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Expression and functional analysis of mussel taurine transporter, as a key molecule in cellular osmoconforming

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Summary

Most aquatic invertebrates adapt to environmental osmotic changes primarily by the cellular osmoconforming process, in which osmolytes accumulated in their cells play an essential role. Taurine is one of the most widely utilized osmolytes and the most abundant in many molluscs. Here, we report the structure, function and expression of the taurine transporter in the Mediterranean blue mussel (muTAUT), as a key molecule in the cellular osmoconforming process. Deduced amino acid sequence identity among muTAUT and vertebrate taurine transporters is lower (47–51%) than that among vertebrate taurine transporters (>78%). muTAUT has a lower affinity and specificity for taurine and a requirement for higher NaCl concentration than vertebrate taurine transporters. This seems to reflect the internal environment of the mussel; higher NaCl and taurine concentrations. In addition to the hyperosmotic induction that has been reported for cloned taurine transporters, the increase in muTAUT mRNA was unexpectedly observed under hypoosmolality, which was depressed by the addition of taurine to ambient seawater. In view of the decrease in taurine content in mussel tissue under conditions of hypoosmolality reported previously, our results lead to the conclusion that muTAUT does not respond directly to hypoosmolality, but to the consequent decrease in taurine content. By immunohistochemistry, intensive expression of muTAUT was observed in the gill and epithelium of the mantle, which were directly exposed to intensive osmotic changes of ambient seawater.

Key words: taurine, taurine transporter, osmolyte, osmoconforming, adaptation, Mediterranean blue mussel.

Introduction

In aquatic invertebrates such as molluscs, not equipped with advanced mechanisms for regulating their internal environment, osmotic adaptation at a cellular level using an ‘osmolyte system’ plays an essential role. This osmotic adaptation involves accumulation and quantitative regulation of a large amount of organic solutes called ‘osmolytes’ in the intracellular space, to enable cells to respond to the high and variable salinity of the internal environment while maintaining the cell volume and low, stable intracellular salinity concentration (Lockwood, 1963; Somero and Bowlus, 1983).

Whereas information on the osmotic adaptation and regulation at both the cellular and whole-body levels, and the related molecular mechanisms in teleost fish, has been collected (Karnaky et al., 1998; Wood et al., 1995), osmotic adaptation at a cellular level in aquatic invertebrates, which is probably a more vital process than that in teleost fish or mammals, has almost been ignored and is still poorly understood. Although the studies dealing with the biochemical analysis of enzymes related to osmolyte metabolism (Nomura et al., 2001; Yoshikawa et al., 2002), change in cell volume on exposure to salinity change (Neufeld and Wright, 1998, 1996a,b), and transport activity of osmolytes (Neufeld and Wright, 1995; Petty and Lucero, 1999; Silva and Wright, 1992) have been reported, the related molecular mechanisms have never been studied.

Here, we report the molecular cloning, function and expression analysis of a taurine transporter involved in taurine uptake in the cell of the Mediterranean blue mussel. Most molluscs cannot entirely regulate their internal environments (Somero and Bowlus, 1983). Among these, brackish species, including mussels, have to adapt to a wide range of osmolality (almost 0–1000 mOsm kg^{-1}). Taurine is found to be the most abundant and thus, is an important osmolyte not only in mussels (Deaton et al., 1985a,b; Gills, 1972; Livingstone et al., 1979; Potts, 1954; Toyohara and Hosoi, 2004; Zurburg and DeZwaan, 1981) but also in numerous other invertebrates, fishes and mammals (Huxtable, 1992). Intracellular taurine concentration in one species of mussels was estimated to be approximately 200 mmol l^{-1}, corresponding to one-fifth of the total intracellular osmolality (Neufeld and Wright, 1995). The intracellular taurine content is mainly regulated via a transmembrane transport (Huxtable, 1992). Taking these facts

into consideration, the taurine transporter in animals such as mussels, which mainly use taurine as an osmolyte, could be a key molecule of the cellular osmoconforming process. In some mammals and fishes, taurine transporters have already been cloned (Han et al., 1998; Jhiang et al., 1993; Liu et al., 1992; Miyamoto et al., 1996; Ramamoorthy et al., 1994; Smith et al., 1992; Takeuchi et al., 2000a,b; Uchida et al., 1992; Vinnakota et al., 1997) and the involvement of some of them in cellular osmotic adaptation were confirmed (Takeuchi et al., 2000a,b; Uchida et al., 1992), although the taurine content and its contribution to the intracellular osmolality and osmotic adaptation in these animals are relatively lower than that in mussels.

In 1937, Baldwin summarized the regulation of the internal environment, namely the solute composition and the consequent osmotic pressure, in aquatic animals (Baldwin, 1937). Even though the detailed mechanisms were unclear, it was already known that some brackish invertebrates that were not equipped with advanced mechanisms for regulating their internal environment, could adapt to significant changes in environmental salinity. This present study is a major contribution to our understanding of the mechanism of osmotic adaptation at a cellular level in aquatic invertebrates, which has been known about since the beginning of the last century, but is only now being elucidated.

Materials and methods

Animals

Mediterranean blue mussel *Mytilus galloprovincialis* Lamarck (50–70 mm shell length) cultured in Matoya Bay, central Honshu, Japan, were commercially purchased and maintained in artificial seawater of 3.0% salinity without feeding. Approximately 20 mussels were maintained in a 50 l tank with aeration.

Cloning and sequencing

Total RNA was prepared from the mantle of mussels by the guanidine isothiocyanate/cesium chloride method, and poly(A)$^+$ RNA was purified using Oligotex-dT30 (Takara Bio Inc., Shiga, Japan). cDNA fragments were amplified by reverse transcription-polymerase chain reaction (RT-PCR). To obtain cDNAs as PCR templates, 1 μg of poly(A)$^+$ RNA was reverse-transcribed with oligo(dT)$_{20}$ primer. For PCR, the degenerate primers with *Eco*RI sites were prepared on the basis of the conserved amino acid sequence in the putative fourth extracellular loop and ninth transmembrane domain of mammalian and fish taurine transporters (Han et al., 1998; Jhiang et al., 1993; Liu et al., 1992; Miyamoto et al., 1996; Ramamoorthy et al., 1994; Smith et al., 1992; Takeuchi et al., 2000a; Takeuchi et al., 2000b; Uchida et al., 1992; Vinnakota et al., 1997). The sequences of the forward and reverse primers were 5′-dCGGAATTCTTYATGGCNCARGARCARGGN-3′ and 5′-dCGGAATTCCATNCCNCCYTCNGTNACCAT-3′, respectively. The amplified PCR product (324 base pairs) was subcloned into the *Eco*RI site of pBluescript II KS$^+$ (Stratagene, La Jolla, CA, USA) and the sequence was determined. In order to identify the 5′ and 3′ ends of the cDNA corresponding to this fragment, 5′ and 3′ rapid amplification of cDNA ends (RACE) was performed using the SMART^{TM} RACE cDNA Amplification Kit (BD Biosciences, San Jose, CA, USA). Amplified fragments were directly subcloned into pGEM-T Easy vector (Promega Corporation, Madison, WI, USA) and sequenced on both strands. The determined sequences were assembled into one contig with an open reading frame, called muTAUT for the mussel taurine transporter. For the expression of muTAUT, the sequence of the complete open reading frame was amplified and subcloned into the pcDNA3.1/myc-His B expression vector (Invitrogen Corporation, Carlsbad, CA, USA) and the pCS2+ vector (Turner and Weintraub, 1994) for expression in HEK293T cells and *Xenopus laevis* oocytes, respectively. The constructs were then verified by sequencing. The nucleotide sequence for muTAUT has been deposited in the DDBJ/EMBL/GenBank databases under accession number AB190909.

Expression in HEK293T cells

HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 25 mmol l^{-1} Hepes, 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin at 37°C in air containing 5% CO_2. Cells were plated in 24-well plates and transiently transfected with the muTAUT-pcDNA3.1/myc-His B vector or the mock vector, using cationic liposomes (LipofectAMINE, Invitrogen Corporation) in accordance with the manufacturer's instructions. The transfection was carried out for 16–18 h, and the medium was then replaced. Cells were assayed for taurine uptake activity 48 h post-transfection as described previously by Takeuchi et al. (2000b). Since the taurine uptake was almost linear for at least the first 45 min of incubation (data not shown), we used a 30 min uptake incubation period for the experiments.

Expression in Xenopus laevis *oocytes*

cRNA of muTAUT was obtained by *in vitro* transcription from linearized muTAUT-pCS2+ vector using SP6 RNA polymerase. Stage IV–V *Xenopus laevis* oocytes were isolated and the follicle removed using Collagenase A (Roche Diagnostics, Mannheim, Germany). They were then injected with 25 ng cRNA diluted with 50 nl diethyl pyrocarbonate-treated water per oocyte and incubated in modified Barth's solution supplemented with 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin for 2–3 days. The oocytes were briefly rinsed in a standard uptake buffer (Utsunomiya-Tate et al., 1996) and incubated in 24-well plates containing 1 ml of standard uptake buffer for 30 min at room temperature. Since a higher concentration of NaCl in the medium was required to allow sufficient taurine uptake *via* muTAUT for detailed functional analysis (described in Results and Discussion), the taurine uptake activity of the oocytes was measured in NaCl-added uptake buffer (standard uptake buffer containing 200 mmol l^{-1} NaCl). The oocytes were incubated in 0.5 ml of

NaCl-added uptake buffer containing 0.5 μCi ml^{-1} [^{3}H]taurine (NEN Life Science Products, Inc., Boston, MA, USA) for 30 min. Taurine and NaCl concentrations in the buffer were varied and a portion or all of the Na^+ and the Cl^- ions were replaced with choline ions and either gluconate or NO_3^- ions, respectively, depending on the purpose of the experiment. The oocytes were rinsed three times with standard uptake buffer and each of them was solubilized by pipetting in 200 μl of water. The radioactivity was measured by liquid scintillation counting.

The tilapia taurine transporter (tTAUT, accession no. AB033497) was also subcloned into pCS2+ vector and expressed in the oocytes using the same method as for the comparative analysis of the NaCl concentration requisite for taurine uptake activity.

Northern blot analysis

Twenty micrograms of total RNA purified from the mussels were electrophoresed in 1% agarose gels containing 2 M formaldehyde. RNAs were transferred to a nylon membrane (Gene Screen Plus, NEN Life Science Products, Inc.) and muTAUT mRNA was hybridized with the 324 base fragment of the RT-PCR product labeled with [α-^{32}P]dCTP using a Megaprime DNA labeling system (Amersham Biosciences Corp., Piscataway, NJ, USA) for 15 h at 65°C in 0.25 M Na_2PO_4, 1 mmol l^{-1} EDTA, and 7% SDS (Church and Gilbert, 1984). This filter was rinsed three times with 2× standard saline citrate (SSC) and washed three times with 0.2× SSC containing 0.1% SDS for 20 min at 65°C, it was then subjected to autoradiography.

Immunohistochemistry

The mantle and gill tissues of the mussels exposed to osmotic change were fixed in Bouin's fixative. Fixed tissues were dehydrated in an ethanol series and embedded in paraffin wax. Thin sections of approximately 7 μm were prepared using a microtome PR-50 (Yamato Koki, Tokyo, Japan). The sections were pretreated with 1.5% H_2O_2 in phosphate-buffered saline (PBS; 137 mmol l^{-1} NaCl, 8.1 mmol l^{-1} Na_2HPO_4, 2.68 mmol l^{-1} KCl, 1.47 mmol l^{-1} KH_2PO_4) and then washed several times with 1× PBS and 1× PBST (PBS with 0.1% Triton X-100). Tissues were blocked in 10% fetal bovine serum in 1× PBST for 1 h and then incubated with the diluted muTAUT polyclonal antibody. The antibody was raised against the synthetic peptide of (C)SMEYEKFLQKDSNV, which corresponded to the carboxyl-terminal end of muTAUT with an additional amino-terminal cysteine. The antibody was purified using an affinity column to which the synthetic peptide was bound, and was then further diluted 1:1000 in 1% skimmed milk in 1× PBST. After 12 h incubation with the diluted antibody, sections were washed in 1× PBST for 30 min (three changes) and incubated for 30 min at room temperature with the second antibody labeled with horseradish peroxidase (Nichirei Corporation, Tokyo, Japan). After 30 min of PBST washes (three changes), the colorimetric reaction was initiated by adding diaminobentidine substrate (0.5 mg ml^{-1} 3,3-diaminobenzidine and 0.005% H_2O_2 in 1× PBS). Stained sections were observed using a light microscope (FX-PH-21, Nikon Corporation, Tokyo, Japan).

Results

Structure of muTAUT

As a result of RT-PCR performed with degenerate primers using poly(A)$^+$ RNA from the mussel mantle, the fragment with 324 base pairs was amplified and the deduced amino acid sequence showed significant identity with taurine transporters, which belong to the sodium- and chloride-dependent neurotransmitter transporter superfamily. Based on the high sequence identity with reported taurine transporters, there was every possibility that this fragment was the partial sequence of the taurine transporter in the Mediterranean blue mussel (muTAUT). Identification of the full-length coding sequence (2001 bases), encoding 666 amino acids with a predicted relative molecular mass of 75,100 Da (Fig. 1) was based on the 5′- and 3′-RACE results. The Kyte-Doolittle hydropathy profile of the deduced amino acid sequence predicts 12 putative transmembrane domains (data not shown), which is a structure common to the superfamily of sodium- and chloride-dependent neurotransmitter transporters (Palacin et al., 1998). The transmembrane domains predicted by TMHMM (Krogh et al., 2001) occupy similar positions to those of vertebrate taurine transporters with regard to the alignment of amino acid sequences (Fig. 1). However, the predicted amino acid sequence of muTAUT shows 47–51% identity to that of vertebrate taurine transporters, whereas vertebrate taurine transporters have 78% and greater identity among themselves. The second extracellular loop of muTAUT contains five potential *N*-glycosylation sites.

Functional characterization of muTAUT

The functional characterization of muTAUT was determined by a transient expression in HEK293T cells. Taurine uptake

Table 1. *Taurine transport activity and the Na^+ and Cl^- dependence of muTAUT expressed in HEK-293T cells*

	Taurine uptake (pmol/30 min/10^6 cells)	
Medium	Control vector	pcDNA3.1 myc/His B-muTAUT
NaCl	213±56.2 (100)	811±32.1 (100)
Na^+ free (choline chloride)	8.3±1.0 (3.9)	10.0±2.0 (1.2)
Cl^- free (sodium gluconate)	9.4±0.3 (4.4)	17.0±1.3 (2.1)

Control vector (pcDNA3.1myc/His B) and cDNA construct (pcDNA3.1myc/His B-muTAUT) were introduced to HEK-293T cells, and uptake of 10 μmol l^{-1} taurine containing 0.5 μCi ml^{-1} [^{3}H]taurine was measured. Values are mean ± standard deviation of four samples from a single experiment. The values in parentheses represent the percentage of the uptake in NaCl medium.

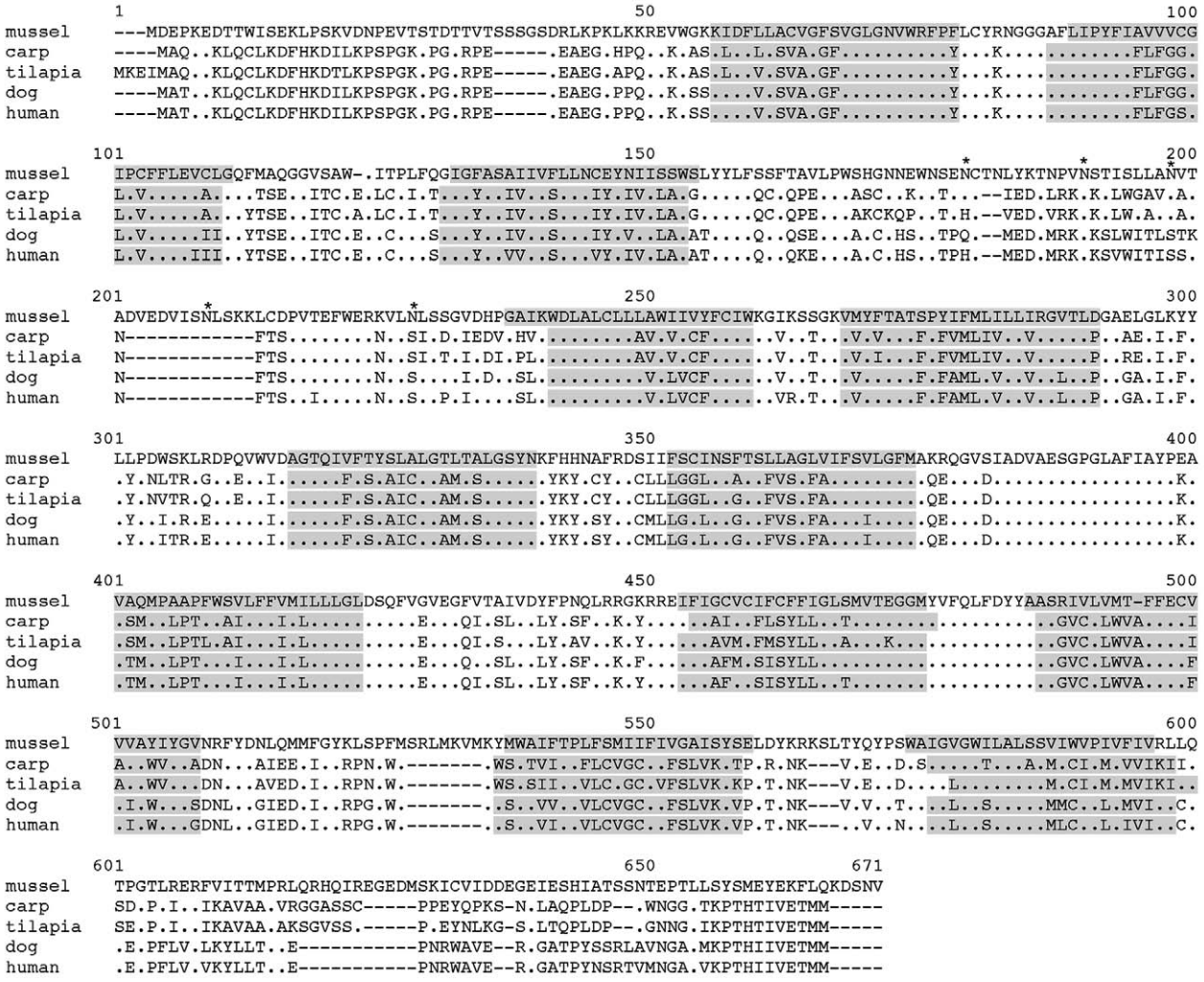

Fig. 1. Amino acid sequence alignment of mussel taurine transporter (muTAUT) with fish and mammalian taurine transporters. Dots indicate amino acids identical to those of muTAUT. Hyphens indicate gaps. Gray boxes indicate the 12 putative membrane-spanning domains. Asterisks in the second extracellular domain show the potential *N*-glycosylation sites of muTAUT. Accession numbers of each sequence are as follows, carp: AB006986, tilapia: AB033497, dog: M95495, and human: U09220.

activity in muTAUT cDNA-transfected cells was approximately 3.8-fold higher than that in mock vector-transfected cells (Table 1). Uptake of taurine by the cDNA-transfected cells decreased by <98.6% when Na^+ in the uptake medium was replaced by choline or Cl^- was replaced by gluconate.

Functional analysis in Xenopus *oocytes*

Fig. 2 shows the kinetics of the taurine transport of muTAUT expressed in *Xenopus* oocytes. Eadie-Hofstee plots revealed an apparent K_m of 41 μmol l^{-1}. The Na^+ and Cl^- stoichiometry of taurine uptake estimated by the 'activated

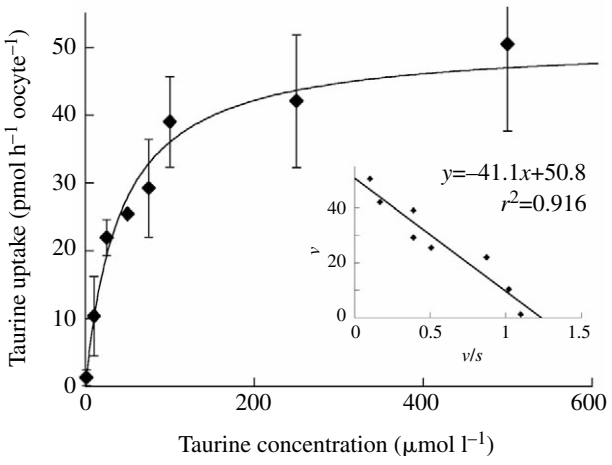

Fig. 2. Kinetics of taurine uptake into *Xenopus* oocytes injected with mussel taurine transporter (muTAUT) synthetic RNA. Uptake of taurine into cRNA-injected oocytes was measured at indicated taurine concentrations with 0.5 μCi ml^{-1} [^{3}H]taurine. Values obtained under the same conditions with uninjected oocytes were subtracted from values of corresponding injected samples. An Eadie-Hofstee plot of the data is depicted in the inset.

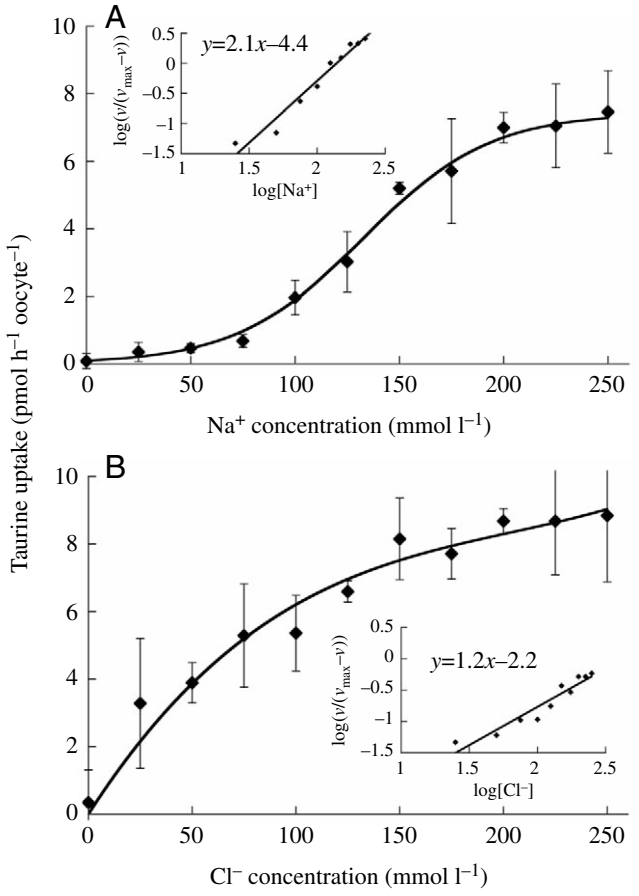

Fig. 3. Dependence of taurine uptake on [Na^+] and [Cl^-] in mussel taurine transporter (muTAUT)-injected oocytes. Taurine uptake was measured at (A) various Na^+ (1–250 mmol l^{-1}) concentrations and constant Cl^- concentration (250 mmol l^{-1}) or (B) various Cl^- (1–250 mmol l^{-1}) concentrations and constant Na^+ concentration (250 mmol l^{-1}), with 10 μmol l^{-1} taurine containing 0.5 μCi ml^{-1} [^{3}H]taurine. Isotonicity of the medium was maintained with choline chloride or sodium gluconate. Plotted data were adjusted by subtraction of uptake values by water-injected oocytes from those by muTAUT-injected oocytes (N=6–8). Error bars represent the standard deviation. The Hill coefficient and K_{50} were estimated by linear fitting of Hill plots (insets).

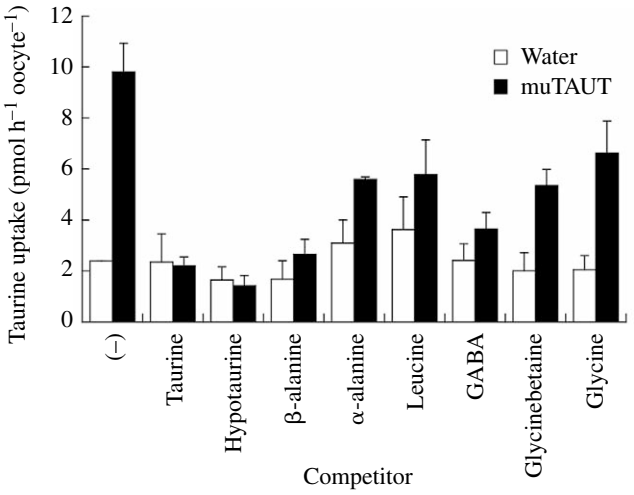

Fig. 4. Competition analysis of taurine uptake by mussel taurine transporter (muTAUT)-injected oocytes. Taurine uptake in muTAUT- and water-injected oocytes was measured with 10 μmol l^{-1} taurine containing 0.5 μCi ml^{-1} [^{3}H]taurine in the presence of 1 mmol l^{-1} unlabeled compounds as the competitor. Each column represents the mean ± standard deviation of 5–8 oocytes.

method' is shown in Fig. 3. In order to estimate the Na^+ stoichiometry, taurine uptake induced by muTAUT was measured at various Na^+ concentrations with a constant Cl^- concentration. The relationship between taurine uptake and Na^+ concentration was sigmoidal (Fig. 3A). Hill-type analysis of the data revealed a Hill coefficient of 2.1 with a K_{50} value of 158 mmol l^{-1} Na^+. These data suggest two or more Na^+ ions are coupled with the transport of one taurine molecule. By the same analysis, taurine uptake resulting from muTAUT exhibited a hyperbolic dependence on Cl^- concentration, and the coefficient was 1.2 with a K_{50} value of 81 mmol l^{-1} Cl^-, suggesting the involvement of one Cl^- ion per transport of one taurine molecule (Fig. 3B).

In order to analyze the substrate specificity of the muTAUT, various agents were examined for their ability to inhibit the [^{3}H]taurine uptake activity (Fig. 4). Taurine uptake of muTAUT-injected oocytes was markedly inhibited by unlabeled taurine, hypotaurine and β-alanine. Gamma aminobutyric acid (GABA) inhibited taurine uptake to a smaller extent than these β-amino acids. All the other agents of α-amino acids and glycinebetaine also inhibited taurine uptake with a statistical significance.

Since sufficient taurine uptake from muTAUT had not been detected in the standard uptake medium, taurine uptake activity of muTAUT-injected oocytes was measured in various medium conditions in a preliminary analysis. As a result, it was revealed that taurine uptake due to muTAUT necessitated higher NaCl concentration. Fig. 5 shows the relationship between taurine uptake and NaCl concentration in muTAUT- and tilapia taurine transporter (tTAUT)-injected oocytes. muTAUT-injected oocytes showed hardly any activity in 50 mmol l^{-1} or lesser concentration of NaCl in the uptake medium, and only 30.6% uptake was exhibited in the standard uptake medium (100 mmol l^{-1} NaCl). By contrast, tTAUT-injected oocytes showed adequate activity in the standard uptake medium. Since the medium had NaCl concentration above 100 mmol l^{-1}, the medium was hyperosmotic for the *Xenopus* oocytes. Therefore, the effect of the medium osmolality on taurine uptake activity was also measured in the hyperosmotic medium containing 100 mmol l^{-1} constant concentration of NaCl by adding various concentrations of glycerol. Uptake activities of both muTAUT-injected and tTAUT-injected oocytes were unaffected by a hyperosmolality below 400 mOsm kg^{-1}·H_2O. In medium with a higher osmolality than 500 mOsm kg^{-1}·H_2O, significant decrease in the uptake activity was observed, probably because of hyperosmotic stress on the oocytes and thus, the analysis was impossible.

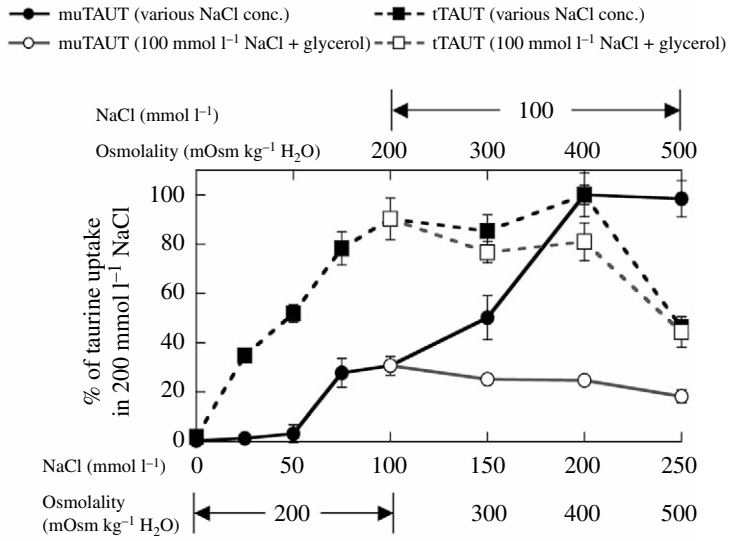

Fig. 5. Relationship of taurine uptake and NaCl concentration/medium osmolality in mussel taurine transporter (muTAUT)- and tilapia taurine transporter (tTAUT)-injected oocytes. Closed symbols indicate taurine uptake in various NaCl concentrations indicated in the lower horizontal axis. Open symbols represent taurine uptake in 100 mmol l^{-1} NaCl concentration with various medium osmolalities adjusted by the addition of glycerol (upper horizontal axis). Values obtained under the same conditions with uninjected oocytes were subtracted from those of corresponding injected samples.

Expression of muTAUT

Northern blot analysis with RNAs isolated from the mantle, gill and adductor muscle of the mussel exposed to a change in ambient osmolality caused by the salinity change is shown in Fig. 6. Abundance of muTAUT RNA increased in all analyzed tissues at least 2 h after the exposure to 2× seawater and was maintained at a level higher than that at 0 h (Fig. 6A). In the mussel exposed to 0.5× seawater, induction of muTAUT RNA was also observed (Fig. 6B); this was depressed by the addition of 25 mmol l^{-1} taurine to the ambient seawater in the mantle of mussels exposed to 0.5× seawater for 24 h. (Fig. 6C).

Immunohistochemistry with anti-muTAUT antibody detected the expression of muTAUT in the mantle epithelium and the gill (Fig. 7). In the mantle, the expression of muTAUT was detected in the basolateral region of the epithelial cells, whereas no staining for muTAUT was detectable in the apical membrane because of the endogenous brownish pigments on the epithelium surface. More intensive staining was detected basolaterally in the epithelial cells after the exposure to 0.5× seawater for 48 h (Fig. 7A,B). The staining on the gills also revealed the hypoosmotic responsiveness of muTAUT expression (Fig. 7C,D). It should also be noted that mussel tissues exposed to 0.5× seawater swelled, probably as a result of the permeation of water (Fig. 7B,D).

Discussion

muTAUT is the first taurine transporter cloned from an organism belonging to classes other than mammals and fishes. Considering the importance of taurine as an osmolyte in aquatic invertebrates, the taurine transporter is probably one of the most essential molecules in the osmotic conforming process in these animals.

Although muTAUT has the same transport mechanism as previously cloned taurine transporters, i.e. coupling with Na^+ and Cl^- ions and the stoichiometry of these ions (Table 1, Fig. 3), it has also become apparent in this study that muTAUT, which has structural features distinct from those of other cloned taurine transporters, has three characteristic functional properties. The first is that the muTAUT K_m value for taurine transport is much higher than that of mammalian taurine transporters. The muTAUT K_m value for taurine transport is 41 μmol l^{-1} (Fig. 2), and that for taurine transport in mouse brain (Liu et al., 1992), mouse retina (Vinnakota et al., 1997), dog (MDCK cells; Uchida et al., 1992), human retina (Miyamoto et al., 1996) and human placenta (Ramamoorthy et al., 1994) is 4.5, 13.2, 9.1, 2.0 and 5.9 μmol l^{-1}, respectively. K_m values of taurine transporters in rat (Smith et al., 1992) and carp (Takeuchi et al., 2000b),

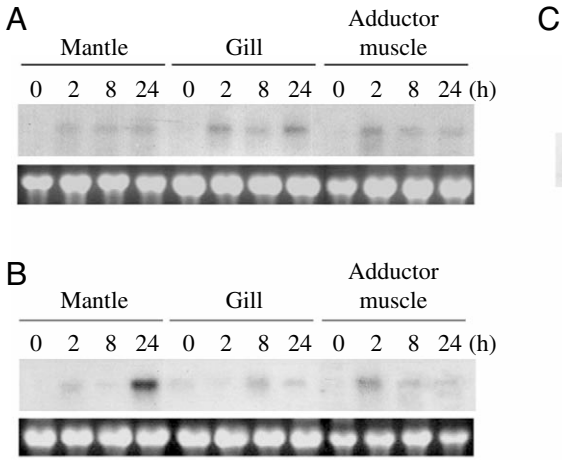

Fig. 6. Northern blot analysis of taurine transporter mRNA in mussels exposed to changes in ambient salinity. (A,B) Time course of mussel taurine transporter (muTAUT) mRNA abundance in the mantle, gill and adductor muscle of mussels exposed to 2× seawater (A), 0.5× seawater (B), respectively. Total RNAs extracted from three mussels and mixed for minimization of individual variability. Lower panels show the rRNA signals of ethidium bromide-stained gel. (C) Depression of muTAUT mRNA expression by the addition of taurine. Northern blot analysis was performed using 20 μg of total RNAs from the mantle of mussels exposed to 0.5× seawater with or without 25 mmol l^{-1} taurine for 24 h. RNAs loaded in each lane were extracted from different specimens.

estimated using COS-7 cells, are excluded from the discussion because there is some indication that the use of COS-7 cells affected the K_m values (Takeuchi et al., 2000b). It should be noted that the K_m value for taurine transport of muTAUT is in good agreement with the K_m value of taurine transport in the basolateral surface of the excised gill cells (35.3 μmol l^{-1}) of California mussel *Mytilus californianus* (Neufeld and Wright, 1995).

The second distinct functional property is the competition of various α-amino acids and glycinebetaine to taurine transport (Fig. 4). A much smaller competitive effect from α-alanine and proline has been reported for the taurine transporter of human placenta (Ramamoorthy et al., 1994) and tilapia (Takeuchi et al., 2000a). However, other studies in human placental cell (Miyamoto et al., 1988), rabbit jejunal cells (Miyamoto et al., 1989), brush-border membrane vesicle cells (Miyamoto et al., 1989), human neuroblastoma × glioma hybrid cells (Kurzinger and Hamprecht, 1981), MDCK cells (Uchida et al., 1991) and cloned transporters (Liu et al., 1992; Smith et al., 1992; Takeuchi et al., 2000b; Uchida et al., 1992) have reported competition only by hypotaurine, β-alanine and GABA. In addition, competition analysis of the tilapia taurine transporter under the same conditions as those in this study revealed that the taurine transport activity was completely unaffected by α-amino acids (data not shown). Considering these facts, our results suggest that taurine specificity of muTAUT is lower than that of other reported taurine transporters. However, there is also evidence that in two species of mussel, α-amino acids had no effect on epidermal taurine transport (Wright and Secomb, 1984).

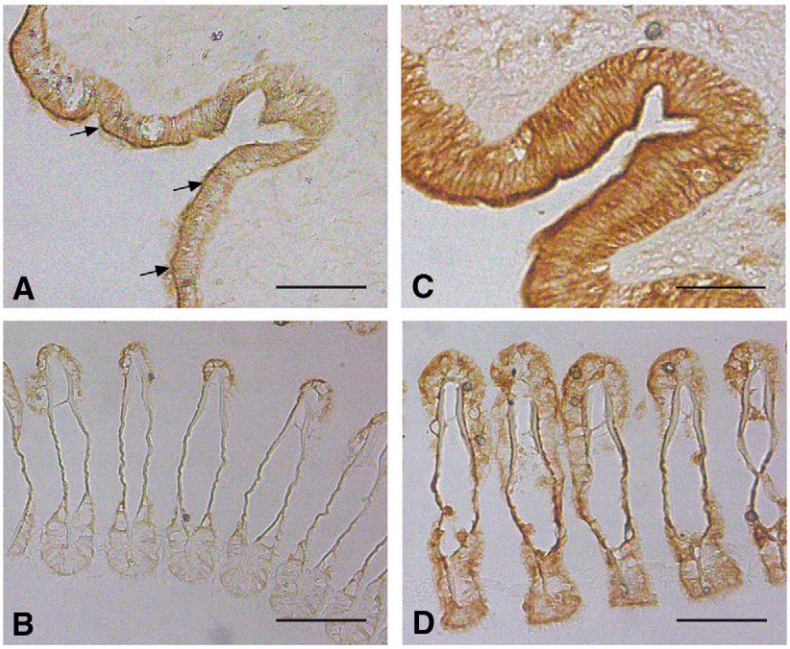

Fig. 7. Immunohistochemical detection of taurine transporter on the mantle of mussels exposed to changes in ambient salinity. (A,B) Mantle sections and (C,D) gill sections of mussels acclimated to 1× seawater (A,C) or exposed to 0.5× seawater for 48 h (B,D). The brownish color on the surface of the mantle (indicated by arrows in A) is not staining but endogenous pigments. Scale bars, 50 μm.

The third characteristic is that muTAUT taurine transport requires a higher NaCl concentration (Fig. 5). Although the relationship between the NaCl concentration in the external medium and the taurine transport activity of the cloned taurine transporter expressed in *Xenopus* oocytes has never been determined, kinetic analysis of epithelial taurine transport activity in *Mytilus californianus* gill revealed an apparent K_{50} value of 200–400 mmol l^{-1} Na^+ (Silva and Wright, 1992; Neufeld and Wright, 1995). Data obtained in the present study was interpreted as confirmation, at the molecular level, of previous results obtained at a tissue level.

Why does muTAUT have these distinctive functional properties? The most obvious answer to this question is because of differences in the primary structure: there is relatively lower percentage identity between the deduced amino acid sequence of muTAUT and other cloned vertebrate taurine transporters than there is among the vertebrate taurine transporters themselves. In particular, some mutations exist on the residues that are completely conserved among vertebrates. Of these, mutations in the regions important for the transport activity, such as the first extracellular loop and the adjacent domain involved in the reaction with Na^+ and Cl^- ions (Nelson and Lill, 1994) and the transmembrane region involved in the recognition and selectivity of the transport substrate (Palacin et al., 1998), possibly influence the functional properties. Even though the elucidation of this question requires further studies, such as site-directed mutagenesis, to analyze the structure–function relationship, muTAUT provides useful information on the functional features of taurine transporters.

Molecular evolution of muTAUT that causes these structural and functional differences should be considerably influenced by the internal environment of the mussel where muTAUT is functioning. The mussel mainly utilizes taurine as an osmolyte, as described above, and therefore, taurine is accumulated at a high concentration in the mussel cells. Taurine in hemolymph is also highly concentrated, for example, in the common mussel, taurine concentration in the hemolymph is approximately 500 μmol l^{-1} (Zurburg and DeZwaan, 1981), which is one order of magnitude higher than that in mammals (approximately 50–80 μmol l^{-1} in plasma; Cuisinier et al., 2002; Delaney et al., 2003; Pacioretty et al., 2001). In addition, taurine is dominant and accounts for approximately 80% of the total amino acid pool in the mussel (Toyohara and Hosoi, 2004), suggesting that the concentration and proportion of taurine is much higher than in mammals. It is assumed that this concentrated taurine condition is reflected in the lower taurine affinity and specificity of muTAUT. Furthermore, because the mussel is an osmoconformer, Na^+ and Cl^- concentration of the hemolymph is at the same level as in seawater. This means that

internal Na^+ and Cl^- concentrations of the mussel are three times as high as those in mammals and fishes. Considering these facts, the requisite for higher NaCl concentration by muTAUT is also in good agreement with the internal environment of the mussel.

Northern blot analysis demonstrated that the expression of the muTAUT gene at the transcriptional level was induced not only by the hyperosmotic condition but also by the hypoosmotic condition (Fig. 6). Hyperosmotic responsive expression of the taurine transporter has been reported in tilapia (Takeuchi et al., 2000a) and cell lines established from dog (Uchida et al., 1992) and carp (Takeuchi et al., 2000b), and therefore, one of the important roles of the taurine transporter is considered to be the modulation of intracellular osmolality by taurine uptake when the osmolality of the extracellular medium increases (Uchida et al., 1992). It is assumed that the muTAUT gene is induced in conditions of hyperosmolality to increase intracellular osmolality thus preventing the cell from shrinking and the intracellular concentration of Na^+ and Cl^- ions from increasing.

Hypoosmotic responsiveness of the taurine transporter, on the other hand, has never been reported. It has not been analyzed in studies on mammalian taurine transporters, but only in the EPC (*Epithelioma papulosum cyprini*) cell line established from carp epithelium. It has been confirmed in this cell line that mRNA abundance of carp taurine transporter was not increased by hypoosmotic stress (Takeuchi et al., 2000b). Considering that the primary function of the taurine transporter is uptake and accumulation of taurine in the cells, it was an unexpected finding that the muTAUT gene is induced under hypoosmotic conditions, in which the mussel cells should release taurine to decrease the intracellular osmolality. In fact, it had been revealed by our previous study (Toyohara and Hosoi, 2004) that taurine content in the mantle tissue decreased considerably from 8 to 24 h after exposure to hypoosmolality. Subsequently, we analyzed the relationship between the hypoosmotic induction of muTAUT and taurine concentration, and it was revealed that the induction of the muTAUT gene in hypoosmolality was depressed by the addition of 25 mmol l^{-1} taurine to the ambient seawater. This suggests that the expression of the muTAUT gene is not directly regulated by hypoosmolality, but by a consequent decrease in the taurine content caused by the decrease in ambient osmolality (Fig. 6C). It also supports the hypothesis that the pattern of muTAUT induction in the mantle, revealed by northern blot analysis in this study, was in good agreement with the pattern of decrease in taurine content in the mantle, under similar hypoosmotic condition, in our previous study (Toyohara and Hosoi, 2004). In addition, the regulation at the transcriptional level of the taurine transporter by taurine concentration has been demonstrated in some mammals (Han et al., 1997a,b, 1998).

We suggest that, as in mammals, muTAUT expression at a transcriptional level is regulated by the taurine concentration of the mussel. The remarkable decrease in taurine content caused by the considerable decrease in hemolymph osmolality seems to be specific for the osmoconforming animals such as molluscs. Therefore, the hypoosmotic induction of the taurine transporter was not observed in the carp (Takeuchi et al., 2000b). In other words, these facts demonstrate that the extensive decrease in intracellular taurine content occurs in hypoosmolality, suggesting the importance of taurine as an 'adaptive' osmolyte, which mediates the change in intracellular osmolality.

Immunohistochemistry using anti-muTAUT antibody revealed the intense expression of muTAUT in the basolateral region of the mantle epithelium and the gill (Fig. 7), where the cells are directly in contact with the external seawater. Considering that taurine is apparently absent in the environmental seawater (Braven et al., 1984) despite its high concentration in the hemolymph (approximately 500 μmol l^{-1}; Zurburg and DeZwaan, 1981) and cytoplasm (approximately 200 mmol l^{-1}; Neufeld and Wright, 1995), the cells exposed directly to environmental seawater will need to maintain a much higher concentration gradient of taurine than the cells surrounded entirely by hemolymph. Our results suggest that the high expression of muTAUT in these cells corresponds to the accumulation of intracellular taurine from hemolymph to cope with the steep taurine gradient between the cytoplasm and seawater, and consequent high adaptability to environmental osmotic change. Although the expression of muTAUT in the apical membrane of the mantle epithelium could not be detected, it would seem to be appropriate to assume that apical taurine transport resulting from muTAUT is likely to be insignificant regardless of the apical expression of muTAUT, because the K_m value for taurine transport of muTAUT (41 μmol l^{-1}) is much higher than the taurine concentration in the seawater. However, a previous study by Wright and Secomb (1989) demonstrated that the apical surface of the gill cells of *Mytilus edulis* and *M. californianus* certainly show taurine transport activity with an apparent K_m value of approximately 5 to 8 μmol l^{-1}. They reached the conclusion that apical taurine transport with a taurine affinity that is not suited to uptake from much low concentrations of taurine in seawater may play a role in recovering taurine lost from the gill surface. *In vivo* dynamics of taurine and the involvement of muTAUT is one of the important future assignments.

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