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Effect of osmotic stress on expression of a putative facilitative urea transporter in the kidney and urinary bladder of the marine toad, *Bufo marinus*

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Summary

Anuran amphibians accumulate a large amount of urea in their extracellular fluids to avoid a severe dehydration under dry and hyper-saline environments. To clarify the mechanisms of urea retention, we examined structure and distribution of the urea transporter (UT) in the kidney of the marine toad (*Bufo marinus*), and its expression in the kidney and urinary bladder following exposure to dry and hyper-saline conditions by means of cDNA cloning, semi-quantitative RT-PCR, immunoblot analysis and immunohistochemistry. The *Bufo* UT cDNA cloned from the kidney encodes a 390-amino-acid residue protein, which is 80% identical to *Rana esculenta* UT with the functional characteristics of a urea transporter. The *Bufo* UT mRNA was abundantly expressed in the kidney and urinary bladder, but not in the skin. In immunoblot analysis using a specific antibody raised against the *Bufo* UT, a 52 kDa protein similar to the glycosylated forms of mammalian UT-A2 (~55 kDa) was detected in extracts from plasma membrane fractions of the kidney and urinary bladder. When toads were acclimated to dry and hyper-saline environments for 7 days, UT mRNA expression was upregulated in the kidney and urinary bladder and there was an elevated plasma urea concentration and osmolality. Immunohistochemistry showed that the UT was specifically localized on the apical membrane of the early distal tubule, known to be the diluting segment, in the kidney and the epithelial cells of urinary bladder. Immunoreactive cells were not detected along the late distal tubule, the connecting tubule or the collecting duct in the kidney. The present findings suggest that the *Bufo* UT probably contributes to urea transport in the kidney and urinary bladder in response to hyperosmotic stresses such as body fluid hypertonicity and dehydration.

Key words: urea transporter (UT), kidney, diluting segment, *Bufo marinus*, osmoregulation.

Introduction

Urea is known to be a major end product of nitrogen metabolism in adult amphibians, except for aquatic amphibians such as *Xenopus laevis*. Urea has a relatively low toxicity and can be accumulated at high concentrations in the body fluids of amphibians (Jorgensen, 1997; Withers, 1998). When frogs and toads are acclimated to dry and hyper-saline environments, they accumulate urea in their body fluids as a result of increases in hepatic urea production and/or decreases in renal excretion (Funkhouser and Goldstein, 1973; Balinsky, 1981). In conditions of osmotic stress, dry and hyper-saline acclimation, urea accumulation contributes to retention of body water and helps avoid dehydration. This osmotic strategy is known to be used by desiccating and aestivating toads, such as the desert spadefoot toad *Scaphipus couchi* (McClanahan, 1967) and the urodele *Siren lacertian* (Etheridge, 1990), and by hyper-saline-acclimated anurans such as *Rana cancrivora* and *Bufo* sp. (Gordon et al., 1961; Katz, 1973).

In short-term acclimation to a hyper-saline environment, urea retention is associated with a decrease in urine production and a modest increase in the rate of urea synthesis (McBean and Goldstein, 1970a; McBean and Goldstein, 1970b). By contrast, long-term acclimation is associated with accelerated urea synthesis and increased activity of urea cycle enzymes (Balinsky, 1981). Renal retention is also an important mechanism for accumulation of urea in anuran amphibians (Schmidt-Nielsen and Lee, 1962; Shpun and Katz, 1995). Urea clearance is probably influenced by the glomerular filtration rate (GFR) and by renal tubule function, secretion and reabsorption in the kidney. It has been reported that in anurans, urea is reabsorbed across the renal tubule in the kidney and also in the urinary bladder (Schmidt-Nielsen and Lee, 1962; Shpun and Katz, 1995).

Previously, although urea was proposed to cross cell membranes by lipid-phase permeation, it has been found that passive urea movement occurs through facilitative urea transporters, which have been identified and characterized in cell membranes from mammalian kidney (Smith and Rousselet, 2001; Bagnasco, 2005), amphibian urinary bladder (Couriaud et al., 1999) and fish gill and kidney (Smith and Wright, 1999; Mistry et al., 2001, 2005). In mammals, facilitative urea transporters are the renal urea transporter (UT-A) and the erythrocyte urea transporter (UT-B). The renal urea transporter UT-A has four isoforms: UT-A1 (Shayakul et al., 1996), UT-A2 (Smith et al., 1995), UT-A3 (Karakashian et al., 1999) and UT-A4 (Karakashian et al., 1999). Mammalian UT-As are expressed in the renal medulla of the kidney and stimulated by several hormones such as arginine-vasopressin (AVP), glucocorticoids and mineralocorticoids (Wade et al., 2000; Shayakul et al., 2000; Peng et al., 2002; Gertner et al., 2004), and also in various physiological and pathological states (Shayakul et al., 2000; Sands, 2004).

In amphibians, the presence of a facilitative urea transporter has been previously demonstrated in studies of functional urea transporters, which were found to be expressed after microinjection of *Xenopus* oocytes with total or fractionated poly(A)$^+$ RNA isolated from amphibian urinary bladder epithelial cells (Martial et al., 1991). In addition, a cDNA encoding frog UT has been cloned from the urinary bladder of *Rana esculenta* (Couriaud et al., 1999). The frog UT protein has high identity to rat UT-A2 and UT-B1, and the urea uptake mediated by the protein is inhibited by both phloretin and the mercurial reagent *para*-chloromercuribenzene sulfonate (pCMBS). However, to our knowledge, there has been no report of the expression and localization of UTs in the amphibian kidney, which, as well as the urinary bladder, is an important osmoregulatory organ in anurans.

In this study, we identified a full-length cDNA encoding the urea transporter from the kidney of the marine toad *Bufo marinus*, and investigated changes in the expression of UT mRNA in the kidney and urinary bladder of the toad under hyperosmotic stress. In addition, we clarified cellular localization of *Bufo* UT along the nephron segments of the kidney.

Materials and methods

Animals

Adult male specimens of the marine toad *Bufo marinus* L. were collected in the field on Ishigaki Island, Okinawa, and used in this study. Fifteen toads, weighing 161.9±11.5 g (mean ± s.e.m.), were housed in containers (45 cm×50 cm×40 cm) with moist soil, in the laboratory and maintained on a 12 h:12 h light:dark cycle at about 25°C until use. They were allowed free access to water, and fed European crickets or bovine liver once a week. All tissue samples were taken under anesthesia with diethyl ether, and samples except those for histological observation were frozen quickly in liquid nitrogen and stored at –80°C until use. All animal experiments were conducted according to the Guideline for Care and Use of Animals approved by the ethics committee of the University of Toyama. Two female toads were used for the tissue distribution of *Bufo* UT mRNA experiment.

Osmotic treatments and analysis of plasma and urine components

Experimental protocols were described in detail in our previous paper (Konno et al., 2005). Briefly, toads in the control group were maintained on moist soil and allowed free access to water, and toads in two experimental groups were subjected to osmotic treatments (dry and hyper-saline acclimation). In one group, toads were kept on dried sponge fragments, whereas in the other, toads were immersed in 300 mOsmol kg^{-1} H_2O NaCl solution. Toads in each group were starved during the 7-day experiment, and their body mass was measured every day. Blood samples were collected by cardiac puncture using heparinized 1-ml syringes and centrifuged at 2000 ***g*** for 20 min at 4°C. Urine samples were collected from the urinary bladder using syringes. Osmolality, Na^+, Cl^-, K^+ and urea concentration were measured in plasma and urine samples.

Molecular cloning of Bufo UT cDNA

Total RNA was extracted from the *Bufo* kidney using acid guanidinium thiocyanate–phenol–chloroform extraction, as described previously (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was purified from the total RNA with an Oligotex-dT30 super mRNA purification kit (TaKaRa, Otsu, Japan). Adaptor-ligated double-strand kidney cDNA was synthesized with a SMART cDNA Library Construction Kit (Clontech, Mountain View, CA, USA). Degenerate primers for the urea transporter (UT) were designed based on the UT cDNA sequences of the dogfish, *Triakis scyllium* (Hyodo et al., 2004), the edible frog, *Rana esculenta* (Couriaud et al., 1999), and mammalian UT-A2 sequences. The primer sequences were 5′-GTNCARAAYCCNTGGTGGRC-3′ (sense) and 5′-CCANGGRTTRTCRCANCCRTA-3′ (antisense). PCR was performed using high-fidelity Ex-Taq DNA polymerase (TaKaRa) using the following schedule: 94°C for 2 min, 35 cycles of 94°C for 1 min, 55°C for 30 s, 72°C for 1 min and finally 72°C for 10 min. The PCR products were separated electrophoretically on 1% agarose gel containing ethidium bromide (0.5 μg ml^{-1}), and the major band of the expected size was cut from the gel. The cDNA fragment purified from the sliced gel was ligated into pT7Blue T-Vector (Novagen, San Diego, CA, USA) and the resulting plasmid was transformed into the competent cell (XL1-Blue, Invitrogen, Carlsbad, CA, USA). Blue/white screening on Luriabroth (LB) ampicillin–agar plates identified white colonies with the potential insert, and the identified colonies were liquid cultured. The plasmid DNA was isolated by a modified alkaline/SDS method (Rapid Plasmid Purification Systems, Marligen Bioscience, Ijamsville, MD, USA). The sequencing reaction was performed with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

The nucleotide sequence was determined using an ABI Prism 310 or 3100 DNA sequencer (Applied Biosystems). The full-length *Bufo* UT cDNA was obtained by 5′- or 3′-rapid amplification of cDNA ends (RACE) with adaptor primers (Clontech) and UT gene-specific primers, which were designed on the basis of the sequences of cDNA fragments obtained by degenerate PCR. The specific primers were 5′-AAGCAGTGGTATCAACGCAGAGT-3′ (5′ adaptor primer), 5′-CTGTCAGCGTGGACACTACAGT-3′ (antisense), 5′-GCTCACCTGGCAAACTCAC-3′ (sense), and 5′-ATTCTAGAGGCCGAGGCGGCCGACA-d$(T)_{30}N_{-1}$N-3′ (3′ adaptor primer).

Tissue distribution of Bufo UT mRNA

Tissue expression of UT mRNA was examined by RT-PCR. Total RNA was isolated from various tissues (brain, tongue, heart, lung, liver, stomach, spleen, small intestine, large intestine, kidney, urinary bladder, testis, ovary, ventral pelvic skin and dorsal skin) using Isogen (Nippon gene, Tokyo, Japan). To prepare the first-strand cDNA, 1 μg of total RNA was reverse-transcribed with a First Strand cDNA Synthesis Kit (Roche Diagnostics, Basel, Switzerland). The specific PCR primers (sense: 5′-ACCTGGTCTGATGTCCACATAC-3′ and antisense: 5′-GCAGCTCCTAGGTAGGCACA-3′) were synthesized based on sequences 637–658 and 955–974 of the *Bufo* UT cDNA sequence (GenBank accession no. AB212932). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal standard. The GAPDH primer pair (sense: 5′-CTGGCTCCTCTTGCAAA-GGT-3′ and antisense: 5′-GTGTATCCCAGGATTCCCTTC-3′) was designed based on the GAPDH sequences of the marsh frog, *Rana ridibunda* (GenBank accession no. AY072703), and the African clawed frog, *Xenopus laevis* (GenBank accession no. U41753). PCR was performed with 27 cycles (UT) and 24 cycles (GAPDH) of denaturation (94°C, 1 min), annealing (55°C, 30 s) and extension (72°C, 1 min). The PCR products were separated electrophoretically in 3% agarose gel containing ethidium bromide, and detected by a gel photograph instrument (Printgraph, Atto, Tokyo, Japan). Band densities were analyzed using Scion Image software (Scion Corporation, Frederick, MD, USA).

Preparation of urea transporter polyclonal antibody

A polyclonal antibody against the *Bufo* UT was raised by immunizing Japanese white rabbits subcutaneously with a synthetic peptide that included cysteine at the COOH terminus, NH_2-LSKVTYPEC-COOH, corresponding to amino acids 364–370 of the *Bufo* UT. The antiserum was collected and purified using an affinity column bearing the immobilized synthetic peptide with affinity gel beads (Affi-Gel 10, Bio-Rad, Hercules, CA, USA).

Immunoblotting

Tissue samples from the kidney, urinary bladder, heart and liver were homogenized in ice-cold membrane isolation solution (250 mmol l^{-1} sucrose, 10 mmol l^{-1} triethanolamine, containing 1 μg ml^{-1} leupeptin, and 0.1 mg ml^{-1} phenylmethyl sulfonyl fluoride, adjusted to pH 7.6) using a tissue homogenizer (Physcotron NS-310E, Nition, Chiba, Japan). The homogenates were centrifuged at 2000 ***g*** for 20 min at 4°C to remove whole cells, nuclei and mitochondria, and the supernatant was centrifuged at 17,000 ***g*** for 1 h at 4°C to collect a pellet containing the plasma membrane fractions. The pellet was suspended in ice-cold membrane isolation solution including 1% Triton X-100. Total protein concentration in the samples was measured with a Bio-Rad Protein Assay reagent (Bio-Rad, Japan) using the Bradford method (Bradford, 1976). The samples were solubilized at 60°C for 15 min in Laemmli buffer. SDS–PAGE was performed on 12.5% polyacrylamide gel, and the proteins were then transferred from the gel to a nitrocellulose membrane (Hybond-C, Amersham Biosciences, Piscataway, NJ, USA). To prevent non-specific binding, the membranes were blocked with 5% skimmed milk for 2 h at room temperature, and then probed overnight at 4°C with the *Bufo* UT polyclonal antibody (dilution 1:2000 with 1% BSA–PBS) raised against *Bufo* UT. After washing with TBS–Tween 20, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (ECL plus Western Blotting Detection System, Amersham Biosciences, USA) for 2 h at room temperature. After further washing of the membranes with TBS–Tween 20, secondary antibody binding was visualized using an enhanced chemiluminescence kit (ECL plus Western Blotting Detection System, Amersham Biosciences, USA).

Immunohistochemistry

Kidney and urinary bladder were perfusion-fixed *in situ* via the dorsal aorta with Bouin's fixative, and then removed and post-fixed in the same solution overnight at 4°C. The tissues were dehydrated and embedded in paraffin, and then serial sections (6 μm) were cut and stained using the immunoperoxidase technique (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA). The sections were incubated overnight at 4°C with the *Bufo* UT antibody (dilution 1:4000 with 1% BSA–PBS). Adjacent sections were stained with vacuolar type H^+-ATPase antiserum (dilution 1:8000 with 1% BSA–PBS) that identifies specifically intercalated cells of the late distal tubule and the collecting duct (Uchiyama and Yoshizawa, 2002). Immunoreactivity for UT was visualized with DAB solution (3,3′-diaminobenzidine, Sigma-aldrich, St Louis, MO, USA) containing 0.02% H_2O_2. Sites showing immunoreactivity for UT or vacuolar-type H^+-ATPase were confirmed by omitting the primary antibodies, and replacing the respective antibodies with rabbit preimmune sera, and immunoreabsorption of antibodies with the synthetic antigens (5 μg ml^{-1}) for 24 h at 4°C. All control preparations were negative for immunostaining.

Statistical analysis

Data are presented as means ± s.e.m. Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test and

Student's paired *t*-test. Differences at $P<0.05$ were considered statistically significant.

Results

Effects of acclimation to dry and hyper-saline environments

With acclimation to dry conditions for 7 days, the toads showed a significant loss of body mass from 162.8±6.6 g to 128.4±6.1 g ($P<0.001$, Student's paired *t*-test), and a percentage change in body mass before and after the treatment was –21.2%. Hematocrit (Ht) as an indicator of plasma volume was significantly increased relative to that of the control group ($P<0.01$, ANOVA), suggesting a decrease in plasma volume. In desiccated toads, plasma osmolality, urea and ion concentrations (Na^+, Cl^- and K^+) were significantly increased relative to the control ($P<0.05$ or $P<0.01$, ANOVA). By contrast, toads placed in hypertonic NaCl solution showed a significant gain of body mass from 158.8±17.7 g to 177.8±20.1 g ($P<0.01$, Student's paired *t*-test), and the percentage change in body mass before and after the treatment was 12.2%. Ht was significantly decreased relative to the control ($P<0.01$, ANOVA), suggesting an increase in plasma volume. Plasma osmolality and urea concentration were significantly increased, and the plasma concentrations of Na^+ and Cl^-, but not K^+, were also significantly increased ($P<0.01$, ANOVA). In hyper-saline-acclimated toads, plasma osmolality was higher than the ambient osmolality (300 mOsm). In control toads, the mean urinary bladder content was about 5 ml, which was 3.2% of total body mass. In desiccated toads, the mean bladder content was markedly decreased to less than 1 ml, whereas in hyper-saline-acclimated toads, a large increase to over 15 ml was observed. In all toads examined, urine was hypo-osmotic relative to the plasma. Urea accounted for about 8%, 27% and 16% of the plasma osmolality in control, desiccated and hyper-saline-acclimated toads, respectively, whereas sodium accounted for about 46%, 36% and 42%. However, urea accounted for about 70%, 68% and 19% of the urine osmolality in control, desiccated and hyper-saline-acclimated toads, respectively, whereas sodium accounted for about 16%, 40% and 42%. The concentration ratios of urine/plasma sodium (U/P_{Na+}) in hyper-saline-acclimated toads were significantly higher than those of control and desiccated toads, whereas the concentration ratios of urine/plasma urea (U/P_{urea}) in hyper-saline-acclimated toads were significantly lower than those of both the other groups. These data are shown in Table 1.

Molecular cloning of Bufo UT

A full-length cDNA sequence for *Bufo* UT is 1363 bp long, and has a polyadenylation signal (AATAAA) at position 1290–1295. A putative open reading frame (109–1278) encodes a protein of 390 amino acid (GenBank accession no. AB212932; Fig. 1A). Hydropathy analysis using the Kyte–Doolittle algorithm predicted 10 putative transmembrane regions with an N terminus and a C terminus located in the cytoplasm (Fig. 1B). There was one putative *N*-glycosylation site at amino acids 211–213 (NIT) in the putative central extracellular loop (Fig. 1A). The *Bufo* UT protein has high identity to *Rana* UT (80%) and also has 76% and 68% amino acid identity with rat UT-A2 and UT-B2, respectively.

Table 1. *Plasma and bladder urine parameters in various osmotic conditions*

Parameter	Control	Dry	Hyper-saline
Change in body mass (%)	–2.3±0.3	–21.2±1.4**	12.2±0.9**
Hematocrit (%)	31.4±0.7	44.2±1.7**	22.8±0.9**
Plasma			
Osmolality (mOsmol kg^{-1} H_2O)	224.0±3.1	408.4±5.2**	345.0±2.9**
Na^+ (mmol l^{-1})	102.6±0.7	147.4±5.0**	143.6±1.2**
Cl^- (mmol l^{-1})	68.6±2.0	97.0±1.7**	131.4±3.5**
K^+ (mmol l^{-1})	3.8±0.2	6.7±0.2*	3.6±0.1
Urea (mmol l^{-1})	17.0±1.4	111.6±7.8**	56.6±2.1**
Bladder urine			
Osmolality (mOsmol kg^{-1} H_2O)	59.2±3.9	378.8±5.6**	323.6±2.5**
Na^+ (mmol l^{-1})	9.6±0.7	15.4±1.5*	136.2±5.0**
Cl^- (mmol l^{-1})	5.2±0.6	7.9±0.3	115.4±3.8**
K^+ (mmol l^{-1})	1.7±0.2	27.2±0.8**	1.5±0.1
Urea (mmol l^{-1})	41.1±2.1	256.9±17.7**	60.3±3.1*
Bladder urine content	±	–	++
Ratio of urine to plasma			
U/PNa^+	0.09±0.01	0.10±0.01	0.95±0.04**
U/Purea	2.49±0.24	2.31±0.05	1.07±0.02**

Values are means ± s.e.m. All groups $N=5$. *$P<0.05$ and **$P<0.01$, significantly different from corresponding control values (ANOVA followed by Bonferroni's test). –, ± and ++ indicate that urine volume is less than 1 ml, about 5 ml, and over 15 ml, respectively.

A

```
          1                                                          60
Bufo UT:  MESHIPINKEEMEEKKPEESPDRCSHGVGIFCTALHYISGEMKEFGAWLRDKPVPFQFID
Rana UT:  MESHIPINMAEKELKKPVESPNHCSSGLGFFCTAIHYISGEMKEFGNWLKDKPIIFQFID
rUT-A2:   -MEESSEIKVETNTTRTTWIQSSMIAGGKRVSKALSYITGEMKECGEGLKDKSPVFQFLD
rUT-B2:   -----MEDIPTMVKVDRGESQILSCRGRRWGLKVLGYVTGDMKEFANWLKDEPVGLQFMD
                                     *     ..: *::*:*** .  *:*:.  :**:*

          61              1                         2                120
Bufo UT:  WVLRGTSQVMFVNNPLSGLIIIAGLFLQNPWWAIAGCLGTVVSTLTALILSQDRSAIAAG
Rana UT:  WVLRGASQVMFVSNPLSGLIIIAALFLQNPWWAIAGCLGTAVSTLTALLLSQDRSAIAAG
rUT-A2:   WVLRGTSQVMFVNNPLSGILIVLGLFVQNPWWAISGCLGTIMSTLTALILSQDKSAIAAG
rUT-B2:   WILRGISQVVFVSNPISGILILAGLLVQNPWWALCGCVGTVVSTLTALLLSQDRSAIAAG
          *:*** ***:**.**:**::*: .*::******:.**:** :******:****:******

          121    3                      4                            180
Bufo UT:  LHGYNGILVGLLMAVFSDKGDWYWWLLIPVTVMSMTCPILSSALASIFSKWDLPVFTLPF
Rana UT:   LHGYSAMLVGLLMAVFSTKGDWYGWLLFPVAVMSMTCPLLSSALGNIFSKWDLPVFTLPF
rUT-A2:   LHGYNGVLVGLLMAVFSDKGNYYWWLLLPVIVMSMTCPILSSALSTVFSKWDLPVFTLPF
rUT-B2:   LQGYNATLVGILMAVFSDKGDYFWWLIFPVSAMSMTCPVFSSALSSLFSKWDLPVFTLPF
          *:**.. ***:****** **::: **::** .******::****..:*************

          181  5                                                     240
Bufo UT:  NIAVCLHIAATGHYNIFFPTADIKPIDAVPNITWSDVHIPSLLKAIPVGVGQVYGCDNPW
Rana UT:  NIAVCLHLAATGPNNEFFPTVDFRPTGVVPNISWSDVEISQLLKAVPVGVGQVYGCDNPW
rUT-A2:   NIAVTLYLAATGHYNLFFPTKLLQPAVTTPNITWSDVQVPLLLRAIPVGIGQVYGCDNPW
rUT-B2:   NMALALYLSATGHYNTFFPSKLFMPVSSVPNITWSELSALELLKSLPVGVGQIYGCDNPW
          *:*: *:::***  * ***:  : *   .***:**::     **:::***:**:*******

          241     6                   7                    8         300
Bufo UT:  TGGIFLASLFLSSPIICMHAAIGSAFGMIAGLSLATPFEKIYFGLWGYNSVLACIAVGGM
Rana UT:   SGGIMLVGLFISSPIICMHAAIGSAMGMLAGLSIAAPFESIYFGLWGYNSVLGCIAVGGM
rUT-A2:   TGGIFLVALFVSSPLICLHAAIGSTIGMLAALSIATPFDSIYFGLCGFNSTLACIAIGGM
rUT-B2:   TGAIFLCAILLSSPLMCLHAAIGSLLGVIARLSLAAPFKDIYSGLWGFNSSLACIAIGGM
          :*.*:* .:::***::*:****** :*::* **:*:**..** ** *:** *.***:***

          301            9                        10                 360
Bufo UT:  FYALTWQTHLLAIACALFCAYLGAALGNMMSVFGLPSCTWPFCLSALIFLLITTNNEGIY
Rana UT:   FYALTWQTHLLAIACALMCAYLGAALANVMAVIGLPSCTWPFCLTTLIFVLLTTNNPAIY
rUT-A2:   FYVITWQTHLLAIACALFAAYLGAALANMLSVFGLPPCTWPFCLSALTFLLLTTNNPGIY
rUT-B2:   FMALTWQTHLLALACALFTAYFGACMTHLIAAVHLPACTWSFCFATLLFLLLTTENPNIY
          * .:********:****: **:**.: ::::.. **.***.**:::* *:*:**:*  **

          361                               398
Bufo UT:  KLPLSKVTYPEANRIFFQNLKKDRKDKCNI--------
Rana UT:   KLPLSKVTYPEDNRIYYLNLKKERKKQ-----------
rUT-A2:   KLPLSKVTYPEANRIYFLSQEKNRRASMITKYQAYDVS
rUT-B2:   RMPLSKVTYSEENRIFYLQNKKSAVDRPL---------
          ::*******.* ***:: . :*.
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B

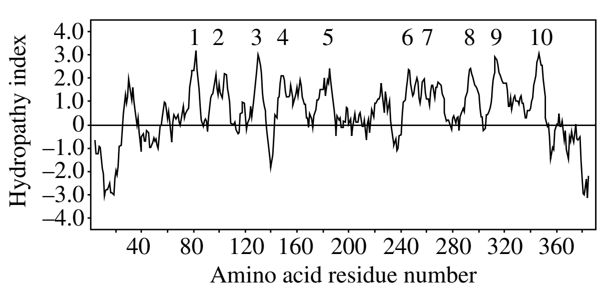

Fig. 1. Primary structure of the urea transporter (UT) isolated from the kidney of *Bufo marinus*. (A) The deduced amino acid sequence is aligned with those of *Rana esculenta* UT (GenBank accession no. Y12784), rat UT-A2 (U09957) and rat UT-B2 (U81518) using the Clustal algorithm. Asterisks denote identical amino acid residues; the horizontal bars indicate the predicted transmembrane regions; the box indicates putative *N*-glycosylation sites; the underline indicates the ALE domain, which is considered to be a signature sequence for UT-B. (B) Kyte-Doolittle hydropathy profile of the deduced *Bufo* UT amino acid sequence predicts the presence of trans-membrane regions (1–10).

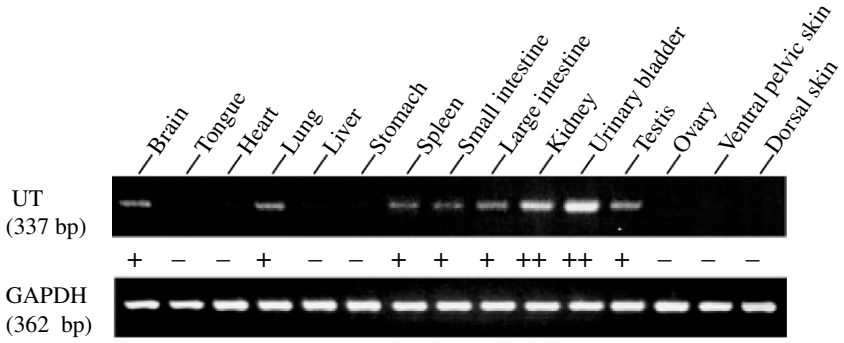

Fig. 2. RT–PCR analysis of tissue distribution of *Bufo* urea transporter (UT) mRNA. PCR was performed using specific primers for *Bufo* UT and frog GAPDH. ++, Strong expression; +, weak expression; –, absence of the mRNA.

Tissue distribution of the Bufo UT mRNA

The tissue distribution of *Bufo* UT mRNA was examined by RT–PCR with total RNA from various tissues. The *Bufo* UT mRNA was strongly expressed in the kidney and urinary bladder, and was weakly expressed in the large and small intestine, brain, lung, spleen and testis (Fig. 2). However, no *Bufo* UT mRNA expression was detected in the tongue, heart, liver, stomach, ovary and ventral pelvic and dorsal skin.

Effects of dry and hyper-saline acclimation to UT mRNA expression in the kidney and the urinary bladder

We performed semi-quantitative RT–PCR to clarify whether UT mRNA expression in the kidney and urinary bladder is regulated in response to dry and hyper-saline environments. The levels of UT mRNA expression in the kidney and urinary bladder were normalized to the expression of GAPDH mRNA. The expression of UT mRNA in the kidney and urinary bladder was significantly increased by both dry and hyper-saline acclimation for 7 days (Fig. 3). There was no significant difference in UT mRNA expression in the kidney and urinary bladder between toads acclimated to dry conditions and toads acclimated to hyper-saline environment.

Identification of immunoreactive Bufo UT proteins

To detect expression of UT protein in the kidney and urinary bladder, and to confirm specificity of *Bufo* UT antibody, we performed immunoblot analysis using extracts from both organs. In the membrane fractions from the kidney and urinary bladder, the affinity-purified antibody demonstrated prominently labeled a single band at 52 kDa (Fig. 4, lanes 1 and 2). However, this immunopositive band was not detected in the extracts from the heart and liver (Fig. 4, lanes 3 and 4). The molecular mass of the *Bufo* UT protein was similar to that of mammalian UT-A2 (~55 kDa). As shown in Fig. 4 (lanes 5 and 6), no immunopositive band was observed when the antibody was preincubated with an excess of the immunizing peptide (5 μg ml^{-1}).

Localization of UT in the Bufo kidney

To investigate the distribution of *Bufo* UT along the nephron of the kidney in the marine toad, we carried out immunohistochemical analysis of toad kidney sections using an affinity-purified antibody raised against the C-terminal peptide of *Bufo* UT. We found that *Bufo* UT was expressed in the early distal tubule (Fig. 5A), in the ventral zone of the kidney. The *Bufo* UT antibody predominantly labeled the

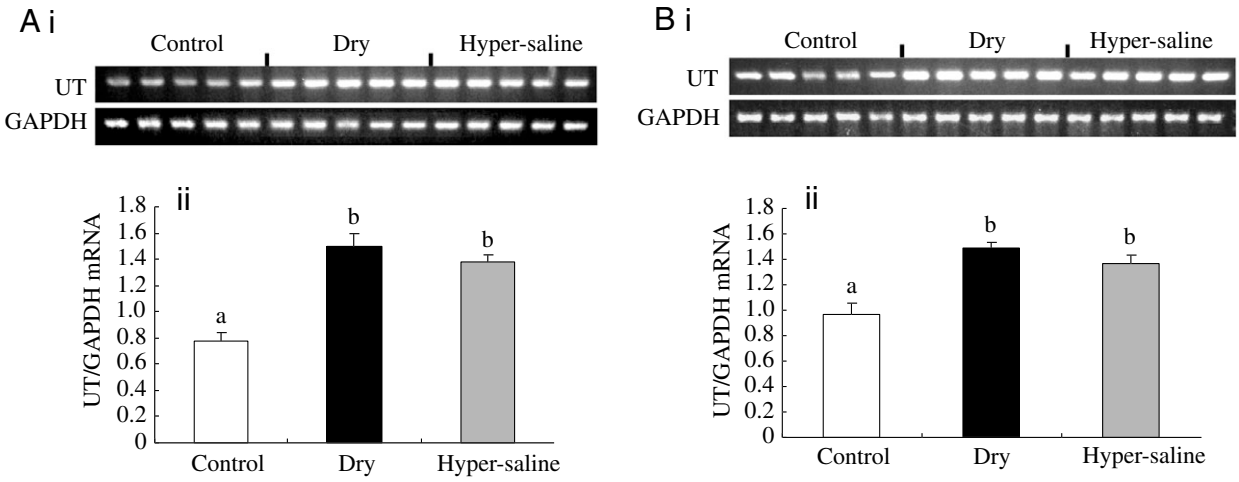

Fig. 3. Expression of *Bufo* urea transporter (UT) mRNA relative to GAPDH mRNA in the kidney (A) and urinary bladder (B) of marine toads exposed to dry or hyper-saline conditions. The signal level of each band in Ai, Bi is presented as a ratio of *Bufo* UT/GAPDH mRNA in Aii, Bii, respectively. Values are means ± s.e.m. N=5. Bars with different letters have significantly different values (ANOVA, P<0.05).

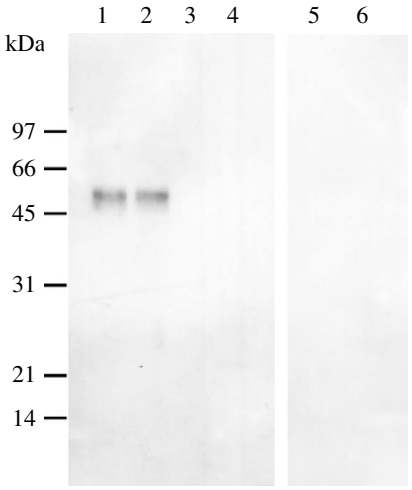

Fig. 4. Immunoblot analysis of urea transporter (UT) protein expressed in the kidney and urinary bladder of the marine toad. The affinity-purified *Bufo* UT antibody recognized a single band of 52 kDa in extracts of the kidney (lane 1) and urinary bladder (lane 2), but not of heart (lane 3) and liver (lane 4) on negative control tissues. These bands disappeared after preabsorption with the synthetic immunogen (kidney, lane 5; urinary bladder, lane 6).

apical membrane of the cells in the early distal tubule (Fig. 5B,C). An absorption test showed that an excess of the peptide (5 μg ml^{-1}) blocked the labeling by the *Bufo* UT antibody. We also performed immunohistochemical analysis using both *Bufo* UT and vacuolar H^+-ATPase antibodies in adjacent sections (Fig. 5C,D). It has been reported that in the kidney of anuran amphibians, vacuolar H^+-ATPase is only present on intercalated cells in the late distal, connecting and collecting tubules (Uchiyama and Yoshizawa, 2002). The vacuolar H^+-ATPase antibody sharply labeled the intercalated cells along the late distal tubule, connecting and collecting tubules (Fig. 5D), whereas immunolabeling for *Bufo* UT was not observed in these tubules. The scheme in Fig. 5E,F summarizes the distribution of *Bufo* UT and vacuolar H^+-ATPase proteins in the marine toad, *Bufo marinus*.

Discussion

In the present study, we report the cloning of a toad homologue (*Bufo* UT) of the putative renal urea transporter. We also found expression of the mRNA in the kidney and urinary bladder on exposure to dry and hyper-saline environments. Using immunohistochemical studies, distribution of the *Bufo* UT in the kidney nephron was examined.

In our acclimation study, we found that urine volume in the urinary bladder decreased and increased in response to dry and hyper-saline acclimations, respectively. Similar responses have been reported in *Bufo viridis* (Katz, 1986; Shpun and Katz, 1989). It is known that in conditions of dehydration, the whole kidney glomerular filtration rate decreases and a large fraction of the filtered fluid is reabsorbed by the renal tubule and the urinary bladder (Shoemaker and Bickler, 1979). Hyper-saline-acclimated anurans such as the crab-eating frog, *Rana cancrivora* (Chew et al., 1972), and *Bufo viridis* (Katz, 1986) develop severe hypernatremia resulting in saliuresis, and store a large volume of bladder urine but do not void it. This indicates reabsorption of water and urea by recycling bladder urine. Urea is freely filtered in the glomerulus and its excretion is determined by filtration and varying degrees of reabsorption in the kidney and urinary bladder. It seems that urea reabsorption from tubular to extracellular fluid occur through facilitative urea transporters, following a urea gradient between plasma and the renal tubular fluid. In the urinary bladder, urine is stored and a substantial amount of urea is facilitatively reabsorbed (Katz and Ben-Sasson., 1984; Shpun and Katz, 1989). In *Rana ridibunda*, the U/P_{urea} ratios were 2.29 and 0.62 in fresh water and 300 mOsm in saline, respectively. Renal handling of urea may change from tubular secretion to reabsorption during hyper-saline acclimation (Shpun and Katz, 1995). In the present study, the U/P_{urea} in bladder urine was 2.49, 2.31 and 1.07 in toads under control, dry and hyper-saline conditions, respectively. This result shows that toads in a hyper-saline environment increased their reabsorption of urea. It is also assumed that net urea reabsorption is stimulated in the kidney, considering that GFR is decreased and water reabsorption is increased significantly in desiccated toads under dry conditions.

Although specific urea transporters are thought to be present in the amphibian kidney and urinary bladder (see Jorgensen, 1997), very little is known about the molecular mechanism of the urea transport process. In the present study, the *Bufo* UT protein was found to have 80% amino acid identity with the frog UT and 76% with rat UT-A2 and a similar membrane topology (Couriaud et al., 1999; Smith et al., 1995). The *Bufo* UT protein lacks the ALE domain, which is considered to be a signature sequence for UT-B. These data suggest that *Bufo* UT belongs to the facilitative UT-A2 family. In immunoblot analysis, *Bufo* UT protein was identified as a single band of 52 kDa in extracts from plasma membrane fractions of the kidney and urinary bladder, but not of the heart and liver. Similar to the glycosylated forms of mammalian UT-A2 (molecular mass ~55 kDa), *Bufo* UT probably has one potential *N*-glycosylation site.

Under dry and hyper-saline acclimation, UT mRNA expression in the kidney and urinary bladder as well as plasma urea concentrations was significantly increased relative to that in control toads. In mammals and fish, changes in UT mRNA expression under a variety of osmotic environments have been reported. Smith et al. (Smith et al., 1995) and Bagnasco (Bagnasco, 2000) demonstrated that dehydration increases the expression of UT-A2 mRNA in rat kidney. Morgan et al. (Morgan et al., 2003) found that renal osmolytes and urea concentration were decreased and that renal UT transcripts (SkUT) were significantly diminished in response to environmental dilution in a marine elasmobranch. These data support the possibility that UT expression in the kidney and

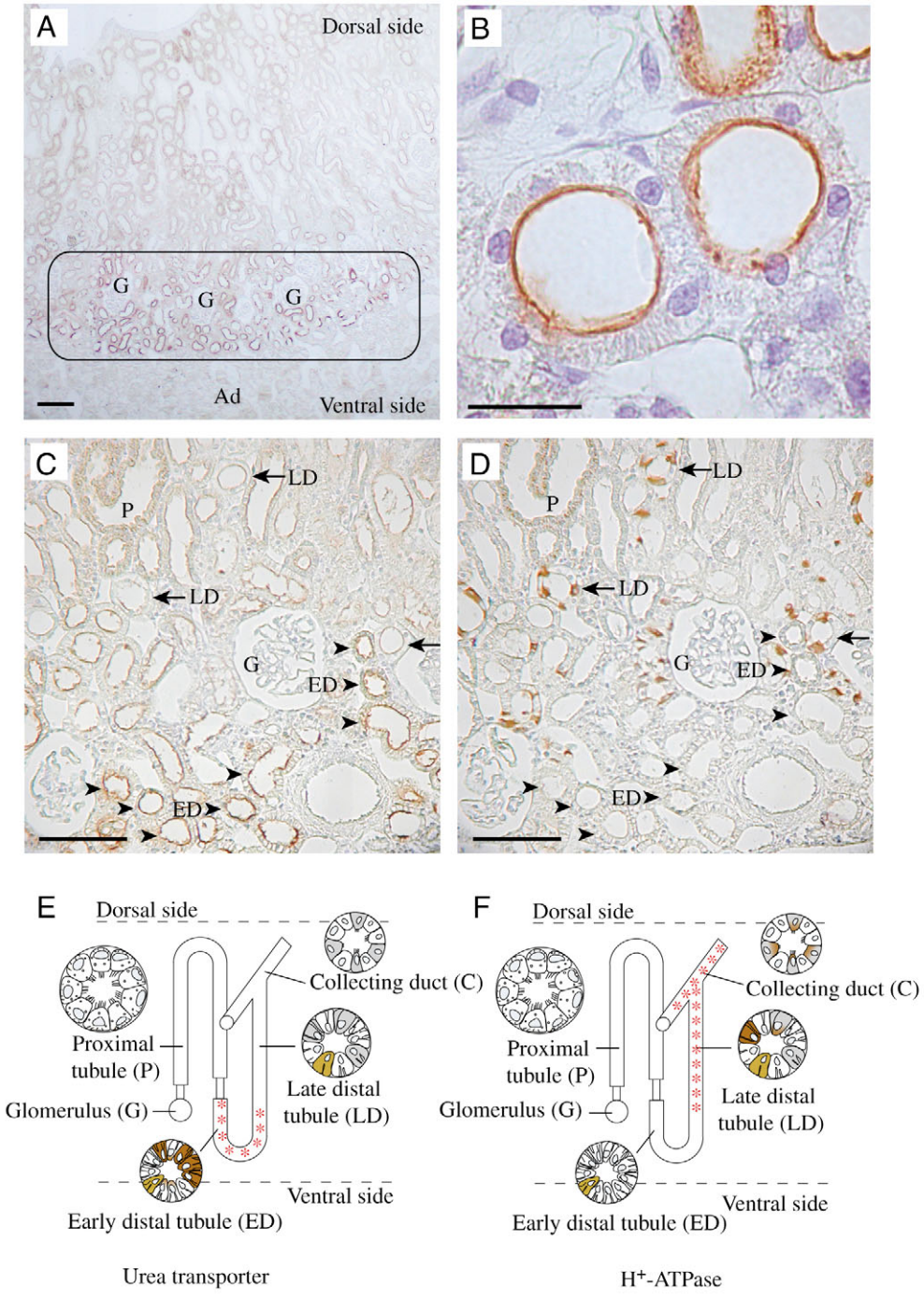

Fig. 5. Immunohistochemical localization of the urea transporter (UT; stained brown) in the kidney of *Bufo* using affinity-purified *Bufo* UT antibody. (A) Immunoreactivity was detected on the apical cell membrane of epithelia in the early distal tubules, located in the ventral zone (boxed) of the kidney. (B) Higher magnification of the immunoreactive apical cell membrane in the early distal tubules. (C,D) In adjacent sections, no UT immunoreactivity was detected in the late distal tubule and collecting duct where H^+-ATPase was expressed. (E,F) Diagrams showing the distribution of UT and H^+-ATPase in the renal nephron, indicated by red asterisk in each scheme, respectively. Arrowheads in C and D, early distal tubule (ED); arrows in C and D, late distal tubule (LD). Ad, adrenal gland; G, glomerulus; P, proximal tubule. Scale bars, (A,C,D) 100 μm; (B) 20 μm.

urinary bladder of *Bufo* is regulated in response to hyperosmosis of body fluid, and that up-regulation of UT expression may contribute to urea accumulation in extracellular fluid.

The sites of passive urea absorption and the amounts reabsorbed have never been clearly defined in the nephron of the amphibian kidney. In the present immunohistochemical study, we showed that *Bufo* UT is localized in a limited

segment of the nephron known as the early distal tubule, or diluting segment, in the ventral zone of the kidney. The UT was present on the apical membrane but not on the basolateral membrane of epithelial cells in the early distal tubule. In addition, no *Bufo* UT immunoreactivity was observed along the late distal tubule, the connecting tubule and collecting tubule, where vacuolar-type H^+-ATPase is expressed in the intercalated cells (Uchiyama and Yoshizawa, 2002). In a renal micropuncture study in *Rana catesbeiana*, Long (Long, 1973) suggested that the amphibian collecting tubule has a very low permeability to urea, so that essentially no urea is secreted into or reabsorbed from the tubule fluid in this segment. Accordingly, our results suggest that urea may be transported from the tubule to intracellular fluid by passive permeation through the facilitative urea transporter located on the apical cell membrane of the early distal tubule in the amphibian kidney.

However, movement of urea from the intracellular fluid to blood and/or the extracellular space across the basolateral membrane has yet to be demonstrated clearly. Kato and Sands (1998) and Sands (1999) suggested that an absorptive sodium/urea counter-transporter is located in the basolateral membrane of the rat inner medullary collecting duct ($IMCD_1$). In addition, Walsh and Smith (Walsh and Smith, 2001) have pointed out two hypothetical components for active urea transport in the elasmobranch kidney; an apical sodium/urea cotransporter and a basolateral sodium/urea counter-transporter. In these models, an inwardly directed sodium gradient in the epithelial cell, established by the basolateral Na^+,K^+-ATPase, provides the driving force for urea movement through the transporters. The early distal tubule of the amphibian nephron has been demonstrated to show extremely low water permeability and significant solute absorption (Stoner, 1977; Stoner, 1985; Oberleithner et al., 1982). In this nephron segment, Na^+,K^+-ATPase is located in the basolateral membranes of cells (Uchiyama and Yoshizawa, 2002; Dantzler, 2003). Thus, in this segment expressing Na^+,K^+-ATPase, movement of urea across the basolateral membrane may occur through other urea transporters such as sodium/urea counter-transporter. However, with regard to urea transport in the amphibian urinary bladder, which has high water permeability, there may be not only a facilitative urea transporter, but also several candidates such as aquaglyceroporins, including aquaporin 3, and active urea transporters.

In this study, we found that *Bufo* UT mRNA was abundantly expressed in the kidney and urinary bladder, but not in the ventral and dorsal skin. It has been reported that the skins of frogs and toads develop inwardly directed urea transport when the animals are adapted to hyperosmotic saline solutions or to dehydration (Katz et al., 1981; Garcia-Romeu et al., 1981; Rapoport et al., 1988). However, kinetic and pharmacological studies, and investigations of hormonal sensitivity, have shown that urea transport in the skin of amphibians differs from that in the urinary bladder (see Ehrenfeld, 1998). Further study of UT in the kidney and urinary bladder may help to characterize differences in urea transport between the urinary system and the skin.

In conclusion, we have demonstrated that the UT cloned from the kidney of *Bufo* belongs to the UT-A2 family of facilitative urea transporter proteins. Immunohistochemically, we showed for the first time that the UT is located in the apical membrane of epithelia along the early distal tubule or the diluting segment of the amphibian kidney. Upon acclimation to dry and hyper-saline conditions, the plasma urea concentration and osmolality were significantly elevated, and these physiological changes were correlated with significant increases in the levels of *Bufo* UT mRNA in both the kidney and urinary bladder. Thus, the putative facilitative urea transporter expressed in the kidney and urinary bladder probably plays an important role in the urea retention response to hyperosmotic stress in the marine toad.

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