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Evidence for facilitated diffusion of urea across the gill basolateral membrane of the rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Recent in vivo evidence suggests that the mechanism of branchial urea excretion in the ammoniotelic rainbow trout (*Oncorhynchus mykiss*) is carrier-mediated. Further characterization of this proposed mechanism was achieved by using an in vitro isolated basolateral membrane vesicle (BLMV) preparation in which isolated gill membranes were used to determine a variety of physiological properties of the transporter. BLMV demonstrated two components of urea uptake, a linear component at concentrations up to 17.5 mmol·l^{-1} and a saturable component ($K_{0.5}=0.35\pm0.01$ mmol·l^{-1}; $V_{max}=0.14\pm0.02$ μmol mg protein^{-1} h^{-1}) with a Hill constant of 1.35 ± 0.18 at low, physiologically relevant urea concentrations (<2 mmol·l^{-1}). Saturable uptake of urea at 1 mmol·l^{-1} by BLMV was reduced by 88.5% when incubated with 0.25 mmol·l^{-1} phloretin, a potent blocker of UT-type facilitated diffusion urea transport mechanisms. BLMV also demonstrated differential handling of urea versus urea analogues at 1 mmol·l^{-1} concentrations and total analogue/total urea uptake ratios were 32% for acetamide and 84% for thiourea. Saturable urea uptake at 1 mmol·l^{-1} was significantly reduced by almost 100% in the presence of 5 mmol·l^{-1} thiourea but was not affected by 5 mmol·l^{-1} acetamide or 5 mmol·l^{-1} *N*-methylurea. Lastly, total urea uptake at 1 mmol·l^{-1} by BLMV was sensitive to temperatures above and below the temperature of acclimation with a Q_{10}>2 suggesting a protein carrier-mediated process. Combined, this evidence indicates that a facilitated diffusion urea transport mechanism is likely present in the basolateral membrane of the rainbow trout gill.

Keywords: Nitrogen excretion; UT-A; Urea analogue; Phloretin; Membrane vesicle; Teleost

1. Introduction

Like most other freshwater teleosts, the rainbow trout (*Oncorhynchus mykiss*) excretes the majority of its nitrogenous wastes across the gills as ammonia. However, a handful of teleosts expend energy to detoxify ammonia and excrete urea as their primary waste product [1–4], made possible by a fully functional ornithine-urea cycle (OUC) in internal tissues (especially the liver) and specialized facilitated diffusion urea transport mechanisms in the gills (UT transporters; [5,6]). While adult rainbow trout do not have a full complement of OUC enzymes, they do maintain a surprisingly high level of circulating urea compared to endogenous ammonia concentrations (5–50× more urea) and urea makes up approximately 10% of total nitrogen waste excretion (reviewed in Ref. [7]). Recent physiological evidence suggests that, as in ureotelic fish, urea excretion is carrier-mediated in ammoniotelic organisms such as the rainbow trout [8,9] and the plainfin midshipman (*Porichthys notatus*; [10]). This physiological evidence is supported by preliminary molecular analysis of both rainbow trout (P.A. Wright, C. Rexroad, M.D. McDonald, P.J. Walsh, unpublished data) and midshipman gill [11], suggesting the presence of mRNA for UT transporters.

Amongst teleosts where branchial UT genes have been cloned (*tUT*; gulf toadfish [5] and *mtUT*; Lake Magadi tilapia [6]) or are suspected (plainfin midshipman [11]), a consistent pattern of urea and analogue handling is observed in vivo where acetamide clearance at the gills is 35–60% of urea clearance and thiourea clearance is only 16–19% [6,10,12]. This highly conserved pattern of differential urea

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and analogue handling was also observed in the rainbow trout where the ratio of analogue/urea branchial clearance was 48% for acetamide and only 22% for thiourea, strongly suggesting the presence of a UT diffusion mechanism [9].

Upon infusion with exogenous urea loads, the gill of the trout effectively cleared the plasma of excess urea, suggesting a role for a facilitated diffusion UT transport mechanism for urea [8]. Furthermore, saturation of this mechanism was suggested when branchial excretion rate could not keep up with infusion rate [8], a similar observation to that in the toadfish, where *tUT* is expressed in the gill [13]. Recently, saturation of several urea transporters has been described in fish [14,15], in contrast to their mammalian counterparts that have proven difficult to saturate [16].

We therefore employed an in vitro protocol to further characterize the mechanism(s) by which urea is excreted across this epithelium. The purpose of the present study was to characterize any urea transport mechanism that might be present in the basolateral membrane of the trout gill through the use of the branchial basolateral membrane vesicle (BLMV) preparation originally developed by Perry and Flik [17]. Our results indicate the presence of a phloretin-sensitive, facilitated diffusion urea transport mechanism in the basolateral membrane of the gill that is saturated at low, physiologically relevant urea concentrations ($K_{0.5}$ = 0.35 mmol·l^{-1}, V_{max} = 0.14 μmol mg protein^{-1} h^{-1}; Hill constant = 1.35).

2. Materials and methods

2.1. Experimental animals

Rainbow trout (*O. mykiss*) were obtained from Humber Springs Trout Farm in Mono Mills, Ontario. The fish were acclimated to 14 °C and were fed with commercial trout pellets every second day. Acclimation was carried out in dechlorinated Hamilton tapwater [in mmol·l^{-1}: Ca^+ = 1.8; Cl^- = 0.8; Na^+ = 0.6; Mg^+ = 0.5; K^+ = 0.04; titration alkalinity (to pH 4.0) = 1.9; total hardness = 140 mg l^{-1} as $CaCO_3$; pH 8.0].

2.2. Preparation of gill BLMVs

BLMV were prepared using the method of Perry and Flik [17] as modified by Bury et al. [18]. For each preparation, five to seven adult rainbow trout (250–400 g) were stunned by a blow to the head and killed by severance of the spine. The gills were then perfused with approximately 60 ml ice-cold perfusion saline (0.9% NaCl, 0.5 mM ethylenediaminetetraacetic acid-disodium salt (Na_2-EDTA), 20 i.u.·ml^{-1} heparin; adjusted to pH 7.8 with Tris), quickly removed from the fish and placed in ice-cold perfusion saline without heparin. All subsequent procedures were performed at 1–4 °C.

The gill epithelium without red blood cells was scraped from the cartilage of the filaments with a glass slide and a small aliquot from each fish was frozen at −80 °C for later analysis of enzyme activity. The remaining tissue (5–10 g wet weight) was combined and homogenized with a Dounce homogenizer (Kontes) with 30 strokes of a loose-fitting pestle in 30 ml of a hypotonic solution (25 mM NaCl, 1 mM HEPES, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonylfluoride (PMSF); adjusted to pH 8.0 with Tris). After homogenization, the volume was adjusted to a final value of 80 ml with the hypotonic solution, and then centrifuged at 550 × *g* for 15 min to remove nuclei and cellular debris. Floating debris on the supernatant were removed and the supernatant was then decanted into a clean centrifuge tube and centrifuged at 50 000 × *g* for 30 min. The resulting pellet consisted of a top fluffy white layer (plasma membranes) and a firm brownish bottom (mitochondria). The white portion of the pellet was carefully resuspended in 5–10 ml of isotonic buffer (250 mM sucrose, 5 mM $MgSO_4$, 5 mM HEPES; adjusted to pH 7.4 with Tris) by gentle agitation. The resuspended pellet was adjusted to a volume of 20 ml and homogenized for 100 strokes using the tight fitting pestle of a Dounce homogenizer (Kontes). This second homogenate was centrifuged at 1000 × *g* for 10 min and then at 10 000 × *g* for 10 min, producing a pellet containing the remainder of contaminating membranes. The supernatant was decanted into a clean centrifuge tube and centrifuged at 50 000 × *g* for 20 min to produce a final pellet of enriched basolateral membranes. A small sample of this final pellet was taken and stored at −80 °C for subsequent enzyme analysis. The remainder of the pellet was resuspended in 1–3 ml of resuspension buffer (250 mM sucrose, 10 mM KNO_3, 0.8 mM $MgSO_4$, 0.5 mM Na_2-EDTA, 20 mM HEPES; adjusted to pH 7.4 with Tris) and was used immediately for protein concentration determination and urea transport assays. Typically, such a procedure yields the BLMV preparation as 20% inside-out vesicles, 30% right side out vesicles and the remaining 50% of the vesicle membranes as unsealed [17].

2.3. Transport measurements

Transport of [^{14}C]-urea was measured at trout acclimation temperature (14 °C) by a rapid filtration technique described by van Heeswijk et al. [19]. Freshly prepared BLMV pellets were resuspended at a protein concentration of approximately 1 mg membrane protein·ml^{-1} in resuspension buffer and allowed to equilibrate for 30 min on ice. Thorough mixing and formation of vesicles was achieved by 10 passages through a 23-gauge needle both before and after the equilibration period. BLMV urea uptake was assessed by prewarming the 135 μl of assay media (250 mM sucrose, 10 mM KNO_3, 11 mM $MgSO_4$, 6 mM Na_2-EDTA, 20 mM HEPES, 1 mM urea plus 5 μCi/ml [^{14}C]-urea (specific activity of 5 mCi/mmol); adjusted to pH 7.4 with Tris) to 14 °C, after which 35 μl of vesicle solution was added, the solution mixed on a vortex mixer and routinely incubated for 5 s. Different incubation duration and temperatures were

used in some tests, as outlined below. After incubation, 150 μl of the vesicle/assay mixture was immediately subjected to rapid filtration and the vesicles collected on nitrocellulose filters (Schleicher and Schüell Ltd.; 25 mm, 0.45 μm). The filters had been pre-incubated in ice-cold stop solution with a high "cold" urea content (200 mM sucrose, 10 mM KNO_3, 10 mM $MgSO_4$, 20 mM HEPES, 50 mM urea; adjusted to pH 7.4 with Tris) to reduce the nonspecific binding of the [^{14}C]-urea to the filter. The filtered membranes were washed twice with 1-ml aliquots of this ice-cold stop solution to displace surface-bound [^{14}C]-urea then placed in individual glass scintillation vials. While 1 mmol·l^{-1} urea, typical of blood and tissue levels in vivo [8], was used routinely in transport assays, other concentrations were used in some trials, as outlined below.

2.4. Urea transport assays

Time dependence of [^{14}C]-urea uptake was determined by incubating BLMV in assay solution for 5, 8, 10 or 20 s. In order to verify that urea had been transported into the vesicular space, BLMV were loaded with urea using the standard resuspension and assay conditions over a 30-s period, after which either 10 μl of 10% Triton-X in assay solution (to lyse the vesicles) or 10 μl of assay solution alone (control) was added to the vesicles. The concentration of radiolabeled urea present in the vesicles was measured at 3 and then 10 min after this point.

In order to establish concentration-dependence, urea uptake was measured over a range of urea concentrations (in mmol·l^{-1}: 0.09, 0.18, 0.51, 1.0, 2.0, 5.9, 11.7, 17.5). Part of the sucrose in the assay solution was reciprocally substituted with urea, keeping the osmolality of every solution the same and eliminating the effects of osmotic differences. Inhibitory effects of analogues on urea transport were examined by adding 5 mmol·l^{-1} of either thiourea, acetamide or *N*-methylurea to the assay solution in reciprocal substitution for sucrose. To measure acetamide or thiourea uptake, 1 mmol·l^{-1} of respective analogue plus [^{14}C]-analogue replaced urea in the assay solution. Analogue uptake was then measured as described above. Inhibition of urea uptake by phloretin was investigated by resuspending the isolated membranes in a resuspension media with 0.25 mmol·l^{-1} of phloretin dissolved in ethanol so that the total ethanol concentration in the media did not exceed 0.04%. To measure urea uptake, phloretin-exposed vesicles were then incubated in assay media with the same concentration of phloretin. Vehicle control vesicles were resuspended and then incubated in 0.04% ethanol alone. In order to determine temperature sensitivity, urea uptake was measured in BLMV incubated in assay solutions at temperatures of 1, 7, 14 and 23 °C.

2.5. Analytical techniques and calculations

The activity of Na^+K^+-ATPase, found only on the basolateral membrane of the gill, was used as an indicator of the relative enrichment of the final BLMV preparation. Na^+K^+-ATPase activity was measured as described by McCormick [20] in the initial gill homogenate and the final pellet of BLMV.

Urea concentration in the assay solution was measured using the diacetyl monoxime method of Rahmatullah and Boyde [21], with appropriate adjustments of reagent strength for the different urea concentration ranges and correction for the presence of thiourea and acetamide in the competition studies. This correction was done by adding the experimental concentration of analogue in the same volume as the sample to each point of the standard curve of the urea assay. Any interference to the colorimetric assay by thiourea or acetamide would thus be automatically accounted for when using the respective standard curve, a point that was validated by urea addition/recovery tests. Protein concentration of the BLMV was determined by a commercial kit (Bradfords reagent; Sigma-Aldrich Canada) using bovine serum albumin standards. Nonspecific binding of [^{14}C]-urea to the nitrocellulose filters was measured by filtering through 150 μl of assay solution only. For measurements of [^{14}C]-urea or [^{14}C]-analogue uptake by BLMV, the nitrocellulose filters in glass scintillation vials received 10 ml of ACS fluor (Amersham) and were analyzed by scintillation counting on an LKB Rackbeta 1217 Counter. Tests confirmed that quench was uniform, therefore no correction was necessary.

Uptake of [^{14}C]-labeled urea or analogue (U_X; μmol mg protein^{-1} h^{-1}) by BLMVs was determined by the equation:

$$U_X = \frac{\mathrm{cpm_f}}{\mathrm{SA} \times [P] \times t}, \quad (1)$$

where $\mathrm{cpm_f}$ is the counts present on the nitrocellulose filters, SA is the specific activity of the media (cpm μmol^{-1}), [P] represents the concentration of protein in the BLMV filtered (mg ml^{-1}) and t is the time of incubation (hours). The specific activity (cpm/μmol) of the radiolabelled assay media was measured directly for each experiment using the scintillation counter and colorimetric urea assay described above. In measuring the specific activity directly, the efficiency of the scintillation counter is taken into account when calculating U_X.

Differentiation between linear and saturable components of urea uptake was accomplished as described below. Analysis of cooperative kinetic constants after subtraction of the linear component (described below) gave a value for the maximum rate of uptake (V_{max}), the urea concentration at 50% of the maximum rate ($K_{0.5}$) and a measure of cooperativity (the Hill constant, h). The value for h was then corroborated using the Hill equation as follows:

$$\mathrm{Log}\frac{v}{V_{max} - v} = h\mathrm{Log}[S] - \mathrm{Log}\ K_{0.5}, \quad (2)$$

where v is the transport rate at any given urea concentration, S. If $h = 1$ there is no cooperativity, if $h < 1$ there is negative cooperativity and if $h > 1$ there is positive cooperativity.

The Q_{10} factor is defined as the ratio of two rates for a 10 °C difference in temperature and was calculated as follows:

$$Q_{10} = \frac{J_{K2}}{J_{K1}}^{(10/(K2-K1))} \quad (3)$$

where J_{K1} and J_{K2} are the mean flux rates at a low and high temperature, respectively, in μmol mg protein^{-1} h^{-1} and $K1$ and $K2$ are the low and high temperatures, respectively, in °C. The Q_{10} factor for a physical process such as diffusion is about 1. Q_{10} factors for biochemical reactions including carrier-mediated transport are typically 2 to 3 [22].

2.6. Statistical analysis

Values are expressed as mean ± 1 S.E. where N = replicates of basolateral membrane preparations combined from five to seven fish. Statistical comparisons were made by Student's two-tailed, unpaired t-test as appropriate, with a significance level of $P < 0.05$. Differentiation between the linear and saturable components of urea movement was accomplished using a nonlinear, three-parameter equation available in the SigmaPlot 2000 computer package. Analysis of cooperative kinetic constants after subtraction of the linear component was carried out using a sigmoidal, Hill three-parameter analysis and corroborated by the Hill equation. Best fit curves and regressions were generated using the SigmaPlot 2000 computer package.

3. Results

An approximate sixfold enrichment of Na^+K^+-ATPase activity was measured in trout gill BLMV preparations (99.7 ± 18.5 (6) μmol·mg protein^{-1}·h^{-1}) when compared to the initial crude homogenate (16.9 ± 1.5 (6) μmol·mg protein^{-1}·h^{-1}).

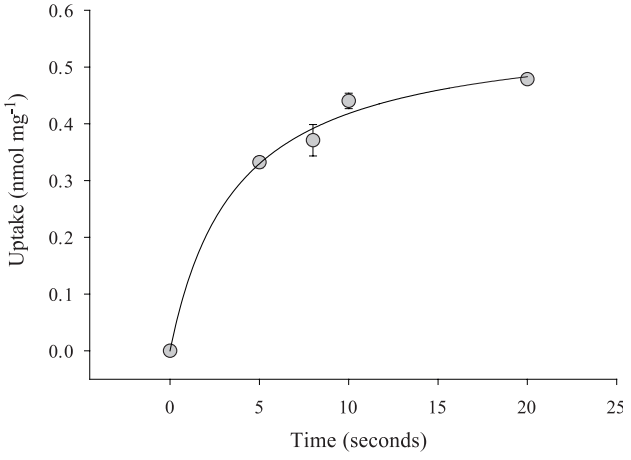

Fig. 1. Time course of total [^{14}C]-urea uptake by BLMV of rainbow trout gill. Each data point is a mean ± 1 S.E. (N = 3 replications) of basolateral membrane preparations combined from five to seven fish.

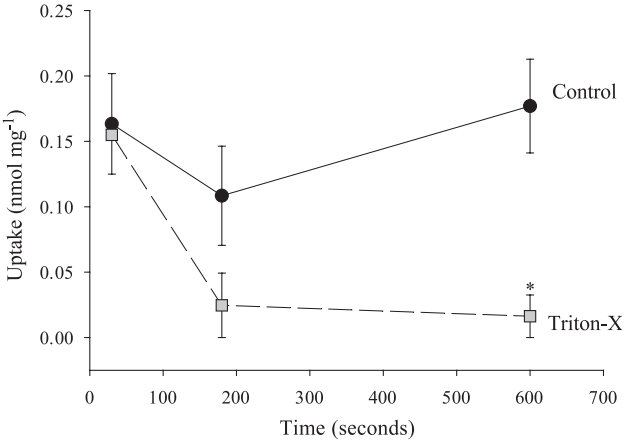

Fig. 2. Total [^{14}C]-urea uptake by trout gill BLMV after 30 s, 3 min and 10 min under control conditions and when incubated in Triton-X after the initial 30-s sample. Each data point is a mean ± 1 S.E. (N = 3 replicates) of basolateral membrane preparations combined from five to seven fish; *$P < 0.05$, significantly different from the corresponding control value.

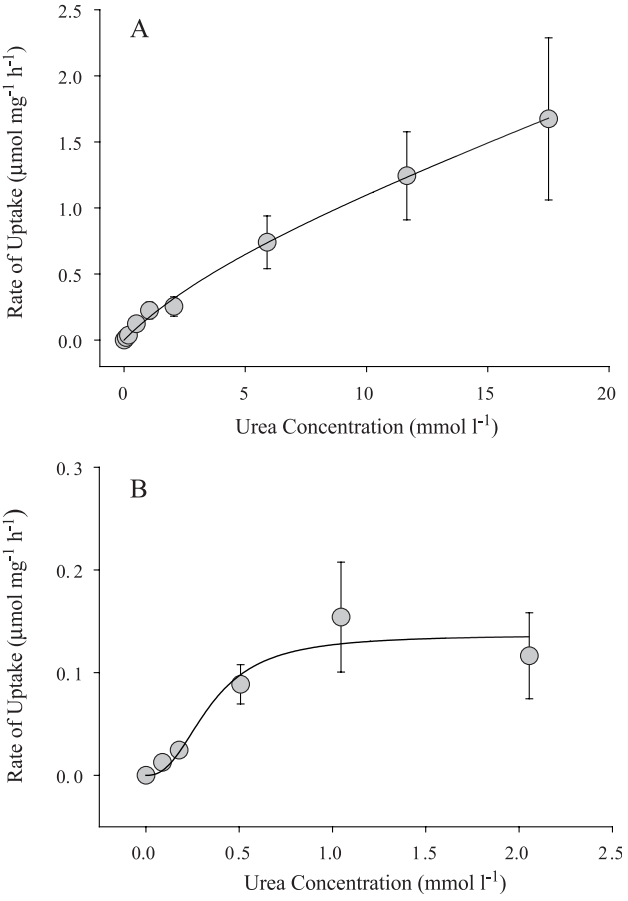

Fig. 3. (A) The rate of [^{14}C]-urea uptake by trout gill BLMV demonstrating two components of urea uptake. The equation for the linear component of uptake is $y = 0.067x$, $r = 0.99$, $P < 0.01$. (B) With the linear component subtracted away, a saturable component is evident at concentrations of up to 2 mmol·l^{-1} with a $K_{0.5}$ of 0.35 mmol·l^{-1} and a V_{max} of 0.14 μmol·mg protein^{-1}·h^{-1}. The equation for the saturable component of uptake is $y = (0.14x^{2.44})(0.35^{2.44} + x^{2.44})^{-1}$, $r = 0.97$, $P < 0.02$. Each data point is a mean ± 1 S.E. (N = 6 replicates) of basolateral membrane preparations combined from five to seven fish.

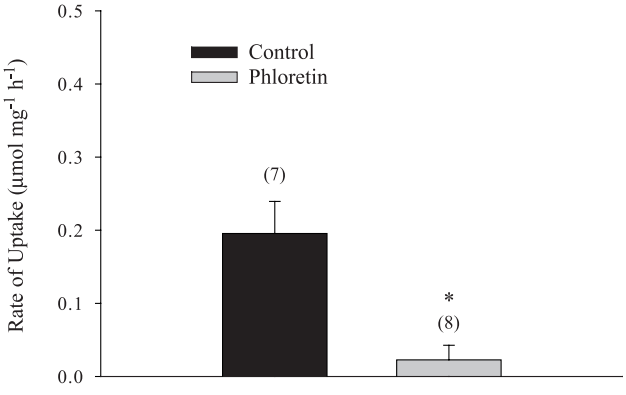

Fig. 4. The rate of saturable [^{14}C]-urea uptake by BLMV incubated in 0.25 mmol·l^{-1} phloretin, a urea transport blocker, was significantly less than for vehicle controls. In the experimental treatment, vesicles were pre-incubated for 1 h in standard incubation media with 0.04% ethanol ± 0.25 mmol·l^{-1} phloretin and added to a standard assay media with 0.04% ethanol ± 0.25 mmol·l^{-1} phloretin. The protocol was identical in the controls, except phloretin was omitted. Each data point is a mean ± 1 S.E. (N = replicates) of basolateral membrane preparations combined from five to seven fish; *P < 0.05, significantly different from controls.

The standard incubation period for BLMV for use in most assays was determined by first investigating the time dependence of urea uptake (Fig. 1). The total uptake of urea (at 1 mmol·l^{-1} in the incubation media) into the vesicles at 14 °C was fast and readily reached a plateau by about 20 s (1/2 time to plateau = 3.7 s). An incubation time of 5 s was therefore selected for further assays as it was before the vesicles reached their maximum filling capacity. Urea appeared to be accumulating in the vesicular space of BLMV, rather than adsorbing to the outside, as exposure to the detergent Triton-X for either 3 or 10 min essentially eliminated total urea uptake when compared to control vesicles kept for the same period of time (Fig. 2).

Total urea uptake by BLMV was measured over a range of urea concentrations in the incubation medium, revealing two components of urea uptake (Fig. 3). Over the entire range of urea concentration used (up to 17.5 mmol·l^{-1}), urea uptake was linearly dependent on the urea concentration (r = 0.99) with a slope of 0.067 μmol·mg protein^{-1}·h^{-1} mmol·l^{-1} (P < 0.01; Fig. 3A). However, at urea concentrations in the physiological range (0.1–2 mmol·l^{-1}), urea uptake demonstrated nonlinear concentration kinetics (Fig. 3B). The nonlinear fit revealed that transport was significantly correlated with urea concentration (r = 0.97; P < 0.02) and yielded a $K_{0.5}$ of 0.35 ± 0.1 mmol·l^{-1}, a V_{max} of 0.14 ± 0.01 μmol·mg protein^{-1}·h^{-1} and a Hill constant (h) of 2.44 ± 1.22 (P = 0.139). For a second estimate of the degree of cooperativity, a linear Hill plot was fit (not shown) with 0.14 as the value for V_{max} in the Hill equation. Using this equation, the $K_{0.5}$ was confirmed as 0.36 and h was calculated to be 1.36 ± 0.18 (P < 0.02). The values calculated for h using either the nonlinear fit or the Hill equation were not significantly different than 1.0, indicating an absence of cooperativity.

Saturable urea uptake by BLMV at 1 mmol·l^{-1} urea was sensitive to the urea transport blocker, phloretin. After subtraction of the linear component, urea uptake in the ethanol controls (0.200 ± 0.043 (7) μmol·mg protein^{-1}·h^{-1}) was 8.6 times greater than the uptake by the vesicles in the presence of 0.25 mM phloretin plus ethanol (0.023 ± 0.022 (8) μmol·mg protein^{-1}·h^{-1}; Fig. 4).

The basolateral membrane of the gill differentiated between urea and acetamide but not between urea and thiourea. At a concentration of 1 mmol·l^{-1}, the total uptake of urea into the vesicles was approximately three times greater than the uptake of acetamide, while the total uptake of urea and thiourea was not significantly different (Fig. 5A). When expressed as a total uptake ratio (analogue/urea), the ratios were 0.84 for thiourea and 0.32 for acetamide. Reflecting these results, thiourea inhibited the saturable component of urea uptake by BLMV (at 1 mmol·l^{-1}). Saturable urea uptake in the presence of 5 mmol·l^{-1} thiourea was reduced

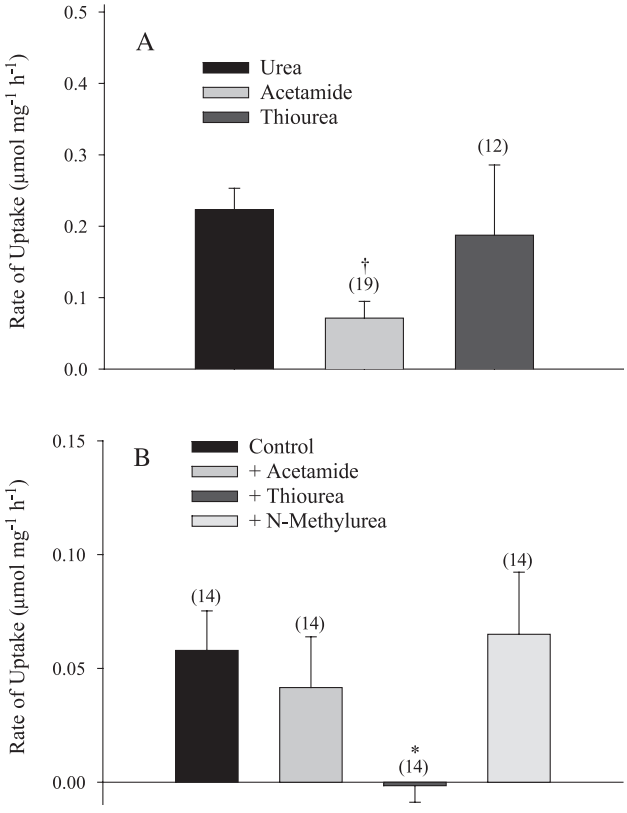

Fig. 5. (A) Mean total uptake rates of [^{14}C]-urea, [^{14}C]-acetamide and [^{14}C]-thiourea at 1 mmol·l^{-1} concentration in the incubation media demonstrating a total uptake of [^{14}C]-urea that was significantly greater than total [^{14}C]-acetamide uptake. However, the total uptake rates of [^{14}C]-thiourea and [^{14}C]-urea were not significantly different. The similar handling of thiourea and urea was reflected in (B) the inhibition by 5 mmol·l^{-1} thiourea on saturable [^{14}C]-urea uptake at 1 mmol·l^{-1}. The presence of 5 mmol·l^{-1} acetamide and 5 mmol·l^{-1} N-methylurea in the assay solution had no significant effect. Each data point is a mean ± 1 S.E. (N = replicates) of basolateral membrane preparations combined from five to seven fish; †P < 0.05, significantly different from urea uptake rate; *P < 0.05, significantly different from the corresponding control value.

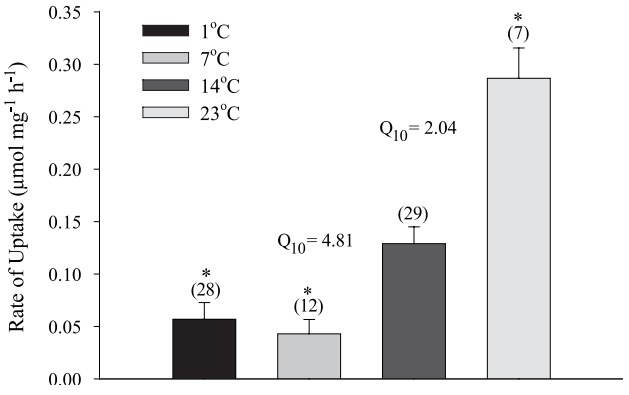

Fig. 6. Mean total uptake rates of [^{14}C]-urea by BLMV incubated at temperatures above or below the temperature of acclimation (14 °C). An increase in temperature caused a significant increase in uptake by a Q_{10} of 2.04. A decrease in temperature to 7 °C caused a significant decrease in urea uptake by a Q_{10} of 4.81 with no further decrease observed at 1 °C. Each data point is a mean ± 1 S.E. (N = replicates) of basolateral membrane preparations combined from five to seven fish; *P < 0.05, significantly different from the temperature of acclimation.

by almost 100% relative to the respective control (Fig. 5B). However, saturable urea uptake was not significantly reduced by the presence of 5 mmol·l^{-1} acetamide or 5 mmol·l^{-1} *N*-methylurea (Fig. 5B) in the assay solution.

Total uptake of urea (1 mmol·l^{-1}), looking at both linear and nonlinear components by BLMV, was substantially altered by changes in temperature. A decrease in temperature from 14 °C (temperature of acclimation) down to 7 °C caused a decrease in total uptake with a Q_{10} of 4.81 (Fig. 6). However, total urea uptake was not inhibited further when temperature was reduced to 1 °C. When temperature was increased to 23 °C, uptake was significantly stimulated with a Q_{10} of 2.04 (Fig. 6).

4. Discussion

By using the in vitro isolated gill membrane approach, it was possible to characterize potential urea transport mechanisms within a specific membrane without the confounding effects of the whole animal. In the present study, rainbow trout BLMV preparations showed a significant 5.9-fold enrichment in Na^+K^+-ATPase activity compared to the initial crude homogenate. This value was similar to those reported by both Perry and Flik [17] and Bury et al. [18] for the same preparation, indicating that it is made up of concentrated basolateral membranes. In previous studies, the BLMV preparation has been shown to consist of 20% inside-out vesicles, 30% right-side out vesicles and 50% unsealed membranes [17,18,23]. Thus, the facilitated diffusion urea transporter characterized in the present study would be assayed in about 50% of the total protein, since facilitated diffusion transporters are typically bidirectional.

While facilitated diffusion urea transport mechanisms are characteristically difficult to saturate in mammalian systems [16], saturation of several urea transporters has recently been described in fish [13–15]. Consistent with these studies, urea transport by BLMV exhibited saturation kinetics at low urea concentrations (< 2 mmol·l^{-1}) with a $K_{0.5}$ (0.35 mmol·l^{-1}) similar to physiological urea concentrations (1–2 mmol·l^{-1}; [8]) and a V_{max} of 0.14 μmol·mg protein^{-1}·h^{-1}. Since approximately 50% of the vesicles are unsealed in the preparation [17,18,23], the V_{max} is underestimated by roughly the same amount.

Up until now, only in vivo approaches have been used to investigate urea handling by the gills in adult rainbow trout, and it has not been possible to clearly demonstrate saturation kinetics of the urea transporter [8,9]. In retrospect, both the saturable and non-saturable components of urea transport, which were apparent using BLMV, may have been evident in trout infused with exogenous urea loads in vivo [8]. Specifically, upon exogenous urea loading at low rates (< 60 μmol·kg^{-1}·h^{-1}), branchial urea excretion rate in trout matched infusion rate; the excess urea was effectively eliminated across the gill and plasma urea concentrations remained close to physiological levels (< 2 mmol·l^{-1}; [8]). During this time, the effective branchial clearance of urea at these low plasma urea concentrations was likely achieved by the facilitated diffusion mechanism described by the present study, operating below saturation. Yet as urea loading rates increased (up to 420 μmol·kg^{-1}·h^{-1}), plasma urea concentrations rose and the branchial excretion of urea subsequently fell to 75% and then 55% of infusion rate [8]. However, only partial saturation of urea excretion in vivo was observed, probably because the linear component (i.e. passive diffusion or nonspecific pathways) was recruited by the high plasma urea levels (>3 mmol·l^{-1}). Thus, in trout infused with urea in vivo, the relationship between excretion rate and plasma urea concentration remained linear over the concentration range of 0.77 to 11.7 mmol·l^{-1}, correspon ding to the linear component above 2 mmol·l^{-1} identified in the BLMV preparation (Fig. 3A).

Similar to the adult rainbow trout, a UT mechanism is also suspected in rainbow trout embryos, in which the production and effective excretion of urea is believed to be an essential mechanism for ammonia detoxification during early life stages [14]. In embryos, there again appears to be two components of urea transport, a saturable component at low concentrations, believed to be facilitated diffusion of urea, and a second, non-saturable component at concentrations of up to 20 mmol·l^{-1}, which could be simple diffusion or movement through nonspecific pathways [14]. Urea uptake by trout embryos displayed saturation kinetics with a K_m = 2.0 mmol·l^{-1} that was similar to internal urea concentrations (1.8 mmol·l^{-1}) and a V_{max} = 10.5 nmol·g embryo^{-1}·h^{-1}. The V_{max} of embryonic trout is much lower than that measured in adult BLMV (800-fold lower capacity assuming 60 mg protein·g embryo^{-1} [24], and correcting for the underestimated V_{max} in BLMV), despite similar circulating levels of urea. Likely, only a small fraction of protein in the embryo is associated

with urea transport, in contrast to the BLMV preparation, accounting for the difference in V_{max}. Regardless, both embryonic and adult mechanisms have half-maximal constants, K, which are close to circulating urea concentrations, indicating that the transporter has a relatively high affinity for urea.

Phloretin, a potent inhibitor of UT transport mechanisms [25], caused a significant inhibition of urea uptake by BLMV in the saturable, physiologically relevant range of urea concentration, suggesting the presence of this type of mechanism on the basolateral membrane of the trout gill. Phloretin sensitivity has been observed in other preparations where UT mechanisms from teleost fish have been studied. When *tUT* of the gulf toadfish was injected into oocytes and exposed to 0.5 mmol·l^{-1} of phloretin, there was a 75% inhibition in urea transport [5]. Additionally, phloretin sensitivity was apparent in oocytes injected with *mtUT* of Lake Magadi tilapia, 1.0 mmol·l^{-1} of phloretin causing an approximate 70% reduction in urea uptake [6], and in the trout embryo, where 0.05 mmol·l^{-1} caused a 100% reduction in urea uptake [14].

Consistent with findings in vivo, there was differential uptake of urea and urea analogues. However, the in vitro BLMV pattern of handling was different in that urea uptake ≥ thiourea uptake>acetamide uptake, in contrast to the in vivo branchial clearance pattern where urea transport>acetamide transport>thiourea transport in both the excretion and the uptake direction [9]. This in vivo pattern of urea versus analogue handling is observed not only in the rainbow trout but also by the gill of the plainfin midshipman [10], by *tUT* of the gulf toadfish [12] and by *mtUT* of the lake Magadi tilapia [6]. However, the BLMV pattern of handling is representative of the basolateral membrane alone, while the in vivo pattern of analogue handling is a combination of both apical and basolateral membranes. Hypothetically, the difference could reflect the contribution of the apical membrane, suggesting the possibility of a second urea transport mechanism on the apical membrane of the gill.

Supporting the pattern observed in the uptake of analogues was the inhibition of urea transport by urea analogues, a well-described characteristic of UT mechanisms [5,25–27]. In the present study, the presence of thiourea in concentrations five times greater than urea significantly inhibited urea uptake by BLMV. Since thiourea and urea uptake rates by BLMV are similar, the inhibitory effect of thiourea on urea uptake suggests that thiourea and urea are moving via the same mechanism and are interacting with each other. In contrast, neither acetamide nor *N*-methylurea inhibited urea uptake when present in fivefold greater concentrations. The differential handling between urea and acetamide (only 30% of urea transport at the same 1 mmol·l^{-1} concentration) could therefore be a consequence of urea moving through a specific transport mechanism while acetamide is moving less effectively by simple diffusion, thus not interfering with urea movement. Thiourea has also proven to be a potent inhibitor of urea transport in vivo via the toadfish gill (which contains *tUT*), causing a 73% reduction in total urea uptake when present in twofold excess in the external media of the gulf toadfish [27]. Similarly, total urea influx across the Lake Magadi tilapia gill in vivo (which contains *mtUT*) was inhibited by approximately 40% in the presence of thiourea, by only 30% in the presence of *N*-methylurea and by 15% in the presence of acetamide, all in threefold excess of urea [6].

Lastly, a change in incubation temperature, either above or below the temperature of acclimation, caused an alteration in urea uptake rate by BLMV with a Q_{10} factor >2, indicative of the involvement of a carrier protein. While the increased uptake rate measured at 23 °C could possibly be due to a lipid phase transition, the rate change measured at more physiological temperatures (from 14 to 7 °C: Q_{10} factor >4) supports the presence of a urea transporter in the basolateral membrane. Urea transport by BLMV was insensitive to a further decrease in temperature from 7 to 1 °C but conceivably, at these low temperatures, carrier-mediated transport of urea may have slowed to a point where urea movement across the membrane occurs solely by simple diffusion. Temperature sensitivity of urea efflux has also been measured in toadfish hepatocytes, shown to have phloretin-sensitive urea efflux [28]. Indeed, if simple diffusion was predominant at temperatures below that of acclimation, it would become evident through a complete concentration dependence experiment at a lower temperature. This would allow for a separate Q_{10} for the non-saturable component of uptake to be established.

Thus, the present study has demonstrated that urea uptake by BLMV (1) displays saturation kinetics at physiological urea concentrations, (2) is phloretin-sensitive, (3) is greater than acetamide uptake, (4) is inhibited by thiourea and (5) demonstrates temperature sensitivity typical of carrier-mediated transport. Taken together, these results strongly suggest the involvement of a facilitated diffusion transport mechanism for urea across the basolateral membrane of the trout gill, possibly similar to those cloned in the toadfish (*tUT*) and the tilapia (*mtUT*) and those suspected in the trout embryo [14] and midshipman [10,11]. The finding of urea transporters in ammoniotelic fish is not unprecedented as Mistry et al. [29] have recently cloned and localized *eUT* to chloride cells in the gills of the Japanese eel (*Anguilla japonica*). Future cloning and expression studies on the urea transport mechanism in the trout gill should lead to a greater understanding of the functional significance of urea transporters in ammoniotelic organisms.

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