Analysis of Double Knockout Mice Lacking Aquaporin-1 and Urea Transporter UT-B

EVIDENCE FOR UT-B-FACILITATED WATER TRANSPORT IN ERYTHROCYTES*

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We reported increased water permeability and a low urea reflection coefficient in Xenopus oocytes expressing urea transporter UT-B (former name UT3), suggesting that water and urea share a common aqueous pathway (Yang, B., and Verkman, A. S. (1998) J. Biol. Chem. 273, 9369–9372). Although increased water permeability was confirmed in the Xenopus oocyte expression system, it has been argued (Sidoux-Walter, F., Lucien, N., Olives, B., Gobin, R., Rousselet, G., Kamsteeg, E. J., Ripoche, P., Deen, P. M., Cartron, J. P., and Bailly, P. (1999) J. Biol. Chem. 274, 30228–30235) that UT-B does not transport water when expressed at normal levels in mammalian cells such as erythrocytes. To quantify UT-B-mediated water transport, we generated double knockout mice lacking UT-B and the major erythrocyte water channel, aquaporin-1 (AQP1). The mice had reduced survival, retarded growth, and defective urinary concentrating ability. However, erythrocyte size and morphology were not affected. Stopped-flow light scattering measurements indicated erythrocyte osmotic water permeabilities (in cm/s × 0.01, 10 °C): 2.1 ± 0.2 (wild-type mice), 2.1 ± 0.05 (UT-B null), 0.19 ± 0.02 (AQP1 null), and 0.045 ± 0.009 (AQP1/UT-B null). The low water permeability found in AQP1/UT-B null erythrocytes was also seen after HgCl2 treatment of UT-B null erythrocytes or phloretin treatment of AQP1 null erythrocytes. The apparent activation energy for UT-B-mediated water transport was low, <2 kcal/mol. Estimating 14,000 UT-B molecules per mouse erythrocyte, the UT-B-dependent P0 of 0.15 × 10−4 cm/s indicated a substantial single channel water permeability of UT-B of 7.5 × 10−14 cm²/s, similar to that of AQP1. These results provide direct functional evidence for UT-B-facilitated water transport in erythrocytes and suggest that urea traverses an aqueous pore in the UT-B protein.

UT-B† (original names UT3 and UT11) is a 42-kDa facilitated urea transporter expressed in mammalian erythrocytes, renal vasa recta, and other sites (1–4). Humans lacking UT-B (Jk null blood group) have low urea permeability in erythrocytes and manifest a mild impairment in maximal urinary concentrating ability (5–9). Transgenic mice lacking UT-B also have very low erythrocyte urea permeability (45-fold lower than normal) and were found to have a urea-selective urinary concentrating defect (4). Members of a related family of facilitated urea transporters (UT-A) are expressed primarily in kidney and are encoded by a different gene producing multiple protein isoforms by alternative splicing (10–12).

In screening membrane transporters for intrinsic water permeability, we found that UT-B (but not UT-A isoforms) increased osmotically driven water transport when expressed heterologously in Xenopus oocytes (13). UT-B-facilitated water transport was blocked by urea transport inhibitors, and reflection coefficient measurements indicated a common water/urea pathway. Increased water permeability in Xenopus oocytes expressing UT-B was subsequently confirmed by Sidoux-Walter et al. (14); however, they concluded that UT-B-facilitated water transport does not occur under physiological conditions. They reasoned, based on water versus urea permeability measurements in oocytes expressing different levels of UT-B, that UT-B-associated water permeability occurs only when UT-B is expressed at non-physiological high levels. The issue has remained unsettled as to whether UT-B can transport water in natively UT-B expressing cells such as erythrocytes.

The mechanism of water transport in erythrocytes has been a subject of long-standing interest. Although it is now established from measurements in AQP1 null erythrocytes that AQP1 provides the major pathway for water transport (15–17), there remains unsubstantiated older evidence that erythrocytes have significant protein-mediated mercurial-insensitive water permeability that is presumably AQP1-independent (18). There is also older conflicting data about whether some water and urea share a common pathway across the erythrocyte plasma membrane with a wide range of reported urea reflection coefficients (σw,urea) from <0.5 to 1.0 (19–22). Technically, the high urea permeability in erythrocytes probably precludes an accurate biophysical determination of σw,urea. The value of σw,urea must be near 1.0 if water moves only through AQP1 and membrane lipids, because AQP1 has been shown to be urea-impermeable (23, 24). However, if some water moves through a common aqueous pathway through UT-B shared by urea, then σw,urea might be less than 1.0.

The purpose of this study was to resolve the question of whether UT-B transports water in erythrocytes and hence whether the UT-B protein contains an aqueous pore and whether erythrocyte σw,urea must be near unity. We generated double knockout mice lacking UT-B and AQP1, reasoning that UT-B-mediated water transport might be measurable in erythrocytes lacking the principal erythrocyte water transporter, AQP1. We found significantly reduced water permeability in

* This work was supported by National Institutes of Health Grants DK53512, HL58198, HL60288, EB00415, and EY13574 and Grant P01 DK35124, HL58198, HL60288, EB00415, and EY13574 from the National Cystic Fibrosis Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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† The abbreviations used are: UT, urea transporter; AQP, aquaporin.

Published, JBC Papers in Press, July 19, 2002, DOI 10.1074/jbc.M206948200

This paper is available online at http://www.jbc.org
AQP1/UT-B-deficient erythrocytes compared with AQP1-deficient erythrocytes, providing quantitative information on the single channel water permeability of UT-B. A secondary purpose of this study was to investigate urinary concentrating ability in mice lacking AQP1 and UT-B together. As in erythrocytes, AQP1 and UT-B are coexpressed in microvascular endothelial cell membranes in descending renal vasa recta. We hypothesized that the double knockout mice may have a profound urinary concentrating defect because of extensive disruption of the countercurrent multiplication and exchange mechanisms.

MATERIALS AND METHODS

Generation of AQP1/UT-B Double Knockout Mice—AQP1/UT-B double knockout mice were generated by intercross of the single AQP1 and UT-B knockouts. The breeding of F2 generation double heterozygous mice yielded 11 AQP1/UT-B knockout mice of 270 pups.

Water Permeability Measurements—Fresh erythrocytes obtained by tail bleeding (~50 µl/bleed) were washed three times in phosphate-buffered saline to remove plasma and the cellularuffy coat. Stopped-flow measurements were done on a Hi-Tech SF-51 instrument. Suspensions of erythrocytes (~0.5% hematocrit) in phosphate-buffered saline were subjected to a 250-mM inwardly directed gradient of sucrose. The kinetics of decreasing cell volume was measured from the time course of 90° scattered light intensity at 530 nm wavelength. Osmotic water permeability coefficients (Pf) were computed from the light scattering time course as described previously (24). In some experiments, 0.3 mM HgCl2 or 0.7 mM phloretin was added to the erythrocyte suspension before stopped-flow experiments.

Erythrocyte Size and Morphology—Erythrocyte number, hematocrit, and mean cell volume were measured by the Hematology Laboratory at San Francisco General Hospital. For light microscopy, blood cells were smeared onto glass slides and stained with eosin-y-methylene blue using the HEMA3 stain set (Biochemical Sciences Inc.).

Immunofluorescence and Immunoblot Analysis—Red blood cell smears on glass slides were fixed in acetone/methanol (1:1) and incubated for 30 min with phosphate-buffered saline containing 1% bovine serum albumin and then with anti-AQP1 (1:1000) or UT-B antisera (1:500) for 1 h at 23 °C in phosphate-buffered saline containing 1% bovine serum albumin. Slides were rinsed with 2.7% NaCl followed by phosphate-buffered saline and incubated with a secondary Cy3-conjugated sheep anti-rabbit F(ab’2) fragment (1:200, Sigma) for visualization by fluorescence microscopy. Immunoblot analysis of ghost membranes from erythrocytes, prepared by hypotonic lysis and washing, was carried out by standard procedures as described (25).

Renal Function Studies—For analysis of urine osmolalities, urine samples were collected by gentle bladder massage. Urine osmolalities were measured by freezing point depression osmometry (Micro-osmometer, Precision Systems, Inc.).

RESULTS

AQP1/UT-B double knockout mice were generated by intercross of AQP1 and UT-B null mice. The double knockout mice were grossly phenotypically normal just after birth. Of 270 mice born in 32 litters from breeding of double heterozygous mice, there were 11 double knockout mice, consistent with the predicted 1:16 Mendelian ratio. However, the growth and survival of the double knockout mice were impaired. Fig. 1A shows that the double knockout mice were ~30% smaller by body weight than wild-type littersmates at age 11 days. Although >90% of living AQP1 and UT-B null mice that were genotyped at 5 days remained alive to adulthood, only 50% of the double knockout mice were alive at 10 days, and all double knockout mice died by 2 weeks. Urinary concentrating function was compared by measurement of urine osmolality. As shown in Fig. 1B, urine osmolality in double knockout mice (553 ± 55 mosM) was similar to that in AQP1 knockout mice (604 ± 18 mosM) but significantly lower than in wild-type mice (816 ± 34 mosM). Because of the small size of the mice, detailed serum analyses were not done.

Blood was analyzed for erythrocyte number, size, and AQP1 and UT-B expression. Table I summarizes peripheral blood analysis. Erythrocyte number and hematocrit were slightly greater in AQP1 and AQP1/UT-B null mice than wild-type and UT-B null mice, which may be a consequence of relative mild dehydration. The mean corpuscular size was not significantly different among the genotypes. Morphology was similar in erythrocytes from mice of the four genotypes as examined in stained smears by light microscopy (Fig. 2A, top panels).

Immunocytochemistry of the permeabilized erythrocytes showed a plasma membrane staining pattern for AQP1 protein in erythrocytes from wild-type and UT-B null mice (Fig. 2A, middle panels). Examination of multiple smears showed similar AQP1 staining in the wild-type and UT-B null mice. No AQP1 staining was seen in AQP1 and AQP1/UT-B null erythrocytes. Comparable UT-B immunostaining was seen in erythrocytes from wild-type and AQP1 null mice (Fig. 2A, bottom panels). The weaker UT-B versus AQP1 staining is because of lower UT-B expression and differences in antibodies. No UT-B immunostaining was seen in UT-B and AQP1/UT-B null erythrocytes. Immunoblot analysis confirmed these findings (Fig. 2B). AQP1 protein was seen in erythrocyte membranes from wild-type and UT-B null mice as a band at ~28 kDa, representing non-glycosylated protein and a more diffuse band at 34–40 kDa representing the glycosylated protein. UT-B protein was seen as a band of 40–46 kDa in erythrocytes from wild-type and AQP1 null mice. An ~90-kDa nonspecific band was seen in erythrocytes of all genotypes.

Osmotic water permeability was measured by stopped-flow fluorescence from the time course of scattered light intensity in response to a 250-mM inwardly directed osmotic gradient of sucrose. The light scattering signal amplitude is inversely related to erythrocyte cell volume. Representative original light scattering data are shown for erythrocytes from mice of the four genotypes in Fig. 3A under control conditions and after brief incubation with the AQP1 inhibitor, HgCl2, and the UT-B inhibitor, phloretin. Experiments were done initially at a low temperature (10 °C) where the strongly temperature-depend-
TABLE I
Hematological properties of erythrocytes
Data are mean ± S.E.M. for five sets of measurements on erythrocytes from different mice. RBC, red blood cell count; HCT, hematocrit; MCV, mean cell volume.

| Genotype | RBC × 10^6/µl | HCT | MCV |
|----------|---------------|-----|-----|
| Wild type | 8.0 ± 0.5 | 40.2 ± 2.6 | 50.6 ± 1.2 |
| AQP1 −/− | 8.9 ± 0.2 | 46.3 ± 1.4 | 49.6 ± 1.4 |
| UT-B −/− | 8.4 ± 0.5 | 43.6 ± 2.1 | 52.4 ± 2.1 |
| AQP1/UT-B −/− | 9.2 ± 0.1 | 45.5 ± 1.1 | 52.3 ± 2.9 |

*p < 0.05 compared to wild-type.

Fig. 2. AQP1 and UT-B expression in erythrocytes. A, erythrocyte morphology in eosin γ-methylene blue-stained smears (top). Scale bar, 10 µm. Immunofluorescence localization of AQP1 and UT-B in erythrocyte smears (bottom). B, immunoblot analysis of erythrocyte plasma membranes from mice of indicated genotype as probed by anti-AQP1 and anti-UT-B antibodies.

ent lipid-mediated water permeability should be minimized.

The decrease in scattered light intensity at late times probably represents slow sucrose (and water) influx due to perturbation of the membrane by phloretin. There was less of a decrease at late times using 0.1 mM phloretin (instead of 0.7 mM) but similar inhibition of water permeability in AQP1 null erythrocytes.

Averaged data are summarized in Fig. 3B. Erythrocytes from wild-type and UT-B null mice had high water permeability (P f ̃ 0.02 cm/s) that was not inhibited significantly by phloretin. Water permeability in AQP1 null erythrocytes (P f ̃ 0.0019 cm/s) was similar to that in wild-type erythrocytes after HgCl2. Water permeability in AQP1/UT-B null erythrocytes was quite low (P f ̃ 0.00045 cm/s), insensitive to inhibitors, and similar to that in UT-B null erythrocytes after HgCl2 and in AQP1 null erythrocytes after phloretin. Together these data provide strong evidence for UT-B-facilitated water transport in erythrocytes.

Temperature dependence measurements were done to further investigate the mechanisms of erythrocyte water transport and the nature of UT-B-facilitated water transport. Fig. 4A shows representative original light scattering data for erythrocyte water permeability at four temperatures. Water transport was fast in erythrocytes from wild-type and UT-B null mice and weakly temperature-dependent. The lower water permeability in AQP1 null erythrocytes was more strongly temperature-dependent, increasing ~3-fold from 10 to 35 °C. Water permeability in AQP1/UT-B null erythrocytes was even more strongly temperature-dependent, increasing ~9-fold from 10 to 35 °C. Fig. 4B (top) summarizes the data as an Arrhenius plot of lnP f versus reciprocal absolute temperature, where the slope is proportional to the activation energy E a. P f was weakly temperature-dependent for wild-type and UT-B null erythrocytes. Stronger temperature dependence was observed in AQP1 null erythrocytes (apparent E a 7.3 kcal/mol) and AQP1/UT-B null erythrocytes (E a 19 kcal/mol). The high E a of 19 kcal/mol strongly suggests water diffusion through the lipid bilayer.

Fig. 4B (bottom) shows an Arrhenius plot in which the temperature dependence of the UT-B-mediated component of erythrocyte water permeability (P f/UT-B) was computed from the difference in P f measured in AQP1 null versus AQP1/UT-B null erythrocytes. E a for the UT-B-mediated water permeability was ~2 kcal/mol, providing biophysical evidence for a water passage through a UT-B-associated aqueous channel.

DISCUSSION

This study reports direct functional evidence that UT-B in mammalian erythrocytes is able to transport water. We found that osmotic water permeability in erythrocytes from mice lacking both AQP1 and UT-B was 4.2-fold lower than that in erythrocytes from mice lacking AQP1 alone. A similar low water permeability was found in erythrocytes from AQP1 null mice after UT-B inhibition by phloretin and in erythrocytes from UT-B null mice after inhibition of AQP1 by HgCl2. UT-B-facilitated water transport was weakly temperature-dependent, as found in Xenopus oocytes expressing UT-B (13). These results explain why the activation energy of erythrocyte water transport after mercurial inhibition or AQP1 deletion is substantially lower than expected for lipid-mediated water transport (26). They also provide a molecular basis for the conclusion of Dix and Solomon (18), based on studies of membrane perturbing agents, that the mercurial-insensitive water permeability in erythrocytes involves in large part a protein pathway. Based on the quantitative data here, Fig. 5 summarizes the contributions of protein and lipid pathways for water and urea transport in mouse erythrocytes. At 10 °C, ~90% of water is transported through AQP1, 8% through UT-B, and the remainder through the lipid membrane. The vast majority of urea is transported through UT-B. At 37 °C, ~79% of water is trans-
ported through AQP1, 6% through UT-B, and the remainder through the lipid membrane.

The results obtained here using a genetic approach permit computation of a lower limit for erythrocyte $\sigma_{\text{urea}}$. Using the data in Fig. 5 and assuming a $\sigma_{\text{urea}}$ of zero for UT-B-mediated water transport, erythrocyte $\sigma_{\text{urea}}$ is predicted to be 0.92. This is probably a significant underestimate of erythrocyte $\sigma_{\text{urea}}$ because $\sigma_{\text{urea}}$ for UT-B-mediated water transport is at least 0.3 based on oocyte data (13), giving erythrocyte $\sigma_{\text{urea}} \approx 0.95$. These values are consistent with the data of Levitt and Mlukody (19), reporting a $\sigma_{\text{urea}}$ of near unity, but disagree with low $\sigma_{\text{urea}}$ values of 0.5–0.7 reported in a series of studies by Solomon and colleagues (18, 22, 27), where $\sigma_{\text{urea}}$ was probably underestimated because of the confounding effect of rapid diffusional urea transport.

Urine osmolalities in AQP1/UT-B double knockout mice and AQP1 null mice were similar, indicating that impairment of vasa recta water and urea transport together does not produce a profound nephrogenic diabetes insipidus with hypotonic urine as found in mice lacking AQP3 (17, 28). However, the limited survival of the double knockout mice precluded studies of urinary concentrating ability in adult mice after maximal urinary concentrating function has developed. Analysis of proximal tubule (29, 30), thin descending limb of Henle (31), and descending vasa recta (32) function has indicated that the impaired urinary concentrating ability in AQP1 null mice results primarily from defective countercurrent multiplication and exchange, producing a hypoosmolar renal medullary interstitium (33). The urea-selective urinary concentrating defect in UT-B null mice appears to result from impairment of urea recycling from the renal medullary interstitium to the descending vasa recta (4). Recognizing the caveat that adult double knockout mice could not be studied, our finding that the urin-
nary concentrating defect in AQP1/UT-B is similar to that in AQP1 null mice suggests that little urea recycling occurs after disruption of the medullary concentration gradients of NaCl and urea.

It is interesting to compare the intrinsic (single channel) water permeabilities of AQP1 versus UT-B. We measured similar osmotic water and urea permeabilities in mouse and human erythrocytes (not shown). Assuming, as in human erythrocytes, that there are 14,000 copies of UT-B and 200,000 copies of AQP1 (9) per mouse erythrocyte plasma membrane, then there is ~1 UT-B molecule per 14 AQP1 molecules. From the data in Fig. 3, AQP1 contributes 13 times more than UT-B to erythrocyte water permeability. Thus, the single channel (per molecule) water permeability of UT-B in erythrocytes is very similar to that of AQP1 (7.5 × 10⁻¹⁴ cm²/s). The presence of a continuous aqueous pathway through UT-B that efficiently facilitates osmosis is an interesting observation that may account for the exceptionally high transport turnover rate of UT-B (2–6 × 10⁶ s⁻¹, Ref. 34), as high as that of ion channels and >2–3 orders of magnitude greater than that of classic carriers and active transporters. Atomic resolution structural analysis of UT-B, as recently completed for AQP1 (35), should define the aqueous pore traversing the UT-B protein.

The AQP1/UT-B double knockout mice generated for the experiments here had significant impairment in their growth compared with single knockout mice deficient in AQP1 or UT-B. The AQP1/UT-B double knockout mice died in their first 2 weeks of life. The reason(s) for the impaired growth and survival of AQP1/UT-B null mice were not established from the data presented here. AQP1 is expressed in erythrocytes, as well as in epithelial cells in kidney, choroid plexus, ciliary body, and in multiple endothelial capillaries including renal vasa recta. UT-B is expressed in erythrocytes, urinary bladder epithelium, brain astrocytes, renal vasa recta endothelia, and as yet unidentified sites in colon, heart, liver, and testis (1–4, 36, 37). AQP1 and UT-B are thus coexpressed only in erythrocytes and renal vasa recta. With no evidence of hemolysis (normal hematocrit, reticulocyte count, serum haptoglobin, and lactate dehydrogenase), it seems unlikely that defective erythrocyte function is responsible for impaired mouse growth and survival. Defective urinary concentrating ability may be responsible for failure of the double knockout mice to thrive, based on the observation that mouse kidneys readily develop obstructive renal failure after polycystia, as found in transgenic mice lacking functional aquaporins 2 and 3 (17, 38), the vasopressin V₂-receptor (39), and the NKCC2 (40). We did not investigate in detail the cause of impaired survival in AQP1/UT-B double knockout mice, given the focus of this study on erythrocyte UT-B function.

In summary, the data here indicate that erythrocyte UT-B functions as an efficient water transporter, although the absolute contribution of UT-B to total water transport in normal erythrocytes is small because of the fewer copies of UT-B than AQP1. Although we believe that water movement through UT-B is unlikely to be physiologically important in erythrocytes, kidney, or other tissues in which UT-B is expressed, we propose that the aqueous pore through UT-B is important in its highly efficient urea transport function. Mutagenesis and structural studies should be informative in this regard.

Acknowledgments—We thank Dr. L. Vetrivel for help in computations and Liman Qian for mouse breeding.

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J. Biol. Chem. 2002, 277:36782-36786.
doi: 10.1074/jbc.M206948200 originally published online July 19, 2002

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