; Zhou et al., 2003; Zhou et al., 2004). A gill cell culture model from other fish species including salmon and eel, however, has not yet been established. In this study, we aimed to investigate the effects of hypertonic stress on the cellular responses of freshwater gill epithelial cells. Using purified pavement cells (PVCs), we demonstrated the effect of osmotic shrinkage on cell regulatory volume increase (RVI) as well as the expressions of the three important ion transporters: $Na^+/K^+$-ATPase, $Na^+/K^+/2Cl^-$ cotransporter (NKCC), $Na^+/H^+$ exchanger-1 (NHE-1) in the cells. The involvement of the three ion transporters in RVI was demonstrated using three specific blockers [i.e. ouabain, bumetanide and 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA)]. In addition, we established the primary epithelial gill culture from freshwater-adapted eels. Using the cultured cells, we measured and compared the direct effects of dexamethasone (DEX), prolactin (PRL), thyroid hormone ($T_3$), insulin-like growth factor-1 (IGF-1), dibutyryl cAMP (dbcAMP) and dibutyryl cGMP (cGMP) to the expressions of the ion transporters.

## Materials and methods
### Cell isolation, sizing and counting

Japanese eels (*Anguilla japonica* Temminck & Schlegel) weighing between 500–600 g, were reared in fibreglass tanks supplied with charcoal-filtered aerated tap-water at 18–20°C under a 12 h:12 h L:D photoperiod for at least 3 weeks. The eels were anaesthetized by 1–2% tricaine methanosulfonate (Sigma, St Louis, MO, USA) and the gill epithelial cells were isolated using a three-step discontinuous Percoll gradient system as described previously (Wong and Chan, 1999b; Tse et al., 2006). Briefly, fish gills were perfused with buffered saline (130 mmol l$^{-1}$ NaCl, 2.5 mmol l$^{-1}$ KCl, 5 mmol l$^{-1}$ NaHCO$_3$, 2.5 mmol l$^{-1}$ glucose, 2 mmol l$^{-1}$ EDTA, 10 mmol l$^{-1}$ Hepes, pH 7.7) to remove blood cells, and the fish were then decapitated. Gill arches were excised, washed and cut into small fragments for tryptic digestion (0.5% trypsin + 5.3 mmol l$^{-1}$ EDTA). The cell suspension was then filtered, washed and finally resuspended in 1.6 g ml$^{-1}$ of Percoll solution for pavement cell (PVC) isolation. The purity and the identity of PVCs was determined by their weak staining of $Na^+/K^+$-ATPase as compared to chloride cells (Tse et al., 2006;

Wong and Chan, 1999b). Isolated PVCs were then washed and resuspended in Leibovitz's L-15 medium (Sigma) supplemented with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin and 0.5% fungizone (Gibco-BRL).

The cells were maintained in the medium for 1 h at room temperature before cell sizing and counting. Thereafter the cells were either resuspended in a normal (317 mOsmol l$^{-1}$) or modified (500 mOsmol l$^{-1}$, adjusted with 5 mol l$^{-1}$ NaCl) Leibovitz's L-15 medium. Blockers for three ion transporters [1 mmol l$^{-1}$ ouabain, 100 μmol l$^{-1}$ bumetanide or 100 μmol l$^{-1}$ 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA); Sigma] were added to the cells before the application of hyperosmotic stress. The medium osmolarity was determined using a vapour pressure osmometer (Wescor, 5500XR, Logan, UT, USA). The two groups of cells were counted and sized using a Coulter Multisizer II [with an orifice tube of 70 μm diameter and Isoton II (Beckamn, Coulter, Miami, FL, USA) as electrolyte] in every 5–10 min interval for 60 min. The cell count signal was the change of conductance of the electrolyte induced by particle resistance. Isoton II was used as the blank and calibration was carried out with monodiameter particles (P.D.V.B. latex 5.06 μm, Beckman Coulter). An aperture coincidence correction was below 2%.

### Primary culture of freshwater gill epithelial cells

The cell suspension obtain from trypsin digestion was filtered and washed. The cells were then seeded at a density of $5\times10^6$ cells cm$^{-1}$ onto collagen-coated culture plate. The cells were incubated at 22°C in a growth chamber with a humidified air atmosphere. One day after seeding, the plate was rinsed to remove mucous cells. Gill epithelial cells cultured for 1 day were then exposed to either hypertonic stress or drug treatments. In preliminary studies, we conducted time-course experiments to investigate the effects of the treatments on the expression of the ion transporters. The cultured cells were incubated in the different conditions for varying periods of time (1, 2, 6, 12 and 24 h). A significant induction of the ion transporter protein was observed after 6 h of hypertonic treatment and only after 24 h of hormonal or cell signaling molecule treatment. No induction of the ion-transporter mRNA was observed. Hence we selected the 6 h treatment for the hypertonic stress (500 mOsmol l$^{-1}$) but 24 h exposure to each of the following drug treatments: (a) 0.5–2 μmol l$^{-1}$ dexamethasone (DEX; Calbiochem, Darmstadt, Germany), (b) 0.1–10 nmol l$^{-1}$ prolactin (PRL; Calbiochem), (c) 0.1–10 nmol l$^{-1}$ insulin-like growth factor-1 (IGF-1; Sigma), (d) 0.05–0.2 μmol l$^{-1}$ thyroid hormone ($T_3$), (e) 1–4 mmol l$^{-1}$ dibutyryl cGMP (cGMP) or (f) 1–4 mmol l$^{-1}$ dibutyryl cAMP (dbcAMP; Calbiochem). For real-time PCR analysis, the isolated gill epithelial cells were dissolved in Trizol Reagent (Gibco-BRL) for total RNA extraction. For western blots, the samples were resuspended in cold lysis buffer containing 250 mmol l$^{-1}$ Tris–HCl, pH 8.0, 1% NP-40 and 150 mmol l$^{-1}$ NaCl and were assayed for protein concentration (DC Protein Assay Kit II, Bio-Rad Pacific Ltd, Hercules, CA, USA).
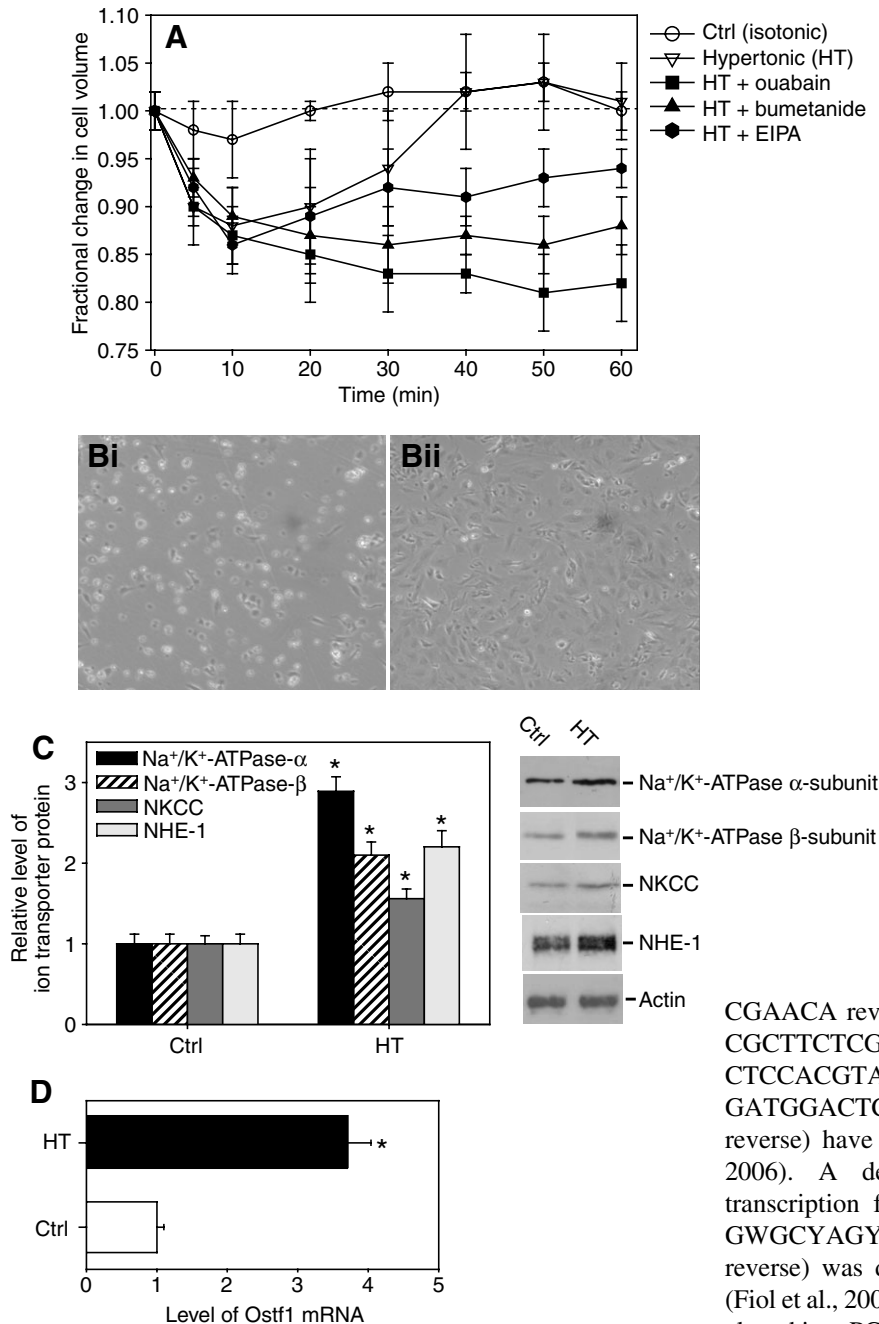
Fig. 1. Hypertonicity-induced cell volume change and the expression of Na+/K+-ATPase, Na+/K+/2Cl− cotransporter (NKCC) and Na+/H+ exchanger-1 (NHE-1) in freshwater gill pavement cells (PVCs). (A) Percoll-purified PVCs were incubated in either normal (317 mOsmol l−1) or hypertonic (500 mOsmol l−1) medium. Blockers for three ion transporters (ouabain, bumetanide or EIPA) were added to the cells before the application of hyperosmotic stress. The change in cell volume was monitored using a Multisizer for a period of 60 min. Note that the three blockers reduced the RVI response in the cells. (B) A primary freshwater PVC culture was established. Over 80% of gill epithelial cells attached after overnight incubation (Bi); the cell suspension was obtained from trypsin digestion. (Bii) Gill epithelial cells cultured for 1 day. (C) Cell lysates were obtained from 6 h hypertonic treatment (HT) of the PVC culture and analysed by western blot (right). The amounts of the respective ion transporter protein were quantified and tabulated in graphical form (left). Significant induction of α and β subunits of Na+/K+-ATPase, NKCC and NHE-1 were observed. (D) The gene expression levels for Ostf1 in the control and hypertonic-treated cells. *$P<0.05$ compared with the control. The results were obtained from more than five independent experiments.

CGAACA reverse), Na+/H+ exchanger-1 (NHE-1, AJ006917: CGCTTCTCGTCTTCGTCTACAG forward, CATGTTGGC-CTCCACGTATGG reverse), and actin (CTGGTATCGT-GATGGACTCT forward, AGCTCATAGCTCTTCTCCAG reverse) have been tested in our previous study (Tse et al., 2006). A degenerative primer set for osmotic stress transcription factor 1 (Ostf1, AY679524: AGTGCCTCYG-GWGCYAGYGT forward, TGGAACTTYTCCAGYTGYTC reverse) was designed according to the published sequences (Fiol et al., 2006; Fiol and Kultz, 2005). The PCR products were cloned into PCRII-TOPO (Invitrogen, Carlsbad, CA, USA) and subjected to dideoxy sequencing for verification. The copy number of the transcripts for each sample was calculated in reference to the parallel amplifications of known concentrations of the respective cloned PCR fragments. Standard curves were constructed and the amplification efficiencies were about 0.9–0.95. The occurrence of primer-dimers and secondary products was examined using melting curve analysis. Our data indicated that the amplification was specific. There was only one PCR product amplified for each individual set of primers. Control amplification was done either without reverse transcriptase or without RNA. We had determined the actin mRNA levels per same quantity of cDNA from PVCs exposed to the different treatments. The mRNA expression levels were comparable with the threshold cycle (Ct) values of around

### Real-time PCR

Purified sample RNA with a ratio of 1.8–2.0 at $A_{260}/A_{280}$ was used. Briefly, 0.5 μg of total cellular RNA was reversed transcribed (iScript, Bio-Rad). PCR reactions were conducted with the iCycler iQ real-time PCR detection system using iQ™ SYBR® Green Supermix (Bio-Rad). Primers for Na+/K+-ATPase α-subunit (X76108: TCTGATGTCTCCAAGCAGGC forward, CTGGTCAGGGTGTAGGC reverse) and β233-subunits (AJ239317: ATGTCAGGAAATAAAGACAGT forward, TGCGTGGGTTTGTAGTTGCTCA reverse), Na+/K+/2Cl− co-transporter-1 (NKCC-1; AJ486858: CCCATCA-TCTCCAACTTCTTCCT forward, CCCACCAGTTGATGA-

20–21 cycles. Hence in this study the expression levels of actin mRNA were used for data normalization.

### Western blot analysis

Samples were subjected to electrophoresis in 10% polyacrylamide gels. The gels were then blotted onto PVDF membranes (PerkinElmer Life Sciences, Boston, MA, USA). Western blot was conducted using mouse antibodies against Na$^+$/K$^+$-ATPase α (α5; 1:100), β-subunits (β4B4; 1:100), NKCC (T4; 1:100; Developmental Studies Hybridoma Bank, University of Iowa) and NHE-1 (1:1000; Chemicon Int., Temecula, CA, USA), followed by incubation with (1:4000) horseradish peroxidase-conjugated goat anti-mouse antibody. Specific bands were visualized using chemiluminescent reagent (Western-lightening Plus, PerkinElmer Life Sciences). The blots were then washed in phosphate-buffered saline (PBS) and re-probed with (1:100) mouse anti-actin serum (JLA20, Developmental Studies Hybridoma Bank, the University of Iowa, IA, USA). Images of the blots were digitally captured by a gel documentation system (UVP). The optical density of each band was quantified using image analyzing software (Metamorph, Universal Imaging Corp.). The data were then normalized using the expression levels of actin.

### Statistical analysis

All data are represented as means ± s.e.m. Statistical significance was tested using Student's t-test. Groups were considered significantly different if P<0.05.

### Results

#### Effect of hypertonic treatment on the cell size and the expressions of NHE-1, NKCC and Na$^+$/K$^+$-ATPase in PVCs

We conducted preliminary studies to test the viability of the gill epithelial cells maintained in different hypertonic media, with osmolarities of 500, 700 and 900 mOsmol l$^{-1}$. Significant cell death was observed in media of 700 and 900 mOsmol l$^{-1}$, as evaluated by Trypan Blue exclusion test. The viability of the gill epithelial cells maintained in 500 mOsmol l$^{-1}$ was similar to the control (317 mOsmol l$^{-1}$). Hence the osmolarity of the hypertonic medium used in the subsequent experiments was 500 mOsmol l$^{-1}$. Multisizer counting demonstrated that the hypertonic-treated PVCs decreased in size significantly in 5 min, by about 13%. Regulatory volume increase (RVI) was induced afterwards as the cell size recovered and stabilized at about 30–40 min post-treatment (Fig. 1A). All three blockers reduced the RVI response in the cells. Bumetanide or ouabain treatment produced the most remarkable effect. There was no significant change in the size of the cells that were maintained in the control medium.

Primary freshwater gill epithelial cell culture was established (Fig. 1B). Over 80% of gill epithelial cells attached after overnight incubation. In the culture, the percentage of chloride cells was very low (about 1%). The major of the cell population was found to be the PVCs. Using the cell culture model, we tested the effect of hypertonic stress and hormones/cell signaling
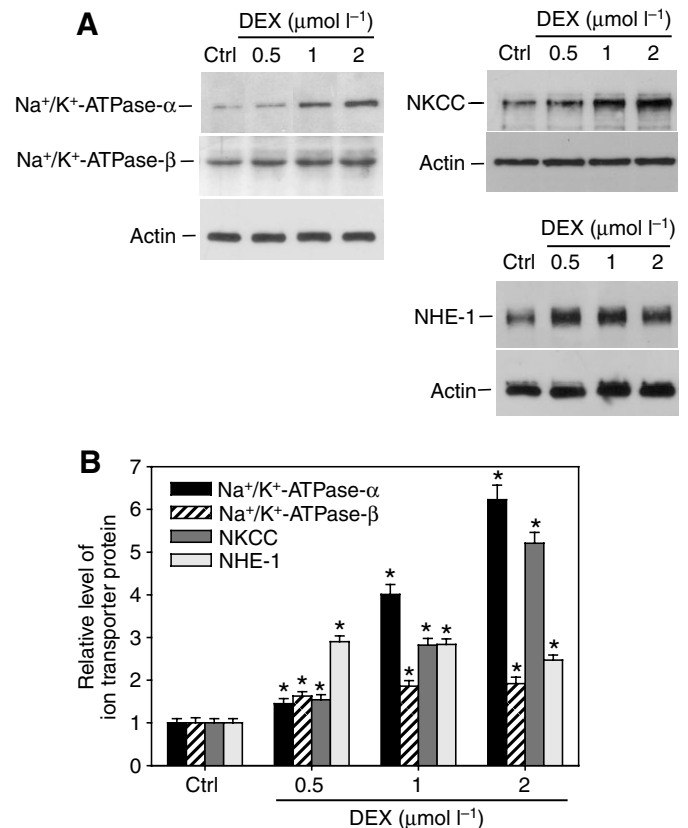


Fig. 2. Effect of DEX on the expression of Na$^+$/K$^+$-ATPase, Na$^+$/K$^+$/2Cl$^-$ cotransporter (NKCC) and Na$^+$/H$^+$ exchanger-1 (NHE-1) in pavement cells (PVCs) in culture. The cells were exposed to 0.5–2 μmol l$^{-1}$ DEX for 24 h. (A) Cell lysates were collected for western blot analysis and (B) the amounts of the respective ion transporter proteins were quantified. Note that the three transporters were significantly induced. *P<0.05 compared with the control. The results were obtained from more than four independent experiments.

molecules on the expression levels of the three ion transporters (i.e. NHE-1, NKCC, α and β subunits of Na$^+$/K$^+$-ATPase). Our data indicated that hypertonic treatment in PVC cultures for 6 h notably stimulated the expression of the ion transporter proteins (Fig. 1C). Comparatively, the catalytic subunit of Na$^+$/K$^+$-ATPase, α-subunit was induced threefold. The β-subunit and NHE-1 were induced twofold, whereas NKCC increased by about 0.7 fold. Expression of Na$^+$/K$^+$-ATPase α- and β-subunits was stimulated, suggesting the induction provided the subunits required for making the functional enzymes. There was no significant change in the transcript levels of the ion transporters (data not shown). However about 3.5-fold induction of Osft1 mRNA was observed at 6 h post-treatment (Fig. 1D).

#### Effect of the hormones (DEX, prolactin, T$_3$ and IGF-1), dbcAMP and dbcGMP on the expression of ion transporters in the primary PVC culture

DEX-exposed PVCs showed dose-dependent induction of Na$^+$/K$^+$-ATPase α- and β-subunits, NKCC and NHE1 as revealed by western blotting (Fig. 2). Among those, the
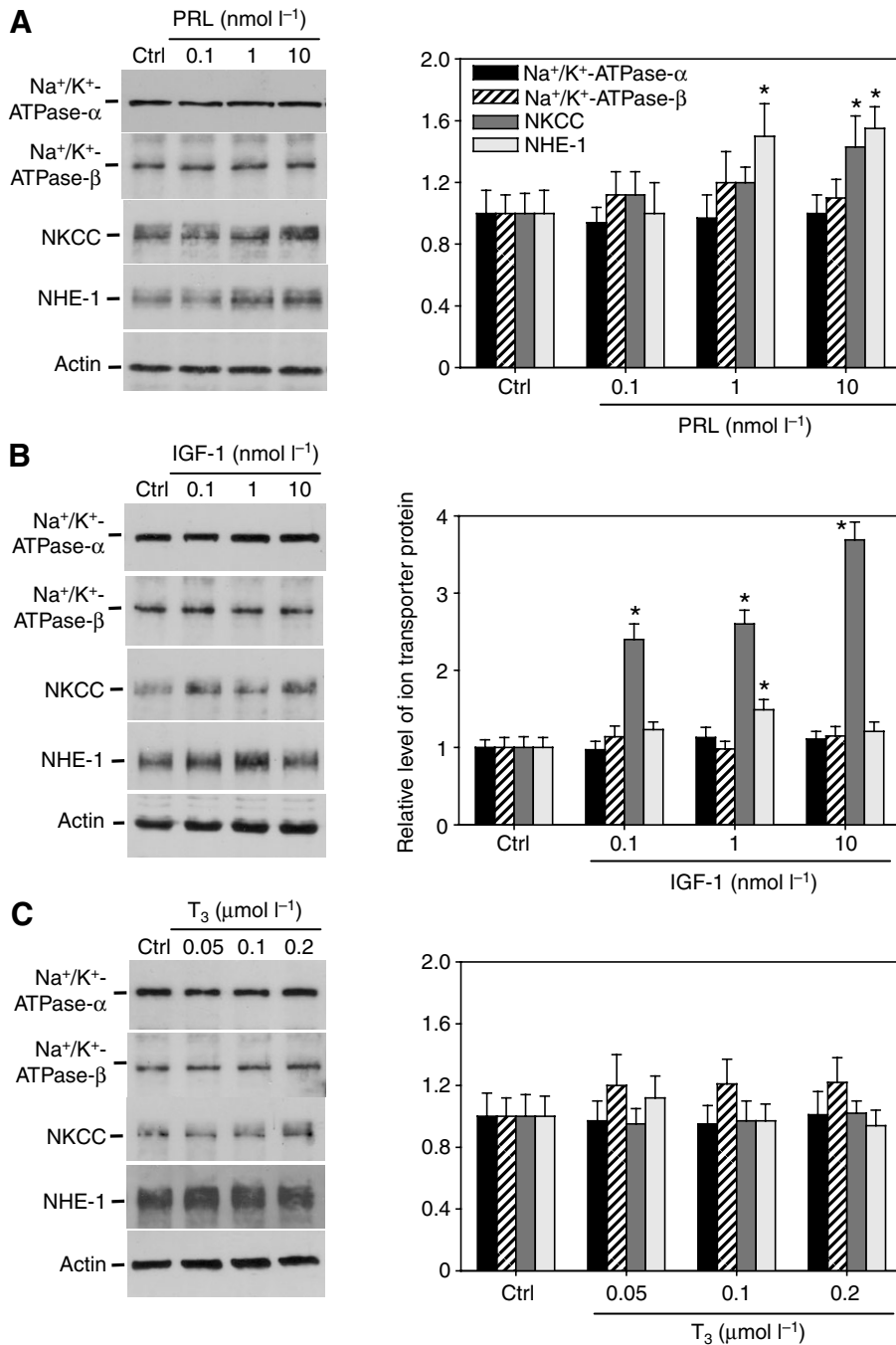
**A**



**B**



**C**



Fig. 3. Effect of prolactin (PRL), thyroid hormone (T$_3$) or insulin-like growth factor-1 (IGF-1) on the expression of Na$^+$/K$^+$-ATPase, Na$^+$/K$^+$/2Cl$^-$ cotransporter (NKCC) and Na$^+$/H$^+$ exchanger-1 (NHE-1) in pavement cells (PVCs) in culture. The cells were exposed to the respective hormones for 24 h. Cell lysates were collected for western blot analysis (left) and the amounts of the respective ion transporter proteins were quantified (right). (A) PRL at high dose stimulated the expression of NKCC and NHE-1. (B) IGF-1 elicited a dose-dependent induction of NKCC. (C) T$_3$ had no observable effect to the expression of the ion transporters. *$P<0.05$ compared with the control. The results were obtained from more than six independent experiments.

stimulatory effect on Na$^+$/K$^+$-ATPase $\alpha$-subunit and NKCC were the most striking, of approximately four- to sixfold induction. The induction was found to be at the translational level as there was no significant change in the mRNA levels (data not shown).

PRL had no effect on the protein levels of Na$^+$/K$^+$-ATPase $\alpha$- and $\beta$-subunits (Fig. 3A). At the higher doses (1–10 nmol l$^{-1}$) of PRL, significant induction of NKCC and NHE-1 (~1.6-fold) were observed. However, the induction was significantly lower than that evoked by DEX. IGF-1 showed a dose-dependent stimulatory effect solely on NKCC (two- to fourfold) (Fig. 3B). T$_3$ had no effect on the expression of the three ion transporters (Fig. 3C). The dbcAMP-exposed cells, showed stimulatory effect on the three ion transporters (Fig. 4). The highest induction was observed in NHE-1 expression (four- to sixfold). The induction of the other ion transporters (Na$^+$/K$^+$-ATPase and NKCC) were about two- to threefold. The effect of dbcGMP was not significant. Both hormonally and dbcAMP-mediated effects were found to be at the protein level as there was no significant change in their respective mRNA levels as revealed by the real-time PCR assay (data not shown).

## Discussion

The fish gill epithelium is a very complex transport membrane that simultaneously participates in an assortment of ion and molecule transport activities, including respiratory gases, universal osmolytes, nitrogenous wastes and water (Evans et al., 2005). To achieve ion homeostasis, the branchial ion transport activity is influenced by water salinities and different hormones, so that the osmolarity in the intracellular and extracellular compartments can be maintained. In the past, a considerable number of studies focused on the investigation of how gill cell functions contributed to the regulation of plasma osmolarity. However, studies on the direct osmotic challenge to the ion homeostasis of the gill epithelial cells are limited. Using isolated killifish opercular epithelia, Marshall et al. (Marshall et al., 2005) demonstrated that a hypotonic shock rapidly inhibited Cl$^-$ secretion b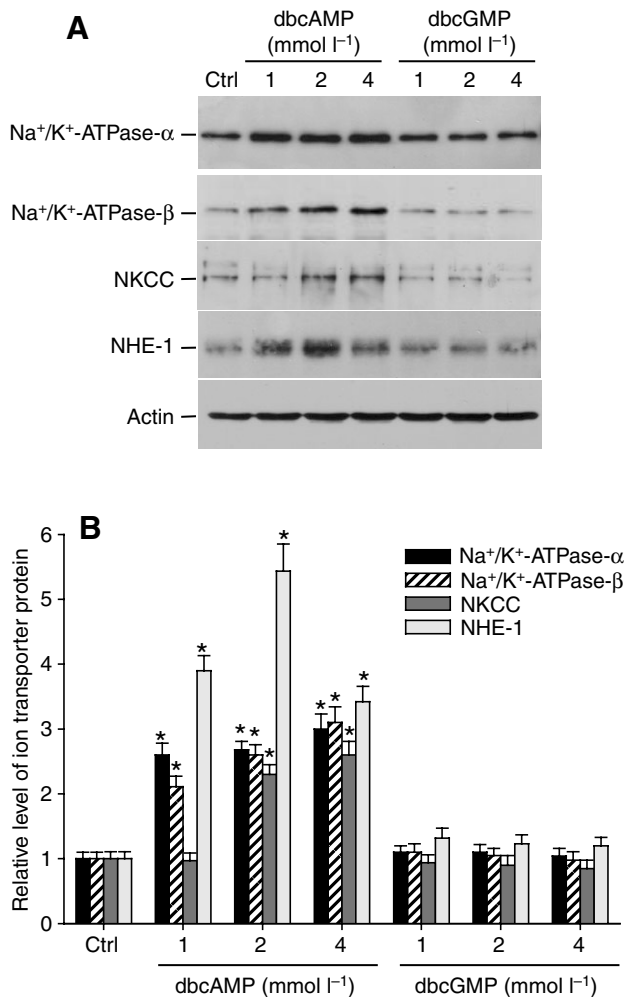y chloride cells, possibly through the phosphorylation of MAPK, JNK, stress protein kinase, oxidation stress response kinase, and focal adhesion kinase. In this study, we aimed to determine the effect of osmotic hypertonicity, hormones and cellular

Fig. 4. Effect of dibutyryl cAMP (dbcAMP) or dibutyryl cGMP (dbcGMP) on the expression of Na$^+$/K$^+$-ATPase, Na$^+$/K$^+$/2Cl$^-$ cotransporter (NKCC) and Na$^+$/H$^+$ exchanger-1 (NHE-1) in pavement cells (PVCs) in culture. The cells were exposed to 1–4 mmol l$^{-1}$ of the drugs for 24 h. (A) Cell lysates were collected for western blot analysis and (B) the amounts of the respective ion transporter proteins were quantified. Dibutyryl cAMP significantly induced the expression of the three ion transporters. The effect of dbcGMP was negligible. *$P<0.05$ compared with the control. The results were obtained from more than four independent experiments.

signaling molecules on the expression of ion transporters in the cultured primary freshwater gill epithelial cells. The modulation of Na$^+$/K$^+$-ATPase, NKCC and NHE-1 expression levels were determined, because they are known to be the important ion transporters involved in maintaining a stable cell volume (Gilles, 1988; Chamberlin and Strange, 1989; Lang et al., 1998b; Lang et al., 1998a).

Our data demonstrated that the hypertonic treatment (500 mOsmol l$^{-1}$) of the isolated PVCs induced cell shrinkage, followed by regulatory volume increase (RVI). The observation was comparable to a similar study using red blood cells obtained from rainbow trout, carp and European flounder (Brauner et al., 2002; Weaver et al., 1999). In addition, the application of the

three specific blockers reduced the RVI response in the cells. Comparatively the effects of ouabain and bumetanide were more remarkable. The data indicated the participation of NHE-1, NKCC and Na$^+$/K$^+$-ATPase in volume regulation during hypertonic treatment. Interestingly, our data indicated that osmotic shrinkage can induce the expression of the ion transporters in PVCs. Western blot analysis of the 6 h hypertonic-treated PVCs revealed significant induction of NHE-1, NKCC and Na$^+$/K$^+$-ATPase $\alpha$ and $\beta$ subunits. As compared to the time frame (30 min) required for cell volume recovery in PVCs, the RVI could possibly be attributed to stimulation of the activities of the existing ion transporter proteins. In mammals, RVI is known to be triggered by shrinkage-activated mechanisms that stimulate the activities of Na$^+$/H$^+$ exchanger-1 (NHE-1), chloride/bicarbonate exchanger (Cl/HCO$_3^-$) and Na$^+$/K$^+$/2Cl$^-$ cotransporter (NKCC) (Hoffmann and Dunham, 1995). NHE1s from mammalian, amphibian and teleost species are highly conserved and are activated by cell shrinkage (Pedersen and Cala, 2004; Pedersen et al., 2003; Pedersen et al., 2006; Holt et al., 2006). The RVI responses in red blood cells of the European flounder was found to involve NHE-1 and Cl/HCO$_3^-$ (Weaver et al., 1999). The importance of the cell shrinkage activation of NKCC-1 expression has been demonstrated in both mammals and the intestine of American eels (Lionetto et al., 2001; Russell, 2000). Nevertheless, RVI is possibly accompanied by the action of NHE-1 in exchanging H$^+$ for an extracellular Na$^+$ as well as the increase of NKCC-1 activity that mediates Cl$^-$ influx. A stable intracellular [Na$^+$] can then be maintained by the activity of the Na$^+$/K$^+$-ATPase (Muto et al., 2000). The processes lead to an increase in Na$^+$ and Cl$^-$ intake and hence restoration of the cell volume. In the present study, the stimulation of protein expression from 6 h onward may imply a long-term adaptation that allows the maintenance of cell volume during continuous exposure to the hypertonic medium. The induction of Na$^+$/K$^+$-ATPase expression was probably related to an increase in intracellular Na$^+$ during RVI, since it was reported that cytosolic [Na$^+$] can directly modulate the expression of Na$^+$/K$^+$-ATPase in rat kidney epithelial cells (Muto et al., 2000). Interestingly our data indicated that the induction of ion transporters was regulated at the translational level. Another study using gill organ culture, also demonstrated that the hypertonic-induced Na$^+$/K$^+$-ATPase activity was similarly regulated at the translational level (Mancera and McCormick, 2000). To ensure that the lack of response in the transcript levels is not due to the limitation of our real-time PCR assay, we determined the mRNA level of Osft1, which is known to response to salinity stress (Fiol et al., 2006; Fiol and Kultz, 2005). Our data indicated that the hypertonic-treated PVCs showed significant induction of Osft1 at 6 h of post treatment. This observation supports the notion that PVCs may possess osmoreceptive function which is important for cell volume regulation.

In nonshrunken, cultured gill epithelial cells that were maintained in L-15 medium (317 mOsmol l$^{-1}$), we found that the stimulatory effects of DEX (an exogenous glucocorticoid) and cAMP were the most remarkable. The expression levels of the

three ion transporters were significantly induced. During seawater transfer, it has been reported that there was a marked transitory increase in plasma cortisol and branchial cAMP levels (Foskett et al., 1983; Marshall and Singer, 2002; Assem and Hanke, 1981; Ball et al., 1971; Mayer et al., 1967). Numerous studies have demonstrated the stimulatory effect of cortisol on $Na^+/K^+$-ATPase and NKCC expressions in different fish species (Pelis and McCormick, 2001; Wong and Chan, 2001; Laiz-Carrion et al., 2003; Richards et al., 2003; Sunny and Oommen, 2001). A cultured gill epithelium study demonstrated a stimulatory effect of cortisol on $Na^+$ and $Cl^-$ transport (Zhou et al., 2003). It has been demonstrated that epinephrine (*via* cAMP and protein kinase A) stimulated $Cl^-$ secretion by the enhancement of chloride conductance in seawater fish opercular membranes (Foskett and Scheffey, 1982; Foskett et al., 1983; Marshall and Singer, 2002). Taking into account the data obtained from the hypertonic stress experiments, our results indicated that DEX and cAMP may participate in gill cell volume regulation in the early phase of seawater adaptation. Although there are only limited fish data in the literature, particularly for NHE-1 regulation, a stimulatory effect by DEX was demonstrated in mammalian models (Baum et al., 1994; Cho et al., 1994; D'Andrea et al., 1996; D'Andrea-Winslow et al., 2001; Yang et al., 2001; Whorwood and Stewart, 1995). It seems that the regulation is comparable between fish and mammals.

By contrast, the effect of PRL, IGF-I, $T_3$ and cGMP were considerably lower in this aspect. PRL is known to be important in freshwater adaptation (Evans et al., 2005). Its biological function is found to be associated with a reduction of ion loss rather than an increase in ion uptake. The general effect of PRL to $Na^+/K^+$-ATPase expression is still controversial (Evans et al., 2005; Madsen et al., 1995; McCormick and Bradshaw, 2006; Miguel et al., 2002). It was also interesting to note that PRL can stimulate $Na^+$ and $Cl^-$ transport in cultured gill epithelium, although it had no effect on $Na^+/K^+$-ATPase activity (Zhou et al., 2003). Our data provide a possible explanation, in that PRL may instead induce NHE-1 and NKCC activities to facilitate the ion transport function. Growth hormone and $T_3$ are believed to be seawater acclimating hormones (Evans, 2002; Evans et al., 2005). Both hormones are able to induce gill $Na^+/K^+$-ATPase activities in different fish species. However, these effects were suggested to be mediated by cortisol and/or IGF-I (Madsen and Bern, 1993; McCormick et al., 1991; McCormick and Bradshaw, 2006). Our data indicated that $T_3$ had no significant effects on the expression levels of the three ion transporters. IGF-1 can only induce the expression of NKCC, which agrees with the results from another study (Pelis and McCormick, 2001). The action of cGMP on the expression of the three ion transporters was not obvious. The use of cGMP in this study was to mimic the action of natriuretic peptide which is recognized to be important in rapid regulation of ion transport in fish (Takei and Hirose, 2002). Although the effects of PRL, IGF-I, $T_3$ and cGMP on the expression of the ion transporters were not apparent, the possibility that their actions are mediated by the direct modulation of the ion transporter activities still cannot be excluded.

The present study highlighted the issue of osmotic shrinkage that may occur in fish gill epithelial cells during seawater acclimation. The direct transfer of a fish from a freshwater to a seawater environment imposes immediate osmotic stress on the gill epithelia. Recently, an osmotic stress transcription factor 1 (Ostf1) and a transcriptional factor IIB (TFIIB) were cloned from seawater-acclimating tilapia (Fiol and Kultz, 2005). The regulation of Ostf1 was demonstrated in both seawater-acclimating intact fish and hypertonic-treated primary gill epithelial cell culture and was reported to be important in the early phase of seawater acclimation (Fiol et al., 2006). Since the fish gill is one of the major osmoregulatory organs, the ability of the gill epithelial cells to recover from osmotic shrinkage/swelling is critical for fish osmoregulation. The osmotic shrinkage, which possibly causes *de novo* activation of Ostf1 and TFIIB in gill epithelial cells, together with the actions of seawater-acclimating hormones (i.e. cortisol, GH/IGF-I, $T_3$, epinephrine and natriuretic peptides) could provide an integrated signal to modulate the expression of different ion transporters for the maintenance of osmotic homeostasis in the gill epithelial cells and the fish. This paper sheds light on the capability of the gill epithelial cells to trigger their own osmoreceptive function in sensing the salinity of the external environment for both cell volume regulation and osmoregulation.

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