NH₃ Is Involved in the NH₄⁺ Transport Induced by the Functional Expression of the Human Rh C Glycoprotein

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Renal ammonium (NH₃ + NH₄⁺) transport is a key process for body acid-base balance. It is well known that several ionic transport systems allow NH₄⁺ transmembrane translocation without high specificity for NH₄⁺, but it is still debated whether NH₃, and more generally, gas, may be transported by transmembrane proteins. The human Rh glycoproteins have been proposed to mediate ammonium transport. Transport of NH₄⁺ and/or NH₃ by the epithelial Rh C glycoprotein (RhCG) may be of physiological importance in renal ammonium excretion because RhCG is mainly expressed in the distal nephron. However, RhCG function is not yet established. In the present study, we search for ammonium transport by RhCG. RhCG function was investigated by electrophysiological approaches in RhCG-expressing Xenopus laevis oocytes. In the submillimolar concentration range, NH₄Cl exposure induced inward currents (I_AM) in voltage-clamped RhCG-expressing cells, but not in untransfected cells. At physiological extracellular pH (pHₑₒₓ) = 7.5, the amplitude of I_AM increased with NH₄Cl concentration and membrane hyperpolarization. The amplitude of I_AM was independent of external Na⁺ or K⁺ concentrations but was enhanced by alkaline pHₑₒₓ and decreased by acidic pHₑₒₓ. The apparent affinity of RhCG for NH₄⁺ was affected by NH₃ concentration and by changing pHₑₒₓ whereas the apparent affinity for NH₄⁺ was unchanged by pHₑₒₓ, consistent with direct NH₄⁺ involvement in RhCG function. The enhancement of methylvammonium-induced current by NH₄⁺ further supported this conclusion. Exposure to 500 μM NH₄Cl induced a biphasic intracellular pH change in RhCG-expressing oocytes, consistent with both NH₃ and NH₄⁺ enhanced influx. Our results support the hypothesis of a specific role for RhCG in NH₃ and NH₄⁺ transport.

The human Rh family is composed of five known proteins: RhD, RhCE, RhAG, RhBG, and RhCG. Whereas RhD, RhCE, and RhAG proteins are expressed in erythroid cells, RhBG and RhCG proteins are expressed in epithelial tissues. Northern blot analyses have shown that RhBG is expressed mainly in the kidney and liver (1) and that RhCG is expressed mainly in the testis and kidney (2, 3). Rh proteins share homologies with the Mep/Amt family from yeasts, bacteria, and plants (4, 5). Despite numerous studies implicating Mep/Amt proteins in ammonium transport (6, 7), the transported substrate is still debated. Recent studies report, on one hand, that AmtB protein facilitates NH₄⁺ diffusion across the cytoplasmic membrane of Salmonella typhimurium (8) and, on the other hand, that LeAMT1;1 acts as an NH₂⁺ uniport after its functional expression in Xenopus laevis oocytes (9).

Consistent with the involvement of Rh proteins in transmembrane ammonium transport, yeasts deficient in endogenous ammonium transport system (ΔMep Saccharomyces cerevisiae) are enabled to grow in a low ammonium-containing medium when transformed by RhAG or by RhCG (3). Based on their finding that Mep and Amt are NH₃ channels, Soupene et al. (10, 11) raised the hypothesis that Rh proteins are involved in gas transport rather than in ionic transport. However, Westhoff et al. (12) concluded that RhAG mediates an electroneutral ionic exchange of NH₄⁺ for H⁺ after its functional expression in X. laevis oocytes. To our knowledge, the mechanistic properties of the human proteins RhBG and RhCG are not yet established. These proteins may have an important role in acid-base balance because ammonium excretion by the kidney plays a major role in acid excretion.

The aim of our study was to functionally express RhCG in X. laevis oocytes and investigate whether RhCG is involved in ammonium (NH₄⁺ and/or NH₃) transport. Exposure of voltage-clamped RhCG-expressing cells to submillimolar concentrations of NH₄Cl ([NH₄Cl]) induced inward currents (I_AM). The amplitude of I_AM increased with [NH₄Cl] and membrane hyperpolarization, consistent with an NH₄⁺-related current. However, the amplitude of I_AM was strongly sensitive to changes in extracellular pH (pHₑₒₓ) of ±0.5 pH unit (pH U), an experimental maneuver that only slightly changes [NH₃] but substantially affects [NH₄⁺]. At alkaline but not physiological pHₑₒₓ, the enhancement of methylammonium-induced current by micro-molar [NH₄Cl] further supports the requirement of the neutral form for NH₃/methylammonium transport in RhCG-expressing oocytes. Exposure to 500 μM NH₄Cl induced a biphasic intracellular pH change in RhCG-expressing oocytes, consistent with both NH₃ and NH₄⁺ influx into the cell. These results are consistent with RhCG-induced NH₃ and NH₄⁺ transport.

MATERIALS AND METHODS

cRNA Synthesis and Expression in X. laevis Oocytes—To routinely control RhCG expression in X. laevis oocytes, we constructed an N...
terminal fusion of the protein RhCG with the green fluorescent protein (GFP; Ref. 13). GFP-RhCG expression was demonstrated by a significant increase in green fluorescence, observable under microscopic control (excitation wavelength, 480 ± 40 nm; emission filter, 505 nm). RhCG cDNA was amplified by PCR from the pRS426-RhCG construct (3) using 5'-CTG-CAGCATGGCTGGAACCAACACT-3' and 5'-CTCCTACCTGCCC-TGGGAACCTAGGG-3' as sense and antisense primers, respectively. The 1466-bp RhCG cDNA was inserted downstream of the GFP coding region into the pQBI25-R1 vector. The in-frame insertion of RhCG cDNA was confirmed by sequencing. To synthesize cDNA coding for GFP-RhCG fusion protein, the GFP-RhCG cDNA was subcloned into pT7TTS plasmid. The pT7TTS-GFP-RhCG construct was linearized with SmaI restriction enzyme and transcribed in vitro from the T7 promoter using SP6C CAP mRNA capping kit. Defolliculated X. laevis oocytes (stage V- VI) were injected with cRNA dissolved in 50 nL of water or with 50 nL of water and then incubated at 18 °C (14). All experiments were performed in RhCG-expressing oocytes (oocytes injected with cRNA of GFP-RhCG) versus control oocytes (oocytes injected with water or cRNA of GFP). No difference was observed between control oocytes expressing GFP alone and control oocytes injected with water.

Voltage-Clamp Experiments—Two-electrode voltage-clamp experiments were performed as described previously (15, 16). Except where stated, the oocyte membrane potential was held at Vm = −50 mV. Oocytes were superfused by a control Ringer solution adapted for amphibian (containing 96 mM NaCl, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 5 mM Hepes, pH 7.5 with NaOH or with NaOH and adjusted to pH 7.5 with NaOH) or by up to seven substitution solutions differing from each other by a single parameter. In all experiments, the first substitution was a 500 μM NH4Cl-containing solution at pH 7.5. Solutions at pH 7.0 or 8.0 were also buffered with Heps/NaOH. Solutions at pH 8.5 were buffered with TAPS/NaOH (switching from Heps to TAPS buffer, at identical pH values). In Na+-free experiments, NaCl was replaced by an equimolar concentration of choline chloride, and pH was adjusted using Trizma (Tris base). To calculate [NH4+] and [NH3] in ammonium-containing solutions, the pKa of NH3 was taken as 9.25.

As reported by other groups (9, 17), we noticed that in control oocytes, even at low millimolar concentrations, NH4Cl (and methylammonium) may elicit endogenous currents that increase at alkaline pH (17). This is likely related to the multiple endogenous cationic conductances that are activated by NH4Cl in native X. laevis oocytes (18). In the present study, such endogenous currents were detected in H2O-injected oocytes upon exposure to [NH4Cl] ≥ 3 mM, [NH4Cl] ≥ 1.5 mM, and [NH4Cl] ≥ 500 μM at pH 7.0, 7.5, and 8.0, respectively. At these low [NH4Cl], the endogenous current amplitude (Iendog) varied from one batch of oocytes to another. Thus, when applying corrections, the NH4Cl-induced response in control cells was subtracted from the NH4Cl-induced response measured in RhCG-expressing oocytes from the same batch. However, because Iendog in oocytes may also vary within the same batch of oocytes, we avoided as far as possible the use of NH4Cl concentrations high enough to induce an endogenous response, except when necessary for further characterization of RhCG functional properties.

Intracellular pH Measurements—Intracellular pH (pHi) and membrane potential (Vm) were simultaneously measured using double-barreled pH-sensitive microelectrodes (filled with the Fluka H+/H11032 TGGGAGCCTAGGG-3') or the Fluka H+/H11001 CAGCATGGCTGGAACCAACACT-3'. The pHi of the patch pipette was monitored by the pH-sensitive dye N,N-diethyl-4-aminocinnamaldehyde (E-DMA) (18), which was in -jected into the patch pipette. The resulting current-voltage relationship (Fig. 2) shows that Iendog increased with hyperpolarization (inside negative), consistent with the enhancement of net entry of positive charges (namely, NH4+) into RhCG-expressing oocytes. Extrapolating the current-voltage relationship to more depolarized values (which were not experimentally assessed due to the activation of a Na+ conductance induced by depolarization; Ref. 20) gives a reversal potential (Erev) near 0 mV. This Erev value may correspond to the combined equilibrium potential of the main cationic species (Na+ + K+) of NH4+ or of H+ ions (18). Because a major involvement of H+ ions was not supported by the pH stability of RhCG-expressing oocytes upon pH change (from pH 7.5 to 8.5, ΔpH = 0.02 ± 0.01; p = 0.3; n = 4), we next determined the specificity of the currents to NH4+ compared with other cationic species.

First, voltage-clamped oocytes (Vm = −50 mV) were exposed pairwise to a Ringer solution supplemented with 500 μM NH4Cl or 500 μM choline chloride or NaCl. Neither NaCl nor choline chloride induced a current in RhCG-expressing oocytes or control cells (data not shown), whereas Iendog was −13.2 ± 1.1 nA in RhCG-expressing oocytes (n = 8) but was not detectable in NaCl- or NH4Cl-added oocytes. The non-involvement of Na+ ions in RhCG function was further confirmed by measuring in a paired fashion that the current induced by 500 μM NH4Cl added to a plain Ringer solution or to a Na+-free solution was the same (n = 10; p = 0.3). This also confirms that RhCG does not mediate a nonselective cationic pathway, in which case Iendog would be expected to increase under Na+-free conditions.

Second, the discrimination between K+ and NH4+ in RhCG-expressing oocytes was investigated. To this end, the effect of adding 500 μM KCl to the Ringer solution was checked. This maneuver did not induce an inward current in RhCG-expressing oocytes, consistent with high selectivity for NH4+ over K+.

This finding is in agreement with studies in S. cerevisiae showing that RhCG did not complement the growth defect of yeast deficient in K+ transport (3). In fact, in this series, we observed a slight KCl-induced outward current in all oocytes (+2.1 ± 0.6 nA in RhCG-expressing oocytes and +0.8 ± 0.7 nA in H2O-injected oocytes; n = 8). This is likely related to the properties of the endogenous oocyte Na,K-pump activity, which is highly sensitive to changes in extracellular [K+] (18), which was increased by 25% in our experiments. The non-involvement of K+ in RhCG function was confirmed by measuring in a paired fashion that the current induced by 500 μM NH4Cl added in a plain Ringer solution or in a K+-free medium was the same (n = 3; p = 0.5).

The above-mentioned results are consistent with the induction (or enhancement) of an ammonium-related, rheogenic process consecutive to RhCG functional expression. This suggests that RhCG has a different function than those proposed for RhAG (the erythroid homologue of RhCG) or for Rh1 protein.
from Chlamydomonas reinhardtii (11, 12). RhAG, after functional expression in X. laevis oocytes, was reported to mediate an electrically silent NH₄⁺/H⁺ exchange (12). In that study, the authors proposed that this electroneutral ionic transport mediated by RhAG would not change pHᵢ, despite the RhAG-induced NH₄⁺ influx into the cell (12). Another group speculated that human Rh proteins mediate CO₂ gas diffusion (11). According to the authors, this hypothesis is supported by their observation that incubation of the green alga C. reinhardtii in a high-CO₂ environment increased the expression of the related RH gene, RH1 (11). Because our study focuses on ammonium transport, we will not discuss our results in the light of putative CO₂ diffusion by RhCG.

According to the authors, the hypothesis is supported by their observation that incubation of the green alga C. reinhardtii in a high-CO₂ environment increased the expression of the related RH gene, RH1 (11). Because our study focuses on ammonium transport, we will not discuss our results in the light of putative CO₂ diffusion by RhCG. With regard to NH₃, which is also a gas, RhCG-mediated transport of this uncharged species cannot account for the observed IAM, at least not in a simple manner. However, the rheogenicity of the ammonium-induced response does not exclude the possibility that NH₃ is transported together with an ion or that NH₃ stimulates an electrogenic transport in RhCG-expressing oocytes. In the experiments reported above, the discrimination observed between NH₄⁺ and K⁺ in RhCG-expressing oocytes, as well as the observation that Na⁺, K⁺, and choline⁺ do not mimic the effect of NH₄⁺ on membrane current, agrees with results reported in X. laevis oocytes expressing LeAMT1;1, an Amt protein from tomato root hair (9). Our results strongly suggest that, under our experimental conditions, RhCG function is specifically related to ammonium transport.

**Effect of Extracellular pH on NH₄Cl-induced Currents**—When it is expressed in X. laevis oocytes, the Amt protein LeAMT1;1 mediates an NH₄⁺/H⁺ uniport (9). The NH₄⁺/H⁺ currents induced by LeAMT1;1 expression were pHₒ-independent (9). To determine whether LeAMT1;1 and RhCG have similar functional properties, we looked for the effect of changing pHₒ on IAM in voltage-clamped oocytes.

We measured the current induced by a given NH₄Cl concentration by exposing the cells in a paired fashion to 500 µM NH₄Cl at pHₙ = 7.5 and then at pHₙ = 7.0 or 8.0. In RhCG-expressing oocytes, at pHₙ = 7.0, IAM was barely measurable (IAM = −1.2 ± 0.5 nA at pHₙ = 7.0 versus −13.2 ± 1.7 nA at pHₙ = 7.5; n = 10; p < 0.05), whereas it was greatly enhanced at pHₙ = 8.0 (IAM = −28.2 ± 2.8 nA at pHₙ = 8.0 versus −8.6 ± 0.6 at pHₙ = 7.5; n = 9; p < 0.05). Such large changes in IAM were surprising.
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**Fig. 2.** NH₄Cl-induced current as a function of the membrane potential across the oocyte membrane. Top panel, original tracing obtained from a single oocyte. Holding potentials (mV) are indicated below the trace. Exposure to [NH₄Cl] = 500 μM, pHₙ = 7.5 is indicated by the bars above the trace. Bottom panel, current (I, in nA)-voltage (V, in mV) relationship. Results from eight oocytes (mean ± S.E.) exposed to 500 μM NH₄Cl. The dashed line was obtained by linear regression (R² = 0.99).

because at constant [NH₄Cl] = 500 μM, changing pHₙ by ± 0.5 pH U only marginally affects NH₄⁺ concentration ([NH₄⁺] decreases by ~25 μM from pHₙ 7.0 to 8.0). However, this change of pHₙ by ± 0.5 pH U causes a ~3-fold change in [NH₃]. To confirm that a pHₙ/NH₃ change directly affects Iₐm, we compared the current induced by [NH₄Cl] = 500 μM at pHₙ 7.5 with the currents induced by NH₄Cl-containing solutions with either the same [NH₃] or the same [NH₃] (see Fig. 3). These solutions were buffered to pH 7.0 (Fig. 3A) or pH 8.0 (Fig. 3B). In both series, Iₐm was the same during exposure of RhCG-expressing oocytes to solutions with different pHₙ, but containing identical [NH₃].

The above-mentioned results show a strong effect of changing pH/NH₃ on NH₄⁺-induced current in RhCG-expressing oocytes. This is in variance with results reported for LeAMT1:1-expressing oocytes (9). In that study, the pHₙ, independence of Iₐm was invoked as a strong argument to conclude that LeAMT1:1 mediates an NH₄⁺ uniport, independent of NH₃ (9). Our results also argue for a putative NH₄⁺-H⁺ co-transport mediating both NH₄⁺ and H⁺ influx into the cell. Such a transport system would induce an Iₐm inward current, but alkaline external pH should reduce Iₐm (conversely, acidic pH should increase Iₐm), whereas we observed the opposite. These results suggest that NH₃ or H⁺ ions are directly involved in RhCG function.

**Substrate Dependence of Iₐm**—Discrimination between H⁺ and NH₃ effects is not obvious because of the equilibrium reaction between NH₄⁺, NH₃, and H⁺. Nonetheless, we attempted to arrive at a better understanding of RhCG function by establishing the concentration dependence of the ammonium-induced currents on each of these three species. To this end, Iₐm was measured while keeping pHₙ, [NH₃], or [NH₄⁺] constant while varying the other two.

First, RhCG-expressing oocytes were exposed to increasing [NH₄Cl] at a constant pHₙ of 7.5 (Fig. 4A) or 7.0 (Fig. 4B). This protocol gives the dependence of Iₐm on [NH₄Cl]. This represents, to a first approximation, the Iₐm dependence on [NH₄⁺] because NH₄⁺ concentration is so much higher than that of NH₃ in this pH range. The current-concentration relationships saturated at relatively low substrate concentrations, suggesting carrier (rather than channel) behavior. The apparent affinity for ammonium (thus, in a first approximation, for [NH₄⁺]) appears to be pHₙ-dependent. This observation is at variance with results reported for the function of RhAG because changes in pHₙ did not modify the kinetics of ammonium inhibition of methylammonium uptake measured in RhAG-expressing oocytes (12). This finding was taken as an argument against the transport by RhAG of NH₃ or of the neutral form of methylammonium (12). Interestingly, in RhCG-expressing oocytes, the apparent NH₃ affinity (~8 μM) seems to be pHₙ-independent.

In another experimental series, RhCG-expressing oocytes were exposed to increasing [NH₃] at constant [NH₄⁺] (pHₙ was changing). This protocol was carried out at constant [NH₃] ~ 491 μM (Fig. 5A) or 246 μM (Fig. 5B). Results confirmed that half-maximal Iₐm was reached for [NH₃] ~ 8 μM (corresponding to different pHₙ) as in the previous series (Fig. 4). This finding suggests NH₃ transport by RhCG and/or its direct involvement in Iₐm.

In a complementary protocol, RhCG-expressing oocytes were exposed to increasing [NH₃] at constant [NH₄⁺] (in the same range of pHₙ values as in the previous protocol, i.e. from pHₙ 7.0 to 8.0 by increments of 0.2 pH U). This experimental series was performed with [NH₃] constant at 8.73 or 4.36 μM. Analysis (by analysis of variance) of the results (data not shown) obtained in n = 5–7 oocytes showed that Iₐm was constant for a given [NH₃], despite the 10-fold change imposed in [NH₃] between pH 7.0 and 8.0. These results agree with results from Fig. 3 and suggest either that [NH₃] acts as an “on-off switch” for Iₐm or that NH₃ was at a saturating concentration. To discriminate between these possibilities, RhCG-expressing oocytes were again exposed to increasing [NH₄⁺] at constant [NH₃], but the previous protocol was changed in two ways. First, to obtain solutions containing less [NH₃], pHₙ was raised to 8.6 or 8.8. To obtain a better resolution of the changes in Iₐm, [NH₃] = 13.09 μM rather than [NH₃] = 4.36 μM was used. Results shown in Fig. 5, C and D, show that half-maximal Iₐm was reached for [NH₃] ~ 140 and 120 μM for [NH₃] = 13.09 and 8.73 μM, respectively. These results show that [NH₃] does not act as an on-off switch for Iₐm. They are consistent with the expression in RhCG-expressing oocytes of a high affinity NH₃ transport system dependent on NH₃.

Finally, to check whether pHₙ, per se affects RhCG function, we measured the current induced by [NH₃] and [NH₄⁺] above their respective saturating concentrations as determined from Figs. 4 and 5. To this end, the current induced by [NH₄Cl] = 5 mM was measured at various pHₙ values. In H₂O-injected oo-
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Fig. 3. NH₄Cl-induced currents in RhCG-expressing oocytes: dependence on pH, [NH₃], and [NH⁴⁺]. Currents (I, in nA) induced in voltage-clamped oocytes (Vᶜ = −50 mV) by NH₄Cl-containing solutions. [NH₃] and [NH⁴⁺] values (in μM) of the solutions and the pH of the solutions are indicated below the graphs. Significance of the results was assessed by paired t test, compared with the results obtained by exposing the oocytes to the 500 μM NH₄Cl-containing solution, pH = 7.5. NS, not significant; and *, p < 0.05. A, currents induced by an NH₄Cl-containing solution ([NH₄Cl] = 500 μM, pH = 7.5, □□) and by solutions buffered at pH 7.0 and with the same [NH₃] (□) or the same [NH⁴⁺] (■). Results are expressed as mean ± S.E. (n = 8 oocytes). B, currents induced by an NH₄Cl-containing solution ([NH₄Cl] = 500 μM, pH = 7.5, □□) and by solutions buffered at pH 8.0 and with the same [NH₃] (□□) or the same [NH⁴⁺] (■). Results are expressed as mean ± S.E. (n = 7 oocytes).

First, we looked for methylammonium-induced current, IₘMeA, at pHₙ = 7.5. Because H₃C-NH₂/H₃C-NH³⁺ has a higher pKₐ than NH₃/NH⁴⁺ (10.65 versus 9.25) for identical total concentrations of NH₄Cl or MeACl at pH 7.5, methylammonium ions (H₃C-NH⁻/H₃C-NH³⁺) are in slightly higher concentration than NH₄⁺ (thus a lower H₃C-NH₂ concentration than NH₃). However, 500 μM MeACl induced no current in either control or RhCG-expressing oocytes (n = 17) and −0.2 ± 0.02 in control oocytes (n = 11). Of note, in RhCG-expressing oocytes, a small IₘMeA was recorded when [MeACl] was increased to 750 (−3.0 ± 0.2 nA; n = 4) or 1000 μM (−3.6 ± 0.9 nA; n = 4), whereas no current was detectable in control cells (n = 3).

Second, we looked for a possible change in the current induced by 500 μM NH₄Cl upon simultaneous exposure to NH₄Cl and MeACl (500 μM each). Whereas this experimental maneuver was without effect in H₂O-injected oocytes (n = 8), exposure of RhCG-expressing oocytes simultaneously to MeACl and NH₄Cl induced a current that was higher than NH₄Cl alone (IₘMeA = −30.9 ± 3.1 versus IₘNH₄⁺ = −23.4 ± 3.8 nA; n = 4; p < 0.05). Increasing
Fig. 4. NH₄Cl concentration-current dependence in RhCG-expressing oocytes. Correlations between currents (I) and NH₄Cl concentration in RhCG-expressing oocytes (Vc = -50 mV). The dashed lines through the points (expressed as means ± S.E. from 3–16 oocytes) were calculated by fitting (using Kaleidagraph or Sigma Plot software) to the data the modified Hill equation $I = I_{\text{max}} [S]^{n} / ([S] + K_m)$, where $I$ is the measured current, $I_{\text{max}}$ is the maximum current, $[S]$ is the substrate concentration, $n$ is the apparent Hill coefficient, and $K_m$ is the apparent concentration of substrate corresponding to half-maximal current, expressed as means ± S.D. $A$, I as a function of [NH₄Cl]/[NH₃], with pH kept constant (pHᵢ = 7.5). Apparent half-maximal current was obtained for [NH₄Cl] = 467.6 ± 60.5 µM (corresponding to [NH₃] = 8.1 µM), with an apparent Hill coefficient > 2, suggesting the presence of two (or more) binding sites, with strong cooperativity. Extrapolated $I_{\text{max}}$ was 27.2 ± 3.2 nA. B, I as a function of [NH₄Cl]/[NH₃], with pH kept constant (pHᵢ = 7.0). Apparent half-maximal current was obtained for [NH₄Cl] = 1291.5 ± 338.8 µM (corresponding to [NH₃] = 7.22 µM). Extrapolated $I_{\text{max}}$ was 21.0 ± 4.3 nA.

[MeACl] to 750 or 1000 µM in the presence of 500 µM NH₄Cl led to a slight increase of $I_{\text{MeA}}$ to ~31.2 ± 3.0 and ~33.7 ± 2.5 nA ($n = 4$), thus saturating. These results suggest that H₃C-NH₂ behaves as a low affinity substrate in RhCG function. Interestingly, our data show that $I_{\text{MeA}}$ > $I_{\text{AM}}$, a higher value than the paired $I_{\text{IAM}}$ (~9.1 ± 0.8 nA; $n = 11$; $p < 0.05$) obtained with 500 µM NH₄Cl/pHᵢ 7.5 (i.e. [NH₃] ~ 8.5 µM). This result confirms that H₃C-NH₂ may substitute for NH₄⁺ and agrees with an effect of the noncharged form of the substrate on the current (reflecting the influx of the charged form of the substrate) amplitude.

Next, we searched for a stimulation of $I_{\text{MeA}}$ when providing a concentration of NH₄⁺ near the value determined for half-maximal $I_{\text{IAM}}$, while taking care to keep [NH₃] as low as possible. To this end, 50 µM NH₄Cl was used at pHᵢ = 8.5 (i.e. [NH₃] ~ 8 µM). As shown in Fig. 7, adding 50 µM NH₄Cl to 500 µM MeACl increased the induced current by an amount similar to that obtained at pHᵢ 7.5 in the presence of 500 µM NH₄Cl (p = 0.6; $n = 14$). At pHᵢ 7.5, the same maneuver was without effect (Fig. 7). This result was expected because at pHᵢ 7.5, in a solution of 50 µM NH₄Cl, the [NH₃] is ~1 µM, which is very low compared with the measured half-maximal concentration of ~8 µM.

Taken together, our results agree with NH₃ involvement in NH₄⁺/H₃C-NH₂-induced current. This finding is specific to RhCG compared with its erythroid homologue. In RhAG-expressing oocytes, methylammonium uptake was reported not to be rheogenic and to be independent of [NH₃] (12). In RhCG-expressing oocytes, our results suggest an electrogenic methylammonium influx activated by [NH₃].

An ammonium-induced activation of the AE2 exchanger after its functional expression in X. laevis oocyte has been documented and was partly explained by an NH₄Cl-induced intracellular Ca²⁺ increase (21). However, a similar explanation cannot account for our results because ammonium-induced activation of AE2 was observed in the presence of [NH₄Cl] = 10–20 mM (21), an experimental condition very different from our conditions (stimulation of $I_{\text{MeA}}$ was observed using micromolar concentrations of NH₄⁺/NH₃). Moreover, in our experiments, $I_{\text{MeA}}$ stimulation appears to be specifically related to RhCG expression because it was only observed in RhCG-expressing oocytes, not in water-injected oocytes. Interestingly, Rh proteins were proposed be "sensors" and to regulate ion transport, by analogy to MEP2, which was shown to regulate the cell differentiation in S. cerevisiae (3, 22–24). According to this proposal, RhCG might be a regulator of ammonium transport, an interesting hypothesis for a protein whose function in native tissue is not yet known. Interactions between the epithelial Rh glycoproteins RhCG and RhBG and acid-equivalent transport systems have also been speculated (21, 23) due to their localization in native tissue. In mouse distal nephron, AE1 and RhBG are co-localized in the basolateral membrane of type A cells (23), and in the rat and mouse kidneys, RhCG is localized in the apical membrane of tubular cells that are involved in ammonium secretion and in net transcellular acid-base transport (23, 25). However, from our results, we can only conclude that transport of NH₄⁺/H₃C-NH₂ is stimulated by the neutral form of these compounds in RhCG-expressing oocytes.

In light of these results suggesting that [NH₃] stimulates $I_{\text{MeA}}$, we further checked our previous conclusion that RhCG discriminates between NH₄⁺ and K⁺ (known to be competitive substrates on various transport systems) because the observed lack of K⁺-induced inward current in RhCG-expressing oocytes was obtained using NH₄⁺-free solutions. Thus, we looked for an induced current when adding 50 µM NH₄Cl to a Ringer solution supplemented with 500 µM KCl at pHᵢ = 8.5. This maneuver did not induce an inward current in RhCG-expressing oocytes (p = 0.5; $n = 3$). This further confirms that RhCG function is specifically related to ammonium (methylammonium) transport.

Effect of NH₄Cl Exposure on Intracellular pH—To better determine whether NH₃ is transported with NH₄⁺, we meas-
Fig. 5. \( \text{NH}_3 \) and \( \text{NH}_4^+ \) current dependence in RhCG-expressing oocytes. Dose-response curves for \( \text{NH}_3 \) and \( \text{NH}_4^+ \) in oocytes expressing RhCG (Vc = −50 mV), exposed to increasing \([\text{NH}_3] \) or \([\text{NH}_4^+]\). Data were normalized to the extrapolated maximal current, and apparent \( K_m \) values (expressed as means ± S.D.) were obtained by fitting the Hill equation to the data (dashed lines through the means ± S.E.; n = 3–18 oocytes). A, normalized I as a function of \([\text{NH}_3]/[\text{pH}] \) with \([\text{NH}_3] \) kept constant (\([\text{NH}_3] = 491.26 \mu M \)). Apparent half-maximal current was obtained for \([\text{NH}_3] = 7.66 \pm 0.47 \mu M (\text{corresponding to pH} = 7.44). B, normalized I as a function of \([\text{NH}_4^+]/[\text{pH}] \) with \([\text{NH}_4^+] \) kept constant (\([\text{NH}_4^+] = 245.63 \mu M \)). Apparent half-maximal current was obtained for \([\text{NH}_4^+] = 7.60 \pm 1.4 \mu M (\text{corresponding to pH} = 7.74). C, normalized I as a function of \([\text{NH}_3]/[\text{pH}] \) with \([\text{NH}_3] \) kept constant (\([\text{NH}_3] = 13.09 \mu M \)). Apparent half-maximal current was obtained for \([\text{NH}_3] = 137 \mu M (\text{corresponding to pH} = 8.23). D, normalized I as a function of \([\text{NH}_4^+]/[\text{pH}] \) with \([\text{NH}_4^+] \) kept constant (\([\text{NH}_4^+] = 8.73 \mu M \)). Apparent half-maximal current was obtained for \([\text{NH}_4^+] = 116 \mu M (\text{corresponding to pH} = 8.13)."

Fig. 6. Effect of \( \text{pH}_H \) on ammonium-induced current in control and RhCG-expressing oocytes. Effect of \([\text{NH}_4\text{Cl}] = 5 \text{ mM} \) in voltage-clamped oocyte (holding potential, Vc = −50 mV) at various \( \text{pH}_H \) values. Recorded current (I) in control oocytes (○) and RhCG-expressing oocytes (●) is indicated on the ordinate. \( \text{pH}_H \) values are indicated on the abscissa. Results are expressed as mean ± S.E. (n = 11–18 control oocytes; n = 10–12 RhCG-expressing oocytes).Measured the effect on \( \text{pH}_H \) of exposing oocytes to 500 \( \mu M \) \( \text{NH}_4\text{Cl} \) (\( \text{pH}_H = 7.5 \)). Control and RhCG-expressing oocytes exhibited the same resting \( \text{pH}_H \) (\( \text{pH}_H = 7.38 ± 0.02 (n = 9) \) versus \( 7.39 ± 0.01 (n = 9) \)), but exposure to \( \text{NH}_4\text{Cl} \) induced a \( \text{pH}_H \) change (biphasic change) only in RhCG-expressing oocytes, as shown in Fig. 8. In RhCG-expressing oocytes, the initial effect of \( \text{NH}_4\text{Cl} \) exposure was a slight transient alkalinization (\( \text{pH}_H \) increased to \( 7.42 ± 0.01 \)), which was significantly different from the resting \( \text{pH}_H \), \( p < 0.05 \), which was not detected in \( \text{H}_2\text{O} \)-injected oocytes (\( \text{pH}_H \) was stable (\( 7.38 ± 0.02 \)) and was not different from the resting \( \text{pH}_H \), \( p = 0.3 \)). During \( \text{NH}_4\text{Cl} \) exposure, \( \text{pH}_H \) significantly decreased to \( 7.34 ± 0.01 \) in RhCG-expressing oocytes but was unchanged in control oocytes (\( 7.38 ± 0.03 \)). Using a 20 \( \mu M \) \( \text{NH}_4\text{Cl} \)-containing solution, we previously reported that the oocyte membrane is only slightly permeable to \( \text{NH}_4^+ \) but that initial \( \text{NH}_3 \) influx, \( \Phi_{\text{NH}_3} \), into the cell is reflected by a small but detectable cell alkalinization preceding the cell acidification due to \( \text{NH}_4^+ \) influx, \( \Phi_{\text{NH}_4^+} \) (18, 26). In the present study, application of 500 \( \mu M \) \( \text{NH}_4\text{Cl} \) had no effect on \( \text{pH}_H \) in control oocytes. This indicates that this \( \text{NH}_4\text{Cl} \) concentration is too low to induce any detectable \( \Phi_{\text{NH}_3} \) or \( \Phi_{\text{NH}_4^+} \) through the oocyte endogenous membrane pathways for ammonium. Thus, in the presence of 500 \( \mu M \) \( \text{NH}_4\text{Cl} \), the biphasic \( \text{pH}_H \) change observed in RhCG-expressing oocytes reflects enhanced \( \Phi_{\text{NH}_3} \) and \( \Phi_{\text{NH}_4^+} \) as compared with control oocytes. One may argue that RhCG expression has simply enhanced endogenous \( \text{NH}_3 \) and \( \text{NH}_4^+ \) transport in the oocyte. However, the saturation of \( I_{\text{AM}} \) as a function of \([\text{NH}_4\text{Cl}]\) (Fig. 4) does not agree with published characteristics of oocyte endogenous \( \text{NH}_3 \).
Functional Expression of RhCG in X. laevis Oocytes

**Fig. 7.** Stimulation of methylammonium-induced current by NH₃. In voltage-clamped RhCG-expressing oocytes (Vc = −50 mV), the effect of adding 50 μM NH₄Cl to a 500 μM methylammonium Cl (MeACl)-containing solution was studied at two different pHₕ values (7.5 and 8.5, as indicated above the graph). The current induced by 500 μM NH₄Cl was systematically recorded at the beginning of each experiment. At pHₕ 7.5, 50 μM NH₄Cl, 500 μM MeACl, and 50 μM NH₄Cl + 500 μM MeACl had no effect. At pHₕ 8.5, 500 μM MeACl induced an inward current at variance to 50 μM NH₄Cl. Adding 50 μM NH₄Cl to 500 μM MeACl stimulated the methylammonium-induced current by an amount similar to that measured at pHₕ 7.5 in the presence of 500 μM NH₄Cl. This increase in methylammonium-induced current is represented by diagonal lines on the gray background bar. Values are given as means ± S.E. (n = 14). The significance of the results was assessed by paired t test (*, p < 0.05) compared with the current measured in the presence of 500 μM NH₄Cl, pH 7.5. The difference $I_{\text{MMeA-MMeCl}} - I_{\text{MMeA}}$, obtained at pHₕ 8.5, was not significantly different from the current recorded at pHₕ 7.5 in the presence of 500 μM NH₄Cl ($I_{\text{MMeA-MMeCl}} - I_{\text{MMeA}} = 8.7 ± 1.3$ nA at pHₕ 8.5; n = 14; p > 0.6). The NH₄H₂NH₄ (pKₐ = 9.25) or H₃C-NH₂/H₃C-NH₃ (pKₐ = 10.65) contents of the solutions are indicated as follows. 500 μM NH₄Cl, pH 7.5: [NH₄⁺] = 8.7 μM and [NH₃⁻] = 491.3 μM; 50 μM NH₄Cl, pH 7.5: [NH₄⁺] = 0.87 μM and [NH₃⁻] = 49.1 μM; 50 μM NH₄Cl, pH 8.5: [NH₄⁺] = 7.55 μM and [NH₃⁻] = 42.4 μM; 500 μM MeACl, pH 7.5: [H₃C-NH₂] = 3.5 μM and [H₃C-NH₃] = 496.5 μM.

**Fig. 8.** Measurement of pHₙ of oocytes during NH₄Cl exposure. Original tracings obtained with intracellular pH-sensitive microelectrodes showing pHₙ change during exposure to a 500 μM NH₄Cl-containing solution, indicated by arrows. A, tracing obtained in an RhCG-expressing oocyte. Please note the slight intracellular alkalinization upon NH₄Cl exposure, followed by cell acidification. Similar results were obtained in nine oocytes. B, tracing obtained in an H₂O-injected oocyte. Please note the lack of pHₙ change during NH₄Cl exposure. Similar results were obtained in nine oocytes.

pathways (18), thus supporting our conclusion that RhCG expression induces a heterologous transport of both NH₃ and NH₄⁺.

Nonetheless, the interpretation of the significance of biphasic NH₄Cl-induced ΔpHₙ for RhCG function is not unequivocal. This ΔpHₙ is consistent with the influx of both NH₃ and NH₄⁺ but raises the question of whether the transport of NH₃ and NH₄⁺ is stoichiometrically coupled. The biphasic ΔpHₙ suggests that RhCG-mediated $\Phi_{\text{NH₃}}$ and $\Phi_{\text{NH₄⁺}}$ both vary during NH₄Cl exposure. This favors the hypothesis of uncoupled fluxes. Associated but uncoupled transport systems have been reported in the literature. For example, glutamate transporters are associated with thermodynamically uncoupled Cl⁻ channels (27), and glutamine transporter SN1 is associated with a non-stoichiometrically coupled proton current (28). However, the observed biphasic ΔpHₙ does not definitively rule out stoichiometric coupling of $\Phi_{\text{NH₃}}$ and $\Phi_{\text{NH₄⁺}}$. It could be that RhCG mediates a bidirectional NH₃ flux, as reported for AmtB protein (8). In that case, during NH₄Cl exposure, the initial cell alkalinization results from inward fluxes of both $\Phi_{\text{NH₃}}$ and $\Phi_{\text{NH₄⁺}}$, (the very partial dissociation of NH₃, only partly tempers the effect on pHₙ of the NH₄⁺ protonation, thus cell alkalinization). If NH₄⁺ reaches transmembrane equilibrium before NH₃, a proton shuttle would be induced (29). If RhCG mediates bidirectional NH₃ flux (as reported for AmtB protein (8)), a reduced NH₃ net influx will occur. Because the NH₃ net influx is higher than NH₄⁺ net influx, the cell acidifies. Additional studies are needed to determine whether RhCG-mediated NH₃ and NH₄⁺ fluxes are stoichiometrically coupled.

In summary, our study shows that, when expressed in X. laevis oocytes, RhCG induces ammonium transport by a novel mechanism: NH₄⁺ transport is enhanced by a mechanism depending on NH₃. Moreover, our results provide evidence that human Rh proteins may not all share a unique functional role: in RhCG-expressing oocytes, NH₄⁺ is involved in electrogenic NH₃ transport, whereas in RhAG-expressing oocytes, an NH₃-dependent, electroneutral NH₃/H⁺ antiporter was reported (12). Because our results demonstrate enhanced NH₄⁺ transmembrane transport in RhCG-expressing oocytes, RhCG may represent a facilitating pathway for transmembrane NH₃ diffusion. Other recent findings support the role of transmembrane proteins in gas transport (30–32), despite the widely admitted theory of free gas diffusion across biological membranes. When expressed in X. laevis oocytes, the human protein RhCG mediates a highly specific ammonium transport with
high substrate affinity but with complex behavior meriting further investigation to determine the mechanism of RhCG effects. RhCG function in the kidney remains to be investigated.

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NH$_3$ Is Involved in the Transport Induced by the Functional Expression of the Human Rh C Glycoprotein

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