Electroneutral ammonium transport by basolateral rhesus B glycoprotein

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The liver and kidney are important tissues for ammonium (NH_4^+/NH_3) metabolism and excretion. The rhesus B glycoprotein (RhBG) is a membrane protein expressed in liver and kidney with similarity to NH_4^+ transporters found in microorganisms, plants and animals. In the kidney, RhBG is predominantly localized to basolateral membranes of distal tubule epithelia, including connecting tubules and collecting ducts. These epithelia display mainly electroneutral ammonium transport, in contrast to other tubular sites, where net NH_4^+ transport occurs. In accordance with its localization, human RhBG mediates saturable, electroneutral transport of the ammonium analogue methylammonium when heterologously expressed in *Xenopus* oocytes. Uptake of methylammonium saturates with a $K_m = 2.6$ mM. Methylammonium uptake is inhibited by ammonium and this inhibition saturates with a $K_i \approx 3$ mM. Electric current measurements and intracellular pH_i determinations suggest that RhBG acts as an electroneutral NH_4^+–H^+ exchanger.

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Ammonium metabolism and transport is of critical importance in microorganisms, plants and animals. As ammonium may be cytotoxic, its sequestration and excretion plays a key role in liver and kidney physiology (Knepper *et al.* 1989; Haussinger *et al.* 1992). Ammonium exists in aqueous solution in two molecular forms, NH_3 and NH_4^+, in pH-dependent equilibrium with a pK_a of 9.25. Thus, at physiological pH, most of the ammonium (~98.6%) is in the charged form. Both species can be transported across plasma membranes, with NH_3 being relatively permeable via lipid diffusion, although its permeability varies according to the lipid and protein composition (Lande *et al.* 1995). Some plasma membranes do not show significant NH_3 fluxes (Kikeri *et al.* 1989; Priver *et al.* 1993). The term ammonium will be used for NH_4^+/NH_3 throughout the manuscript, while the chemical symbols will be used to denote the charged NH_4^+ or uncharged NH_3 species specifically. The same formalism applies to methylammonium.

The charged species, NH_4^+, requires transport proteins for membrane passage. AMT/MEP-type proteins encode NH_4^+ transporters and these proteins were initially molecularly identified from yeast and plants (Marini *et al.* 1994; Ninnemann *et al.* 1994). The sequence similarity of rhesus polypeptides from erythrocytes (Rh) to AMT/MEP-type proteins suggested that the Rh-type proteins also encode NH_4^+ transporters (Marini *et al.* 1997). Indeed, ammonium transport by RhAG glycoprotein was reported after heterologous expression in yeast (Marini *et al.* 2000). However, RhAG and a homologue, RhCG (Liu *et al.* 2000), were functionally different from yeast and plant NH_4^+ transporters (Marini *et al.* 2000). Functional differences between AMT/MEP-type and Rh-type proteins were also found after heterologous expression in oocytes, where RhAG function seems best explained as an NH_4^+–H^+ antiport (Westhoff *et al.* 2002), while RhCG was suggested to be involved in NH_4^+ and NH_3 transport (Bakouh *et al.* 2004). *Xenopus laevis* oocytes provide an ingenious system for analysing most solute and ion transporters, but need to be used with care for analysis of ammonium transporters since they express endogenous NH_4^+-permeable ion channels that are activated by NH_3 influx and intracellular alkalinization (Burckhardt & Fromter, 1992; Cougnon *et al.* 1996; Burckhardt & Burckhardt, 1997; Boldt *et al.* 2003).

Rhesus B glycoprotein (RhBG), another member of the mammalian NH_4^+ transporter family with a molecular weight of the non-glycosylated protein of 40 kDa, is expressed in key organs associated with ammonium transport and metabolism (Liu *et al.* 2001; Quentin *et al.* 2003). Besides expression in ovary and skin, RhBG glycoprotein is detected in the liver, the gatekeeper organ of nitrogen metabolism and nitrogen balance in animals.

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Animals generally excrete surplus nitrogen via the urea cycle but nitrogen can also be excreted as ammonium. This process is performed by the kidney, another site of RhBG expression, and is used to control the blood plasma pH.

Ammonium metabolism in kidney functions to dispose of H^+ in urine. Ammonium is initially produced from glutamine catabolism in the proximal tubule, where it is released into the tubular fluid (Knepper *et al.* 1989). It is then concentrated in the inner medulla, and is filtered from the tubular fluid in the ascending limb of the loop of Henle, where NH_4^+ replaces potassium at several transporters at the potassium-binding site; examples include the Na^+–K^+-ATPase, Na^+–K^+–$2Cl^-$ cotransporter, and potassium channels. RhBG and RhCG glycoproteins are expressed in the distal nephron and collecting duct, where ammonium is finally released in a pH-dependent manner into the urine. RhBG expression is detected in the connecting tubule (CNT), the initial collecting tubule (ICT), the cortical collecting duct (CCD), the outer medullary collecting duct (OMCD) and the inner medullary collecting duct (IMCD) (Quentin *et al.* 2003; Verlander *et al.* 2003). Interestingly, RhBG protein is predominantly identified at basolateral membranes, while the homologous RhCG glycoprotein, which is mostly expressed in the same cells, is strictly located in apical membranes (Eladari *et al.* 2002; Quentin *et al.* 2003; Verlander *et al.* 2003). The distal renal epithelia are thought to be much less permeable to NH_4^+, but they mediate net NH_3 transport (Knepper *et al.* 1989; Flessner *et al.* 1991). Net NH_3 transport may be either by direct NH_3 diffusion or by NH_4^+–H^+ exchange. The major form of acid secretion in the urine is as ammonium, so that each H^+ is exchanged for NH_4^+, which is finally excreted.

In this study, currents were recorded by two-electrode voltage-clamp from RhBG-expressing oocytes. In addition, uptake experiments with the radiolabelled ammonium analogue methylammonium and intracellular pH_i measurements were performed and identifed RhBG as an NH_4^+–H^+ exchanger. We additionally found that NH_4^+–H^+ exchange is associated with small ammonium-dependent and -independent currents in *Xenopus* oocytes.

Methods

Plasmid construct

Human RhBG was cloned from a kidney cDNA library (Clontech) and checked by sequencing. The plasmid pOO2 (Ludewig *et al.* 2002) containing the coding region of human RhBG plus a 9 bp sequence (Kozak motif: GCCGCCACC) upstream the ATG ligated into the *Eco*RI site was kindly provided by J.-P.-Cartron and C. Lopez (INSERIM, Paris, France). Capped cRNA was transcribed by SP6 RNA polymerase *in vitro* using mMessage mMachine (Ambion), after linearizing the plasmid with *Mlu*I.

Preparation and injection of oocytes

Ovarian lobes were removed from adult female *Xenopus* frogs anaesthetized by immersion in 0.2% tricaine methanesulphonate (Sigma) for 45–60 min. Frogs were humanely killed after the final oocyte collection. Animal care and experiments followed approved institutional guidelines at the University of Tübingen. Oocytes (Dumont stage V or VI) were defolliculated using 10 mg ml^{-1} collagenase (Boehringer, Germany) and 5 mg ml^{-1} trypsin inhibitor (Sigma, Germany) for 1–2 h and injected with 15–50 nl of cRNA ($\approx$ 15–50 ng per oocyte). Oocytes were kept after injection for 3–4 days at 16°C in ND96 solution (mM: 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 Hepes (pH 7.4)), supplemented with 2.5 mM sodium pyruvate and gentamycin (20 μg ml^{-1}).

Electrophysiological measurements

Standard bath solutions contained (mM): 100 NaCl, 2 $CaCl_2$, 2 $MgCl_2$, 4 Tris, pH adjusted to 7.5 with Mes. For measurements at higher pH, the standard solution adjusted to pH 8.5 with Tris was used. For measurements at pH 5.5 and 6.5, the solution contained 4 mM Mes and pH was adjusted using Tris. In some solutions, NaCl was replaced by choline chloride. Potassium was omitted from all solutions to minimize effects on the endogenous oocyte Na^+–K^+ pump activity, which is highly sensitive to changes in extracellular [K^+] (Cougnon *et al.* 1996). Oocytes were clamped to −30 mV with a DAGAN CA-1 amplifier and currents were measured after stepping from the holding potential to different test potentials with pCLAMP6.0 software. Short pulses ($<$ 200 ms) were used to repress slowly activating endogenous currents. Pulses were given from +40 to −160 mV in 20 mV steps. Oocytes were placed in a small recording chamber and the gravity-driven solution exchange system allowed full exchange of bath solutions within a few seconds. Ammonium was applied for brief periods of $<$ 2 min, and oocytes were immediately washed with standard bath solution. Data were collected from more than eight batches of oocytes. Each reported experimental result was observed in at least two independent batches of oocytes.

Data analysis

Data were processed using Sigma plot (Jandel Scientific). The available concentration of the charged or the uncharged form was calculated using the Henderson-Hasselbach equation:

$pH = pK_a + \log_{10}[NH_3]/[NH_4^+]$. A pK_a of 9.25 was used for ammonium and a pK_a of 10.66 was used for methylammonium. All data are given as means ± standard deviation. The concentration dependence of methylammonium uptake was fitted using $V = V_{max}/(1 + K_m/c)$, where V_{max} is maximal uptake at a saturating methylammonium concentration, K_m is substrate concentration permitting half-maximal uptake, and c is the experimentally used concentration.

Radiotracer uptake

The ammonium analogue methylammonium (MA; $[^{14}C]CH_3$-NH_3^+; Amersham) was used as a radiotracer. Choline-based bath solutions were used for uptake experiments. Batches of > 10 healthy looking oocytes were incubated for 20 min in 200 μl of standard bath solutions containing radioactive methylammonium at room temperature. Initial tests showed that uptake did not saturate within the first 20 min (Westhoff *et al.* 2002). Oocytes were then carefully washed 5 times in 1 ml ice-cold buffer containing 10 mM unlabelled methylammonium and separated into groups of three per scintillation vial. After solubilization in 10 μl 5% SDS, 4 ml scintillation buffer was added and activity was analysed by liquid scintillation counting.

Intracellular pH changes (pH_i)

Intracellular pH changes (pH_i) were monitored using the pH-sensitive dye 2′,7′-bis-(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) (Sasaki *et al.* 1992). Six to eight healthy oocytes were selected and placed in light-protected 1 ml uptake buffer (ND96, containing 5 μM BCECF-AM) and incubated at room temperature for 30 min. Two BCECF-loaded oocytes were washed in ND96, placed on a glass slide, covered with a coverslip and then placed on an inverted Leica fluorescence microscope. Oocytes were superfused with ND96 or ND96 containing 500 μM NH_4Cl by gravity flow. Changes in BCECF fluorescence were measured using a spinning wheel fluorescence system with excitation at 410 ± 30 and 470 ± 20 nm and emission at 535 ± 20 nm. Pictures were taken every 10 s through an objective lens (×20) focused on the oocyte rim. Fluorescence changes obtained by superfusion of ND96 solution buffered to different pH_o (supplemented with 10 μM of the protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) to collapse pH_i) were used to determine the pH_i/(fluorescence ratio) slope. CCCP was diluted from a 1000× stock in ethanol.

Results

Currents associated with RhBG expression in oocytes

As oocytes are known to express endogenous NH_4^+-permeable channels, they were first tested for endogenous ion channel activities measured between +20 and −140 mV. After oocytes had been voltage clamped, currents elicited by brief pulses were nearly identical in choline-based or sodium-based solution at pH_o 7.5 (Fig. 1*A*). Addition of 1 mM ammonium did not change the currents, but a current of slightly varying magnitude appeared consistently upon addition of ≥ 3 mM NH_4^+ (Fig. 1*A*).

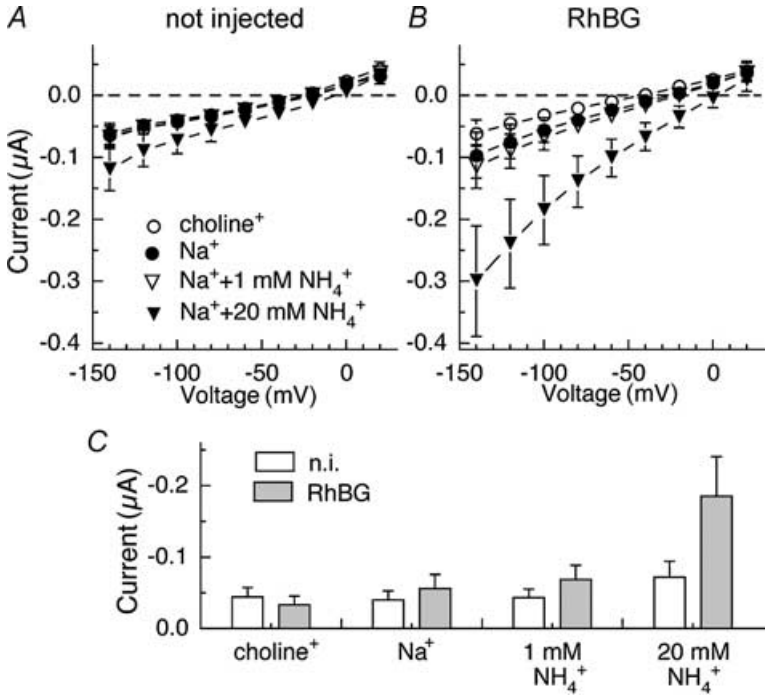

Figure 1. Currents by RhBG expressed in *Xenopus* oocytes at pH_o 7.5 at different ammonium concentrations
Current–voltage relations recorded in choline (○) and sodium-based solutions without ammonium (●), or containing 1 mM (□) and 20 mM (■) ammonium. *A*, background currents from non-injected oocytes ($n = 7$). *B*, means from ($n = 5$) RhBG-expressing oocytes. *C*, effect of extracellular cations on inward currents at −100 mV (n.i. = not injected).

Oocytes expressing RhBG glycoprotein had very similar conductances in choline-based solution, but the inward current magnitude was larger in sodium-based solution ($P < 0.05$). When 1 mM ammonium was added, inward currents slightly increased, but higher ammonium concentrations, e.g. 20 mM NH_4Cl, elicited a three-fold current increase ($P < 0.001$, Fig. 1*B*). A comparison of currents elicited at −100 mV from non-injected and RhBG-expressing oocytes is shown in Fig. 1*C*. The NH_4^+-induced currents in 20 mM NH_4^+ in RhBG-expressing oocytes may represent direct NH_4^+ transport by RhBG, or may be due to endogenous NH_4^+-permeable channels activated by RhBG function. It has been shown that endogenous cation channels are activated by influx of NH_3 (Boldt *et al.* 2003). As the NH_3/NH_4^+ ratio decreases at lower pH, higher ammonium concentrations are needed to activate these endogenous channels at acidic pH_o (data not shown) (Boldt *et al.* 2003).

Methylammonium does not induce currents in RhBG-expressing oocytes

In order to minimize the effects of endogenous oocyte ammonium currents, the commonly used transport analogue methylammonium ($H_3C\text{-}NH_3^+$, $pK_a = 10.66$) was used. Methylammonium does not activate endogenous currents at low (mM) concentrations (Fig. 2; data not shown). In non-injected control oocytes, currents were similar if recorded at pH_o 5.5 or pH_o 8.5 and addition of 1 mM methylammonium did not change the current magnitude at either pH_o. Inward currents recorded from RhBG-expressing oocytes were slightly larger, but were again similar at both pH_o values. Addition of methylammonium did not induce electrical currents (Fig. 2).

Methylammonium transport by RhBG

Methylammonium has the additional advantage that it can be used as a radiolabelled tracer. Uptake of [^{14}C]methylammonium was first measured in non-injected oocytes. Methylammonium uptake (1 mM) increased at higher pH_o values and higher $H_3C\text{-}NH_2$ concentrations in accordance with lipid diffusion of the uncharged species (Fig. 3). Uptake rates were several-fold higher at each pH_o in RhBG-expressing oocytes (Fig. 3). RhBG-mediated methylammonium transport was calculated by subtracting endogenous background uptake. RhBG-mediated [^{14}C]methylammonium transport increased at higher pH_o (Fig. 3). Such pH_o dependence would be expected for a transporter that exchanges $H_3C\text{-}NH_3^+$ with H^+, as the driving force for H^+, and thus the driving force for the coupled transport, increases at alkaline pH_o. This pH_o dependence, however, might also be expected for a transporter that solely transports uncharged $H_3C\text{-}NH_2$, since the substrate concentration increases at higher pH_o.

Saturable RhBG-mediated methylammonium transport is blocked by ammonium

At pH_o 7.5, [^{14}C]methylammonium uptake increased ~7-fold upon RhBG expression, and was similar in sodium- or choline-based solutions (Fig. 4*A*). Thus the small sodium current described above (Fig. 1) is stoichiometrically uncoupled to methylammonium uptake. Uptake of [^{14}C]methylammonium by RhBG-expressing oocytes was again calculated by subtracting endogenous background and saturated with a $K_m^{H_3C-NH_3^+} = 2.6$ mM for charged $H_3C\text{-}NH_3^+$ (Fig. 4*B*). Ammonium efficiently suppressed [^{14}C]methylammonium uptake by RhBG, suggesting that ammonium is the natural substrate of RhBG

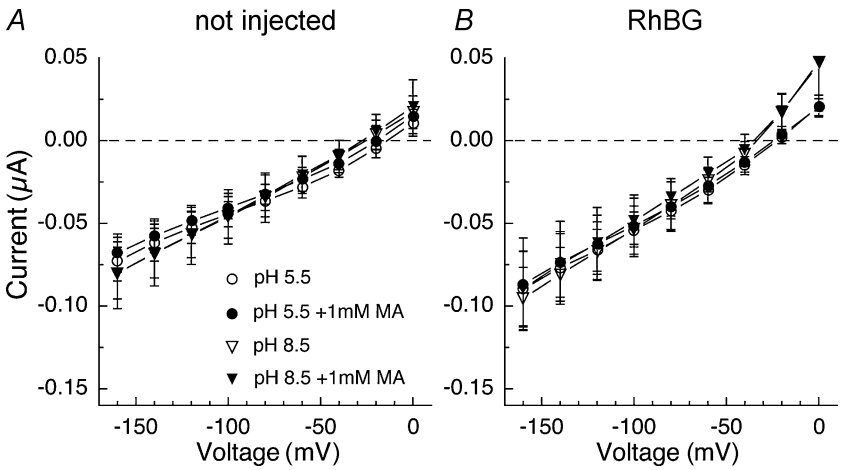

Figure 2. Voltage-dependent currents in oocytes expressing human RhBG at low and high pH_o in sodium-based solutions
Recordings from non-injected oocytes (*A*) and RhBG-expressing oocytes (*B*) at pH_o 5.5 and pH_o 8.5 with or without 1 mM methylammonium ($n = 4$).

and binds with a similar affinity (Fig. 4*B* and *C*). Ammonium inhibited [^{14}C]methylammonium uptake with a $K_i^{NH_4^+} = 3$ mM (Fig. 4*C*). If the uncharged species ($H_3C\text{-}NH_2$) is the substrate transported by RhBG, its affinity can be recalculated for experiments at constant pH_o 7.5, which gives $K_m^{H_3C-NH_2} = 1.8\ \mu$M. Experiments at constant $H_3C\text{-}NH_3^+$ (1 mM), but different pH_o values, yield $K_m^{H_3C-NH_2} = 0.4\ \mu$M (Fig. 4*D*).

NH_4^+ induced alkalinization by RhBG expression

Ammonium, not methylammonium, is likely to be the natural substrate of RhBG and the previous results suggest that electroneutral NH_3 transport or NH_4^+–H^+ counter-transport is the mechanism involved. Ammonium transport was directly measured using the pH-sensitive dye 2′,7′-bis-(carboxyethyl)-5(6)-carboxyfluorescein (BCECF). If RhBG mediates net NH_3 transport (either as NH_3 transport or NH_4^+–H^+ counter-transport), pH_i alkalinization is expected. In contrast, if NH_4^+ is significantly transported, pH_i acidification would be expected. Brief exposure to 500 μM NH_4Cl induced a fluorescence ratio change in RhBG-expressing oocytes, but not in non-injected controls (Fig. 5), which suggested pH_i alkalinization. The fluorescence ratio change was in the range of 0.15 ± 0.04 pH_i units, as approximated by control measurements using calibrated pH_o solutions in the presence of the protonophore CCCP. These experiments identify ammonium as the natural substrate of RhBG and do not support significant net NH_4^+ transport by RhBG. However, the results do not allow us to distinguish between NH_4^+–H^+ counter-transport and NH_3 transport, since both mechanisms reduce intracellular H^+ concentrations, either by H^+ efflux or, if NH_3 is transported, by intracellular protonation of NH_3 which consumes a H^+ to form NH_4^+.

Discussion

The rhesus B glycoprotein (RhBG) is abundant in specific basolateral membranes of renal and hepatic epithelia that are known for their high net NH_3 transport and secretion (Weiner & Verlander, 2003). In order to test a possible role for RhBG in ammonium transport, RhBG was expressed and functionally analysed in *Xenopus* oocytes. In accordance with its tissue localization, RhBG mediates electroneutral methylammonium fluxes when expressed in oocytes.

Electroneutral methylammonium transport by RhBG seems at first to be inconsistent with the fact that expression of RhBG in oocytes induced NH_4^+ currents (Fig. 1). However, it is well known that analyses of ammonium transporters in oocytes are hampered by the occurrence of distinct endogenous ammonium-activated, NH_4^+-permeable channels. Activation of these channels is preceded by diffusive entry of NH_3, followed by internal alkalinization (Burckhardt & Fromter, 1992; Cougnon *et al.* 1996; Burckhardt & Burckhardt, 1997; Boldt *et al.* 2003). The appearance of such currents is dependent on millimolar external ammonium, while low (μM) concentrations do not activate these channels (Ludewig *et al.* 2002, 2003; Bakouh *et al.* 2004). The threshold of activation at pH 7.5 is just above 1 mM external ammonium (Ludewig *et al.* 2002; Boldt *et al.* 2003; Bakouh *et al.* 2004). Expression of any net NH_3 transporter will increase ammonium entry, and hence internal alkalinization, and will thus lower the external ammonium concentrations needed to activate endogenous NH_4^+-permeable channels. As a consequence, it follows that even expression of an electroneutral net NH_3 transporter inevitably leads to activation of endogenous NH_4^+ currents. This hypothesis may need further substantiation in future work, and as long as no specific inhibitor exists, it may remain speculative whether the NH_4^+ currents are carried by endogenous channels or in part of the RhBG protein itself. However, endogenous currents are largely suppressed at low pH, since the ammonium concentration needed to activate endogenous NH_4^+ currents is much higher (Boldt *et al.* 2003; Bakouh *et al.* 2004). Support for the hypothesis that the NH_4^+ currents are carried by endogenous channels comes from the fact that 1 mM ammonium did not induce currents in RhBG-expressing oocytes at pH 5.5 (data not shown).

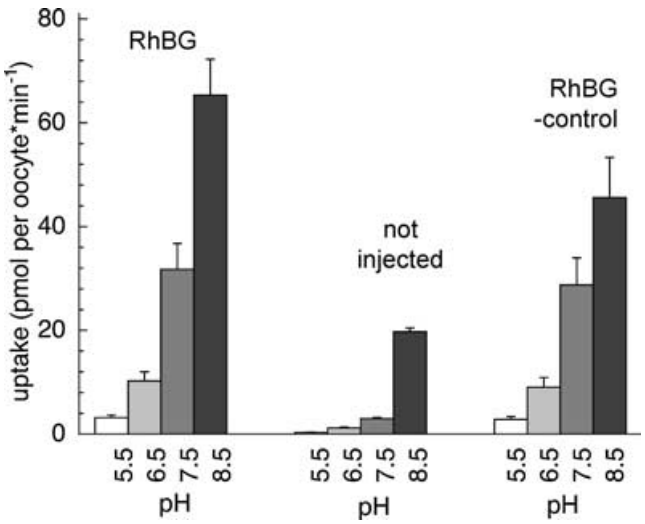

Figure 3. Methylammonium is taken up in a pH_o-dependent manner
Uptake of 1 mM methylammonium by RhBG-expressing oocytes (left panel) and uninjected controls (middle) at different pH_o in choline-based solutions. In the right panel, RhBG-induced uptake was corrected for endogenous uptake by subtracting uptake of non-injected oocytes. The concentrations of uncharged $H_3C\text{-}NH_2$ are 0.18 μM at pH_o 5.5, 1.8 μM at pH_o 6.5, 18 μM at pH_o 7.5 and 178 μM at pH_o 8.5.

Activation of endogenous currents may also explain why a GFP-fused RhCG protein appeared to transport NH_3 and NH_4^+ at the same time (Bakouh *et al.* 2004). Another preliminary study has identified ammonium-induced currents mediated by RhBG (Nakhoul & Hamm, 2004). Clearly, a more rigorous analysis of the endogenous NH_4^+-permeable channels and their relationship to Rh protein expression is needed.

Net NH_3 transport by RhBG was monitored using the fluorescent dye BCECF. No changes were observed upon application of 500 μM NH_4Cl in non-injected controls, while a pH_i increase was observed in RhBG-expressing oocytes. Other methods to determine pH_i, such as pH-sensitive impalement electrodes have identified a transient alkalinization followed by sustained acidification of pH_i (Burckhardt & Fromter, 1992; Cougnon *et al.* 1996). A disadvantage of conventional pH-sensitive electrodes may be that the impalement depth is hard to quantify and pH-sensitive electrodes may measure bulk cytosolic pH_i rather than the submembraneous pH_i. As the oocyte cytoplasm is not highly transparent, BCECF probably measures the pH_i close to the surface.

Methylammonium appears to be superior to ammonium as substrate for evaluating the transport mechanism and was thus mainly used in the present study to investigate RhBG function. RhBG transports methylammonium in an electroneutral manner, since at the resting potential of ~ -20 mV, the uptake of ~ 20 pmol min^{-1} would correspond to methylammonium currents of $\sim$32 nA, if the charged species is solely transported. As shown in Fig. 2, such a current is absent, indicating an electroneutral transport of methylammonium. Increased transport at higher pH_o and higher $H_3C\text{-}NH_2$ concentrations may superficially support the conclusion that RhBG transports the uncharged substrate, but it should be noted that electroneutral NH_4^+–H^+ exchange explains the data in Fig. 3 equally well. Different pH_o not only change $H_3C\text{-}NH_2$ concentrations, but also change pH gradients across the oocyte membrane.

RhBG is a member of the AMT/MEP/Rh family of NH_4^+ transporters (Marini *et al.* 1997; Ludewig *et al.* 2001). Proteins of this family have been grouped into three branches according to sequence similarity: AMT-, MEP- and Rh-like proteins (Ludewig *et al.* 2001). Despite unambiguous evidence for NH_4^+ transport by bacterial and plant AMT/MEP-type proteins (Meier-Wagner *et al.* 2001; Ludewig *et al.* 2002), it has recently been suggested that NH_4^+ transporters are in fact channels for the gas NH_3 (Soupene *et al.* 2002). This is surprising, as no direct binding or transport measurements have been presented to support the hypothesis (Soupene *et al.* 2002). In our hands, the growth tests in batch culture performed by Soupene *et al.* (2002) do not distinguish

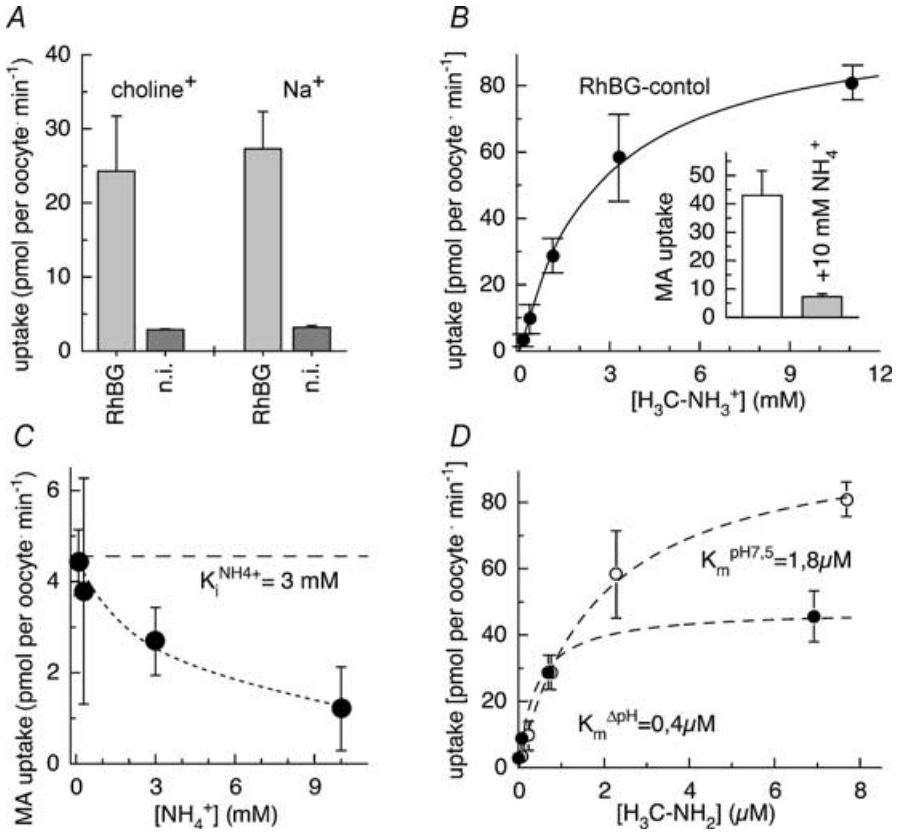

Figure 4. High affinity methylammonium uptake by RhBG and inhibition by NH_4^+
A, methylammonium (1 mM) uptake by RhBG-expressing oocytes in choline- and sodium-based solutions (n.i. = not injected). *B*, methylammonium ($H_3C\text{-}NH_3^+$) uptake by RhBG-expressing oocytes measured at different concentrations at pH_o 7.5. The data reveal a half-maximal concentration of 2.6 mM $H_3C\text{-}NH_3^+$ (RhBG-expressing oocytes minus controls). Inset: inhibition of methylammonium uptake (1 mM) by RhBG-expressing oocytes by NH_4^+ at pH_o 8.5. Open bar, RhBG-induced uptake of 1 mM methylammonium without NH_4^+; grey bar, uptake with 10 mM NH_4^+. *C*, inhibition of RhBG-mediated methylammonium uptake (2 mM, controls subtracted) by NH_4^+ at pH_o 7.5 reveal a $K_i^{NH_4^+}$ of 3.0 mM. *D*, uptake calculated for uncharged $H_3C\text{-}NH_2$ at constant pH_o 7.5 (○) and at different pH_o (●). Resulting saturation constants are $K_m^{pH7.5 H_3C-NH_2} = 1.8\ \mu M$ and $K_m^{\Delta pH H_3C-NH_2} = 0.4\ \mu M$.

between NH_4^+ and NH_3 transport. However, the many detailed studies on NH_4^+ transporters in bacteria, fungi and plants seem to provide unambiguous evidence that a general feature of these proteins is to bind and transport charged NH_4^+. While most studies used the NH_4^+ analogue methylammonium (Siewe *et al.* 1996; Marini *et al.* 1997; Meier-Wagner *et al.* 2001; Sohlenkamp *et al.* 2002), recent direct electrophysiological measurements of plant NH_4^+ transporters expressed in oocytes showed that the electrically charged NH_4^+ binds to the transporter and is transported (Ludewig *et al.* 2002, 2003). Interestingly, neither binding of NH_4^+ to that site, nor NH_4^+ transport is affected by thousand-fold changes in NH_3 (Ludewig *et al.* 2002). Thus NH_3 does not seem to bind the NH_4^+ transporter LeAMT1;1. This fact may not be too surprising, as NH_4^+ and NH_3 are chemically distinct molecules and may require different binding sites: in addition to their charge difference, NH_4^+ has a tetrahedral structure with 109.5 deg bond angle, whereas NH_3 has a pyramidal structure with 107 deg bonds.

It may be of minor importance in liver and kidney physiology whether RhBG exchanges NH_4^+–H^+ or directly transports NH_3. Although a final convincing biophysical demonstration for either suggestion may still be lacking, there are several arguments in favour of NH_4^+–H^+ exchange. Homologous proteins (AMT/MEP/Rh family) often have similar binding sites, and in accordance with that, the affinity of $H_3C\text{-}NH_3^+$ for RhBG is in a similar range to its affinity for LeAMT1;2, a NH_4^+ transporter from tomato (~4-fold lower at −20 mV; Ludewig, U unpublished). Saturating binding sites are a typical feature of transporters and a binding site for charged NH_4^+ may be a structural feature of these structurally related proteins. As discussed above, NH_3 does not influence binding of NH_4^+ to LeAMT1;1, and thus does not itself seem to bind to LeAMT1;1 (Ludewig *et al.* 2002). Binding of NH_4^+ to the related RhAG transporter is identical at different pH_o values, but when it is assumed that the uncharged species binds, the binding constant varies 10-fold (Westhoff *et al.* 2002). The calculated affinity of uncharged $H_3C\text{-}NH_2$ for RhBG would be ~1 μM. Such a high affinity seems to be inconsistent with a gas channel, as channels generally allow only loose binding to establish high flux rates (Hille, 2001). In addition, the binding constant of a substrate to its transporter should not vary under different conditions. However, when RhBG is assumed to bind and transport the uncharged substrate $H_3C\text{-}NH_2$, the affinities assayed at various pH_o values or at constant pH_o differ by a factor of 4.5 (Fig. 4*D*). As suggested for RhBG by the data presented in this study, electroneutral net NH_3 transport has also been suggested for RhAG and RhCG transport proteins (Marini *et al.* 2000; Ludewig *et al.* 2001; Westhoff *et al.* 2002).

How do the results of this paper relate to the physiological role of RhBG? Electroneutral ammonium transport by RhBG is important in the liver, where two distinct metabolic pathways for ammonium exist (Haussinger *et al.* 1992). Periportal or midzonal hepatocytes mediate low-affinity ammonium absorption and metabolism to urea. These cells do not express significant amounts of either RhBG or RhCG. Perivenous hepatocytes, however, express basolateral RhBG in combination with a high-affinity, low-capacity metabolic pathway that converts ammonium to glutamine (Weiner *et al.* 2003). This suggests a major function of RhBG in uptake and scavenging of ammonium, and in hepatocyte pH regulation, probably in combination with bicarbonate transporters.

In the kidney, with few exceptions, those cells that express basolateral RhBG also express apical RhCG (Quentin *et al.* 2003; Verlander *et al.* 2003), suggesting that both transporters are corporately involved in trans-epithelial net NH_3 secretion. RhBG and RhCG appear strongly expressed in acid-secreting intercalated cells in the collecting duct, which also express an apical H^+-V-type ATPase. Active proton secretion may drive apical NH_4^+–H^+ exchange and will concentrate NH_4^+ in the urine (Star *et al.* 1987; Wall *et al.* 1991). Basolateral RhBG may exchange NH_4^+ and H^+ according to the gradients actively established at the opposite apical membrane and will, in the absence of a basolateral H^+ leak, lead to acidification of the blood during transepithelial ammonium transport. Strong expression of RhBG and RhCG in acid-secreting intercalated cells are thus likely to maximize the efficiency of transepithelial ammonium secretion.

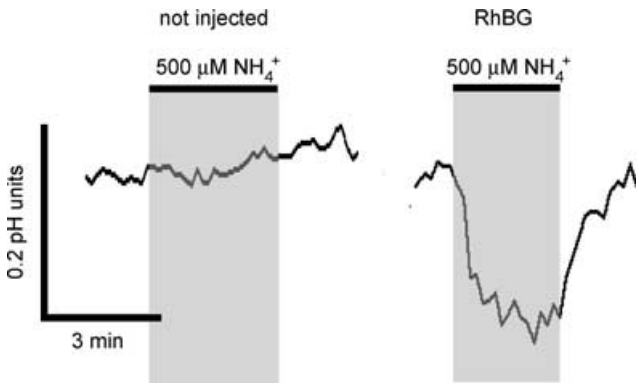

Figure 5. Intracellular alkalinization by ammonium in RhBG-injected oocytes
BCECF-loaded oocytes were superfused with ND96, containing 500 μM NH_4Cl for the indicated period. Left trace: non-injected oocyte, right trace: RhBG-expressing oocyte. Similar results were obtained in $n = 6$ oocytes and yield a pH_i change of 0.15 ± 0.2.

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