PHYSIOLOGICAL AND MOLECULAR CHARACTERIZATION OF UREA TRANSPORT BY THE GILLS OF THE LAKE MAGADI TILAPIA (ALCOLAPIA GRAHAMI)

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Summary

The Lake Magadi tilapia (Alcolapia grahami) is an unusual fish, excreting all its nitrogenous waste as urea because of its highly alkaline and buffered aquatic habitat. Here, using both physiological and molecular studies, we describe the mechanism of branchial urea excretion in this species. In vivo, repeated short-interval sampling revealed that urea excretion is continuous. The computed urea permeability of A. grahami gill is 4.74×10^{-5}±0.38×10^{-5}cm\text{s}^{-1} (mean ± S.E.M., N=11), some 10 times higher than passive permeability through a lipid bilayer and some five times higher than that of even the most urea-permeable teleosts studied to date (e.g. the gulf toadfish). Transport of urea was bidirectional, as demonstrated by experiments in which external [urea] was elevated. Furthermore, urea transport was inhibited by classic inhibitors of mammalian and piscine urea transporters in the order thiourea>N-methylurea>acetamide. A 1700 base pair cDNA for a putative Magadi tilapia urea transporter (mtUT) was cloned, sequenced and found to display high homology with urea transporters from mammals, amphibians and other fishes. When cRNA transcribed from mtUT cDNA was injected into Xenopus laevis oocytes, phloretin-inhibitable urea uptake was enhanced 3.4-fold relative to water-injected controls. Northern analysis of gill, red blood cells, liver, muscle and brain using a portion of mtUT as a probe revealed that gill is the only tissue in which mtUT RNA is expressed. Magadi tilapia gill pavement cells exhibited a trafficking of dense-cored vesicles between the well-developed Golgi cisternae and the apical membrane. The absence of this trafficking and the poor development of the Golgi system in a non-ureotelic relative (Oreochromis niloticus) suggest that vesicle trafficking could be related to urea excretion in Alcolapia grahami. Taken together, the above findings suggest that the gills of this alkaline-lake-adapted species excrete urea constitutively via the specific facilitated urea transporter mtUT.

Key words: Alcolapia grahami, urea transporter gene, UT-A2, gills, nitrogen excretion, Lake Magadi tilapia.

Introduction

The Lake Magadi tilapia (Oreochromis alcalicus grahami, now Alcolapia grahami; Seegers and Tichy, 1999) is a truly remarkable fish. It is the only fish species that survives (and thrives) in the highly alkaline (pH10) and highly buffered (CO₂ content 180 mmol l⁻¹) waters of Lake Magadi in the Rift Valley of Kenya (Coe, 1966). These waters would rapidly kill standard teleostean species, largely because most fish are unable to excrete ammonia under these conditions, resulting in neurotoxicity. Several publications have now documented that a central part of the survival/adaptational strategy of this species is to be completely ureotelic, i.e. to excrete all their nitrogen waste as urea and none as ammonia, an unusual condition for a fully aquatic teleostean fish (Randall et al., 1989; Wood et al., 1989; Wood et al., 1994; Wright et al., 1990). Furthermore, the rates of urea synthesis and excretion approach mammalian excretion rates, perhaps because of the...
high temperature of the environment (Narahara et al., 1996) and nitrogen loading by the predominant diet, cyanobacteria (Wood et al., 1994).

The biochemical mechanisms of urea production in this species are becoming to be relatively well understood. The fish possess a fully functional hepatic ornithine–urea cycle (O-UC) (Randall et al., 1989; Walsh et al., 1993), but two unusual aspects of urea synthesis have recently been discovered. First, the key O-UC enzyme carbamoyl phosphate synthetase (CPSase) and other O-UC enzymes are also expressed in muscle, and the total muscular CPSase activity far outweighs the total hepatic activity (Lindley et al., 1999). This observation led these authors to suggest that most urea is indeed synthesized in the muscle. They further postulated that this architecture was due to the inefficiency of vertebrate CPSase; if CPSase were packaged in the liver, the mass of liver would be incompatible with a laterally compressed perciform body plan (Lindley et al., 1999). The second unusual aspect of urea synthesis in this species is that the enzyme CPSase, while appearing to be of the piscine CPSase III isoenzyme type, uncharacteristically has a high capacity to use ammonia as a substrate, in addition to the more usual substrate glutamine (Lindley et al., 1999).

While information is accumulating on the mechanisms of urea production in this unusual species, very little is known about the mechanism of urea excretion. To date, only one study has addressed urea excretion. Using a divided chamber design separating the anterior and posterior of the fish, Wood et al. (Wood et al., 1994) demonstrated that approximately 80% of urea was excreted from the anterior half of the fish, with 20% being excreted from the posterior half, suggesting a minimal role for urinary urea excretion. Given the rapid progress being made on the physiological and molecular mechanisms of urea excretion in both mammalian and piscine systems (for reviews, see Sands et al., 1997; Walsh and Smith, 2000), the aims of the present study were to begin to analyze mechanisms of urea excretion in the Lake Magadi tilapia. Derived from a combination of physiological and molecular approaches, our results suggest that urea excretion is by the continuous operation of a specific facilitated transport mechanism in the gills. Furthermore, since the specific cellular organization of the gill may be related to this transport, we undertook ultrastructural studies of the cells lining the gills in a comparison between the urea-excreting fish Alcolapia grahami and its ammoniotelic relative Oreochromis niloticus.

Materials and methods

Animals and holding conditions

Lake Magadi tilapia (Alcolapia grahami, Seegers and Tichy, 1999; formerly Oreochromis alcalicus grahami) were captured from the Fish Springs Lagoon site (Narahara et al., 1996) at Lake Magadi, Kenya, by seine net in January and February 1997. The fish were immediately transported to an outdoor laboratory site several kilometers away (at the Magadi Soda Co., PLC) in large plastic containers filled with aerated water from Fish Spring Lagoon (typical water chemistry is reported in Table 1 of Wood et al., 1989). Water freshly collected from this site was also used for all experiments. Typically, fish were used in physiological experiments within 24 h of capture. In addition, selected tissues were frozen on site in liquid N2 and returned to North America at liquid N2 temperatures in a dry shipper. A simple saline, 200 mmol l−1 NaCl (similar to Magadi tilapia plasma; Wright et al., 1990), was used for all physiological procedures, and metomidate-HCl (5 mg l−1 in lagoon water) was used for anesthesia. All blood samples were taken by caudal puncture using a 100 μl gas-tight Hamilton syringe with a customized needle. The syringe was wetted with sodium heparin (1000 i.u. ml−1 in saline).

In vivo physiological studies

Series 1: pattern of urea excretion

To establish normal temporal patterns and rates of urea-N excretion, 11 tilapia (1.3–4.4 g) were placed in individual amber Tusker chambers (Wood et al., 1994) filled with 500 ml of continuously aerated lagoon water and allowed to settle for 1 h. Water samples (5 ml) for urea-N analysis were drawn every 15 min for the next 6.5 h. The temperature averaged 37.5 °C (36–39 °C). At the end of the experiment, the fish were quickly anesthetised and weighed, and the blood was sampled (10–20 μl) for plasma urea-N analysis. Plasma was separated by allowing the blood to settle at 4 °C in vertical hemocrit tubes (as the power failed that day), and then frozen for later analysis.

Series 2: thiourea versus urea permeability

To compare the relative permeability of the gills to urea and the urea analog thiourea, nine larger tilapia (4.9–15.7 g) were anesthetized briefly, weighed and injected with [14C]thiourea (NEN; specific activity 8.60 mCi mmol−1). The [14C]thiourea stock was made up at a concentration of 20 μCi ml−1 in saline; each fish received 10 μl g−1 injected into the hemal arch (i.e. 0.2 μCi g−1) using the customized Hamilton syringe. The fish were transferred first to fresh lagoon water for 1 h for recovery, to allow the radiolabel to distribute in the bloodstream and to wash off any leaking radiolabel, and then to individual shielded beakers, each containing 300 ml of continually aerated lagoon water. Two water samples (each 5 ml) were drawn at 0, 1.5 and 3 h for simultaneous measurements of urea-N and [14C]thiourea fluxes, and then the fish were anesthetized again for terminal blood sampling (50–150 μl) by caudal puncture. Plasma was separated by centrifugation at 10 000 g for 2 min and then frozen for later analysis of urea-N and [14C]thiourea radioactivity. The temperature was 34 °C during the flux measurements in the outdoor laboratory.

Series 3: urea influx and the effects of urea analogs

This series employed an elevated external urea concentration (20 mmol l−1) radiolabeled with [14C]urea (NEN; specific activity 8.60 mCi mmol−1), to test whether
gill urea flux was bidirectional, and evaluated whether urea influx could be inhibited by the simultaneous presence of urea analogs in the external water at three times the concentration of water urea. Influx rates were determined by measuring the appearance of $[^{14}C]$urea in the fish. There were five different treatment groups, each containing 7–10 fish (0.61–2.54 g), placed in shielded beakers containing 1.0 l of continuously aerated lagoon water spiked with 20 mmol l$^{-1}$ urea (40 mmol l$^{-1}$ urea-N) plus 20 µCi l$^{-1}$ $[^{14}C]$urea. This water came from a single stock to give identical concentrations of urea and $[^{14}C]$urea. In addition, the water contained 60 mmol l$^{-1}$ acetamide, 60 mmol l$^{-1}$ N-methylurea, 60 mmol l$^{-1}$ thiourea, 30 mmol l$^{-1}$ NaCl (as an osmotic pressure control) or no addition (as an absolute control). Three water samples (each 5 ml) were taken at 0, 1.5 and 3 h for measurement of water urea and $[^{14}C]$urea radioactivity; values did not change appreciably. Immediately after the 3 h sample, the fish were rapidly rinsed with fresh lagoon water (to remove surface-bound radioactivity), anesthetized, weighed and preserved in liquid N$_2$ for later measurement of $[^{14}C]$urea radioactivity. Because of a procedural error, most of the samples from the thiourea test were lost, so this treatment, together with a simultaneous control, was repeated. The results of the tests were identical, and the data were therefore combined. The temperature during these tests averaged 32°C (31–33°C). For the sake of comparison, normal urea flux rates to the water over a 3 h period (in the absence of added external urea or analogs) and plasma urea-N levels were measured in 10 fish of the same size, and at the same temperature, by the methods outlined in series 1.

**Calculations**

Urea flux rates and concentrations were expressed in units of nitrogen for consistency with previous studies (e.g. Wood et al., 1989; Wood et al., 1994). Urea-N flux rates to the water (in µmol-N kg$^{-1}$ body mass hour$^{-1}$) in series 1, 2 and 3 were calculated from changes in concentration in the water (µmol-N l$^{-1}$) multiplied by volume (l) and factored by time (h) and fish mass (kg). Urea permeabilities (in cm s$^{-1}$) of the gill in series 1 and series 2 were obtained by assuming that 80% of the measured flux rate (µmol-N kg$^{-1}$ body mass h$^{-1}$) occurred across the gills (Wood et al., 1994). This value was divided by the measured plasma urea-N concentration (µmol-N l$^{-1}$) and the gill area (cm$^2$ kg$^{-1}$), while converting hours (×3600) to seconds and liters (×1000) to cm$^3$. The thiourea permeability of the gill in series 2 was calculated using an identical approach, substituting counts of $[^{14}C]$thiourea for µmol urea-N. The gill area of each fish was estimated from its body mass using the allometric relationship of total secondary lamellar surface area (SLSA) to body mass established by Maina et al. (Maina et al., 1996) for this species at Fish Springs Lagoon. Urea-N influx rates in series 3 were calculated by dividing the terminal concentration of $[^{14}C]$urea radioactivity in the whole bodies (counts g$^{-1}$) by the mean specific activity of the water (counts mmol$^{-1}$) and time (h).

**Analytical techniques**

Urea-N in lagoon water (1 ml samples) and plasma (10 µl added to 1 ml of lagoon water) was measured using the diacetyl monoxime method (Rahmatullah and Boyd, 1980; Price and Harrison, 1987) with standards made up in lagoon water. The final volume (1 ml of sample plus 1 ml of reagents) was adjusted to 3 ml by the addition of 1 ml of lagoon water so that the assay could be read on a Spectronic 20 spectrophotometer (Bausch & Lomb).

$[^{14}C]$urea and $[^{14}C]$thiourea radioactivity were measured by scintillation counting (LKB Rackbeta 1217) in samples of lagoon water (1 ml) and plasma (20–100 µl plus 1 ml of lagoon water) made up to 11 ml with Ready-safe aqueous fluor (Beckman). $[^{14}C]$Urea in whole bodies was measured by first solubilizing the whole carcass in 6 ml g$^{-1}$ NCS tissue digest media (Amersham) in a sealed vial overnight at 42°C; 2 ml samples of clear digest were then neutralized with 30 µl of glacial acetic acid and made up to 20 ml with OCS organic fluor (Amersham). Quench correction was performed by the external standard method, and equalization of counting efficiencies in the two fluor systems was performed by internal standardization.

**Molecular studies**

**Polymerase chain reaction cloning**

Initial attempts to screen a Magadi tilapia (whole-body) cDNA library using the toadfish urea transporter (tUT; Walsh et al., 2000) as a probe were unsuccessful, so a reverse transcription/polymerase chain reaction (RT–PCR) approach was taken. Total RNA was isolated from the gill by homogenization of 0.2 g of tissue in 1.2 ml of phenol-guanidinium thiocyanate (Trizol Reagent, Gibco BRL) followed by standard extraction with chloroform and precipitation with isopropanol (Sambrook et al., 1989). mRNA was enriched from total RNA using an mRNA purification kit based on oligo(dT)–cellulose column chromatography (Pharmacia Biotech). cDNA was synthesized using MMLV reverse transcriptase and oligo(dt) primers (Stratagene). PCR was performed on this cDNA using degenerate primers designed using sequences for mammalian, piscine and amphibian urea transporters. These were sense (UTPS) (corresponding to the third base pair coding for amino acid residue 175 to the second base pair coding for amino acid residue 183 of tUT), 5′GGAYTTICCGNTNTYACNYTITCC3′; antisense (UTDA2) (corresponding to amino acid residues 316–310 of tUT), 5′GCAIRTRTGIRYYTGCC-AIGT3′. PCR conditions were 94°C for 4 min, followed by 50°C for 5 min, at which point Taq polymerase was added followed by the mineral oil layer in a hot-start protocol, and then 30 cycles of 94°C for 30 s, 50°C for 1 min, 72°C for 1 min, followed by 72°C for 7 min in a Perkin-Elmer 480 PCR machine using 0.5 ml GeneAmp tubes (Perkin-Elmer).

PCR products were separated by gel electrophoresis (1% agarose gel in TAE buffer (40 mmol l$^{-1}$ Tris-acetate, 1 mmol l$^{-1}$ EDTA, pH 8.0), and the major band of 420 base pairs (bp) was gel-purified and subcloned into the plasmid vector pCR 2.1.
(Invitrogen, Carlsbad, CA, USA). The resultant plasmid (m17-1) was transfected into Epicurean Ultracompetent Escherichia coli (Stratagene, La Jolla, CA, USA). Standard blue/white screening on Luria Broth (LB) containing 25 mg ml\(^{-1}\) kanamycin plates identified colonies with the potential insert, which were then cultured in LB. The plasmid DNA was isolated by the alkaline lysis method (Qiagen Kit, Chatsworth, CA, USA), and restriction digests were performed to ensure the presence of an appropriately sized insert. Both strands of insert DNA of several clones were sequenced using an automated dideoxy chain-termination sequencing method (Sanger et al., 1977) and found to be homologous to the toadfish urea transporter (tUT) gene (see Results).

**Rapid amplification of cDNA ends PCR**

5' and 3' rapid amplification of cDNA ends (RACE) PCR was performed to amplify 5' and 3' ends using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA, USA) and adaptor-ligated gill cDNA (adapt or from the kit). Gene-specific primers were synthesized on the basis of the known sequences of m17-1 and were mtUTGSP1 (the antisense-gene-specific primer for 5'RACE), 5'CCAATGGCGATGCAGGCTA-ACACACAGT3', corresponding to base pairs 380–352 of m17-1, and mtUTGSP2 (the sense-gene-specific primer for 3'RACE), 5'GGAGGCATCTTCATCATCTCCCTTTA3', corresponding to base pairs 211–238 of m17-1. The sense primer for 5'RACE and the antisense primer for 3'RACE were complementary to an adaptor supplied with the kit (which was ligated to the 5' and 3' cDNA ends). PCR conditions were: 94°C for 1 min, followed by five cycles of 94°C for 30 s, 72°C for 4 min, followed by five cycles of 94°C for 30 s, 70°C for 4 min, followed by 25 cycles of 94°C for 30 s, 68°C for 4 min with machine and tubes as above. Generally, PCR reactions were performed in duplicate to exclude PCR errors. The 5'RACE reactions produced a band of approximately 1.2 kilobase pairs (kb) and the 3'RACE reactions produced a band of approximately 1 kb, which were purified, subcloned and sequenced as above. Once sequences were confirmed to overlap, gene-specific primers near to the 5' and 3' ends were designed to obtain a ‘full-length’ clone, and a PCR product of 1.6 kb (mtUT 2Ex-2), which included the putative open reading frame (ORF), was generated and similarly cloned into pCR 2.1.

**Northern analysis**

Total RNA was extracted from several Magadi tilapia tissues (gill, liver, skeletal muscle, blood cells and brain) and poly(A\(^{+}\))-enriched as above. Equal amounts (3 \(\mu\)g) of poly(A\(^{+}\)) RNA were loaded onto a formaldehyde–agarose gel, electrophoresed and transferred to a nylon membrane (Hybond-N, Amersham, Arlington Heights, IL, USA) using standard methods (Sambrook et al., 1989). Following prehybridization, the membrane was hybridized at 42°C in hybridization buffer containing 30% formamide with random-prime \(^{32}\)P-labeled m17-1. \(^{32}\)Pm17-1 was produced by extracting the excised bands from EcoRI-digested minipreps electrophoresed as above, labeling with the RadPrime kit (Gibco, Frederick, MD, USA) and \([\alpha-^{32}\]P]dCTP (ICN, Irvine, CA, USA) and purification on G-50 Sephadex. Final washes were in 0.1X SSC/0.1% SDS at 65°C (1X SSC is 0.15 mol l\(^{-1}\) NaCl, 0.015 mol l\(^{-1}\) sodium citrate).

**Functional expression in Xenopus laevis oocytes**

After linearization of mtUT plasmid with KpnI, cRNA was synthesized in vitro using T7 polymerase (T7-mMessage mMachine, Ambion) and microinjected (30 ng) into collagenase-treated defolliculated Xenopus laevis oocytes, as described previously (Smith et al., 1995). Parallel groups of oocytes were injected with water (50 nl) as a negative control. Oocytes were incubated in isotonic frog saline (in mmol l\(^{-1}\): Na\(^{+}\) 104, Cl\(^{-}\) 104, Ca\(^{2+}\) 1.8, Mg\(^{2+}\) 1, pyruvate 2.5, Hepes 5, pH 7.4, containing 50 \(\mu\)g ml\(^{-1}\) gentamycin) for 2–3 days at 18°C prior to experimentation. For urea flux studies, oocytes were preincubated in buffer (in mmol l\(^{-1}\): 200 mannitol, 2 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 10 Hepes, 5 Tris, pH 7.4) for 15 min and then transferred to uptake solution consisting of the preincubation medium with added urea (1 mmol l\(^{-1}\) and \([^{14}\]C\)urea (2.7 \(\mu\)Ci ml\(^{-1}\)). After 1 min, oocytes were rapidly washed three times with ice-cold uptake solution containing 10 mmol l\(^{-1}\) unlabeled urea and dissolved in 0.5 ml of 10% SDS, and the radioactivity was measured by scintillation counting. Inhibition of \([^{14}\]C\)urea uptake by 1.0 mmol l\(^{-1}\) phloretin dissolved in ethanol was measured by addition of phloretin to the preincubation solution, as described previously (Smith et al., 1995). As a vehicle control, ethanol was included in the control experiments. In some experiments, oocytes were preincubated for 15 min with the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (PMA) (a potent activator of protein kinase C), or with forskolin (an activator of adenyl cyclase used to investigate the effect of cyclic-AMP-dependent protein kinase A, PKA), prior to uptake measurements in the continued presence of these drugs. Both drugs were added as a solution in dimethylsulfoxide (DMSO).

**Ultrastructure of the gill lining**

Fish weighing 1–5 g were captured early in the morning, killed with an overdose of MS222 (0.5 g l\(^{-1}\) in Fish Spring Lagoon water) and processed immediately for transmission electron microscopy of the gill filaments according to methods described previously (Laurent et al., 1995).

**Chemicals, analyses of sequence information and statistical analyses**

Unless stated otherwise, all chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA). Routine analyses of DNA and protein sequences was accomplished by MacVector and DNAStar programs. The sequences of urea transporters for other animals were obtained by a basic BLAST 2.0 search (Altschul et al., 1997) of GenBank. Statistical significance was tested by Student’s t-test or analysis of variance (ANOVA), and post-hoc comparisons were made using the Student–Newman–Keuls or Bonferroni methods as
Urea transport in Magadi tilapia

Results

In vivo physiology

Series 1: pattern of urea excretion

Serial sampling at closely timed intervals (15 min) demonstrated that urea-N accumulates in the external water at a more-or-less constant rate (Fig. 1), rather than as discrete pulses. Overall net urea-N excretion rate in this series at 37.5 °C was 6603±526 m mol-N kg⁻¹ h⁻¹ (N=11), and plasma urea-N was 7900±841 m mol-N l⁻¹ (N=11), yielding an estimated branchial permeability of 4.74·10⁻⁵ ± 0.38·10⁻⁵ cm s⁻¹ (N=11) (means ±1 S.E.M.). Compared with the gills of most teleosts and the even lower permeability of elasmobranch gills, the value in the Magadi tilapia is extremely high (Fig. 2). Indeed, the value is over 10 times that for standard lipid bilayers and approximately five times higher than that of the actively pulsing gulf toadfish (Fig. 2).

Series 2: thiourea versus urea permeability

In individual tilapia, the permeability of the gills to urea was closely correlated with that of its analog thiourea (Fig. 3). However, in this series at 34 °C, absolute thiourea permeability (6.92·10⁻⁶±0.89·10⁻⁶ cm s⁻¹, N=9) was less than 20% of urea permeability (3.69·10⁻⁵±0.37·10⁻⁵ cm s⁻¹, N=9). Notably, this low ratio of thiourea-to-urea permeability in the continuously excreting Magadi tilapia is nearly identical to that of the gulf toadfish during an active pulse event (Fig. 4). At the same time, it is very different from the situation in the non-pulsing toadfish, in which the ratio is close to 1.2, the expected value for these very similar molecules when simple diffusion alone is involved in their movement.

Series 3: urea influx and the effects of urea analogs

Unidirectional influx rates measured in the presence of a high external urea concentration (20 mmol l⁻¹=40 000 μmol-N l⁻¹) in the control group were 6718±125 μmol-N kg⁻¹ h⁻¹ (N=19). In the reference group run at the same temperature (32 °C), net efflux rates were 4346±538 μmol-N kg⁻¹ h⁻¹ (N=10) and plasma urea-N concentration was...
Thus, in the presence of an inward concentration gradient (from water to blood plasma), influx was larger than efflux and in approximate proportion to the relative concentrations on the two sides of the gill, consistent with a bidirectional transport system. When the effects of urea analogs (at 60 mmol l\(^{-1}\), three times the urea concentration in the water) on this influx were examined, influx was significantly reduced by all three, with the potency order thiourea > N-methylurea > acetamide (Fig. 5). Addition of 30 mmol l\(^{-1}\) NaCl had no effect relative to control rates (Fig. 5), indicating that the effects were unrelated to the small elevation of osmotic pressure.

**Molecular cloning**

PCR and RACE PCR yielded a sequence of 1699 bp (GenBank accession no. AF278537) with high homology to published sequences for urea transporters, specifically the UT-A2 of mammals (rabbit and rat), the shark kidney urea transporter (ShUT) and the toadfish gill urea transporter (Smith et al., 1995; Smith and Wright, 1999; Walsh et al., 2000; You et al., 1993). The Magadi tilapia urea transporter has modified polyadenylation sequences at base pairs 1552–1557 (ATTAAT) and at base pairs 1654–1659 (ATTTAA). The longest open reading frame (ORF) begins at nucleotide 103 and has a modified Kozak site upstream of the ATG (GCCCTG) and a G at position +4 (whereas the consensus site is GCCA/GCC and a G at position +4). This ORF ends at base pair 1530, encoding a 475-amino-acid-residue protein that we have named ‘mtUT’ (Magadi tilapia urea transporter) (Fig. 6). A second possible initiation codon in the same frame occurs at nucleotide 244 and is preceded by the sequence GGAGAC and a G at position +4. Although the first 47 amino acid residues of the 475-residue protein do not have high homology with other vertebrate urea transporter proteins, a region of higher homology with other vertebrate urea transporter proteins begins at the second and third methionines (amino acid residues 46 and 53, respectively) of the deduced polypeptide sequence (Fig. 6). Overall, mtUT has 75% amino acid identity with tUT (toadfish UT) and 56–61% amino acid identity with human UT-A2, Rana UT, rat UT-A2, rat UT-B and ShUT. This homology approximates the level of identity between ShUT or tUT and mammalian urea transporters (Smith and Wright, 1999; Walsh et al., 2000).

**Oocyte studies**

Injection of mtUT cRNA into *Xenopus laevis* oocytes resulted in a 3.4-fold increase in urea uptake rates relative to water-injected controls, and this increase was inhibitable by phloretin (Fig. 7A). Furthermore, when the effectors PMA and forskolin were applied to oocytes injected with mtUT cRNA, PMA had the effect of depressing urea uptake to approximately 60%, whereas the effect of forskolin was not significant (Fig. 7B).

**Northern analysis**

Of all the tissues studied (gill, liver, muscle, red blood cells and brain), only the gill showed a signal in northern analyses.

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**Fig. 4.** The ratio of thiourea-to-urea permeability in the gills of the Lake Magadi tilapia (series 2 data) in comparison with the ratio in the gills of the gulf toadfish (Wood et al., 1998) when the latter’s facilitated diffusion transporter is either inactive or activated (means +1 S.E.M., N=6). Values sharing the same letter are not significantly different.

20584±735 µmol-N l\(^{-1}\) (N=10). Thus, in the presence of an inward concentration gradient (from water to blood plasma), influx was larger than efflux and in approximate proportion to the relative concentrations on the two sides of the gill, consistent with a bidirectional transport system. When the effects of urea analogs (at 60 mmol l\(^{-1}\), three times the urea concentration in the water) on this influx were examined, influx was significantly reduced by all three, with the potency order thiourea > N-methylurea > acetamide (Fig. 5). Addition of 30 mmol l\(^{-1}\) NaCl had no effect relative to control rates (Fig. 5), indicating that the effects were unrelated to the small elevation of osmotic pressure.

**Fig. 5.** The effects of three urea analogs (acetamide, N-methylurea and thiourea, present in the external water at a concentration of 60 mmol l\(^{-1}\)) on unidirectional urea-N influx (urea was present in the external water at a concentration of 20 mmol l\(^{-1}\)) in the Magadi tilapia of series 3. Values are given as µmol-N kg\(^{-1}\) h\(^{-1}\), equivalent to nmol-N g\(^{-1}\) h\(^{-1}\). The 30 mmol l\(^{-1}\) NaCl treatment served as an osmotic pressure control. Values are means +1 S.E.M. (control, N=19; NaCl, N=9; acetamide, N=8; N-methylurea, N=7; thiourea, N=13). Values sharing the same letter are not significantly different.
Fig. 6. Alignment of the Lake Magadi tilapia urea transport protein (mtUT) with other urea transporter (UT) proteins (references and accession numbers are as follows: UT, toadfish, Walsh et al., 2000, AF165893; ShUT, shark, Smith and Wright, 1999, AF257331; RanUT, UT Rana, Verbavatz et al., 1996, Y12784.1; ratUT-A, ratUT-A2, rat, Smith et al., 1995, Q62668; humanUT-A2, HUT11, Olives et al., 1994, Q15849; ratUT-B, UT11, Couriaud et al., 1996, X98399.1). Black boxes enclose identical amino acid residues, grey boxes enclose conservative substitutions, numbers are as follows: tUT, toadfish, Walsh et al., 2000, AF165893; ShUT, shark, Smith and Wright, 1999, AF257331; RanUT, UT Rana, Verbavatz et al., 1996, Y12784.1; ratUT-A, ratUT-A2, rat, Smith et al., 1995, Q62668; humanUT-A2, HUT11, Olives et al., 1994, Q15849; ratUT-B, UT11, Couriaud et al., 1996, X98399.1). Black boxes enclose identical amino acid residues, grey boxes enclose conservative substitutions, dashes represent deletions and white areas represent non-identity.
under high stringency (Fig. 8). A single band was seen at approximately 1.9 kb, which we believe corresponds to mtUT cDNA. Notably, a signal near to 4.0 kb often seen in other species was absent from \textit{A. grahami}. When lower-stringency washes (e.g. 1\% SSC, 50 °C) were used, the pattern was the same (results not shown).

\textit{Gill ultrastucture}

Two cell types are examined when the function of gills is studied as a barrier between the fish and the milieu (Laurent and Perry, 1995). The chloride cell has previously been studied in some detail (Laurent et al., 1995) and will not be considered further in the present study because it did not appear to display any particular specific features that might be related to urea secretion. However, pavement cells (PVCs) showed more interesting details. Fig. 9A displays an ultrathin section of a pavement cell of the Lake Magadi tilapia in which three Golgi apparatus profiles are visible side by side in the same cell. This density was representative of the great density of Golgi stacks in the PVCs of this species. Numerous vesicles (50–200 nm in diameter) are scattered around the Golgi apparatus and between these organelles and the apical membrane. They are variably filled with electron-dense material. Fig. 9B demonstrates the relationships between the Golgi saccules and vesicles pinching off from them. Some vesicles, usually the smallest, display a

Fig. 7. Effects of Lake Magadi tilapia urea transport protein (mtUT) expression on urea uptake by \textit{Xenopus laevis} oocytes. (A) Summary of the urea accumulated in a 1 min period by water-injected control oocytes or oocytes expressing mtUT. Hatched columns show uptakes under control conditions. Open columns show uptakes in the presence of 1.0 mmol l\textsuperscript{-1} phloretin for 15 min. An asterisk indicates a significant increase ($P<$0.01) in urea uptake compared with the water-injected control group and a double dagger denotes that phloretin caused a significant reduction in uptake in comparison with the paired control group. (B) Effects of the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (PMA) (10\textsuperscript{-6} mol l\textsuperscript{-1}) and forskolin (2x10\textsuperscript{-5} mol l\textsuperscript{-1}) in a 15 min preincubation on 1 min urea uptake rates. An asterisk indicates a significant decrease ($P<$0.01) in urea uptake compared with the control group. Values are presented as means ± S.E.M. with the number of oocytes tested in parentheses.

Fig. 8. Tissue distribution of Lake Magadi tilapia urea transport mRNA (mtUT) using high-stringency northern analysis. Membranes were probed using \textsuperscript{32}P-labeled full-length random-primed m17-1 cDNA probe and hybridized at 42 °C with 30 % formamide. Final washes were in 0.1\% SSC, 0.1 % SDS at 65 °C. Each lane contains 3\% of poly(A\textsuperscript{+}) RNA. G, gill; L, liver; M, muscle; R, red blood cells; B, brain; X, a negative control with no tissue. Numbers on the left are the positions of known molecular mass RNA standards (in kb), and the arrowhead marks the band in gill at approximately 1.9 kb. Ethidium-bromide-stained gels were examined prior to blotting for RNA degradation, particularly for 18S and 28S rRNA, and degradation was not evident.
well-contrasted electron-dense periphery but an electron-lucent center. Some others, usually the largest, are almost completely filled with an electron-dense material. In some cases, a dense core floats inside an electron-lucent vesicle. Lysosomes are absent from, or at least very rare in, the PVCs.

For comparison, Fig. 9C shows a representative ultrathin section of a pavement cell of *Oreochromis niloticus*, a fully ammoniotelic related species. In this case, the PVC is contiguous with a chloride cell (CC). Note the non-active Golgi apparatus and its associated reticulum. Vesicles are scanty and clear. Note the presence of glycocalyx on the plasma membrane indicating a better preservation of ultrastructure due to appropriate working conditions. Scale bars, 1 μm (A,B), 500 nm (C).

Discussion

**In vivo physiology**

The present physiological studies on the Lake Magadi tilapia reveal several new facets of urea excretion mechanisms. First, urea is excreted continuously in this species (Fig. 1), rather than in discrete pulses as has been observed for the gulf toadfish (Wood et al., 1995). It is likely that the extremely high rates of nitrogen metabolism and urea-N production in the Magadi tilapia necessitate continuous nitrogen excretion, and this necessity is reflected in the exceptionally high urea permeability values in the gills compared with those of other fishes, including ureoteles (Fig. 2). The observation of a urea permeability more than 10 times greater than traditional ‘passive’ values (Fig. 2) by itself suggests that a transport system for urea must be present in the gills, a conclusion supported by the other data presented here.

When external urea concentrations were experimentally elevated above plasma levels, unidirectional influx measurements revealed a bidirectional transport system, with rates proportional to the relative concentrations on the two sides of the gill epithelium. This result is identical to that of a similar experiment performed on the ‘pulsing’ gulf toadfish (Wood et al., 1998), in which a facilitated diffusion
mechanism is present in the gills. Both the inhibitory effects of urea analogs on urea transport (Fig. 5) and the correlation between the permeability for thiourea and urea (Fig. 3) further suggest a specific urea-transport mechanism. Indeed, the greater inhibitory potency of thiourea than acetamide is identical to the response of toadfish gill urea transport (McDonald et al., 2000). In the rat kidney inner medullary collecting duct, from which a facilitated diffusion urea transporter has been cloned and fully characterized (Smith et al., 1995), all three of the analogs tested here (Fig. 5) inhibited urea transport with the same potency order (thiourea>N-methylurea>acetamide; Chou and Knepper, 1989). The low thiourea-to-urea permeability (0.18; Fig. 4) continuously present in the gills of the Magadi tilapia was identical to the ratio seen in the gulf toadfish when its facilitated diffusion urea transporter is activated during pulsatile excretion events (Wood et al., 1998; McDonald et al., 2000) and very different from the value of close to 1.0 seen when the transporter is inactive. A value of 1.0 (or higher) is expected when only passive diffusion is occurring in phospholipid and cholesterol membranes (Lippe, 1969). In summary, all these pieces of physiological evidence point to the presence of a continuously active facilitated diffusion transport mechanism for urea in the gills of the Magadi tilapia similar to that present in the rat kidney and the toadfish gill.

**Molecular and oocyte expression studies**

The molecular data presented here provide further confirmation of this conclusion. A transporter was cloned that, when expressed in *Xenopus laevis* oocytes, facilitated urea transport and showed the characteristic phloretin inhibition of urea transporters (Fig. 7A). Several signature sequences of characteristic domains of urea transporters (Karakashian et al., 1999) are highly conserved in the Lake Magadi urea transporter (mtUT; Fig. 6), e.g. the LP box (amino acid residues 215–217–220). Notably, the ALE domain, which is diagnostic of Aquaporin 9 (Ishibashi et al., 1998; Tsukaguchi, 1998), may allow urea exit from tissues that are the sites of urea production. However, Walsh et al. (Walsh et al., 2000) noted a similar expression pattern in *Xenopus laevis* oocytes, facilitating urea transport, and showed the characteristic phloretin inhibition of the oocyte expression studies offer some preliminary information. First, the effect of PMA was to downregulate urea transport by 60% (Fig. 7B). Whether this deactivation occurred by the action of PKC on a PKC phosphorylation site on mtUT is the subject of further studies because an alternative mechanism in our studies may be via PKC effects which increase membrane recycling and possibly reduce the number of mtUT molecules in the membrane. There was no significant effect of forskolin on urea transport (Fig. 7B), and this result is not surprising given the lack of typical PKA sites (RRXS) in the mtUT sequence.

The lack of detectable UT-A2 mRNA in liver and muscle (Fig. 8) demands attention, given that these are the proposed sites of urea production (Lindley et al., 1999). However, Walsh et al. (Walsh et al., 2000) noted a similar expression pattern in the toadfish gill and speculated that other pathways, e.g. a piscine analog of Aquaporin 9 (Ishibashi et al., 1998; Tsukaguchi, 1998), may allow urea exit from tissues that are the sites of urea production.

**Gill ultrastructure**

Comparative gill morphological evidence points to the involvement of the Golgi apparatus and vesicular trafficking in the urea excretion process. Fig. 9A,B demonstrates that PVCs are the site of vesicular trafficking between the Golgi cisternae and the apical membrane in *Alcolapia grahami*. As for the uroteretic gulf toadfish (Walsh et al., 2000; Laurent et al., 2000), we speculate that this rich Golgi apparatus and the intense vesicular trafficking are the structural manifestations of the facilitated diffusion urea-transport process, representing either insertion of the transporter into the apical membrane and/or the packaging of urea into vesicles for transport. Studies to examine urea transporter localization by immunocytochemistry with urea transporter antibodies would test these hypotheses and clarify the precise role of vesicular trafficking in urea transport.
Concluding remarks

In summary, we have presented evidence supporting the hypothesis that urea excretion in the Lake Magadi tilapia is a specific facilitated transport mechanism at the gill. We have cloned and functionally expressed mUT, a candidate mediator of this urea-transport mechanism. Several characteristics of this transport system (e.g. high capacity, continuous operation) appear to differ from other systems (e.g. the toadfish and elasmobranchs), suggesting that additional study will yield clues into the evolution of urea transport mechanisms in the vertebrates.

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