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Urea transport in kidney brush-border membrane vesicles from an elasmobranch, *Raja erinacea*

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Summary

Marine elasmobranch fishes maintain high urea concentrations and therefore must minimize urea loss to the environment in order to reduce the energetic costs of urea production. Previous studies have identified a facilitated urea transporter in the kidney of the dogfish. We examined mechanisms of urea transport in the kidney of the little skate *Raja erinacea* using an isolated brush-border membrane vesicle preparation. Urea uptake by brush-border membrane vesicles is by a phloretin-sensitive, non-saturable uniporter in the dorsal section and a phloretin-sensitive, sodium-linked urea transporter (K_m=0.70 mmol l^{-1}, V_{max}=1.18 μmol h^{-1} mg^{-1} protein) in the ventral section of the kidney. This provides evidence for two separate urea transporters in the dorsal *versus* ventral sections of the kidney. We propose that these two mechanisms of urea transport are critical for renal urea reabsorption in the little skate.

Key words: brush-border plasma membrane, phloretin, urea permeability, little skate, *Raja erinacea*.

Introduction

Since the classic work of Homer Smith (Smith, 1929, 1936), it has been recognized that marine elasmobranchs retain large amounts of urea in order to maintain the osmolality of their body fluids slightly above that of the surrounding environment. At present, it is accepted that urea produced in the liver is retained in the blood by the very low permeability of the gills and by reabsorptive mechanisms in the kidney (Boylan, 1967; Wood et al., 1995; Fines et al., 2001; Walsh and Smith, 2001). The exact mechanisms in the gill and kidney that allow elasmobranchs to retain urea against a very large urea gradient with seawater, however, are not well understood.

The gill, with its large surface area, is the dominant site of urea loss to the environment, accounting for 93–96% of urea excretion in elasmobranchs (Payan et al., 1973; Wood et al., 1995). The relatively low permeability of the gill to urea has long been known (Boylan, 1967), and is estimated to be 82 times less permeable to urea relative to the rainbow trout gill (Pärt et al., 1998). Recent studies have demonstrated the presence of a secondary active gill basolateral Na^+–urea antiporter moving urea from the gill back into the blood against the gradient (Fines et al., 2001). In addition, the extraordinarily high cholesterol content in the basolateral membrane would retard passive urea loss from the gill (Fines et al., 2001). The other potential site of urea loss is the kidney, where approximately 90–96% of the urea in the glomerular filtrate is reabsorbed (Clark and Smith, 1932; Goldstein and Forster, 1971; Payan et al., 1973), ultimately resulting in only 4–7% of urea excretion to the environment (Payan et al., 1973; Wood et al., 1995).

The elasmobranch nephron consists of five loops, which appear to be arranged in a countercurrent fashion, and can be separated into two distinct anatomical regions: the dorsal–lateral bundle, enclosed by connective tissue, and the ventral mass, where loops wind convolutedly in a blood sinus (Lacy and Reale, 1985). A possibly extensive capillary network and a ‘central vessel’ are also present in a countercurrent arrangement to many of the nephron segments (Hentschel, 1988). Based on the countercurrent arrangement of tubules and micropuncture data, Boylan (1972) suggested a model of passive reabsorption of urea, where the fluid in the terminal segment has a higher urea concentration than the surrounding environment, allowing for reabsorption passively down the concentration gradient.

Another aspect of urea reabsorption in the elasmobranch kidney that is not clearly understood is the involvement of carrier-mediated urea transporters in some or all tubule segments. Experiments on whole animals have indicated that in the dogfish kidney, urea reabsorption is active and selective for amide or amide-like compounds (Schmidt-Nielsen and Rabinowitz, 1964), inhibitable by phloretin (Hays et al., 1976), and appears to be linked to sodium reabsorption (Schmidt-Nielsen et al., 1972). Although several models have been suggested for the possible arrangement of urea transporters in the elasmobranch kidney (Walsh and Smith, 2001), these models are based on very little experimental evidence. In a companion study, we have isolated a partial cDNA for a skate kidney urea transporter (SkUT), similar to other facilitated UTs, in both the dorsal and ventral regions of the kidney

(Morgan et al., 2003). Renal SkUT mRNA levels are downregulated with exposure to environmental dilution, suggesting a possible role for SkUT in urea reabsorption and retention.

To further characterize urea transport in the elasmobranch kidney, rates of urea uptake were measured using a rapid filtration method on resealed vesicles prepared from purified brush-border membranes from the two regions of the kidney. Characterization of urea transport measured the ^{14}C-urea uptake in the presence of various urea concentrations, competitive and non-competitive urea transport inhibitors, and energy sources (ATP or ion gradients).

Materials and methods

Experimental animals

Little skates *Raja erinacea* Mitchill 1825 were obtained by otter trawling in Passamaquoddy Bay, New Brunswick, Canada during the months of July and August, 2001 and maintained at the Huntsman Marine Science Center in 1000 liter outdoor tanks under natural photoperiod and supplied with filtered seawater (10°C). For some experiments, skates were transported to the Hagen Aqualab, University of Guelph, where they were maintained under natural photoperiod in artificial seawater (10°C). The skates were fed (filleted, chopped herring) on alternate days and were observed to feed.

Kidney brush-border plasma membrane vesicles

Brush-border membrane vesicles (BBMV) were prepared using methods involving calcium precipitation and differential centrifugation outlined by Bevan et al. (1989) and Kipp et al. (1997), with some modifications. All steps were carried out at 0–4°C. Skates were killed by a blow to the head, followed by severance of the spinal cord, after which both kidneys were rapidly excised and placed on ice. The kidneys were then divided into dorsolateral and ventral sections, based on the appearance of the sections as described by Hentschel et al. (1986). Approximately 0.75 g tissue (wet mass) was homogenized in 15 ml of homogenizing buffer containing 10 mmol l^{-1} D-mannitol, 2 mmol l^{-1} Tris-HCl, pH 7.1, using a Polytron (Brinkmann, Mississauga, ON, Canada) homogenizer for 15 s, followed by a 30 s interval and then an additional homogenization of 15 s. A sample (1 ml) was taken from this homogenate and used immediately for enzymatic analysis. The rest of the homogenate was filtered through cheesecloth and $CaCl_2$ was added to a final concentration of 30 mmol l^{-1}. After 15 min, the preparation was centrifuged at 1400 ***g*** for 12 min, the supernatant decanted into a clean centrifuge tube, and centrifuged for 20 min at 15 800 ***g***. The resulting pellet was resuspended in buffer and homogenized in a glass–Teflon homogenizer at low speed. The volume was adjusted to a final volume of 15 ml with buffer and $CaCl_2$ was added to a final concentration of 30 mmol l^{-1}. After 15 min, the preparation was centrifuged at 2200 ***g*** for 12 min, the supernatant decanted and centrifuged for 20 min at 20 000 ***g***. The resulting pellet was suspended in 10 ml vesicle buffer containing 100 mmol l^{-1} D-mannitol, 1.5 mmol l^{-1} $CaCl_2$, 20 mmol l^{-1} Hepes, 20 mmol l^{-1} Tris, pH 7.4, and centrifuged at 47 800 ***g*** for 20 min. The final pellet of enriched brush-border membranes was resuspended in 0.5 ml of vesicle buffer by passage through a 23-gauge needle (10 times) to aid vesicle formation, and used immediately in further studies.

Validation of membrane preparation

The relative purity of the final preparation and the relative contamination of the membrane preparation by other cellular membranes were assessed by enzymatic assays on the initial homogenate and the final pellet of enriched BBMV. Alkaline phosphatase (Gasser and Kirschner, 1987), Na^+,K^+-ATPase (McCormick, 1993), cytochrome *c* oxidase (Blier and Guderley, 1988), and glucose-6-phosphatase (Stio et al., 1988) were used as marker enzymes for brush-border membrane, basolateral membrane, inner mitochondrial membrane and endoplasmic reticulum, respectively. All measurements were made in duplicate at 25°C using a spectrophotometer (Hewlett Packard, Mississauga, ON, Canada).

Orientation of brush-border membrane vesicles

The orientation of the membrane vesicles was determined using the methods of Giudicelli et al. (1985) and Drai et al. (1990). This method is based on the inability of starch to cross the membrane and on the orientation of maltase–glucoamylase in the brush-border membrane. In brief, enriched BBMV were incubated for 10 min with or without detergent (0.04% *n*-octyl β-D-glucopyranoside). The activity of maltase-glucoamylase (a brush-border membrane enzyme) was determined by evaluating the hydrolysis of soluble starch (10 mg ml^{-1}) in sodium citrate buffer (0.1 mol l^{-1}, pH 6.5). Glucose production after 20 min was measured using a glucose oxidase reaction (Sigma kit). The percentage of inside-out vesicles was calculated as the difference in the enzyme activity with and without treatment in detergent. With detergent treatment, all possible enzyme activity is revealed (i.e. right-side-out, inside-out and leaky vesicles) while without detergent treatment only enzyme activity in right-side-out and leaky vesicles will be present.

Urea transport assays

Transport of ^{14}C-urea was performed at 10°C by a rapid filtration method as previously described (Fines et al., 2001). Freshly prepared BBMV were suspended in 10 ml of buffer containing 300 mmol l^{-1} NaCl, 5.2 mmol l^{-1} KCl, 2.7 mmol l^{-1} $MgSO_4$, 5 mmol l^{-1} $CaCl_2$, 15 mmol l^{-1} Tris-HCl, pH 7.4, 370 mmol l^{-1} D-mannitol and equilibrated on ice for 30 min. The BBMV were then collected by centrifugation at 47800 ***g*** for 20 min and resuspended in the same buffer at a protein concentration of approximately 0.3 mg ml^{-1}. Thorough mixing was achieved by passage through a 23-gauge needle (10 times). Transport experiments were initiated by the addition of 40 µl of radioactive elasmobranch isolation medium (EIM) (containing 1.85 MBq ^{14}C-urea) to 10 µl of BBMV suspension. EIM contained 300 mmol l^{-1} NaCl, 5.2 mmol l^{-1} KCl, 2.7 mmol l^{-1} $MgSO_4$, 5 mmol l^{-1} $CaCl_2$,

370 mmol l^{-1} urea, 15 mmol l^{-1} Tris-HCl, pH 7.4. Urea uptake was measured over a range of urea concentrations (0.2–370 mmol l^{-1}). In EIM containing less than 370 mmol l^{-1} urea, total osmolarities of the EIM solutions were maintained using a balance of D-mannitol, which functions as an osmotic replacement. Uptake was terminated at 8 s (dorsal) and 5 s (ventral) intervals by rapid dilution of the incubation solution with 1 ml of ice-cold stop solution (EIM containing 370 mmol l^{-1} urea). These times were shown to provide valid estimates of the initial rates of transport (data not shown). The diluted sample was immediately filtered through pre-wetted filters (Millipore Isopore, 0.4 μm HTTP type). Filters were washed with 2× 3 ml of ice-cold stop solution and placed in a scintillation vial with 15 ml of ScintiSafe Econo F scintillation fluid (Fisher). Each preparation was measured in duplicate.

Inhibition assays

Inhibition of urea transport was examined to further define the properties of the transporter. The urea analogues thiourea, acetamide, *N*-methylurea and 1-(4-nitrophenyl)-2-thiourea (NPTU), were tested as known competitive inhibitors in other urea transport systems. BBMVs were prepared in the same manner as above, except vesicles were preincubated with analogues, by adding thiourea, acetamide, or *N*-methylurea (370 mmol l^{-1}) to EIM solution, instead of the mannitol suspension buffer. These vesicles were then incubated with 0.5 mmol l^{-1} urea (with 74 kBq of ^{14}C-urea) and treated as described above. The analogue NPTU was used at a final concentration of 0.08 mmol l^{-1} in EIM due to its low solubility. Prior to the addition of the incubation mixture, 10 μl of the NPTU solution was added to the BBMV and rapidly mixed. Urea uptake was then measured as described above.

The inhibitors phloretin (0.25–0.5 mmol l^{-1}) in ethanol (0.75%) and $HgCl_2$ (0.3 mmol l^{-1} in EIM solution) and a combination of both inhibitors (0.5 mmol l^{-1} phloretin and 0.3 mmol l^{-1} $HgCl_2$ in 0.75% ethanol) were tested. Prior to the addition of 0.5 mmol l^{-1} urea (containing 74 kBq of ^{14}C-urea), 10 μl of the inhibitor or control (0.75% ethanol or EIM solution) was added to the BBMV and mixed. Urea uptake was measured as described above.

ATP dependence

The ATP dependence was determined to evaluate the requirement for ATP as an energy source. In previous studies from our laboratories, elasmobranch gill urea transport was found to be dependent on ATP and a Na^+ gradient (Fines et al., 2001). Urea uptake was measured in EIM containing 4 mmol l^{-1} urea (containing 74 kBq of ^{14}C-urea). This concentration of urea was used to examine transport below the non-saturable range. Individual solutions contained ATP (10 mmol l^{-1}), or ATP (10 mmol l^{-1}) and ouabain (1 mmol l^{-1}), or ATP (10 mmol l^{-1}) and *N*-ethylmaleimide (NEM) (1 mmol l^{-1}), and a control (no additions). If urea transport was altered by NEM, this would indicate the presence of V-type and P-type ATPases (i.e. proton pumps; Ehrenfeld, 1998). Urea uptake was measured as described above.

Cation specificity

Cation specificity of urea transport was also examined in the BBMVs, using modified resuspension buffer and radioactive mixture containing only one of the following salts: KCl or NaCl. BBMV were prepared as described above and then separated for use with individual cations.

Sodium

The final pellet was suspended in a medium containing 370 mmol l^{-1} D-mannitol, 2.7 mmol l^{-1} $MgSO_4$, 5 mmol l^{-1} $CaCl_2$, 15 mmol l^{-1} Tris-HCl, 25 mmol l^{-1} NaCl and 225 mmol l^{-1} *N*-methyl-D-glucamine (NMDG, an osmotic replacement). The BBMV were allowed to equilibrate on ice for 30 min before being collected by centrifugation at 47800 ***g*** for 20 min and resuspended in the same medium at a known protein concentration of approximately 0.3 mg ml^{-1}. Control incubations used the same medium in which the final pellet was resuspended. Gradient incubations contained 250 mmol l^{-1} NaCl and no NMDG, in order to produce an inwardly-directed gradient across the BBM, similar to that found in the skate kidney. The incubation medium contained 4 mmol l^{-1} urea and 366 mmol l^{-1} mannitol in place of the 370 mmol l^{-1} mannitol and 1.85 MBq ml^{-1} ^{14}C-urea.

Potassium

The final pellet was suspended in a medium containing 370 mmol l^{-1} D-mannitol, 2.7 mmol l^{-1} $MgSO_4$, 5 mmol l^{-1} $CaCl_2$, 15 mmol l^{-1} Tris-HCl, 250 mmol l^{-1} KCl. The BBMV were allowed to equilibrate on ice for 30 min before being collected by centrifugation at 47800 ***g*** for 20 min and resuspended in the same medium at a known protein concentration of approximately 0.3 mg ml^{-1}. Control incubations used the same medium in which the final pellet was resuspended. Gradient incubations contained 25 mmol l^{-1} KCl and 225 mmol l^{-1} NMDG, in order to produce an outwardly-directed gradient across the BBM, similar to that found in the skate kidney. The incubation medium contained 4 mmol l^{-1} urea and 366 mmol l^{-1} mannitol in place of the 370 mmol l^{-1} mannitol and 1.85 MBq ml^{-1} ^{14}C-urea.

Protein determination

The protein concentration of the BBMV preparations was determined by the method of Bradford (1976) using a Bio-Rad kit (Richmond, CA, USA) with bovine serum albumin as the standard.

Statistical analysis

Values are expressed as means ± standard error of the mean (S.E.M.). An *F*-test for comparison of curves was used in determination of best fit of regression lines. Data from functional vesicles studies (analogs and inhibitors) were not normally distributed, and therefore a log-transformation was performed to satisfy the assumption of normality before further statistical analysis. Statistical comparisons were made by one-way analysis of variance (ANOVA), and secondary tests were

performed using the Tukey–Kramer multiple comparison test or Student's *t*-test. Values were considered statistically significant if $P<0.05$.

Source of chemicals

N-methylurea was obtained from Fluka through Sigma-Aldrich Chemicals. ^{14}C-urea was obtained either from Amersham Life Science (Baie d'Urfé, Quebec) or Sigma Chemical (St Louis, MO, USA). All other chemical were obtained from either Fisher Scientific (Whitby, ON, Canada) or Sigma Chemicals (Oakville, ON, Canada) and were of reagent grade.

Results

Marker enzymes

The measurement of the four marker enzymes demonstrated that the brush-border membrane preparation was highly purified and only slightly contaminated by other membranes (Tables 1 and 2). Alkaline phosphatase, the marker enzyme for the brush-border membrane, was enriched 63.44-fold and 61.23-fold for the dorsal and ventral sections of the kidney, respectively.

In tissue from the dorsal section of the kidney, the specific activities of glucose-6-phosphatase (endoplasmic reticulum) and cytochrome *c* oxidase (mitochondria) indicated a

Table 1. *Marker enzyme specific activities and magnitude of purification of each membrane*

	Enzyme specific activity (μmol substrate h^{-1} mg^{-1} protein)			
Fraction	Alkaline phosphatase	Na^+,K^+-ATPase	Cytochrome *c* oxidase	Glucose-6-phosphatase
Dorsal				
Homogenate	3.17±0.68	0.53±0.04	1.66±0.20	0.14±0.04
BBMV	173.56±42.75	0.58±0.14	0.58±0.19	0.08±0.04
Magnitude of purification (-fold)	63.44±16.39	1.12±0.20	0.35±0.10	0.44±0.19
Ventral				
Homogenate	3.84±1.13	0.48±0.06	1.77±0.20	0.14±0.03
BBMV	162.99±52.12	0.48±0.08	0.43±0.10	0.19±0.14
Magnitude of purification (-fold)	61.23±19.39	1.12±0.28	0.23±0.04	1.06±0.27

BBMV, brush border membrane vesicles.

Magnitude of purification was calculated by dividing the specific activity in the BBM fraction by the specific activity in the initial tissue homogenate.

Values are means ± S.E.M. (N=6).

Table 2. *Total activity of marker enzymes, percentage recovery and percentage contamination in the final brush border membrane vesicle preparation*

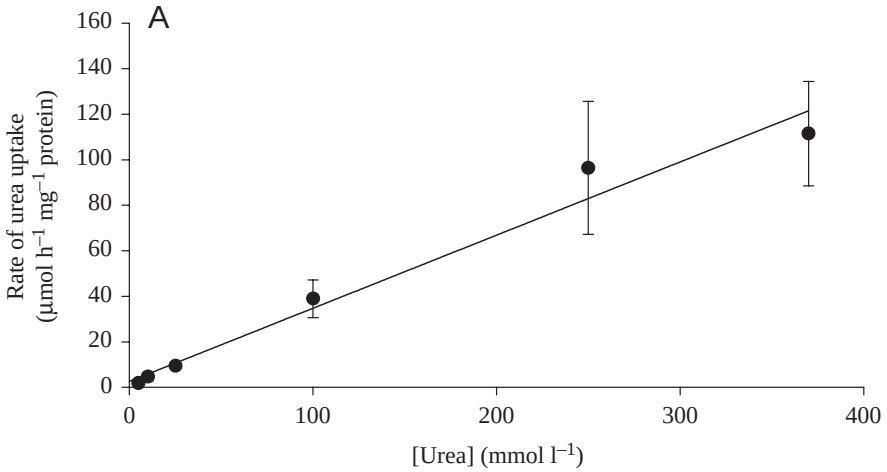
	Enzyme activity (μmol substrate h^{-1})			
Fraction	Alkaline phosphatase	Na^+,K^+-ATPase	Cytochrome *c* oxidase	Glucose-6-phosphatase
Dorsal				
Homogenate	221.21±54.09	36.58±5.73	111.64±16.86	11.39±3.62
BBMV	43.48±13.84	0.11±0.02	0.10±0.02	0.03±0.02
Recovery (%)*	20.52±6.39	0.34±0.04	0.11±0.03	0.15±0.08
Contamination (%)†		0.47±0.20	0.49±0.23	0.03±0.02
Ventral				
Homogenate	273.79±73.92	35.06±8.68	119.40±15.48	12.00±4.45
BBMV	52.77±19.87	0.13±0.02	0.11±0.03	0.11±0.10
Recovery (%)	23.94±8.70	0.42±0.07	0.09±0.02	0.44±0.20
Contamination (%)		0.38±0.09	0.37±0.11	0.09±0.07

BBMV, brush border membrane vesicles.

*Recovery was calculated as the percentage of the total activity in the BBM fraction relative to the total activity in the initial tissue homogenate.

‡Contamination was calculated as the percentage of the total activity in the BBM fraction relative to the total activity of alkaline phosphatase in the BBM fraction.

Values are means ± S.E.M. (N=6).



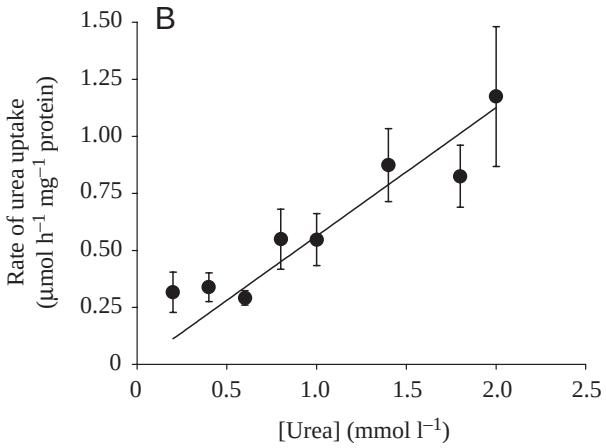


Fig. 1. (A) Rates of urea uptake at various urea concentrations in dorsal BBMV from the kidney of the little skate *Raja erinacea*. The regression is y=0.3309x, r^2=0.9708. Values are means ± S.E.M., N=5. (B) Expansion of the lower end of the urea concentration range, subtracting the linear rate from A. The regression is y=0.563x, r^2=0.8413. Values are means ± S.E.M., N=6.

contamination of these marker enzymes of 0.44-fold and 0.35-fold, respectively (Table 1). The Na^+,K^+-ATPase (basolateral membrane) was slightly enriched in the BBMV preparation with an increase in specific activity of 1.12-fold (Table 1). However, based on the total activity of Na^+,K^+-ATPase, there was only 0.34% recovery of the initial amount of enzyme and 0.47% contamination (percentage of total alkaline phosphatase activity) (Table 2). Marker enzymes for the endoplasmic reticulum and inner mitochondrial membrane had recoveries of 0.15% and 0.11% of the initial amount of enzyme and contributed 0.03% and 0.49% contamination, respectively (Table 2). Analysis of maltose–glucoamylase activity (Table 3) indicates that only 5.0% of vesicles are sealed inside-out, the remaining percentage of vesicles were right-side-out or leaky.

In the ventral section of the kidney, the specific activities of cytochrome *c* oxidase (mitochondria) indicated a contamination with inner mitochondrial membrane enzyme of 0.23-fold (Table 1). The marker enzymes for the basolateral membrane and endoplasmic reticulum were slightly enriched in the BBMV preparation with an increase in specific activity of 1.12- and 1.06-fold, respectively (Table 1). However, based on the total activity of Na^+,K^+-ATPase (basolateral membrane) and glucose-6-phosphatase (endoplasmic reticulum) there was only 0.42% and 0.44% recovery of the initial amount of enzyme and 0.38% and 0.09% contamination (percentage of total alkaline phosphatase activity), respectively (Table 2). Cytochrome *c* oxidase had a recovery of 0.09% of the initial amount of enzyme and contributed 0.37% contamination (Table 2). The activity of maltose–glucoamylase revealed that 4.6% of vesicles have an inside-out orientation (Table 3), so the majority of vesicles are right-side-out or leaky.

Final recovery of brush-border membrane was 20.52% and 23.94% for dorsal and ventral sections, respectively (Table 2), which is similar to that reported by Bijvelds et al. (1997).

Concentration dependence of urea uptake by BBMV

Urea uptake when measured over a range of urea concentrations in the incubation medium revealed one component of urea uptake in the dorsal BBMV and two components in the ventral BBMV (Figs 1, 2). At high concentrations of urea (5–370 mmol l^{-1}), the uptake was linearly dependent upon the urea concentration in both regions (Figs 1A, 2A). At low concentrations of urea (0.2–2 mmol l^{-1}), when the linear rate at high concentrations of urea has been subtracted from the curve, urea uptake exhibits saturation-like kinetics in the ventral BBMV (Fig. 2B). In the dorsal BBMV, however, there is a linear relationship at low urea concentrations, indicating non-saturation (F-test, Fig. 1B); however the apparent linear relationship may be the result of

Table 3. *Orientation of brush-border membrane vesicles from the dorsal and ventral sections of the kidney of the little skate* Raja erinacea

	Enzyme activity (%)*	
	Without detergent	With detergent
Dorsal	100	105.0±7.2
Ventral	100	104.6±11.1

*Enzyme activity without detergent was taken as 100%.
Values are means ± S.E.M., N=3.

multiple saturation curves. When the ventral data are transformed using a Lineweaver–Burk plot, urea uptake at urea concentrations of 0.2–2 mmol l^{-1} had a K_m of 0.70±0.20 mmol l^{-1}, and a V_{max} of 1.18±0.39 μmol h^{-1} mg^{-1} protein for the ventral BBMV (Fig. 2C).

Inhibition of urea uptake

Urea uptake by both dorsal and ventral BBMV demonstrated sensitivity to the non-competitive inhibitors phloretin and mercury chloride (Fig. 3). There was a dose-dependent phloretin inhibition of urea uptake by BBMV, with inhibition at 0.25 mmol l^{-1} of 24% and 22%, for dorsal and ventral, respectively (not shown), and at 0.50 mmol l^{-1} of 37% and 55%, for dorsal and ventral, respectively (P<0.05; Fig. 3). Mercury chloride inhibited urea uptake in both dorsal and ventral BBMV (P<0.05; Fig. 3). As well, the addition of phloretin and mercury chloride together also significantly inhibited urea uptake compared to control rates (P<0.05, Fig. 3). In the dorsal section, this inhibition was significantly greater than that of phloretin alone (P<0.05, Fig. 3). There was no change to the control rate of urea transport with the addition of ethanol vehicle (data not shown). The use of urea analogues changed the rate of urea uptake in the dorsal BBMV (Fig. 4). Here, nitrophenylthiourea (NPTU) significantly reduced urea uptake, but this reduction was not seen in the ventral BBMV. There was no significant effect on urea uptake in either section by the urea analogues, acetamide and *N*-methylurea (Fig. 4). There was a significant difference between the dorsal and ventral regions in response to acetamide (P<0.05, Fig. 4).

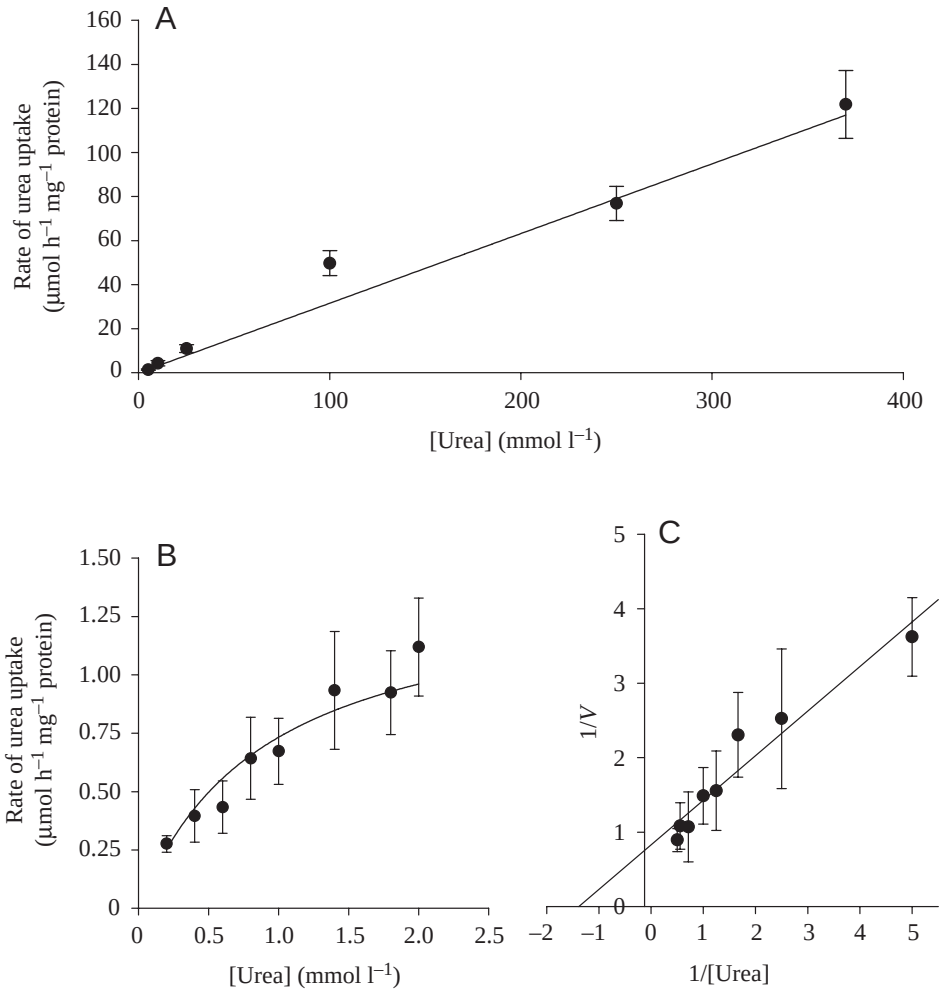


Fig. 2. (A) Rates of urea uptake at various urea concentrations in ventral BBMV from the kidney of the little skate *Raja erinacea*. The regression is y=0.3312x, r^2=0.9724. Values are means ± S.E.M., N=5. (B) Expansion of the lower end of the urea concentration range, subtracting the linear rate from A. The regression is y=1.3941x/(0.9016+x), r^2=0.9561. Values are means ± S.E.M., N=6. (C) Lineweaver–Burk transformation of the relationship between urea concentration and urea uptake V (μmol h^{-1} mg^{-1} protein) by BBMV. The regression is y=0.5988x+0.8308, r^2=0.9330. Values are means ± S.E.M., N=6.

ATP independence of urea uptake by BBMV

The addition of ATP to the incubation medium had no significant effect on the rate of urea uptake into dorsal (86.5±14.4%, N=5) and ventral BBMV (92.7±19.4%, N=5). As well, the addition of ouabain (dorsal 103.1±23.8%, N=5; ventral 80.1±14.3%, N=5) and NEM (dorsal 79.4±15.1%, N=5; ventral 74.9±23.9%, N=5) had no effect on ATP-stimulated urea uptake in BBMV (control dorsal 2.98±0.86 μmol h^{-1} mg^{-1} protein, N=5; control ventral 5.10±1.54 μmol h^{-1} mg^{-1} protein, N=5) (not shown).

Cation dependence of urea uptake by BBMV

There was no significant difference between urea uptake in dorsal and ventral BBMV in media containing only sodium or only potassium ions with no concentration gradient present (data not shown). When urea uptake was measured in the presence of an outwardly-directed potassium concentration gradient, there was no significant change in the rate of urea uptake in either dorsal or ventral BBMV (Fig. 5). As well, in the dorsal BBMV there was no significant change in urea uptake in the presence of an inwardly-directed sodium concentration gradient; however, in the ventral BBMV the rate of urea uptake significantly increased when an inward sodium concentration gradient was present (P<0.05; Fig. 5).

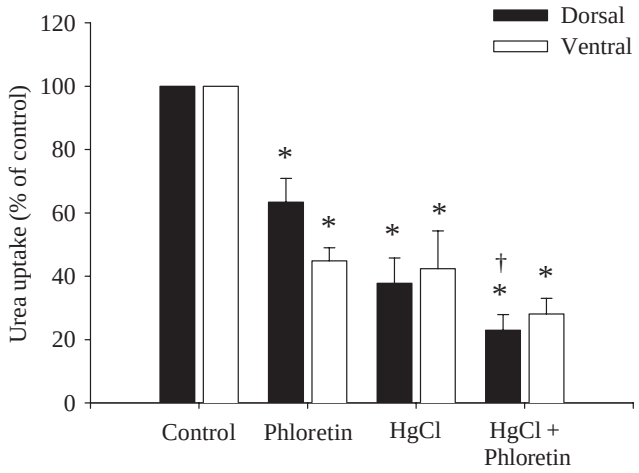


Fig. 3. Inhibition of urea uptake by phloretin (0.50 mmol l^{-1}), mercury chloride ($HgCl_2$; 0.30 mmol l^{-1}) and phloretin (0.50 mmol l^{-1}) + $HgCl_2$ (0.30 mmol l^{-1}) in dorsal and ventral BBMV from the kidney of the little skate *Raja erinacea*. Urea concentration in the incubation medium was 0.5 mmol l^{-1}. Control urea uptake rates are 1.42±0.14 and 1.41±0.18 μmol h^{-1} mg^{-1} protein, for dorsal and ventral sections, respectively. Values are means ± S.E.M., N=5–7. *Significant difference from respective control; †significant difference from dorsal phloretin (ANOVA on log-transformed data, Tukey–Kramer multiple comparison test, P<0.05).

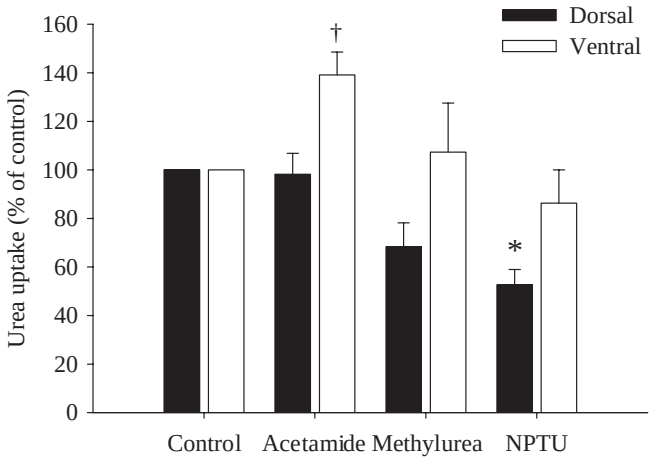


Fig. 4. Effect on urea uptake in the dorsal and ventral BBMV from the kidney of the little skate *Raja erinacea*, by the urea analogs acetamide, *N*-methylurea (370 mmol l^{-1}) and nitrophenylthiourea (NPTU; 0.08 mmol l^{-1}). Urea concentration in the incubation medium was 0.5 mmol l^{-1}. Control rates are 1.32±0.19 and 0.97±0.09 μmol h^{-1} mg^{-1} protein, for dorsal and ventral sections, respectively. Values are means ± S.E.M., N=5. *Significant difference from dorsal control (ANOVA on log-transformed data, Tukey–Kramer multiple comparison test, P<0.05); †significant difference from dorsal acetamide (Student t-test on log-transformed data, P<0.05).

Discussion

Methodology

The method (Kipp et al., 1997) used to isolate brush-border membrane vesicles from the renal tubule epithelium of the little skate *Raja erinacea* yielded a substantial enrichment of the brush border enzyme marker, alkaline phosphatase (Bevan et al., 1989). Although there was only minor contamination (<1%) with endoplasmic reticulum, mitochondrial and basolateral membrane enzyme markers, this may have led to a slight underestimation of urea uptake by BBMV. The enrichment of alkaline phosphatase (63- and 61-fold for dorsal and ventral sections, respectively) was considerably higher than that reported in other studies on elasmobranch kidneys using the same method (12- to 14-fold; Bevan et al., 1989; Kipp et al., 1997). The final recovery of alkaline phosphatase activity (20–24%) was consistent with previous studies on brush-border membranes of the kidneys of rainbow trout (Freire et al., 1995) and tilapia (Bijvelds et al., 1997). The vesicle orientation for all possible configurations of BBMV (inside-out, right-side-out, and leaky) was difficult to measure due to a lack of an appropriate marker enzyme. The percentage of inside-out vesicles in this study (approximately 5.0%) was similar to that demonstrated in tilapia kidney and sea bass intestinal brush-border membrane vesicles using similar methods (Bijvelds et al., 1997; Drai et al., 1990).

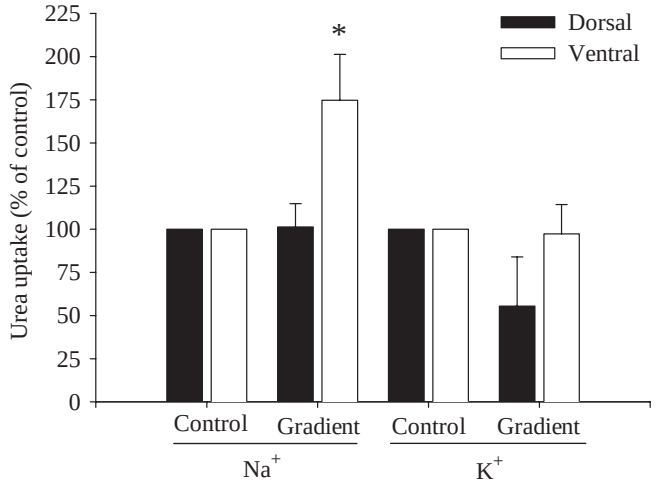


Fig. 5. The effect of Na^+ and K^+ on the rate of urea transport in the dorsal and ventral BBMV from the kidney of the little skate *Raja erinacea*, presented as percentage of control (no ion gradient). Urea concentration in the incubation medium was 4 mmol l^{-1}. Control rates are 1.53±0.32 and 1.56±0.29 μmol h^{-1} mg^{-1} protein, for the dorsal section Na^+ and K^+, respectively, and 1.77±0.44 and 1.42±0.35 μmol h^{-1} mg^{-1} protein, for the ventral section Na^+ and K^+ respectively. Values are means ± S.E.M., N=4. *Significant difference from ventral sodium control (Student t-test, P<0.05).

Characteristics of urea transport

Our data on BBMV provide evidence for different urea transport characteristics in the dorsal and ventral sections of the skate kidney. In the dorsal section, urea transport was not apparently saturable at low or high urea concentrations, but was inhibited by phloretin and $HgCl_2$, and significantly

reduced in the presence of NPTU. These results suggest the presence of one or more facilitated urea transporters, possibly coded for by SkUT (skate urea transporter) or SkUT-homologs, which we have isolated in the kidney of the little skate (Morgan et al., 2003). In the ventral section, urea uptake by BBMV revealed saturation kinetics at low urea concentration (K_m=0.70 mmol l^{-1}, V_{max}=1.18 μmol h^{-1} mg^{-1} protein), but not at high urea concentrations. Urea uptake was inhibited by phloretin and $HgCl_2$, but was stimulated by an inward Na^+ gradient, suggesting the presence of a Na^+-linked urea transporter (UT). The fact that SkUT was expressed in both the dorsal and ventral section (Morgan et al., 2003) indicates that at least two distinct transporters may be active in the ventral BBMV (i.e. a facilitative UT and a Na^+-linked UT). Furthermore, the high non-saturable rate of urea transport in the presence of relatively high concentrations of urea (up to 370 mmol l^{-1} urea), may represent urea movement through nonspecialized aqueous channels, low affinity/high capacity facilitated urea transporters similar to those characterized in mammalian terminal inner medullar collecting ducts (IMCD), the lipid-bilayer membrane, water channels (aquaporins), or possibly a combination of two or more of these pathways.

Water channels facilitate the movement of lipophobic molecules by allowing the molecule to remain in an aqueous phase as it diffuses through the channel, and therefore represent a potential method of diffusion of urea across the membrane. Inhibition of urea transport by mercurial compounds (e.g. $HgCl_2$, *p*-chloromercuribenezesulfonate, pCMBS), was shown to occur in this study, and is generally considered diagnostic of aquaporins (AQP) (Knepper, 1994; Borgnia et al., 1999). In mammals, a subgroup of the aquaporins family (i.e. AQP3, AQP7 and AQP9) has been shown to transport urea and/or glycerol as well as water (Ishibashi et al., 1997; Tsukaguchi et al., 1998). In particular, aquaporin 3 (AQP3), which has been isolated in the basolateral membrane of mammalian kidney tubule cells, has been shown to transport urea as well as water, and this transport is inhibitable by phloretin and $HgCl_2$ (Tsukaguchi et al., 1998). In the present study, the addition of $HgCl_2$ plus phloretin did not inhibit transport relative to $HgCl_2$ alone; however, in the dorsal section, transport in the presence of $HgCl_2$ plus phloretin was significantly lower than with phloretin alone. These results suggest the possibility of AQP-linked urea transport and/or a $HgCl_2$-sensitive UT. Despite being well known for inhibiting aquaporins, however, mercurial compounds are non-specific inhibitors and exert their effects through cysteine residues. In the mammalian UT-B and the frog facilitated urea transporter, pCMBS has been shown to inhibit urea transport (Martial et al., 1996; Couriaud et al., 1999). To our knowledge, the inhibitory effect of mercurial compounds has not been investigated on fish urea transporters, but a $HgCl_2$-sensitive UT in the marine elasmobranch kidney cannot be excluded.

The possibility of a low-affinity, high-capacity facilitated transporter is supported by mammalian studies where the apparent K_m values of urea transporters are extremely high. When urea is present only in the luminal perfusate of the terminal IMCD, urea reabsorption is linear with urea concentrations as high as 800 mmol l^{-1} (Chou et al., 1989), a considerably higher urea concentration than that used in this study. In *Xenopus* oocyte expression studies of the mammalian UT-A2, it was shown that there was no saturation for a range of urea concentrations between 1 and 200 mmol l^{-1} (You et al., 1993). If SkUT is similar to the mammalian UT-A2 in terms of transport kinetics, then it may be responsible for the non-saturable component of urea uptake at high urea concentrations.

The effects of several known inhibitors of urea transport were examined to try to further characterize the saturable component of urea uptake by BBMV. This component of urea transport was examined by using a urea concentration below the apparent K_m. Inhibition by the non-competitive inhibitor phloretin is considered diagnostic of both facilitated and secondary active urea transport systems (Levine et al., 1973; Knepper, 1994; Kato and Sands, 1998; Smith and Wright, 1999; Fines et al., 2001). The inhibition of urea uptake in BBMVs by phloretin observed in this study is consistent with a previous study on urine excretion in free-swimming dogfish (Hays et al., 1976), which demonstrated that phloretin injected into the blood system increased urinary urea excretion and decreased renal urea reabsorption. In BBMVs from the dorsal section, the urea analogue NPTU (80 μmol l^{-1}) significantly reduced urea uptake. This reduction is comparable to that seen in the frog urinary bladder (IC_{50}=79.4 μmol l^{-1}; Martial et al., 1993), indicating a high specificity for this analogue. In the ventral section, acetamide elevated urea uptake above that of the ventral control and the dorsal BBMV in the presence of acetamide. This result was unexpected, but may further indicate that more than one transporter was under study in this section of the kidney, and therefore the transport characteristics are more complex. However, until the analogue concentrations are optimized for this tissue, conclusions concerning analogue responses cannot be drawn (Schmidt-Nielsen and Rabinowitz, 1964; Wood et al., 1995).

The results suggest that urea uptake is not energy dependent in skate renal BBMV, since the addition of adenosine triphosphate (ATP) to the incubation medium did not change the rate of urea uptake. In the presence of NEM, an alkylating agent that binds selectively to sulfhydryl groups blocking V-type and P-type ATPases (i.e. proton pumps; Ehrenfeld, 1998), urea uptake was unchanged. The addition of oubain, a specific inhibitor of Na^+,K^+-ATPase, also had no effect. In contrast, gill basolateral membranes in dogfish showed a significant stimulation of urea uptake in the presence of ATP that was returned to control levels with the addition of ouabain (Fines et al., 2001). In the present study, the lack of effect with ouabain and ATP is not surprising since Na^+,K^+-ATPase is localized to the basolateral, not the brush-border membrane. To examine the effect of Na^+,K^+-ATPase on urea uptake in the BBM, an intact tubule system is required in order to have both the brush-border and basolateral membranes present in the same preparation.

In dorsal BBMV, urea uptake does not appear to be linked

to the sodium or potassium gradient. There is, altogether, little evidence to support the presence of two different high-affinity facilitated transporters in the dorsal section operating at different urea concentrations. This leads to the hypothesis that urea uptake may be due to a single phloretin-sensitive facilitated transporter, possibly that of the SkUT protein (Morgan et al., 2003), and similar to those isolated in the kidney of the dogfish shark and Atlantic stingray (Smith and Wright, 1999; Janech et al., 2003). In the ventral BBMV, the presence of an inwardly directed sodium gradient significantly increased urea uptake relative to the control and potassium gradient experiment. Despite the lack of information on the energy requirements of urea uptake, it is possible that one component of urea uptake in skate renal ventral BBMVs may be due to a Na^+-coupled secondary active urea transporter, similar to the active urea transport described in the mammalian kidney (Kato and Sands, 1998) and in dogfish gills (Fines et al., 2001), while another component of the urea uptake belongs to the same transporter found in the dorsal section. Thus, the inward movement of urea and Na^+ across the brush-border membrane would be linked to the active extrusion of Na^+ from the tubule cell across the basolateral membrane, back to the blood.

In order to completely understand the mechanisms of urea reabsorption, further studies, possibly using tubule isolation techniques and basolateral membrane vesicles, are necessary to complete the hypothesized model. The physiological evidence suggests the presence of a facilitated urea transporter in the brush-border membrane of dorsal and ventral renal tubules, and the additional presence of a sodium-linked urea transporter in ventral tubules. Taken together, our data provide evidence that urea transporter(s) in the skate kidney play a role in urea retention.

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