The Journal of Experimental Biology 214, 1264-1270

doi:10.1242/jeb.050435

## RESEARCH ARTICLE

# Regulatory function of hyperosmotic stress-induced signaling cascades in the expression of transcription factors and osmolyte transporters in freshwater Japanese eel primary gill cell culture

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Accepted 23 December 2010

**SUMMARY**

**In the present study, we investigated the early activation of osmotic stress-related protein kinases, with the aim of characterizing their functional links with downstream effectors (i.e. transcription factors and osmolyte transporters). Freshwater eel primary gill cells were cultured in hypertonic medium (500 mosmol $l^{-1}$) for 6 h. Protein lysates and total RNA were collected for western blotting and quantitative real-time PCR assays. In this study, the osmotic challenge stimulated histone H3 phosphorylation, various signaling pathways (i.e. ERK1/2, p38 MAPK, JNK, CREB, MARCKS and MLCK) and expression of some downstream effectors (i.e. $Na^+/K^+$-ATPase, TauT and Ostf). Increased phosphorylation of acetylated histone is known to promote chromatin relaxation for global gene transcription, probably leading to the activation of downstream effectors for osmotic responses. In addition, the importance of the p38 MAPK and MLCK pathways in the regulation of the expression of $Na^+/K^+$-ATPase and TauT was demonstrated. Inhibition of the p38 MAPK pathway by SB202190 reduced histone H3 phosphorylation and TauT mRNA expression. Moreover, inhibition of the MLCK pathway by ML-7 decreased the expression level of $Na^+/K^+$-ATPase but increased the transcript level of TauT. Collectively, the present study reveals possible functional links of osmosensing signaling cascades to the regulation of downstream effectors.**

Key words: MLCK, p38 MAPK, pERK1/2.

## INTRODUCTION

Osmosensing is a fundamental process that is mediated by a complex regulatory mechanism to integrate signals from (i) the extracellular matrix and cytoskeleton, (ii) intracellular ionic strength/macromolecular crowding and (iii) cell membrane stretch/potential, and form a link with specific effectors to maintain a constant cell volume (Wehner et al., 2003; Lang et al., 1998). A considerable number of studies have been conducted to dissect this fundamental process; however, conclusive roles of the molecular osmosensors have not yet been elucidated. This is probably because of the intricacy of signal crosstalk derived from different osmosensors, which makes it very difficult to independently dissect the individual osmosensing signal cascade. Nevertheless, knowledge of the interaction between activation of osmosensor-elicited signaling molecules, downstream effectors and cell volume homeostasis is important for deciphering this fundamental process, which is involved in a variety of cell functions.

Euryhaline teleost fish are able to inhabit waters of changing salinity. The changes exert considerable osmotic stress on fish gills, which are known to be an excellent target tissue for investigating the osmosensing mechanism. However, studies on signaling cascades activated by osmosensors of fish gills are scarce. In a study of hypertonic stress responses in gill epithelia of the euryhaline teleost *Fundulus heteroclitus*, upregulation of a 14-3-3 protein and mitogen-activated protein kinases (MAPKs) was demonstrated (Fiol and Kültz, 2007; Kültz and Avila, 2001; Kültz et al., 2001; Kültz, 2005). Using isolated killifish opercular epithelia, hypotonic shock was found to modulate phosphorylation of MAPKs, stress-activated protein kinase, oxidation stress response kinase and focal adhesion kinase (Marshall et al., 2005; Marshall et al., 2008). In the characterization of some downstream effectors, the cloning and regulation of branchial sodium chloride–taurine transporter (TauT) (Chow et al., 2009), osmotic stress transcription factors (Ostf) and general transcription factor IIB (TFIIB) were reported (Choi and An, 2008; Fiol and Kültz, 2005; Tse et al., 2008). Nevertheless, the functional significance of the identified signaling pathways and the downstream effectors is not known. There are many signaling molecules that are reported to be involved in osmosensing in animal cells (Hoffmann et al., 2009; Wehner et al., 2003). Activation of MAPKs and myosin light chain kinase (MLCK) cascades has been found to be important for hypertonic stress-induced responses (Hoffmann et al., 2009; Kolch, 2005; Shaul and Seger, 2007). In the present study, using freshwater primary gill cell culture, we reveal the involvement of MAPKs and MLCK in the early phase of hyperosmotic challenge. Their regulatory roles in hypertonicity-activated histone modification and the expression levels of the transcription factors (i.e. Ostf) and osmolyte transporters (i.e. $Na^+/K^+$-ATPase, TauT) were further elucidated.

## MATERIALS AND METHODS

### Animals and gill cell isolation

Japanese eels (*Anguilla japonica*, Temminck and Schlegel 1847), weighing between 500 and 600 g, were kept in a fiberglass tank supplied with charcoal-filtering aerated tap water at 18–20°C under a 12 h L:12 h D photoperiod for at least 3 weeks. The fish were anesthetized with MS222 (Sigma, St Louis, MO, USA) and the

gills were then perfused *via* the ventral aorta with phosphate-buffered saline (PBS, pH 7.7) to remove blood cells. The fish were killed by decapitation and the gill arches excised. The gill tissues were cut into small fragments and were subjected to two cycles of tryptic digestion (0.5% trypsin + 5.3 mmol $l^{-1}$ EDTA in PBS, pH 7.7) for the establishment of a primary gill cell culture as previously described (Tse et al., 2007; Tse et al., 2008). After tryptic digestion, the cell suspension was filtered through stainless steel mesh (104 and 73.7 μm, Sigma) and washed in Leibovitz's L-15 medium (Gibco, Invitrogen, Carlsbad, CA, USA). In general, the cells had high viability (>90%) and consisted of a heterogeneous mixture of mitochondria-rich cells (chloride cells), pavement cells and mucous cells. Cells were seeded in Leibovitz's L-15 medium, supplemented with 10% fetal bovine serum (Hyclone®, Perbio, Thermo Fisher Scientific, Cramlington, UK), 1% penicillin/streptomycin and 0.5% fungizone (Gibco, Invitrogen) at a density of $2\times10^6$ cells $cm^{-2}$ onto collagen-coated culture plates. The cells were incubated at 22°C in a growth chamber with humidified air atmosphere.

### Experiments using the primary gill cell culture

One day after seeding, each culture well was rinsed with PBS (pH 7.7) to remove mucus and unattached cells. The cells were then exposed to hypertonic medium, with or without drug treatment. The hypertonic medium was prepared by the addition of 90 mmol $l^{-1}$ NaCl (Sigma) to Leibovitz's L-15 medium (320 mosmol $l^{-1}$), giving a final osmolarity of 500 mosmol $l^{-1}$ (Chow et al., 2009). The osmolarity of the prepared medium was measured by a vapor pressure osmometer (Wescor, 5500XR, Logan, UT, USA). The cells were incubated in Leibovitz's L-15 medium (isotonic medium) or the hypertonic medium for designated time intervals (i.e. 2 min to 6 h). In some experiments, cell lysates were collected at 0, 2, 5, 10, 20 and 30 min post-incubation for western blot analysis. Total RNA was extracted at 3 and 6 h post-incubation for real-time PCR analysis. The cells were either untreated or treated with the drug solvent (DMSO, Sigma) or kinase inhibitors dissolved in DMSO, i.e. 10 μmol $l^{-1}$ PD98059, 20 μmol $l^{-1}$ SB202190, 10 μmol $l^{-1}$ SP600125, 50 μmol $l^{-1}$ ML-7, 20 μmol $l^{-1}$ H89 or 30 nmol $l^{-1}$ staurosporine (Calbiochem, Merck, Rahway, NJ, USA). In all experiments, the final volume of DMSO did not exceed 0.4% of the culture medium.

### Real-time PCR analysis

Purified sample RNA with an $A_{260}/A_{280}$ ratio of 1.8–2.0 was used. Briefly, 0.5 μg of total cellular RNA was reverse transcribed (iScript, Bio-Rad, Hercules, CA, USA). PCR reactions were conducted with the iCycler iQ real-time PCR detection system using iQ™ SYBR® Green Supermix (Bio-Rad). Gene-specific primers were designed (Table 1) and the sequences of the PCR products were verified (Chow et al., 2009; Tse et al., 2008). Copy numbers of the transcripts for each sample were calculated with reference to a parallel amplification of known concentrations of the respective cloned PCR fragments. The occurrence of primer-dimers and secondary products was inspected 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 RT or without RNA. The relative expression ratio $R$ of target gene/GAPDH was calculated according to the method described by Pfaffl (Pfaffl, 2001):

$$R = E_{\text{target}}^{\text{CPtarget (control – treatment)}} / E_{\text{GAPDH}}^{\text{CPGAPDH (control – treatment)}}, \quad (1)$$

where PCR efficiency $E=10^{(-1/\text{slope})}$ and CP is the crossing point at which fluorescence rises above background.

### Western blot analysis

Sample protein concentrations were measured using the DC protein assay kit (Bio-Rad). A 70 μg sample of protein was loaded per lane and subjected to electrophoresis through 10% polyacrylamide gel. The gels were then blotted onto polyvinylidene fluoride (PVDF) membranes (PerkinElmer Life Sciences, Foster City, CA, USA) at 30 V for 90 min in 1× Novex® tris-glycine transfer buffer (Invitrogen). Western blotting was conducted using rabbit polyclonal antibodies against phospho-extracellular signal-regulated kinase (Thr202/Tyr204) (pERK1/2, cat no. 9101), phospho-c-Jun N-terminal kinase (Thr183/Tyr185) (pJNK, cat no. 9251), phospho-myristoylated alanine-rich C-kinase substrate (Ser152/156) (pMARCKS, cat no. 2741), single phosphorylated myosin light chain (Ser19) (pMLC2, cat no. 3671), total MLC2 (cat no. 3672), acetylated histone 3 (Lys9/Lys14) (histone H3, cat no. 9677), phosphorylated histone H3 (Ser10) (cat no. 9701) and total histone H3 (cat no. 9715) (all from Cell Signaling Technology, Danvers, MA, USA) and phospho-cAMP response element-binding protein (pCREB) (Calbiochem, cat no. 238465), and mouse monoclonal antibodies against phospho-p38 MAPK (Thr180/Tyr182) (pp38 MAPK, cat no. 9216, Cell Signaling Technology), followed by incubation with (1:4000) horseradish peroxidase-conjugated goat anti-rabbit/mouse antibody. Specific bands were visualized using chemiluminescent reagent according to the manufacturer's instructions (Western-lightening Plus, PerkinElmer Life Sciences). The blots were then washed in PBS and re-probed with (1:100) mouse monoclonal anti-actin antibody (JLA20, Developmental Studies Hybridoma Bank, the University of Iowa, IA, USA) for normalization. Band intensity was captured by the gel documentation system (ChemiDoc™ XRS, Bio-Rad) and analyzed by Quantity-One® software (Bio-Rad).

### Statistical analysis

Drug treatments were performed in triplicate in each experiment and every experiment was performed on at least three separate cell isolations. All data are represented as the mean ± s.e.m. Statistical significance was assessed with Student's $t$-test or one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. Groups were considered significantly different if $P<0.05$.

## RESULTS

### Hypertonic stress-induced changes in protein phosphorylation

Hypertonic challenge of primary gill cell cultures elicited the activation of numerous signaling pathways, including ERK1/2, p38 MAPK, JNK, CREB, protein kinase C (PKC) and MLC2 (Fig. 1A). Some of the activations were rapid but transient (pERK1/2 and pp38 MAPK) and some responded slowly (i.e. pJNK, pMARCKS, pCREB and pMLC2). Of these, the activation of pERK1/2, pp38 MAPK and pMLC2 was found to be most prominent. In addition to the activation of the signaling molecules and protein kinases, hypertonic challenge stimulated mRNA expression of the transcription factor Ostf and osmolyte transporters (i.e. $Na^+/K^+$-ATPase α- and β-subunits and TauT) (Fig. 1B). In cells maintained in isotonic medium, the relative expression levels of Ostf, $Na^+/K^+$-ATPase β-subunit and TauT were significantly higher than those of the $Na^+/K^+$-ATPase α-subunit. Moreover, the fold induction of their expression following hypertonic treatment was comparable. The effects of hyperosmotic stress on the transcript levels of cystic fibrosis transmembrane conductance regulator (CFTR), $Na^+$–$K^+$–$Cl^-$ co-transporter (NKCC1a) and TFIIB were negligible at 6 h post-treatment.

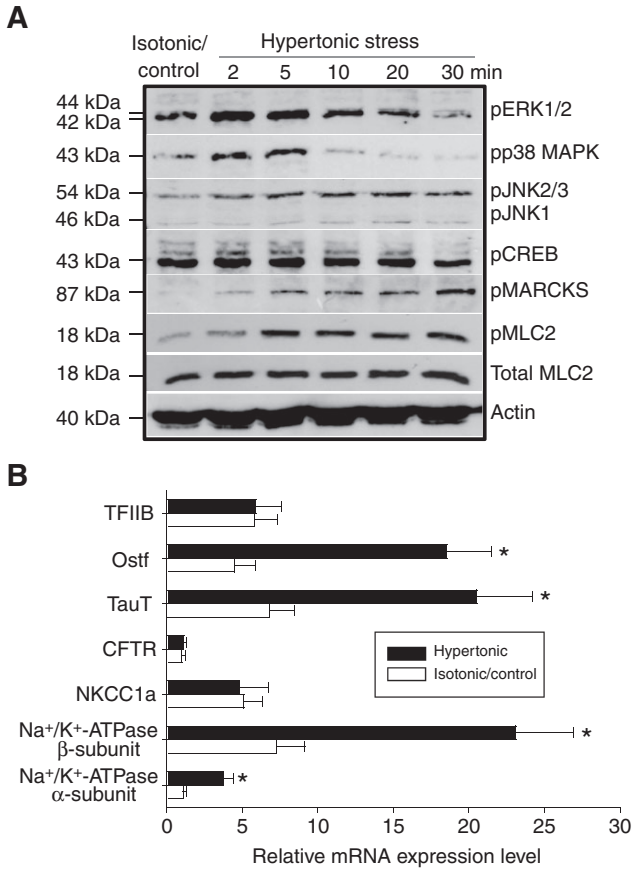


Fig. 1. Activation of protein kinases, and mRNA expression levels of transcription factors and osmolyte transporters in gill cells under hypertonic stress (500 mosmol $l^{-1}$). (A) Representative western blot data showing the changes in the levels of phosphorylated (p) mitogen-activated protein kinases (MAPKs, i.e. ERK1/2, p38 MAPK, JNK), CREB, MARCKS and MLC2 in the gill cells 2–30 min post-treatment. (B) Relative mRNA expression levels of $Na^+/K^+$-ATPase α- and β-subunits, NKCC1a, CFTR, TauT, Ostf and TFIIB in gill cells in isotonic/control and hypertonic conditions after 6 h. *$P$<0.05 compared with the respective controls. For description of protein kinases, transcription factors and osmolyte transporters, see main text.

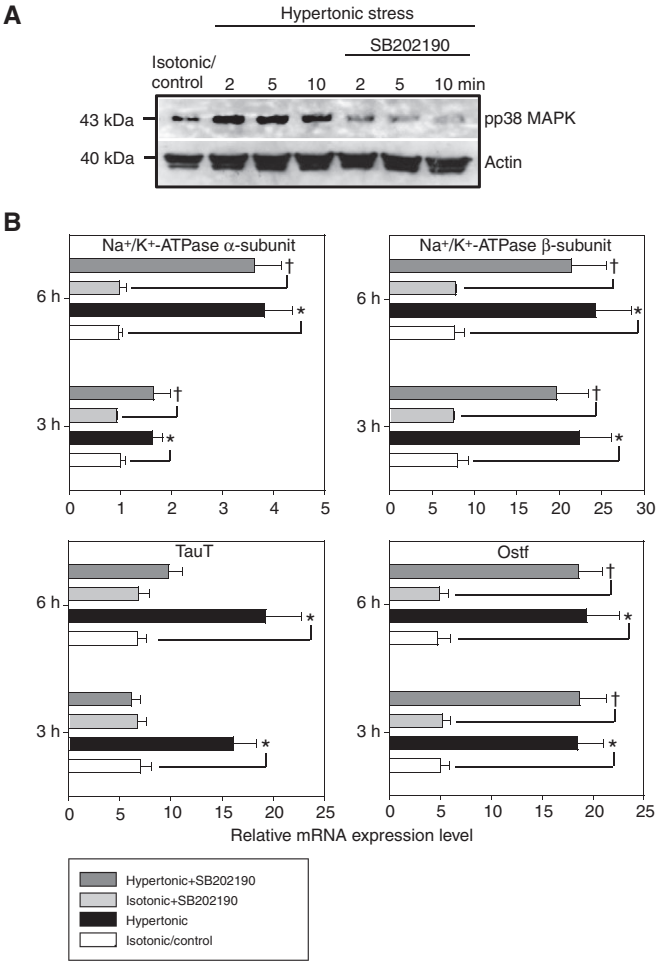


Fig. 2. Effects of the protein kinase inhibitor SB202190 on the phosphorylation of p38 MAPK and the transcript levels of $Na^+/K^+$-ATP α- and β-subunits, TauT and Ostf. (A) Representative western blot data showing the inhibitory effect of SB202190 on the level of hypertonic stress (500 mosmol $l^{-1}$)-induced phosphorylation of p38 MAPK in cultured gill cells. (B) Real-time PCR data showing SB202190-mediated inhibition of the hypertonic stress-induced increase in transcript levels of TauT in gill cells at 3 and 6 h post-treatment. *$P$<0.05 and †$P$<0.05 compared with untreated and inhibitor-treated isotonic controls, respectively.

### Effect of protein kinase inhibitors on expression levels of hypertonic stress-induced transcription factors and osmolyte transporters

The pathway-specific inhibitors SB202190 (Fig. 2) and ML-7 (Fig. 3) significantly modulated the mRNA expression levels of TauT and/or $Na^+/K^+$-ATPase α- and β-subunits. Other inhibitors (i.e. PD98058, SP600125, H89 and staurosporine) had no noticeable effects on the expression levels of these genes (data not shown).

Treatment of the cells with SB202190 significantly decreased the hypertonic stress-induced activation of pp38 MAPK (Fig. 2A) and TauT mRNA expression (Fig. 2B). However, no observable effects on the expression levels of $Na^+/K^+$-ATPase α- and β-subunits and Ostf were detected (Fig. 2B).

Treatment of the cells with ML-7 reduced the hypertonic stress-induced phosphorylation level of MARCKS and MLC2 (Fig. 3A). These changes were accompanied by the downregulation of $Na^+/K^+$-ATPase α- and β-subunits (Fig. 3B). Interestingly, ML-7 treatment upregulated the basal transcript levels of TauT and synergistically enhanced hypertonic stress-induced TauT expression. The effect on Ostf mRNA level was not noticeable.

### Effects of hypertonic stress and protein kinase inhibitors on histone modification

To reveal the effects of hypertonic stress on epigenetic changes in the cells, the levels of total, acetylated and phosphorylated histone H3 were analyzed by western blotting (Fig. 4A). Hypertonic treatment specifically and significantly increased the level of phospho-H3. The levels of acetylated and total histone H3 were unchanged.

Treatment of the hypertonic stress-challenged cells with SB202190 significantly reduced the level of phospho-H3 (Fig. 4B). No noticeable effects were observed in cells cotreated with other inhibitors.

## DISCUSSION

Osmosensing-elicited signaling cascades are important for mediating cellular responses to anisosmotic challenges (Wehner et al., 2003; Hoffmann et al., 2007; Hoffmann et al., 2009). A considerable

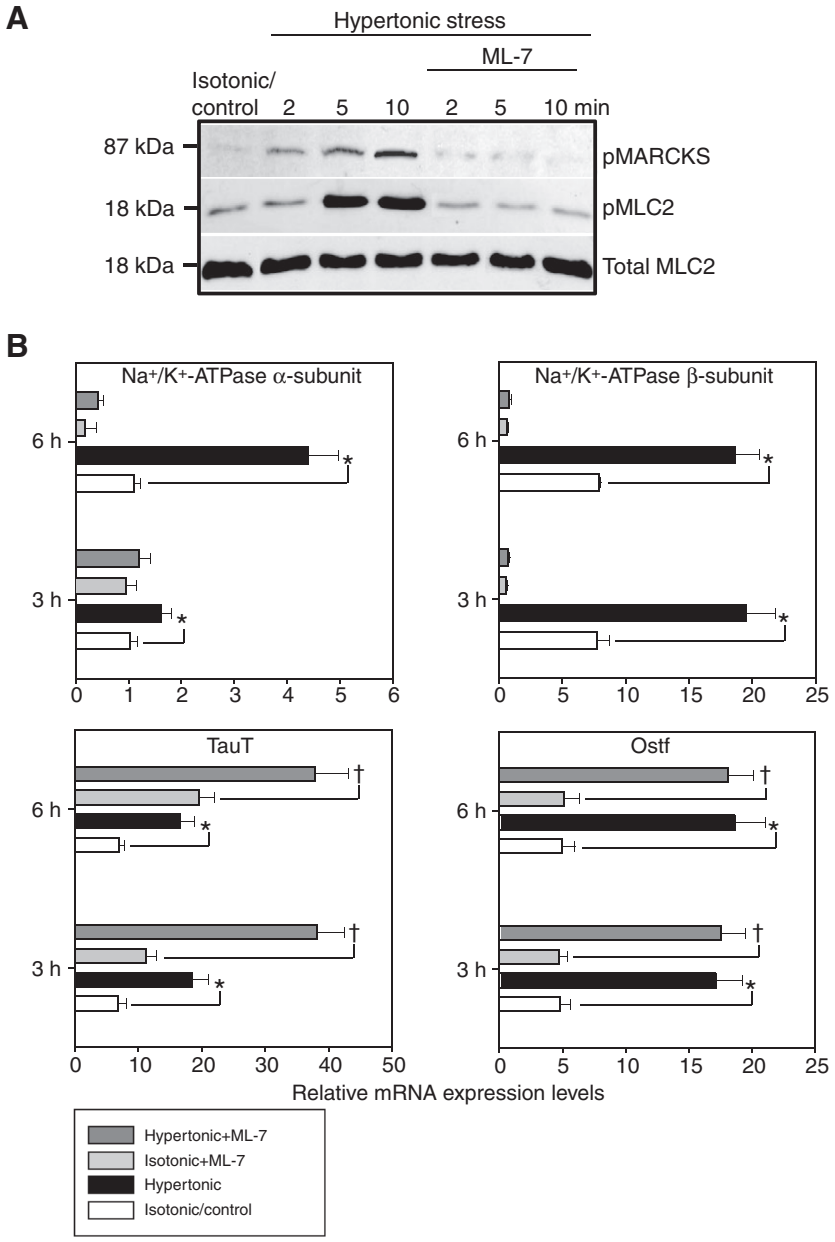


Fig. 3. Effects of the protein kinase inhibitor ML-7 on the phosphorylation of MARCKS and MLC2 and the transcript levels of $Na^+/K^+$-ATP α- and β-subunits, TauT and Ostf. (A) Representative western blot data showing the inhibitory effect of ML-7 on the level of hypertonic stress (500 mosmol $l^{-1}$)-induced phosphorylation of MARCKS and MLC2 in cultured gill cells. (B) Real-time PCR data showing ML-7-mediated inhibition of both basal and hypertonic stress-induced transcript levels of $Na^+/K^+$-ATP α- and β-subunits in the gill cells at 3 and 6 h post-treatment. Synergistic effects on TauT mRNA expression were observed in ML-7 and hypertonic stress-treated cells. *$P$<0.05 and †$P$<0.05 compared with untreated and inhibitor-treated isotonic controls, respectively.

number of studies using mammalian cell models have reported the involvement of various signaling molecules in osmotic challenges. In contrast, cellular osmotic responses in the teleost fish model system have been less well studied. Moreover, it is generally believed that signaling cascades activated by osmosensing systems in gill cells are involved in the functional transformation of gill epithelia. Such transformation may lead to gill cell remodeling to facilitate the processes of cell volume regulation and transepithelial ion transport. The present results help reveal the functional significance of hypertonic stress-activated signaling pathways, histone modification and expression of some downstream effector proteins (i.e. transcription factors and transporters) in gill cells of Japanese eels at an early phase of hyperosmotic challenge (1 min to 6 h).

Hyperosmotic challenge evoked the stimulation of various signaling pathways (i.e. ERK, p38 MAPK, JNK, CREB, MARCKS and MLCK), histone H3 phosphorylation and expression of some downstream targets (i.e. $Na^+/K^+$-ATPase, Ostf and TauT). In the absence of endogenous hormonal and other systemic control factors in the gill culture model, it is interesting to note that $Na^+/K^+$-ATPase, Ostf and TauT can be directly upregulated by hyperosmolarity. The other downstream effectors, like NKCC1a, CFTR and TFIIB, remained unchanged. The lack of upregulation of NKCC1a, CFTR and TFIIB in the hypertonic stress-treated gill cell cultures probably reflects the need for hormonal factors for regulation. The transcript levels of TFIIB in tilapia (Fiol and Kültz, 2005) and NKCC1 and CFTR in killfish gills (Scott et al., 2004) were reported to be significantly increased within 2 or 8 h of seawater acclimation, respectively. Nevertheless, the upregulation of the signaling molecules is in general consistent with other studies in fish and mammals (Hoffmann et al., 2007; Hoffmann et al., 2009; Marshall et al., 2005; Marshall et al., 2008; Sheikh-Hamad and Gustin, 2004). In mammalian studies, hypertonicity induced phosphorylation of MAPKs, including ERK1/2, p38 MAPK and JNK (Wehner et al.,

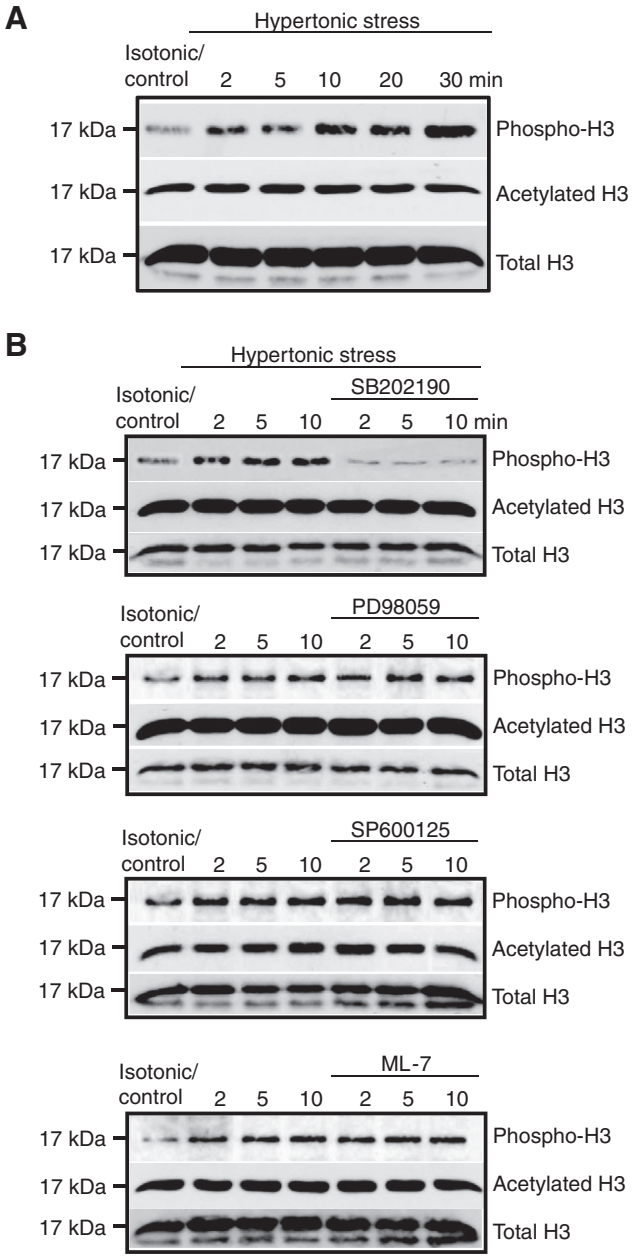


Fig. 4. Effects of hypertonic stress (500 mosmol $l^{-1}$) and protein kinase inhibitors on histone modification. (A) A representative western blot showing hypertonic stress-induced phosphorylation of histone H3 in the gill cells 2–30 min post-treatment. No noticeable change was observed in the level of histone H3 acetylation. (B) Effects of various protein kinase inhibitors on the level of histone H3 phosphorylation. Note the significant reduction of hypertonic stress-induced histone H3 phosphorylation in cells treated with SB202190.

2003). In Madin–Darby canine kidney (MDCK) cells, H4IIE rat hepatoma cells and bovine aortic endothelial cells, activation of ERK1/2 was detectable within 10 min of treatment and reached a maximum level in 20–30 min (Itoh et al., 1994; Duzgun et al., 2000; Schliess et al., 1996). Hypertonic stress-induced activation of p38 MAPK was reported in the human leukemic monocyte lymphoma cell line U937 and in murine inner medullary collecting duct (IMCD) cells (Shrode et al., 1995; Zhang and Cohen, 1996). The time to onset of p38 MAPK activation was short (within 10 min). JNK was also found to be activated by hypertonic challenge in MDCK cells (Itoh et al., 1994), IMCD cells (Berl et al., 1997) and a rat renal proximal tubular cell line (NRK-52E) (Kojima et al., 2010), although the activation of JNK was slower (10–20 min). Other than MAPK pathways, activation of MLCK and the downstream MLC2 in hypertonic stress-challenged rat astrocytes (Shrode et al., 1995) and porcine kidney tubular epithelial (LLC-PK1) cells has been demonstrated (Ciano-Oliveira et al., 2003). In piscine models, using gill tissues of killifish, Kültz and Avila reported the involvement of MAPKs in hyperosmotic challenge (1 h to 4 weeks) (Kültz and Avila, 2001). Using isolated opercular epithelia of killifish, hypotonic stress rapidly and transiently increased levels of phosphorylated p38 MAPK eightfold in 5 min and sevenfold in 60 min (Marshall et al., 2005). These results highlight the importance of MAPKs and MLCK in osmosensing (Hoffmann et al., 2009). Because cellular osmotic responses are rapid, the data from the previous fish studies may not reflect the immediate osmosensing-coupled signaling responses in fish gill cells. Primary gill cell cultures provide an excellent system in which to examine rapid and longer term responses. Hence, in the present study, activation of osmosensing-coupled signaling pathways was investigated at an early phase (within minutes) of hyperosmotic challenge of the primary gill culture. Phosphorylation of ERK1/2, p38 MAPK and MLC2 reached maximum levels within 10 min of hypertonic challenge while JNK phosphorylation increased gradually over 30 min. The profile and the time frame of activation of these protein kinases were comparable to the above-mentioned reports from the mammalian systems (Berl et al., 1997; Duzgun et al., 2000; Itoh et al., 1994; Kojima et al., 2010; Schliess et al., 1996; Shrode et al., 1995; Zhang and Cohen, 1996). Importantly, in addition to the activation of the signaling cascades, our data revealed that the hypertonic challenge induced epigenetic changes in the fish gill cells. Increased phosphorylation of acetylated histone is known to promote chromatin relaxation for global gene transcription, probably leading to upregulation of downstream targets for osmotic responses (Cheung et al., 2000).

In mammalian models, the tonicity-responsive enhancer binding protein (TonEBP) regulates expression levels of the betaine/GABA transporter (BGT), the sodium–*myo*-inositol cotransporter (SMIT) and TauT in mouse kidneys (Bartolo and Donald, 2008). These target genes are responsible for the accumulation of organic osmolytes including betaine, *myo*-inositol and taurine. In fish, the osmolyte transporters $Na^+/K^+$-ATPase and TauT are known to regulate transport of inorganic and organic osmolytes (Chow et al., 2009; Tse et al., 2006; Tse et al., 2007; Tse et al., 2008). The function of Ostf, however, has not been clearly defined yet. Based on its structural similarity to the mammalian TSC-22 domain transcripts, fish Ostf is thought to be involved in the transcriptional regulation of ion transporters or ion channels (Fiol et al., 2007; Tse et al., 2008). The current understanding of $Na^+/K^+$-ATPase, TauT and Ostf regulation comes mainly from anisosmotic challenges; the possible influence of signaling molecules has not been elucidated. The present study aimed to fill this information gap by providing evidence of functional links of the signaling cascades to the regulation of downstream targets. Among various signaling molecules detected in the early phase of hyperosmotic challenge, the p38 MAPK pathway was found to be closely associated with histone H3 phosphorylation and changes in the expression of TauT. The illustration of a functional link between p38 MAPK and TauT supports the notion that the predominant role of p38 MAPK in osmolyte transporter expression is conserved from yeast to mammals (Sheikh-Hamad and Gustin, 2004). In mammalian studies, the p38

Table 1. Real-time PCR primer sequences

| Gene | Primer |
|---|---|
| CFTR | 5′-GCCTACTTCTACTCGGCCTT-3′ forward 5′-TAGGACAGCGTGGTGAAGAT-3′ reverse |
| $Na^+/K^+$-ATPase α-subunit | 5′-GTGCCCATCCTGAAGAGAGA-3′ forward 5′-TGCGATCTTGACATGCTTGT-3′ reverse |
| $Na^+/K^+$-ATPase β-subunit | 5′-TTACGTCATCTTCTACGGCTG-3′ forward 5′-TGGGTTTGTAGTTGCTCAGG-3′ reverse |
| NKCC1a | 5′-CCCATCATCTCCAACTTCTTCCT-3′ forward 5′-CCCACCAGTTGATGACGAACA-3′ reverse |
| Ostf | 5′-TCCGCCAGCTCCTTGATTTG-3′ forward 5′-AGCAGGCAATGGATCTTGTGAA-3′ reverse |
| TauT | 5′-CTTTGTGTCTGGCTTCGCAATAT-3′ forward 5′-GGGGTAGGCAATGAAGGCTAG-3′ reverse |
| TFIIB | 5′-CGCCAGCTTTGATGAGTTTG-3′ forward 5′-TTTGTTCTGTCTACGATGTTTCTG-3′ reverse |
| GAPDH | 5′-GCGCCAGCCAGAACATCATC-3′ forward 5′-CGTTAAGCTCGGGGATGACC-3′ reverse |

CFTR, cystic fibrosis transmembrane conductance regulator; NKCC, $Na^+$–$K^+$–$Cl^-$ co-transporter; Ostf, osmotic stress transcription factors; TauT, sodium chloride–taurine transporter; TFIIB, general transcription factor IIB; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

MAPK pathway was found to stimulate the expression of the osmoprotective transcription factor TonEBP in mouse embryo fibroblasts (Ko et al., 2002) and in a human hepatocellular liver carcinoma (HepG2) cell line (Nadkarni et al., 1999). However, our data indicate that p38 MAPK signaling has no noticeable effect on hypertonicity-induced Ostf expression.

In addition to p38 MAPK signaling, the importance of MLCK pathways in the regulation of $Na^+/K^+$-ATPase and TauT expression was demonstrated in this study. Treatment with the MLCK inhibitor ML-7 prevented phosphorylation of MLC2 and MARCKS in hypertonic stress-treated gill cells. Interestingly, treatment of cells with the PKC inhibitor staurosporine had no effect on the level of pMARCKS. This indicates that the phosphorylation of MARCKS was dependent on MLCK activity. The crosstalk between these two proteins is important as both MARCKS and myosin are necessary for F-actin reorganization and stabilization (Doreian et al., 2009). Our data show that inhibition of the MLCK pathway altered the expression levels of $Na^+/K^+$-ATPase and TauT mRNA. Intriguingly, the osmotic stress response in eel intestinal epithelia was also found to be modulated by inhibitors of MLCK (Lionetto et al., 2002). In the mammalian system, hyperosmotic stress stimulated MLC2 phosphorylation and NKCC activity to facilitate cell volume restoration (Hoffmann and Pedersen, 2007). A decrease of MLC2 phosphorylation may lead to a failure of regulatory volume increase (RVI) through inhibition of NKCC activity. Accordingly, failure of RVI-augmented hyperosmolarity in the cells and so a further stimulation of TauT expression would be expected (Chow et al., 2009).

In summary, short-term hyperosmotic challenge in freshwater gill cells activated a number of signaling pathways, histone phosphorylation and downstream effectors (i.e. Ostf, TauT and $Na^+/K^+$-ATPase). Hypertonicity-induced p38 MAPK-dependent histone H3 phosphorylation and MLC2 phosphorylation correlated with TauT and/or $Na^+/K^+$-ATPase expression in the cells, and these are the important components of hyperosmotic responses in fish gills.

## ACKNOWLEDGEMENTS

This work was supported by the General Research Fund (HKBU 261708), Hong Kong.

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