Molecular characterization of a novel urea transporter from kidney inner medullary collecting ducts

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Received 29 November 1999; accepted in final form 7 November 2000

Shayakul, Chairat, Hiroyasu Tsukaguchi, Urs V. Berger, and Matthias A. Hediger. Molecular characterization of a novel urea transporter from kidney inner medullary collecting ducts. Am J Physiol Renal Physiol 280: F487–F494, 2001.—In the terminal part of the kidney collecting duct, rapid urea reabsorption is essential to maintaining medullary hypertonicity, allowing maximal urinary concentration to occur. This process is mediated by facilitated urea transporters on both apical and basolateral membranes. Our previous studies have identified three rat urea transporters involved in the urinary concentrating mechanism, UT1, UT2 and UT3, herein renamed UrT1-A, UrT1-B, and UrT2, which exhibit distinct spatial distribution in the kidney. Here we report the molecular characterization of an additional urea transporter isoform, UrT1-C, from rat kidney that encodes a 460-amino acid residue protein. UrT1-C has 70 and 62% amino acid identity to rat UrT1-B and UrT2 (UT3), respectively, and 99% identity to a recently reported rat isoform (UT-A3; Karakashian A, Timmer RT, Klein JD, Gunn RB, Sands JM, and Bagnasco SM. J Am Soc Nephrol 10: 230–237, 1999). We report the anatomic distribution of UrT1-C in the rat kidney tubule system as well as a detailed functional characterization. UrT1-C mRNA is primarily expressed in the deep part of the inner medulla. When expressed in Xenopus laevis oocytes, UrT1-C induced a 15-fold stimulation of urea uptake, which was inhibited almost completely by phloretin (0.7 mM) and 60–95% by thiourea analogs (150 mM). The characteristics are consistent with those described in perfusion studies with inner medullary collecting duct (IMCD) segments, but, contrary to UrT1-A, UrT1-C-mediated urea uptake was not stimulated by activation of protein kinase A. Our data show that UrT1-C is a phloretin-inhibitable urea transporter expressed in the terminal collecting duct that likely serves as an exit mechanism for urea at the basolateral membrane of IMCD cells.

Urinary concentration; urea transporter; vasopressin; nomenclature

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Molecular properties of urea transporter isoforms

Table 1.

<table>
<thead>
<tr>
<th>HUGO Gene Symbol</th>
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<th>Tissue Distribution</th>
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<th>Reference No(s.)</th>
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<tr>
<td>SLC14A1</td>
<td>UrT1-A‡</td>
<td>Terminal IMCD, apical membrane</td>
<td>Rat</td>
<td>20, 23 U77971</td>
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<tr>
<td></td>
<td>UrT1-B</td>
<td>tDL</td>
<td>Rat</td>
<td>30 U10358</td>
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<td>24 U99957</td>
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<td></td>
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<td>ND§</td>
<td>Rat</td>
<td>9 AF041788</td>
</tr>
<tr>
<td>SLC14A2</td>
<td>UrT2</td>
<td>Descending vasa recta</td>
<td>Rat</td>
<td>This study AF031642</td>
</tr>
</tbody>
</table>

IMCD, inner medullary collecting duct; tDL, thin descending limb of loop of Henle; ND, not determined. ‡The Human Gene Nomenclature Database (HUGO; http://www.gene.ucl.ac.uk/nomenclature) provides a list of presently approved human gene symbols. †Recommended nomenclature based on discussions with >50 scientists of the urea transporter field (May, 1999). ‡UrT1-A, -B, and -C are alternative splice products of the SLC14A1 gene. §Karakashian et al. (9) reported a 4th putative splice product of rat SLC14A1, but expression of this putative protein could not be detected on Northern analysis.

**METHODS**

cDNA library screening. A rat kidney inner medullary λgt-10 cDNA library was constructed from the oligo(dT)-primed cDNA in the size range from 2.0 to 3.5 kb. Approximately 500,000 clones were screened at high stringency by using the first 652 nucleotides at the NH₂ terminus of rat UrT1-A cDNA as a probe. Inserts from λ-phages were subcloned into the plasmid pBluescript II SK(−), and the sequence from both ends was determined by the dyeoxy chain termination method. A full-length clone containing a 4.0-kb band corresponding to UrT1-A mRNA (23). This observation prompted us to test the hypothesis that there is another splice variant in rat kidney inner medulla. In the present study, we isolated this cDNA from a rat kidney inner medullary cDNA library and characterized its function by using *Xenopus laevis* oocyte expression. The results show that UrT1-C mediates urea transport that is of high capacity and inhibited by phloretin and urea analogs in a manner similar to that of the basolateral urea transporter in the IMCD.

**Fig. 1.** Sequence analysis of UrT1-C cDNA. A: amino acid alignment of members of the UrT family. Sequences of rat UrT1-C (rUrT1-C, GenBank Accession AF031642), rat UrT1-B (rUrT1-B, U09957), rat UrT2 (rUrT2, U81518), and human UrT2 (hUrT2, L36121) were aligned by the PILEUP program (GCG). Identical residues are indicated by shading. The putative transmembrane domains are underlined and indicated by numbers. The consensus sequences for N-linked glycosylation (Asn79, 86), potential protein kinase C (PKC) phosphorylation sites (Ser23, Ser79, Thr477; boxes), and potential protein kinase A (PKA) phosphorylation sites (Ser84, Ser91; boxes) are shown. UrT1-C shares amino acid sequence of UrT1-A at 5' end (1–459) with the exception that UrT1-C ends with an aspartic acid, whereas UrT1-A has a glycine at that position. B: hydropathy analysis of UrT1-C. The hydropathy profile was analyzed by the Kyte-Doolittle algorithm with a window of 21 residues. Putative membrane-spanning domains are shown by numbers. A and C represent the extended hydrophilic domains, and B the hydrophilic domains. C: membrane topology model of UrT isoforms. Membrane-spanning domains of UrT1-C are predicted on the basis of the hydropathy profiles. Putative phosphorylation sites by PKA and PKC are shown. UrT1-A cDNA encodes a large stretch of hydrophobic protein, consisting of the 2 halves, of which the NH₂-terminal half is identical to UrT1-C and the COOH-terminal half is same as UrT1-B. D: schematic representation of the rat UrT gene and putative mechanism of alternate splicing of UrT isoforms. Previous genomic studies of the SLC14A1 gene (23) showed that there are at least 3 groups of exons involved in the splice process, i.e., exon group I (gr.I), group II (gr.II), and group III (gr.III). UrT1-B cDNA consists of 2 exons, including exon group II, which is specific to UrT1-B, and exon group III, which are common to both UrT1-A and UrT1-B. UrT1-C cDNA is composed of at least 2 exons, exon gr.I (gray boxes), which is common to UrT1-A cDNA, and exon group Ia (black boxes), which is specific to UrT1-C. Nos. above boxes indicate nucleotide positions of UT isoform cDNA; underlines, the positions in which alternative splicing takes place.
UREA TRANSPORTER IN INNER MEDULLARY COLLECTING DUCT

A

\[
\begin{align*}
&\text{rUrT1-C} & &\text{rUrT1-B} & &\text{hUrT1-B} & &\text{hUrT2} \\
&\text{MSDHL} & &\text{P} & &\text{S} & &\text{MSDHL} \\
&\text{RNLSS} & &\text{P} & &\text{P} & &\text{RNLSS} \\
&\text{EYI} & &\text{R} & &\text{EYI} & &\text{R} \\
&\text{L} & &\text{L} & &\text{L} & &\text{L} \\
&\text{S} & &\text{S} & &\text{S} & &\text{S} \\
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&\text{S} & &\text{S} & &\text{S} & &\text{S} \\
&\text{T} & &\text{T} & &\text{T} & &\text{T} \\
&\text{H} & &\text{H} & &\text{H} & &\text{H} \\
&\text{L} & &\text{L} & &\text{L} & &\text{L} \\
&\text{E} & &\text{E} & &\text{E} & &\text{E} \\
&\text{S} & &\text{S} & &\text{S} & &\text{S} \\
&\text{T} & &\text{T} & &\text{T} & &\text{T} \\
\end{align*}
\]

B

\[
\begin{align*}
&\text{A} & &\text{B} & &\text{C} \\
&\text{1} & &\text{2} & &\text{3} \\
&\text{4} & &\text{5} & &\text{6} \\
&\text{7} & &\text{8} & &\text{9} \\
&\text{10} & & & & \\
\end{align*}
\]

D

\[
\begin{align*}
&\text{UrT1-A} & &\text{UrT1-C} & &\text{UrT1-B} \\
&\text{Exon gr. I + III} & &\text{PKA (Ser 84, 51)} & &\text{rUrT1-C} \\
&\text{Exon gr. I-Ia} & &\text{PKC (Ser 23, Ser 79, Thr 477)} & &\text{rUrT1-B} \\
&\text{Exon gr. II + III} & &\text{UrT1-A} & &\text{UrT1-A} \\
&\text{Exon gr. I} & &\text{UrT1-C} & &\text{UrT1-B} \\
&\text{2640} & &\text{2640} & &\text{2640} \\
&\text{3988} & &\text{3988} & &\text{3988} \\
&\text{1989} & &\text{1989} & &\text{1989} \\
&\text{1750} & &\text{1750} & &\text{1750} \\
\end{align*}
\]
2.0-kb insert was identified and thereafter designated as UrT1-C. In vitro transcription and oocyte expression. Capped cRNA was synthesized in vitro from the linearized UrT1-C cDNA clone by using T3 RNA polymerase and was microinjected into collagenase-treated and manually defolliculated X. laevis oocytes. Urea transport activity was measured by [14C]-urea uptake as previously described (23, 30). The effects of known urea transporter inhibitors were tested by preincubation for 30 min with various concentrations of phloretin or 150 mM of the urea analogs thionin, 1,3-dimethylurea, and acetamide. The effects of protein kinase A activators were tested by preincubation with a mixture of the cAMP agonists (dibutyryl cAMP, IBMX, and forskolin) for 30 min before the uptake, as described previously (23). The CAMP concentration ranging from 0.1 to 0.5 mM was tested. All studies were performed in sodium-free solution containing 200 mM mannitol, except in studies of sodium-dependent urea uptake, in which 200 mM mannitol was replaced by 96 mM NaCl. Northern analysis. By use of male Sprague-Dawley rats, poly (A)+ RNA was prepared in several tissues including different regions of the kidney, i.e., superficial cortex, deep cortex, outer and inner stripe of outer medulla, inner medulla, and papilla. About 3 μg of each sample were separated on a 1% agarose gel in the presence of 2.2 M formaldehyde and blotted onto a nitrocellulose filter. The filter was hybridized at 42°C with a [32P]-labeled probe synthesized from the full-length rat UrT1-C cDNA and washed in 0.1× standard sodium citrate (SSC)/0.1% SDS at 65°C. In situ hybridization. Digoxigenin-labeled antisense and sense runoff transcripts were synthesized by using the Genius Kit (Boehringer Mannheim) from a PCR fragment that contained ~200 bp of UrT1-C cDNA-specific sequence (sense: 5′-TTTACCGCCAAGCCAAAATTGTT-3′, nucleotides 1760–1781; antisense: 5′-ATGGTCGTACCAGCTTCCTTAAAT-3′, nucleotides 1950–1974) and that was flanked by SP6 and T7 RNA polymerase initiation sites. For comparison of the detection of UrT1-C to UrT1-A and UrT1-B, subtype-specific cRNA probes were generated from UT cDNA (nucleotides 1–652, that recognizes both UrT1-A and UrT1-B, (GenBank AF041788) isolated from rat kidney on the basis of PCR amplification by other investigators (9), except for two amino acids, Pro at position 17 (Leu in UT-A3) and Ala at position 95 (Gly in UT-A3). Compared with UrT1-A cDNA, UrT1-C cDNA is identical at the 5′ ends from position 1 to 1754, whereas it differs at the 3′ end, 219 nucleotides from position 1755 to 1973. The amino acid sequence of UrT1-C is therefore identical to the NH2-terminal half of UrT1-A and has 70 and 62% identity to rat UrT1-B and UrT2, respectively (Fig. 1A). The hydropathy plot of UrT1-C is in accordance with the characteristic pattern of members of the urea transporter protein family, which includes two extended hydrophobic membrane stretches interspersed with hydrophilic regions (Fig. 1, B and C). UrT1-C polypeptide contains one potential N-glycosylation site at Asn279, two potential phosphorylation sites for protein kinase A (PKA; Ser84 and Ser95), and three potential protein kinase C phosphorylation sites (Ser23, Ser79, and Thr447). Tissue distribution and localization of URT1-C mRNA in rat kidney. Northern analysis using full-length UrT1-C as probe revealed a strong hybridization signal of ~2.2 kb and a weak signal of 4.0 kb in rat kidney inner medulla, in particular in the deeper portion (Fig. 2). No signal was detected in the regions of the kidney or in other organs, indicating that the UrT1-C is specific to the inner kidney medulla. Karakashian et al. (9) also observed signals in testis; however, our Northern analysis revealed no signal for testis when using high-stringency washing conditions. To further localize UrT1-C mRNA in rat kidney, we performed in situ hybridization and compared its distribution with the other kidney UT isoforms, UrT1-A and UrT1-B, using adjacent sections. Because the UrT1-C nucleotide sequence is identical to that of the NH2-terminal domain of UrT1-A, we prepared a cRNA probe corresponding to 219 bp of 3′ untranslated region of UrT1-C cDNA, which can distinguish between UrT1-C and UrT1-A transcripts. Hybridization of the antisense URT1-C cRNA probe revealed the strongest signals in the deep portion of the IMCD (Fig. 3), consistent with the distribution observed by Northern analysis. In comparison, hybridization signals obtained with the probe that recognizes both UrT1-A and UrT1-C extended into the basal part of the inner medulla (Fig. 3). Because the UrT1-A probe used is three times longer than the UrT1-C probe, these data do not
reveal the relative proportion of UrT1-A and UrT1-C expression in the IMCD. However, they indicate that the expression levels of both UrT1-A and UrT1-C increase toward the deep IMCD, as has been shown for UrT1-A by quantitative RT-PCR in microdissected kidney tubules (22). Hybridization with UrT1-B-specific cRNA probes showed that the UrT1-B transcript is located in the inner stripe of outer medulla and the outermost portion of the inner medulla. These data indicate that UrT1-C mRNA expression is strongest in the terminal IMCD and that its tubular expression coincides with that of UrT1-A. Its distribution is in good agreement with the regions previously shown by perfusion studies to have extremely high urea permeability (13, 25).

**Functional properties of UrT1-C in X. laevis oocytes.** We examined the functional characteristics of UrT1-C in the X. laevis oocyte expression system. When expressed in the oocytes, UrT1-C mediated an ~15-fold increase in [14C]-urea uptake (1 mM) compared with water-injected control oocytes (Fig. 4A). Urea transport mediated by UrT1-C reached a maximum after incubation for 30 min (Fig. 4B). The level of transport activity of UrT1-A and UrT1-C was compared in the same batch of oocytes (Fig. 4D). Urea transport mediated by UrT1-C was about three- to fourfold higher than that mediated by UrT1-A, 20–30 vs. 5–10 × 10−6 cm/s. Reducing the amount of UrT1-C cRNA for micro-injection to as low as 10 ng/oocyte did not significantly affect UrT1-C transport activity and gave consistently higher uptake levels than UrT1-A. Furthermore, this uptake was about fivefold higher than that mediated by AQP3 (5–10 × 10−6 cm/s; Ref. 29). UrT1-C-mediated urea uptake was not changed by replacing 200 mM mannitol with 96 mM NaCl, indicating that transport is not sodium dependent (Fig. 4A). Uptake of urea (1 mM) was inhibited by preincubation for 30 min with phloretin (0.2–0.7 mM) in a dose-dependent fashion (Fig. 4A). The level of phloretin sensitivity was similar to those observed for UrT1-A and UrT1-B (23, 24) and consistent with the properties of urea transport characterized in perfusion studies of the IMCD (3, 25).

UrT1-C-mediated urea uptake was inhibited by urea analogs (150 mM) including dimethylurea (4.7 ± 0.6% of control), thiourea (44.4 ± 5.9% of control), and acetamide (58.6 ± 5.5% of control) (Fig. 4C). The inhibition was in good agreement with the studies of the perfused IMCD showing that thiourea and methylurea inhibit urea permeability more effectively than acetamide (3). One important characteristic of urea transport on the apical membrane of terminal IMCD segments is its activation by vasopressin via a cAMP-dependent pathway. We demonstrated in previous studies that UrT1-

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**Fig. 2. Tissue distribution of UrT1-C in rat by high-stringency Northern analysis.** Northern blot with rat poly (A+) RNA (3 μg) was hybridized with UrT1-C cDNA probe in a high-stringency condition. Left: positions of size standards (in kb).

**Fig. 3. Localization of UrT1-C mRNA by in situ hybridization in rat kidney.** Freshly frozen kidney sections (10 μm) from rats were hybridized against digoxigenin-labeled cRNA probes to UrT1-A, UrT1-B, or UrT1-C. Left: brightfield micrographs of adjacent sections through inner medulla (UrT1-A+UrT1-C). Right: brightfield micrographs of adjacent sections through inner medulla (top) and inner stripe of outer medulla (UrT1-B, bottom). Sections were developed for identical periods. Hybridization using sense cRNA probes did not reveal any signals (not shown). The UrT1-A probe (650 bp in length), which recognizes both UrT1-A and UrT1-C transcripts, labels the initial and terminal portions of the IMCD in the inner medulla. The UrT1-C-specific probe (219 bp) labels the IMCD primarily in the terminal portion. Though identical development times were used, the labeling intensities for UrT1-A and UrT1-C are not directly comparable because of different probe length. In contrast to UrT1-A and UrT1-C, the UrT1-B probe labels primarily the thin limbs of loop of Henle in the inner stripe of outer medulla. Magnification bar: 100 μm.
A-mediated urea uptake in *X. laevis* oocytes can be stimulated by acute activation of protein kinase A, consistent with the effect of vasopressin stimulation. Preincubation of 30 min with cAMP agonists, however, did not alter transport activities of UrT1-C. These studies indicated that UrT1-C displays functional characteristics of the urea transport previously described in the terminal IMCD with three- to fourfold higher transport activity than UrT1-A but does not appear to be regulated by acute activation of protein kinase A.

**DISCUSSION**

In the present study, we show that another splice variant of the SLC14A1 gene, UrT1-C, is primarily expressed in the terminal IMCD. Sequence analysis indicates that UrT1-C has a hydrophobic transmembrane protein structure similar to the other isoforms UrT1-B (UT2) and UrT1-B (UT3). *X. laevis* oocytes expressing UrT1-C facilitate transport of urea with high capacity, and transport is inhibited by urea analogs and phloretin. The transport characteristics of UrT1-C are consistent with those previously reported for perfused isolated IMCD. In contrast to the apical urea transporter UrT1-A (UT1), the transport activity mediated by UrT1-C is constitutively high, whereas UrT1-A requires activation by protein kinase A to reach similar activity.

We have summarized the properties of urea transporter family members in Table 1. Most of the nucleotide sequences of UrT1-C cDNA correspond to the 5’ part of UrT1-A cDNA. On the basis of genomic analysis (23), we have previously showed that UrT1-A arises by splicing of exon group I and group III, which are located about 7 kb apart (Fig. 1D). UrT1-C mRNA presumably arises as a splice product of exon group I and group Ia. Other investigators recently reported the cloning of two splicing variants of the SLC14A1 gene, UT-A3 and UT-A4, by PCR from rat kidney cDNA (9). Of these isoforms, the physiological role of UT-A4 (UrT1D) remains unclear because its transcript was too low to be detected by Northern analysis. The sequence of UT-A3 cDNA is 99% identical to UrT1-C cDNA and induced phloretin-inhibitable urea transport when expressed in human kidney HEK-293 cells, similar to our expression data in the oocytes.

Our localization and functional characterization data suggest that UrT1-C may be responsible for urea transport on the basolateral membrane of the kidney IMCD cells. Urea reabsorption in the terminal part of the IMCD is an important aspect of urinary concentrating capacity (Fig. 5). To ensure maximal urinary concentrating capacity, urea absorption is delayed to the most terminal part of the IMCD, where effective blood flow is low. This process prevents the escape of urea, through the vasa recta, in the upper part of the inner medulla, where blood flow is much higher (1, 13). Urea permeability measurements in isolated perfused rat and rabbit IMCD segments showed that the urea permeability of the IMCD in the initial part is at least one order of magnitude lower than that in the terminal portions (19). Because transepithelial urea permeability is extremely high in the terminal IMCD, urea movement across the IMCD cells is considered to occur via urea transporters present on both apical and basolateral membranes. Previous perfusion studies indicated that...
both apical and basolateral membranes of the IMCD contain phloretin-sensitive, urea analog-inhibitable urea transporters (25). Immunocytochemical studies showed that UrT1-A is expressed on the apical membranes throughout the entire length of the IMCD (15). Subsequent quantitative measurement of UrT1-A protein in the IMCD segments demonstrated that UrT1-A expression levels are almost comparable between initial and terminal parts (11). These observations suggest that transepithelial urea permeability in the IMCD is mediated by another basolateral transporter, in addition to the apical transporter UrT1-A. Because our in situ hybridization studies demonstrate that the expression of UrT1-C is strongest in the terminal part of the IMCD, where massive urea transport is expected, it is reasonable to speculate that UrT1-C serves as a basolateral transporter to allow basolateral exit of urea in this segment. The greater transport activities of UrT1-C might be crucial for the IMCD to respond promptly to changes in interstitial urea concentration without many changes in cell volume, especially when vasopressin is stimulated during water restriction. Immunolocalization of UrT1-C will be the ultimate approach to confirm the basolateral localization of UrT1-C. However, these studies have been hampered due to the amino acid sequence identity of UrT1-C to UrT1-A at the NH_2 terminus and difficulties in raising specific antibodies against its NH_2 terminus. This study requires comparison of immunocytochemical staining using 1) antibodies made from the NH_2-terminal domain of UrT1-A that also recognize UrT1-C and 2) antibodies that recognize only UrT1-A at the COOH-terminal domain, in parallel with in situ hybridization studies using specific probes for UrT1-A and UrT1-C.

It is possible that basolateral urea exit is mediated by a pathway other than UrT1-C. Several water channels have been reported to be permeable to water as well as urea (6, 7, 29). Among those, aquaporin-3 (AQP3) was shown to be expressed on the basolateral membranes of cells throughout the IMCD (5, 8). However, AQP3 does not appear to play an important role in basolateral exit of urea, because it is expressed most abundantly in the outer half of the inner medulla and it is absent in the terminal part of the IMCD (5). Furthermore, on the basis of studies in X. laevis oocytes, the calculated urea permeability mediated by AQP3 was relatively small (~5 x 10^(-6) cm/s). Finally, on the basis of the perfusion studies with isolated IMCD, separate pathways were shown for urea and water transport, because the urea reflection coefficient was ≈1 and because distinct phloretin sensitivities were observed for water and urea permeability (13, 25). Recently, Kato and Sands (10) showed evidence for a secondary active, sodium-dependent urea transport in the deepest subsegment of the rat IMCD. The actual role of this sodium/urea antiporter in urea secretion and maintenance of water homeostasis remains to be determined.

UrT1-C has two potential PKA sites at the NH_2 terminus. However, despite the findings of Karakashian et al. (9), we did not find PKA activation of UrT1-C transport in the oocytes, making it less likely that UrT1-C is regulated by vasopressin. Because we observed cAMP activation of UrT1-A, which has PKA sites at the same position (Ser^84 and Ser^91) as UrT1-C, activation of UrT1-A must be mediated by other UrT1-A-specific PKA sites located in intracellular loops of the COOH-terminal portion of the transporter (21, 23). Previous studies using video-enhanced contrast microscopy (25) showed that urea permeability of the basolateral membrane of IMCD cells is about twofold greater than that of the apical membrane, when corrected by their surface area. The apical membrane is therefore thought to be rate limiting for overall transepithelial urea transport and the site of vasopressin regulation. However, vasopressin regulation on the basolateral side has not been well examined. Given that the time course of urea permeability stimulated by vasopressin is biphasic (26), this complex response...
might involve the multiple transport steps, including apical urea uptake by UrT1-A and basolateral urea exit by UrT1-C. Further studies are necessary to clarify whether and how UrT1-C is regulated.

In summary, we have isolated and characterized an additional urea transporter isoform, UrT1-C, also known as UTA-3, from the rat kidney inner medulla. UrT1-C mRNA is exclusively expressed in the terminal IMCD, and its transport properties are consistent with those observed in perfusion studies of isolated IMCDs, i.e., in terms of phloretin sensitivities and inhibition by urea analogs. We propose that UrT1-C is the urea transporter on the basolateral side of the IMCD cells allowing basolateral exit of urea and maintenance of medullary hypertonicity that are essential to ensure maximal urinary concentration.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant RO1-DK-46289 (to M. A. Hediger), a research fellowship grant from the Siriraj-China Medical Cooperation, and a National Kidney Foundation Fellowship (to H. Tsukaguchi).

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