Different Transport Mechanisms in Plant and Human AMT/Rh-type Ammonium Transporters

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The conserved family of AMT/Rh proteins facilitates ammonium transport across animal, plant, and microbial membranes. A bacterial homologue, AmtB, forms a channel-like structure and appears to function as an NH3 gas channel. To evaluate the function of eukaryotic homologues, the human RhCG glycoprotein and the tomato plant ammonium transporter LeAMT1;2 were expressed and compared in Xenopus oocytes and yeast. RhCG mediated the electroneutral transport of methylammonium (MeA), which saturated with \( K_m = 3.8 \text{ mM at pH}_0 7.5 \). Uptake was strongly favored by increasing the pHo and was inhibited by ammonium. Ammonium induced rapid cytosolic alkalinization in RhCG-expressing oocytes. Additionally, RhCG expression was associated with an alkali-cation conductance, which was not significantly permeable to NH4+ and was apparently uncoupled from the ammonium transport. In contrast, expression of the homologous LeAMT1;2 induced pH-i-independent MeA+ uptake and specific NH4+ and MeA+ currents that were distinct from endogenous currents. The different mechanisms of transport, including the RhCG-associated alkali-cation conductance, were verified by heterologous expression in appropriate yeast strains. Thus, homologous AMT/Rh-type proteins function in a distinct manner; while LeAMT1;2 carries specifically NH4+, or cotransports NH3/H+, RhCG mediates electroneutral NH3 transport.

INTRODUCTION

Ammonium (NH4+/NH3) is vital for the growth of microorganisms and plants, but it can reach toxic levels when in excess. (The term ammonium is used for the sum of NH4+ and NH3, the chemical symbols are used to denote the molecular species specifically. The same formalism applies to methylammonium [MeA+/MeA]). Most organisms have developed complex ammonium homeostasis mechanisms that comprise ammonium metabolism, import, and export. Proteins involved in ammonium transport were initially identified from yeast and plants and belong to the AMT/MEP/Rh protein family (pfam00909) (Marini et al., 1994; Ninnemann et al., 1994). Later human and animal homologues, the Rh glycoproteins, were identified as ammonium transporters (Marini et al., 2000). Some Rh glycoproteins are involved in the disposal of ammonium in the kidney, probably to maintain acid–base homeostasis (Weiner, 2004).

Plant and yeast AMT/MEPs are involved in the membrane potential–dependent acquisition of the nutrient ammonium (Ninnemann et al., 1994; Marini et al., 1997; Ludewig et al., 2002; Sohlenkamp et al., 2002; Ludewig et al., 2003). This has also been established for many bacterial AMT homologues (Kleiner, 1985; Siewe et al., 1996; Meier-Wagner et al., 2001). An external recruitment site for NH4+ at the pore entrance has been identified in the recently solved crystal structure of the bacterial homologue from Escherichia coli, AmtB (Khademi et al., 2004; Zheng et al., 2004). Based on the rigid high-resolution channel-like structure of AmtB, which represents a milestone in membrane protein structure analysis, Khademi et al. (2004) speculated that AMT/Rh proteins are gas channels that recruit external NH4+ but conduct NH3. This view is supported by recent functional analyses that suggest that AmtB conducts the gas NH3 (Khademi et al., 2004; Javelle et al., 2005). Although an intriguing hypothesis, the gas interpretation may not be valid for eukaryotic members of the family. This “gas” hypothesis is also in opposition to the long-standing view that bacterial ammonium transporters are NH4+ transporters (Kleiner, 1985).

It is of considerable physiological relevance whether neutral NH3 or positively charged NH4+ is transported, since the gradient across the membrane is often opposite for both species. This is the situation at the apical membrane of distal kidney epithelia, where RhCG is co-expressed with the V-type ATPase that actively exports protons into the acidic lumen. Thus, only a small fraction of total luminal ammonium is NH3, which results in an outward NH3 gradient. In contrast, the inside

Abbreviations used in this paper: BCECF, 2',7'-bis-(carboxyethyl)-5(6)-carboxyfluorescein; MeA, methylammonium; MES, 2-[N-morpholino]ethanesulfonic acid.
negative membrane potential of the epithelial cells favors NH$_4^+$ influx along the electrochemical gradient for NH$_4^+$ and, in the presence of a pathway for NH$_3^+$, this will promote accumulation of ammonium in the cytosol. A similar situation is encountered at the plant root epidermis, the site of LeAMT1;2 expression.

The mechanism of ammonium transport is highly controversial for many AMT/Rh homologues. For example, the close AmtB homologue LeAMT1;1 from tomato was suggested to transport NH$_4^+$ (Ludewig et al., 2002), and electrogenic NH$_4^+$ transport has also been conjectured for the mouse homologue Rhbg (Nakhoul et al., 2005) when expressed in oocytes. In contrast, electroneutral transport has been reported for human RhBG (Ludewig, 2004) and RhAG (Westhoff et al., 2002). However, no ionic current measurements have been shown for RhAG (Westhoff et al., 2002), and the transport of the ammonium analogue MeA/MeA$^+$ has not been studied in Rhbg (Nakhoul et al., 2005). A study on genetic variants of human and mouse erythrocytes and erythrocyte ghosts identified RhAG as a major protein involved in rapid ammonium-dependent alkalization. It was concluded that RhAG facilitates NH$_3$ diffusion (Ripoche et al., 2004). RhCG was expressed in oocytes and it was suggested that it transports both NH$_3$ and NH$_4^+$ (Bakouh et al., 2004). A study on RhAG expression in mammalian cell lines similarly suggested both NH$_3$ and NH$_4^+$ transport by RhAG (Benjelloun et al., 2005).

Much of the debate about the mechanism of transport in AMT/Rh proteins originates from the fact that NH$_4^+$ transport had been measured in *Xenopus* oocytes, cells that had been shown to express endogenous NH$_4^+$-inducible currents when exposed to high ammonium concentrations such as 10 mM (Cougnon et al., 1996; Burckhardt and Burckhardt, 1997). However, this view has recently been challenged by several laboratories, and oocytes do not express large endogenous ammonium-inducible currents when exposed to ammonium at <1 mM (Ludewig et al., 2002; Ludewig et al., 2003; Bakouh et al., 2004; Holm et al., 2005).

In this paper we studied two individual eukaryotic AMT/Rh homologues using identical protocols. Human RhCG was chosen as a member of the Rh branch of the family and the plant LeAMT1;2 from *Lycopersicon esculentum* (tomato) as a representative of the AMT branch (Ludewig et al., 2001). Methylammonium and ammonium transport were compared using current and flux measurements. The data identified remarkable differences between the RhCG glycoprotein and the plant AMT transporter and suggest that RhCG functioned as an electroneutral channel or transporter. Transport may involve external recruitment of NH$_4^+/NH_3$, followed by NH$_3$ conduction, as has been suggested for the bacterial homologue AmtB (Khademi et al., 2004; Zheng et al., 2004). Additionally, expression of the RhCG glycoprotein was associated with an ammonium-independent cation conductance in *Xenopus* oocytes. In contrast, tomato AMT proteins evoked specific, saturable, voltage-dependent, pH$_2$-independent NH$_4^+$ (or NH$_3$/H$^+$) currents that were distinct from endogenous ammonium currents. The differences in transport were confirmed by expression in appropriate yeast strains. Thus, despite their sequence similarity and conservation, plant AMT and mammalian Rh glycoproteins are functionally distinct, which may reflect their different physiological roles in ammonium excretion in animals and ammonium acquisition in plants.

**MATERIALS AND METHODS**

**Plasmid Constructs**

All ammonium transporter genes were inserted into plasmid pOO2 (Ludewig et al., 2002). Human RhCG was cloned from a kidney cDNA library (CLONTECH Laboratories, Inc.) and checked by sequencing. The RhCG construct contained a 9-hp sequence (Kozak motif: GCCGCCACC) upstream of the ATG. The plasmid pOO2-LeAMT1;2 has been described in a previous study (Ludewig et al., 2003). Capped cRNA was transcribed by SP6 RNA polymerase in vitro using mMessage mMachine (Ambion), after linearization of the plasmid with MluI.

**Preparation and Injection of Oocytes**

Individual *Xenopus* frogs were kept separately in water tanks in a plant growth room at 21°C and 10 h light/14 h dark cycle. Oocytes were removed from adult female frogs by surgery and manually dissected. Oocytes (Dumont stage V or VI) were defolliculated using collagenase 10 mg/ml (Boehringer) and trypsin inhibitor 5 mg/ml (Sigma-Aldrich) for 1–2 h and injected with ~50 nl of cRNA (~20 ng/per oocyte). Oocytes were kept after injection for 2–3 d at 16°C in ND96 (in mM): 96 NaCl, 2 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 5 HEPES (pH 7.4), supplemented with 2.5 mM Na-pyruvate and gentamycin (20 μg/ml). Oocytes were then used for measurements, or were kept at 4°C for another day and then measured.

**Electrophysiological Measurements**

Standard recording solution was (in mM) 100 NaCl, 2 CaCl$_2$, 2 MgCl$_2$, 4 Tris, pH adjusted to 7.5. In some experiments the pH of this solution was adjusted to pH 5.5, 6.5, or 8.5 with 2-[N-morpholino]ethanesulfonic acid (MES). In potassium- and choline-based solutions, sodium was replaced by the respective chloride salt. In some solutions NaCl was replaced by Naglutamate and recordings were performed using a ground electrode connected to the recording solution via an agar bridge. Oocytes were placed in a small recording chamber and the gravity-driven solution exchange allowed the full exchange of bath solutions within a few seconds. The oocytes were clamped to ~30 mV, which is close to the resting membrane potential of most oocytes used, and the currents were measured after stepping from this holding potential to different test potentials in 20 mV steps for periods of ≥200 ms. Within these short pulses, currents were time independent. At voltages below ~120 mV a slowly activating current was observed in many oocyte batches (also in native oocytes). Therefore only these brief voltage pulses were used to monitor endogenous and Rh-associated currents specifically. All recordings were performed at 20–21°C.

For the current–voltage plots, the constant current at each voltage was averaged and plotted against the voltage. When the slowly
activating current below −120 mV was observed, the current at the beginning of the pulse was used. A complete recording for a single ammonium concentration included (a) brief voltage steps from +40 to −140 mV in control solution without ammonium, (b) addition of ammonium and the identical pulse protocol, (c) followed by complete wash with standard bath solution and acquisition of the current response to an additional pulse protocol. A full recording was completed within <2 min. These brief applications of ammonium minimized secondary effects due to long incubation in ammonium. Each experimental result was observed in at least three independent batches of oocytes. In more than six batches of oocytes, no differences were observed between noninjected or water-injected oocytes and their respective ammonium and methylammonium transport. Means ± standard deviations are given. In some figures the currents “induced by ammonium” are shown; these were obtained by subtracting the current in the respective buffer containing 14C-labeled methylammonium (or at ≤0.9 μA and separated into three per scintillation vial. After solubilization with 10 μl 5% SDS, 4 ml scintillation buffer was added and activity was analyzed by liquid scintillation counting. For the calculation of the concentration dependence of RhCG, the uptake of water-injected controls was subtracted.

Intracellular pH Changes (pH)
Intracellular pH changes (pH) were monitored using the pH-sensitive dye 2',7'-bis-(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) (Sasaki et al., 1992). In brief, several healthy oocytes were placed in uptake buffer (ND96, containing 5 μM BCECF-AM, from a 50 mM stock in DMSO) and incubated at room temperature for 30 min protected from light. Two BCECF-loaded oocytes were placed on a coverslip and mounted on an inverted Leica fluorescence microscope (objective lens ×20). The oocytes were superfused with the standard solution containing 0.5 or 10 mM NH4Cl by gravity flow. Pictures of BCECF fluorescence were taken at maximal rate every 5 s using a spinning wheel fluorescence system with excitation at 410 ± 30 and 470 ± 20 nm and emission at 535 ± 20 nm. The fluorescent ratios were calibrated as in Ludewig (2004).

Expression in Yeast
RhCG and LeAMT1.2 were subcloned into pDR195 and expressed in the yeast Saccharomyces cerevisiae wild-type strain (23344c), the ammonium transporter defective yeast strain (31019b; ΔΔΔmep4·::ura1·31;2·3) (Marini et al., 1997) and the alkali metal cation-sensitive strain AB11c (ena1·::HIS3·ena4; nha1·::LEU2; nhb1·::TRPl1) (Maresova and Sykrova, 2005). The plasmid pNHA1-985, harboring the S. cerevisiae Na+/H+ antipporter gene NHAI under its own promoter was used as a positive control (Maresova and Sykrova, 2005). The growth medium for strain AB11c was YNB (1.7 g/liter yeast nitrogen base w/o amino acid and ammonium sulfate; Difco) supplemented with 3% glucose, 0.1% arginine, 0.01% adenine, 0.01% tryptophane solidified with 2% agar. The pH was adjusted using 50 mM TRIS/MES to pH 5.0 or pH 7.0. The nitrogen-deficient growth medium for strain 31019b was YNB supplemented with 3% glucose and appropriate NH4Cl concentrations and was adjusted to pH 5.3 using 50 mM MES. The methylammonium toxicity medium contained YNB, 3% glucose, 0.1% arginine, and 125 mM methylammonium.

Online Supplemental Material
Fig. S1 shows the pH dependence of MA uptake by RhCG and concentration dependence of NH4+ currents by LeAMT1.2. The online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.200509369/DC1.

RESULTS
Ammonium-independent Rh-associated Currents
RhCG expression in oocytes was associated with ionic currents in standard solutions, even in the absence of ammonium and methylammonium (Fig. 1, A and B). A small increase in background currents compared with noninjected controls was also seen with LeAMT1.2 expression (Fig. 1, A and C). The currents from native oocytes were not affected by a change to sodium-free choline or potassium solutions, while the RhCG-associated currents diminished in solutions in which sodium was replaced by choline, a large cation that is relatively impermeable to most ion channels (Fig. 1 B).
currents in RhCG-expressing oocytes (Fig. 1 B). Although Rh-associated currents were small in choline-based solutions, they were still detectable compared with nonexpressing controls. To test if the inward current was carried by chloride efflux, chloride was partially replaced by glutamate (94%). This did not affect the inward currents and only marginally affected the reversal potential, suggesting that the Rh-associated currents are mainly carried by cations (Fig. 1 B). The maximal RhCG-associated inward currents were ~1 μA. This current will be referred to as the Rh-associated conductance throughout the manuscript.

To test if the Rh-associated cation conductance was associated with RhCG expression in another heterologous host, RhCG was expressed in an alkali metal-sensitive *S. cerevisiae* strain (Δena1-4, Δnha1, Δnhx1) (Maresova and Sychrova, 2005). RhCG increased sodium sensitiv-

Endogenous Transport of Methylammonium Is Electroneutral in *Xenopus* Oocytes

Endogenous background currents were unchanged after addition of the widely used transport analogue methylammonium (10 mM) in all batches tested. Although methylammonium did not affect ionic currents, radio labe-

Figure 2. Current–voltage relations of endogenous ammonium-induced currents. (A) Currents from native oocytes at pH₈, 8.5 without ammonium (open circles), by 300 μM (closed circles), 1 mM (open triangle), and 3 mM ammonium (filled triangle), respectively. (B) Ammonium-induced currents (background subtracted, from A) in a typical oocyte batch. Note that ammonium-induced currents have a reversal potential (arrow) and are outward at positive voltages. Data shown are from oocytes from a single batch, similar data were obtained in two other oocyte batches.
The 14C-methylammonium uptake rate depended linearly on concentration (Fig. 3 B) and increased with more alkaline pHo. The increase was less than the 10-fold increase in the free MeA concentration per pH unit (Fig. 3 C). Interestingly, 14C-methylammonium uptake at pHo 7.4 was partially blocked by ammonium (Fig. 3 D), suggesting that 14C-MeA uptake was only partially mediated by lipid diffusion.

Saturable Methylammonium Transport by RhCG and Inhibition by Ammonium

14C-methylammonium uptake into oocytes was increased by RhCG expression (Fig. 4). The uptake of 14C-methylammonium did not saturate within 20 min, even at 1 or 10 mM concentration (Fig. 4 A). RhCG expression increased the 14C-methylammonium transport in oocytes by ~7–10-fold and was indistinguishable in sodium or choline solutions (Fig. 4 B). Though methylammonium was transported, the addition of methylammonium to RhCG-expressing oocytes did not lead to an increase of the membrane conductance (see below and Fig. 6 D).

The 14C-MeA uptake rates by RhCG were obtained by subtracting the endogenous background uptake at each pHo, using equally treated native oocytes from the same batch. The 14C-methylammonium concentration permitting half-maximal transport rate (Km) was ~3.8 mM at pHo 7.5 (Fig. 4 C). The 14C-methylammonium transport was blocked by ammonium, with a Ki = 3.8 ± 1.2 mM at pHo 7.5. Data from four oocyte batches.

Figure 3. Electroneutral uptake of methylammonium in noninjected control *Xenopus* oocytes is partially blocked by ammonium. (A) Current–voltage relations without methylammonium (open circles) and with various methylammonium concentrations, 1 mM (filled circles), 3 mM (open triangles), 10 mM (filled triangles) at pHo 7.5. Symbols are partially overlapping. (B) Concentration dependence of 14C-methylammonium uptake rate at pHo 7.5. (C) pHo dependence of 14C-methylammonium uptake rate. (D) Block of 14C-methylammonium uptake (1 mM) by ammonium.

Figure 4. Methylammonium uptake by RhCG-expressing oocytes. (A) Linear MeA uptake by noninjected controls (circles) and RhCG (triangles) at 1 mM (open symbols) and 10 mM (filled symbols) at pHo 7.5. (B) MeA uptake (1 mM) of noninjected and RhCG-expressing oocytes in sodium-free choline (left) and sodium solutions (right). (C) Concentration dependence of RhCG at pHo 7.5. (D) Block of MeA uptake (2 mM) by ammonium, KNH4+ = 3.8 ± 1.2 mM at pHo 7.5. Data from four oocyte batches.
Saturable MeA⁺ Currents in LeAMT1;2-expressing Oocytes
In contrast to the situation with RhCG, the external application of MeA⁺ induced time-independent inward currents of up to ∼−100 nA in LeAMT1;2-expressing oocytes. A current–voltage plot of the MeA⁺-induced currents is shown in Fig. 5 A. MeA⁺-induced currents were determined by subtracting the current without MeA⁺ from the currents in MeA⁺-containing solution. The MeA⁺-induced currents were always inwardly directed, even at positive membrane potentials, as to be expected for an externally applied substrate. The currents saturated in the low mM concentration range and half-maximal currents were 6.1 ± 1.1 mM at −140 mV and 5.6 ± 1.0 mM at −100 mV (Fig. 5 B).

Electroneutral MeA Transport by RhCG, but MeA⁺ Currents by LeAMT1;2
The differences in methylammonium transport by RhCG and LeAMT1;2 were evaluated in detail in Fig. 6. The total methylammonium of all experiments in this figure was 1 mM, except for Fig. 6 F, which was performed with 500 μM. As a control, experiments were also performed with native, noninjected, and H₂O-injected oocytes.

In native oocytes, the application of methylammonium did not induce ionic currents at pHₒ 5.5 and pHₒ 8.5 (Fig. 6 A). Similarly, currents in RhCG-expressing oocytes were not changed by methylammonium at low and physiological pHₒ (Fig. 6 B), but a small conductance increase in RhCG-expressing oocytes was observed with methylammonium at alkaline pHₒ 8.5 (Fig. 6 B). Although negligible currents were observed with methylammonium, ¹⁴C-MeA uptake was observed, which strongly increased with more alkaline external pHₒ (Fig. 6 E). The RhCG uptake rate increase was less than 10-fold per pH unit, but resembled the pHₒ-dependent MeA uptake by RhAG (Westhoff et al., 2002) and RhBG (Ludewig, 2004).

The methylammonium uptake rate by LeAMT1;2 was distinct and invariant against pHₒ changes (Fig. 6 F). The ¹⁴C-methylammonium uptake by LeAMT1;2 was paralleled with an MeA⁺ inward current in the LeAMT1;2-expressing oocytes (Fig. 6 C).
Effect of Ammonium on Ionic Currents by RhCG and LeAMT1;2

Original current traces and current–voltage plots in the absence and presence of 10 mM ammonium are shown in Fig. 7 for H2O-injected and RhCG- and LeAMT1;2-expressing oocytes. Currents from H2O-injected and RhCG-expressing oocytes were not substantially changed by 10 mM ammonium, although RhCG-expressing oocytes had larger background current overall. In contrast, a large current increase by ammonium was observed in LeAMT1;2-expressing oocytes. The current increase by ammonium was maximal at the most negative voltages tested and was inward even at positive membrane potentials. This is in agreement with the fact that ammonium was only applied externally, and if ammonium does not rapidly equilibrate with the cytosol, specific NH4+ currents are expected to be exclusively inward.

Current–voltage relations with different ammonium concentrations are shown in Figs. 7 and 8. Ammonium did not affect currents in native oocytes at 1 mM, but the addition of 20 mM ammonium increased the conductance, consistent with the fact that the oocytes used in this study were largely impermeable to NH4+ (Fig. 8 A). The currents of RhCG-expressing oocytes were slightly increased by the addition of 1 or 20 mM ammonium (Fig. 8 B). In accordance with Bakouh et al. (2004), the ammonium-induced currents in RhCG-expressing oocytes were larger at more alkaline pHo. In contrast, even low ammonium, such as 100 μM, induced a large, significant current increase in LeAMT1;2-expressing oocytes (Fig. 8, C and D). The ammonium-induced currents were observed in choline as well as sodium solutions (unpublished data). The differences in the current–voltage relations of ammonium-induced currents (the currents in the absence of ammonium subtracted from the currents in the presence of ammonium) for H2O-injected, RhCG-expressing, and LeAMT1;2-expressing oocytes are shown in Fig. 8 D. While the ammonium-induced currents from H2O-injected and RhCG-expressing oocytes were not substantially different, the NH4+ currents by LeAMT1;2 were large (Figs. 7 and 8), saturated with Km (at −100 mV) = 60 μM (Figs. 7 and S1) and were only inward. In contrast, the small ammonium-induced currents by RhCG were inward at negative voltages, reversed at ~−10 mV and were outward at positive voltages (Fig. 8 D). Interestingly, the magnitude and current–voltage characteristics of the ammonium-induced, RhCG-mediated currents and their pHo dependence were similar to endogenous ammonium-induced oocyte currents (see Fig. 2).
Ammonium-induced pH Alkalization

The transport mechanism was further assayed by monitoring intracellular pH in oocytes using the pH-sensitive dye BCECF. Electroneutral ammonium transport (either as NH$_3$ or NH$_4^+$/$H^+$ exchange) was expected to alkalize the cytosol. Exposure to 500 μM NH$_4$Cl induced a small, rapid fluorescence ratio change in RhCG-expressing oocytes to a steady-state level, but not in H$_2$O-injected controls or in LeAMT1;2-expressing oocytes (Fig. 9). The application of 10 mM ammonium induced a pH alkalization to a similar steady-state level in all oocytes, but alkalization was more rapid in RhCG-expressing oocytes (Fig. 9). A similar pH alkalization by ammonium had been observed with RhBG (Ludewig, 2004). The alkalization by ammonium in RhCG-expressing oocytes is compatible with NH$_3$ transport by RhCG, while the lack of effect of LeAMT1;2 suggests a different transport mechanism in that transporter.

RhCG and LeAMT1;2 Expression in Yeast

Different transport mechanisms of RhCG and LeAMT1;2 were also identified after expression in another heterologous host, the yeast S. cerevisiae. Wild-type yeast growth was impaired on media supplemented with 125 mM methylammonium, a toxic concentration (Fig. 10). The expression of the MeA$^+$ transporter LeAMT1;2 in wild-type yeast increased the sensitivity to methylammonium further, as may be expected from membrane potential–driven MeA$^+$ import by LeAMT1;2. RhCG-expressing wild-type yeast was more resistant to toxic methylammonium concentrations (Fig. 10 A), as previously shown (Marini et al., 2000). This is not compatible with import and accumulation of MeA$^+$ by RhCG, but may rather suggest RhCG-mediated efflux of H$_3$C-NH$_2$. This suggests that RhCG can function not only in import, but also in the export of methylammonium.

A yeast strain that lacks all endogenous ammonium transporters was unable to acquire sufficient ammonium for growth, but RhCG expression permitted weak growth (Fig. 10, B and C), as had been shown previously (Marini et al., 2000). Since the NH$_3$ gradient was probably unfavorable for uptake on acidic, low-ammonium media, passive influx of NH$_3$ may be suboptimal for...
growth, although ammonium assimilation may deplete cytosolic NH$_3$ to very low levels. Membrane potential-driven import of NH$_4^+$ by LeAMT1;2 was expected to maximize uptake on limiting ammonia. The efficient growth complementation by LeAMT1;2 was compatible with this expectation (Fig. 10, B and C).

**DISCUSSION**

Ammonium transporters of human and plant origin were functionally compared in oocytes and yeast using parallel, identical tracer flux and current measurements. The saturable ammonium and methylammonium transport by RhCG was electroneutral, while NH$_4^+$ (or NH$_3$/H$^+$) co- and MeA$^+$ (or MeA/H$^+$) co-transport by LeAMT1;2 was accompanied by ionic currents.

Rh-associated Cation Conductance in the Absence of NH$_4^+$

Even in the absence of ammonium or methylammonium, the expression of RhCG and RhBG increased the background ionic conductance in oocytes (Ludewig, 2004; Mak et al., 2005). Similarly, a small increase in background conductance was also seen in LeAMT1;2-expressing oocytes. The expression of foreign proteins in oocytes is often associated with unrelated background currents (Weber, 1999). This may suggest that the Rh-associated currents were endogenous to oocytes and not intrinsic to Rh proteins. However, time independence, regulation by alkaline pH$_o$, and their impermeability to NH$_4^+$ distinguish these cation currents from other endogenous ionic currents commonly observed by high expression of foreign proteins (Tzounopoulos et al., 1995). In the oocytes used in this study, background currents were not observed with the expression of other foreign proteins, e.g., an iron-phyt siderophore transporter (Schaaf et al., 2004). Furthermore, the expression of RhCG under the control of the strong PMA1 promoter in an alkali metal–sensitive yeast strain affected growth sensitivity, implying cation transport by RhCG in yeast. However, it cannot be excluded that RhCG conferred sodium sensitivity to that strain by an unrelated mechanism.

It is possible that the Rh-associated alkali-cation conductance was endogenous to oocytes and yeast and was activated by Rh expression. We cannot, however, rule out that, e.g., the central pore in the middle of the oligomer of subunits is a pathway for cations. Such a situation is discussed for the, structurally unrelated, aquaporin (water channel) tetramers (Yool and Weinstein, 2002).

**Methylammonium Transport**

The endogenous uptake of $^{14}$C-MeA was linear, electroneutral, and pH$_o$ dependent, in accordance with data obtained previously (Westhoff et al., 2002; Ludewig, 2004). Methylammonium did not affect the membrane potential or ionic currents, although MeA and other amines had previously been found to depolarize a subset of Xenopus oocytes (Burckhardt and Thelen, 1995). Seasonal and laboratory differences between oocytes are well established (Weber, 1999). The MeA uptake in native oocytes was probably not completely due to lipid diffusion of the uncharged species, since ammonium partially competed with MeA transport, indicating the presence of endogenous, electroneutral MeA transporters in Xenopus oocytes.

RhCG expression strongly stimulated $^{14}$C-MeA uptake. In accordance with data from RhAG (Westhoff et al.,
uptake rate by RhCG differed fourfold with a 100-fold transports the uncharged species. The half maximal rent was not observed and suggests that RhCG solely explained by saturation effects (Mak et al., 2005; Zidi-Yahiaoui et al., 2005). Using Avogadro’s number (1 pmol = 6.022 x 10^{23} particles) and the elementary charge (e = 1.602 x 10^{-19} As), the hypothetical current that was expected if the charged species MeA⁺ was transported by RhCG can be calculated: the uptake rate at pHᵢ = 8.5 of ~40 pmol/(oocyte x min) would correspond to an electrical current of ~−64 nA (e = 40 x 6.022 x 10^{11}/60 s) at the resting potential of ~0 mV. This current was not observed and suggests that RhCG solely transports the uncharged species. The half maximal uptake rate by RhCG differed fourfold with a 100-fold change in [H⁺], and H₂C-NH₂ concentration, suggesting that mainly H₂C-NH₃⁺ is recruited by RhCG.

In contrast, methylammonium induced inwardly directed currents by LeAMT1;2. The uptake of ~5 pmol/(oocyte*min) (Fig. 6) was associated with an ionic current of roughly ~7–10 nA (Fig. 3; unpublished data) at the resting potential of methylammonium-exposed oocytes (~−10 mV). The observed currents matched the expected current for exclusive MeA⁺ transport, or MeA/H⁺ cotransport (~8 nA = e × 5 x 6.022 x 10^{11}/60 s). MeA⁺ transport is also compatible with the fact that methylammonium uptake rates were unaffected by pHᵢ.

Ammonium Transport

The electroneutral transport by RhCG was observed for methylammonium, as well as for ammonium. Rapid cytosolic alkalinization is a typical response of many eukaryotic cells to 10 mM ammonium (Roos and Boron, 1981) and has also been observed in oocytes (Sasaki et al., 1992; Burckhardt and Thelen, 1995; Cougnon et al., 1996). In studies that reported a large endogenous NH₄⁺ conductance, however, oocytes were shown to acidify with 10 mM ammonium when measured with pH-sensitive electrodes (Burckhardt and Fromter, 1992; Burckhardt and Thelen, 1995; Cougnon et al., 1996; Boldt et al., 2003). A robust transient alkalinization and subsequent acidification upon addition of 20 mM ammonium has been measured in another study (Holm et al., 2005), suggesting some variability in the ammonium-dependent pHᵢ response in oocytes.

Native oocytes from this study were unaffected by 500 μM ammonium, but alkalinized with 10 mM ammonium, without secondary acidification (Fig. 9). If NH₃ entered the cells primarily, the subsequent formation of NH₄⁺ consumed an H⁺ and lead to cytosolic alkalinization (Roos and Boron, 1981). Rapid alkalinization was observed for RhCG-expressing oocytes, even at low ammonium concentrations (Fig. 9), confirming the electroneutral ammonium transport in Rh glycoproteins. The identical alkalinization was expected for a NH₄⁺/H⁺ exchange and a channel-like NH₃ transport mechanism (Westhoff et al., 2002; Ludewig, 2004). Though we cannot distinguish between these mechanisms, a channel-like NH₃ transport in a hydrophobic pore was suggested from the recently obtained crystal structure in the homologue AmtB (Khademi et al., 2004; Zheng et al., 2004). This mechanism is compatible with the data on Rh glycoproteins. Rapid ammonium-induced alkalinization by RhAG was also found in erythrocyte ghosts (Ripoche et al., 2004) and in kidney cell lines expressing RhBG or RhCG (Zidi-Yahiaoui et al., 2005). The transport mechanism is also compatible with results from RhCG-expressing cell lines (Handlogten et al., 2005) and another study that investigated RhCG and RhBG in oocytes (Mak et al., 2005).

There was no indication for NH₄⁺ currents by RhCG in our study. It remains unclear why others have recorded NH₄⁺ currents associated with RhCG in oocytes (Bakouh et al., 2004; Nakhoul et al., 2005), but it can be speculated that these RhCG-associated currents are related to endogenous currents for the following two reasons. First, the current-voltage relations of ammonium-induced currents are similar for endogenous and RhCG-induced ammonium currents (reversal at ~0 mV), but differ for NH₄⁺ currents by LeAMT1;2 (only inward). Second, NH₃ influx and subsequent internal alkalinization are known to activate endogenous, steeply pH- and NH₃-dependent currents in oocytes (Boldt et al., 2003), and NH₃ influx by RhCG is expected to activate such currents.

In contrast to the situation with RhCG, low concentrations of ammonium neither alkalinized nor acidified LeAMT1:2-expressing oocytes. With NH₄⁺ transport, some acidification was expected, but at physiological pHᵢ only a small fraction (~1%) of the inflowing NH₄⁺ will release H⁺ by forming NH₃. Furthermore, NH₄⁺ depolarizes oocytes that express LeAMT1:2, so NH₄⁺ currents are expected to be exceedingly small in the fluorescence assay. This may explain why no pHᵢ changes with LeAMT1:2 were detected. Despite this, it is of importance that the NH₄⁺ currents by LeAMT1:2 differed from the endogenous currents; LeAMT1:2 currents saturated with Kᵦ (at ~100 mV) ~60 μM (Ludewig et al., 2003)
In contrast, the H3C-NH3 transport by LeAMT1;2 occurs molecularly as NH3/H+ cotransport with 1:1 stoichiometry. Though physiologically equivalent, the NH3/H+ cotransport mechanism appears very attractive based on the hydrophobic nature of the pore in AmtB and the conservation of critical pore residues in other AMTs. Furthermore, it can explain the superb selectivity of AMT transporters against alkali cations.

The different transport mechanisms of RhCG and LeAMT1;2 predicted that yeast growth would be differently affected by either transporter on selective media. As expected for a transporter/channel that equilibrates H3C-NH3+, RhCG rescued growth of a wild-type strain on toxic methylammonium (due to the acidic exterior and low, equilibration of NH3 by RhCG may provide only weakly rescued the growth of the ∆∆∆, mep1, 2, 3) strain (Marini et al., 2000; Westhoff et al., 2004; Javelle et al., 2005). In contrast, the membrane potential–driven NH4+ transport by LeAMT1;2 was expected to allow efficient ammonium acquisition into the cytosol.

That transporters from the same protein family are functionally distinct is not unprecedented. A recent example comes from the CLC family of anion channels/transporters, which comprises anion channels and rapid 2 Cl−/H+ exchangers (Accardi and Miller, 2004). Although the pathway for anions is well apparent from the atomic CLC structures, it has not been resolved how protons are transported in CLCs. Our data cannot distinguish whether the transport by RhCG and LeAMT1;2 is by a “channel-like” or by a “transporter-like” mechanism. Moreover, it is somewhat surprising that the distantly related bacterial AmtB and Rh glycoproteins appear to share the same electroneutral transport mechanism, while AmtB and LeAMT1;2, which are more closely related on sequence level, are functionally distinct (Ludewig et al., 2001).

**Physiological Role of AMT and Rh Ammonium Transporters**

The physiological role of human electroneutral NH3 transporters/channels in erythrocytes, liver, and kidney is associated with acid–base balance and homeostasis. Distal kidney epithelia are long known to be little permeable to NH4+, but mediate net transport of NH4+ (Kleppe et al., 1989). The acid-secreting intercalated cells of the collecting tubules express basolateral RhBG and apical RhCG (Eladari et al., 2002; Quentin et al., 2003; Verlander et al., 2003). As the major form of acid excreted in the urine is in the form of NH4+, each transepitheliaally exported NH3 base binds a proton and NH4+ is finally excreted. Basolateral RhBG and apical RhCG equilibrate the NH3 gradient, thus a >100-fold total ammonium increase can be obtained by the different pH in blood (pH 7.4) and urine (pH 5) across these epithelia.

In contrast, the function of NH4+ transporters in plants is to acquire and accumulate the limiting nutrient ammonium in the strongly negative cytosol. High-affinity electrogenic NH4+ transport and concentrative acquisition of NH4+ has been observed in many plants (Ayling, 1993; Wang et al., 1994) and is probably mediated by AMT proteins.

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**REFERENCES**

Accardi, A., and C. Miller. 2004. Secondary active transport mediated by a prokaryotic homologue of CIC-Cl− channels. Nature. 427:803–807.

Ayling, S.M. 1993. The effect of ammonium ions on membrane potential and anion flux in roots of barley and tomato. Plant Cell Environ. 16:297–303.

Bakouh, N., F. Benjelloun, P. Hulin, F. Brouillard, A. Edelman, B. Cherif-Zahar, and G. Planelles. 2004. NH3 is involved in the NH4+ transport induced by the functional expression of the human RhC glycoprotein. J. Biol. Chem. 279:15975–15983.

Benjelloun, F., N. Bakouh, J. Fritsch, P. Hulin, J. Lippecka, A. Edelman, G. Planelles, S.R. Thomas, and B. Cherif-Zahar. 2005. Expression of the human erythroid Rh glycoprotein (RhAG) enhances both NH3 and NH4+ transport in HeLa cells. Pflügers Arch. 450:155–167.

Boldt, M., G. Burckhardt, and B.C. Burckhardt. 2003. NH4+ conductance in Xenopus laevis oocytes. III. Effect of NH3. Pflügers Arch. 446:652–657.

Burckhardt, B.C., and G. Burckhardt. 1997. NH4+ conductance in Xenopus laevis oocytes. I. Basic observations. Pflügers Arch. 434:306–312.

Burckhardt, B.C., and E. Fromter. 1992. Pathways of NH3/NH4+ permeation across Xenopus laevis oocyte cell membrane. Pflügers Arch. 429:83–86.

Burckhardt, B.C., and P. Thelen. 1995. Effect of primary, secondary and tertiary amines on membrane potential and intracellular pH in Xenopus laevis oocytes. Pflügers Arch. 429:506–512.
Eladari, D., L. Cheval, F. Quentin, O. Bertrand, I. Mouro, B. Cherif-Ludewig, U., N. von Wirén, and W.B. Frommer. 2002. Uniport of ammonium transport by basolateral transporters, along the rat nephron. *J. Am. Soc. Nephrol.* 13:545–554.

Iripchef, P., O. Bertrand, P. Gane, C. Birkenmeier, Y. Colin, and J.P. Cartron. 2004. The human Rhesus-associated RhAG protein mediates facilitated transport of NH₃ into red blood cells. *Proc. Natl. Acad. Sci. USA.* 101:17222–17227.

Roos, A., and W.F. Boron. 1981. Intracellular pH. *Physiol. Rev.* 61:296–434.

Sasaki, S., K. Ishibashi, T. Nagai, and F. Marumo. 1992. Regulation mechanisms of intracellular pH of *Xenopus laevis* oocytes. *Biochim. Biophys. Acta.* 1137:45–51.

Schaaf, G., U. Ludewig, B.E. Erenouglu, S. Mori, T. Kitahara, and N. von Wiren. 2004. ZnYSl functions as a proton-coupled symporter for phytosiderophore- and nicotianamine-chelated metals. *J. Biol. Chem.* 279:9091–9096.

Sieve, R.M., B. Weil, A. Burkowski, B.J. Eikmanns, M. Eikmanns, and R. KrAm. 1996. Functional and genetic characterization of the (methyl)ammonium uptake carrier of *Corynebacterium glutamicum*. *J. Biol. Chem.* 271:5398–5403.

Sohlkenkamp, C., C.C. Wood, G.W. Roeb, and M.K. Udvardi. 2002. Characterization of *Arabidopsis* AtAMT2, a high-affinity ammonium transporter of the plasma membrane. *Plant Physiol.* 130:1788–1796.

Tzounopoulos, T., J. Mayle, and J.P. Adelman. 1995. Induction of endogenous channels by high levels of heterologous membrane proteins in *Xenopus* oocytes. *Biophys. J.* 69:904–908.

Verlander, J.W., R.T. Miller, A.E. Frank, I.E. Royaux, Y.H. Kim, and I.D. Weiner. 2003. Localization of the ammonium transporter proteins RhBG and RhCG in mouse kidney. *Am. J. Physiol. Renal Physiol.* 284:F323–F337.

Wang, M.Y., A.D.M. Glass, J.E. Shaff, and L.V. Kochian. 1994. Ammonium uptake by rice roots III. Electrophysiology. *Plant Physiol.* 104:899–906.

Weber, W.M. 1999. Endogenous ion channels in oocytes of *Xenopus laevis* recent developments. *J. Membr. Biol.* 170:1–12.

Weiner, I.D. 2004. The Rh gene family and renal ammonium transport. *Curr. Opin. Nephrol. Hypertens.* 13:533–540.

Westhoff, C.M., M. Ferreri-Jacobia, D.O. Mak, and J.K. Fossett. 2002. Identification of the erythrocyte Rh blood group glycoprotein as a mammalian ammonium transporter. *J. Biol. Chem.* 277:12499–12502.

Westhoff, C.M., D.L. Siegel, C.G. Burd, and J.K. Fossett. 2004. Mechanism of genetic complementation of ammonium transport in yeast by human erythrocyte Rh-associated glycoprotein (RhAG). *J. Biol. Chem.* 279:17443–17448.

Yool, A.J., and A.M. Weinstein. 2002. New roles for old holes: ion channel function in aquaporin-1. *News Physiol. Sci.* 17:68–72.

Zheng, L., D. Kostrewa, S. Bernache, F.K. Winkler, and X.D. Li. 2004. The mechanism of ammonium transport based on the crystal structure of AmtB of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA.* 101:17900–17905.

Zidi-Yahiaoui, N., I. Mouro-Chanteloup, A.M. D’Ambrosio, C. Lopez, P. Gane, C. Le van Kim, J.P. Cartron, Y. Colin, and P. Ripoche. 2005. Human Rhesus B and Rhesus C glycoproteins: properties of facilitated ammonium transport in recombinant kidney cells. *Biochem. J.* 391:33–40.
During the production process, Fig. S1 was split into two separate figures, Figs. S1 and S2. However, the citations were not updated to reflect this change.

(p. 135) At the end of MATERIALS AND METHODS, the “Online Supplemental Material” section should begin “Figs. S1 and S2 show the pH dependence...”

(p. 139) In RESULTS, in the section “Effect of Ammonium on Ionic Currents by RhCG and LeAMT1;2,” the sentence beginning “While the ammonium-induced currents” should read “While the ammonium-induced currents from H2O-injected and RhCG-expressing oocytes were not substantially different, the NH4+ currents by LeAMT1;2 were large (Figs. 7 and 8), saturated with Km (at −100 mV) = 60 μM (Figs. 7 and S2) and were only inward.”

(p. 142–143) In DISCUSSION, under the section “Ammonium Transport,” the sentence at the end of p. 142 beginning “This may explain why no pH<i> changes” should read “This may explain why no pH<i> changes with LeAMT1;2 were detected. Despite this, it is of importance that the NH4+ currents by LeAMT1;2 differed from the endogenous currents; LeAMT1;2 currents saturated with Km (at −100 mV)~60 μM (Ludewig et al., 2003) (see Fig. S2).”