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Modulation of electroneutral Na transport in sheep rumen epithelium by luminal ammonia

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Abdoun, Khalid, Friederike Stumpff, Katarina Wolf, and Holger Martens. Modulation of electroneutral Na transport in sheep rumen epithelium by luminal ammonia. *Am J Physiol Gastrointest Liver Physiol* 289: G508–G520, 2005. First published April 14, 2005; doi:10.1152/ajpgi.00436.2004.—Ammonia is an abundant fermentation product in the forestomachs of ruminants and the intestine of other species. Uptake as NH_3 or NH_4^+ should modulate cytosolic pH and sodium-proton exchange via Na^+/H^+ exchanger (NHE). Transport rates of Na^+, NH_4^+, and NH_3 across the isolated rumen epithelium were studied at various luminal ammonia concentrations and pH values using the Ussing chamber method. The patch-clamp technique was used to identify an uptake route for NH_4^+. The data show that luminal ammonia inhibits electroneutral Na transport at pH 7.4 and abolishes it at 30 mM ($P < 0.05$). In contrast, at pH 6.4, ammonia stimulates Na transport ($P < 0.05$). Flux data reveal that at pH 6.4, ~70% of ammonia is absorbed in the form of NH_4^+, whereas at pH 7.4, uptake of NH_3 exceeds that of NH_4^+ by a factor of approximately four. The patch-clamp data show a quinidine-sensitive permeability for NH_4^+ and K^+ but not Na^+. Conductance was 135 ± 12 pS in symmetrical NH_4Cl solution (130 mM). Permeability was modulated by the concentration of permeant ions, with $P_K > P_{NH4}$ at high and $P_{NH4} > P_K$ at lower external concentrations. Joint application of both ions led to anomalous mole fraction effects. In conclusion, the luminal pH determines the predominant form of ammonia absorption from the rumen and the effect of ammonia on electroneutral Na transport. Protons that enter the cytosol through potassium channels in the form of NH_4^+ stimulate and nonionic diffusion of NH_3 blocks NHE, thus contributing to sodium transport and regulation of pH.

rumen; ammonia; sodium transport; sodium-hydrogen exchanger; potassium channel

AMMONIA IS PRODUCED IN ALL sections of the gut in animals and humans as a result of the microbial degradation of nitrogenous compounds and of the hydrolysis of recycled urea. Concentrations of ammonia in the ruminal fluid have been reported to range from 4 to 70 mM (11) in ruminants, and >50% of the daily nitrogen intake is absorbed in the form of ammonia (45).

Ammonia influences a variety of epithelial functions. In T84 human intestinal crypt epithelia, ammonia blocks a K^+ channel required for maintaining the driving force for electrogenic apical chloride secretion (30). Acid secretion is reduced in bullfrog oxyntic cells by the possible involvement of an apical K channel (28). Na transport is impaired in rat colon by NH_4^+ competing with the Na-binding site of the Na^+/H^+ exchanger (NHE) (13). Cougnon et al. (17) have shown that NH_4^+ is transported by the colonic H^+/K^+-ATPase of the rat expressed in *Xenopus* oocytes.

Ammonia absorption takes place in the rumen (6), small intestine (41), and the colon (15) and occurs primarily by simple diffusion of the nonionized lipid-soluble NH_3 form. However, ionic diffusion (NH_4^+) has also been demonstrated (5, 8, 12, 15).

In the kidney (31, 52), where NH_4^+ has to be transported against a considerable concentration gradient from the renal cortex (<0.1 mM) to the medulla and, ultimately, the urine, transport of NH_4^+ has to be coupled to other ions and replaces other cations in transport proteins such as the NHE3, Na-K-2Cl exchange, K/H exchangers, or NH_4^+/H^+ exchangers belonging to the Rh gene family (31, 52). Conversely, in the rumen and the colon, where NH_4^+ is in abundant supply, transport via channels is energetically feasible and would explain the increase in short-circuit current observed both in rat colon and rumen epithelium after exposure to this ion (5, 14).

It is generally known that cellular uptake of ammonia affects intracellular pH (pH_i) (7, 46). Predominant NH_3 uptake tends to alkalinize the cytoplasm, whereas the predominant uptake of NH_4^+ acidifies it. The magnitude and direction of this change in pH_i depends on the relative transport rates of NH_3 and NH_4^+, which can be altered by the luminal pH according to the Henderson-Hasselbalch equation. The wide variations in ruminal ammonia concentrations (up to 70 mM) (11) and of pH (5.4–7.4) cause corresponding alterations of NH_3 and NH_4^+ concentrations and flux rates across the rumen epithelium (25). Note that in rumen, NH_4^+ transport can also be influenced by changes in the potential difference (PD) across the apical membrane (PD_a) (5), whereas exchangers are typically not affected by membrane potential (54).

Alteration of pH_i is well known to affect cellular transport of Na^+ via NHE in various systems (7, 46), including the rumen epithelium (42), and a modulation of Na transport is to be anticipated. In a recent study (1), it was demonstrated that interaction between ammonia and Na^+ transport across the isolated rumen epithelium of sheep is modulated by the diet.

The mechanisms underlying this adaptation to an increase in the uptake of nitrogen (urea or protein) are poorly understood. However, it should be noted that luminal application of ammonia induced a significantly higher increase in short-circuit current (I_{sc}) in tissues of concentrate-fed animals than in epithelia of hay-fed animals (1). Therefore, we suggested that the ammonia-induced change in I_{sc} is caused by the uptake of NH_4^+ in its protonated form. Dissociation of NH_4^+ within the cytosol should increase the amount of H^+ available for exchange with sodium.

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Alkalinization of ruminal epithelial cells by uptake of ammonia in the form of NH_3 was demonstrated by Müller et al. (42), whereas experiments by Bödeker and colleagues (5, 6) show uptake of NH_4^+. In these experiments, the ammonia-dependent increase of I_{sc} was abolished by pretreatment with mucosal quinidine, and the authors suggested involvement of a potassium channel (5, 6). There are conflicting reports on the permeability of the apical membrane of rumen epithelium for potassium, depending on whether luminal potassium was high (20, 35, 50), or low (21, 35). This question obviously needs further clarification.

It was therefore the aim of the present study *1*) to determine the flux of ammonia through the rumen epithelium, (*2*) to change the relative flux rates (NH_4^+ or NH_3) by variation of luminal pH and PD_a, *3*) to look for effects of altered ammonia flux rates on Na transport, and *4*) to characterize the NH_4^+ conductance.

The patch-clamp data in this study show that ruminal epithelial cells express quinidine-sensitive channels of 130 pS that conduct both potassium and NH_4^+ but not sodium. Relative permeability of the membrane for potassium rose with concentration. Anomalous mole-fraction effects (29) between K^+ and NH_4^+ suggest competition for a common pathway that may limit apical potassium efflux from the rumen epithelium under in vivo conditions.

Ussing chamber data demonstrate that variations of luminal pH determine the form of ammonia transport as NH_4^+ or NH_3 and consequently affect the absorption of Na^+ depending on the transported form of ammonia. Predominant uptake of NH_4^+ through potassium channels stimulated and predominant diffusion of NH_3 inhibited Na transport across the rumen epithelium of sheep. Under physiological conditions (pH 6.50 or lower), ammonia stimulates Na transport and may be a modulator of Na absorption via NHE, significantly contributing to the classic effect of short-chain fatty acids (24, 33).

MATERIALS AND METHODS

The incubation of rumen epithelium has been described in detail by Martens et al. (39). Briefly, sheep were killed in a local slaughterhouse, and the reticulorumen was removed from the abdominal cavity within 2–3 min. A 250-cm^2 piece of rumen wall was taken from the ventral sac, cleaned in a buffer solution, stripped from the muscle layer, and taken (some 20 min) to the laboratory in a buffer solution maintained at 38°C. The buffer was gassed with 95% O_2-5% CO_2. Pieces of the epithelium (3 × 3 cm) were mounted between the two halves of an Ussing chamber to give an exposed area of 3.14 cm^2.

The mounted tissues were bathed on each side with 18 ml buffer solution by using a gas lift system and were gassed with 95% O_2-5% CO_2 or 100% O_2 (HCO_3^--free buffer solutions) at 38°C. The standard electrolyte solution contained (in mM) 90 Na^+, 5 K^+, 1 Ca^{+2}, 2 Mg^{+2}, 25 HCO_3^-, 59 Cl^-, 1 $H_2PO_4^-$, 2 HPO_4^{2-}, 25 acetate, 10 propionate, 5 butyrate, 10 glucose, and 30 D(−)-*N*-methylglucamine-hydrochloric acid (NMDGCl). In ammonia containing buffer solutions, NMDGCl was replaced by equimolar NH_4Cl (5, 15, and 30 mM). In HCO_3^--free buffer solutions, HCO_3^- was replaced by gluconate, and the solutions were buffered with HEPES (8 mM). Amiloride was obtained from Sigma (Munich, Germany). Radioisotopes (^{22}Na and ^{36}Cl) were purchased from Amersham (Braunschweig Germany). All reagents were of analytical grade.

Electrical Measurements and Calculation of Na and Cl Flux Rates

For details, see Martens et al. (38). The transepithelial conductance (G_t) was determined by briefly applying a 100-μA current across the tissue in both directions and measuring the resulting change in transmural potential difference (PD_t), from which G_t can be calculated using Ohm's law. The sum of all electrogenic ions moving across the tissue was determined by measuring the external current (equivalent to I_{sc}) necessary for clamping PD_t to zero.

Fluxes were measured in the short-circuit mode. ^{22}Na and ^{36}Cl (80 and 100 kBq, respectively) were added to the "hot" side of the epithelium, and three flux periods of 30 min were performed after an equilibration time of 30 min. Samples from the "hot" site were taken before the first and after the last flux period for the calculation of the specific radioactivity. The sample volume was replaced by the corresponding buffer. ^{22}Na and ^{36}Cl fluxes were determined in separate experiments. Total ammonia flux {mucosal-to-serosal flux [J_{ms} (NH_4^+ + NH_3)]} was calculated from aliquots taken from the serosal side at the beginning and the end of the flux period and determined directly by using an ion analyzer (gas-sensitive electrode; EA 940; Orion).

Radioactivity

^{22}Na and ^{36}Cl were assayed by using a well-type crystal counter (LKB Wallace-Perkin-Elmer, Überlingen, Germany) and a β-counter (LKB Wallace-Perkin-Elmer), respectively.

Statistics

Statistical evaluations were carried out by using the SPSS program version 10.0 for Windows. Results are given as means ± SE. The comparison between the groups was carried out in the form of a repeated-measurement analysis of variance with a two-factorial model without interaction. P values <0.05 were considered significant. N refers to the number of experimental animals, and n refers to the number of tissues.

Patch-clamp experiments. Cells from rumen epithelium were cultured on glass coverslips according to established methods (36, 49). Briefly, the stratum corneum (26) was removed from ruminal papillae by fractional trypsinisation, and the last (fourth or fifth) fraction was plated out in cell culture dishes. The number of cornified cells in the culture increased rapidly after seeding. Coverslips were removed from the culture dishes for experiments 3–8 days after seeding or, alternately, 1–5 days after reseeding from primary culture. Previous studies in our lab suggest that cells at this stage have differentiated and express proteins that are found in the apical membrane of ruminal epithelium (36, 48).

Immediately before use, cells were gently cleansed with trypsin (0.02%, Biochrome, Berlin, Germany) for 1 min and introduced into a perfusion chamber on the stage of an inverted microscope where they were superfused by warmed Ringer solution (37.5°C; TC01 and PH01, Lohmann Research Equipment, Castrop-Rauxel, Germany). All patch-clamp experiments were performed essentially as in a previous study (36). Only cells in which washout occurred, demonstrating seal stability, were used for evaluation. Capacitance was measured regularly; cells in which swelling occurred were excluded. Note that usually the seals of such cells ruptured quickly, and stable measurements with washout could not be obtained.

Currents were recorded using an EPC 9 patch-clamp amplifier (HEKA Elektronic, Lambrecht, Germany). Pulse generation, data collection, and analysis was performed using TIDA for Windows software (HEKA Elektronic) and filtered with a 2.9-kHz Bessel filter. Records were corrected for capacitance. Positive ions flowing into the pipette correspond to a negative current and are depicted in figures as going downward. For inside-out patches, the pipette potential corresponds to the negative membrane potential.

Two types of pulse protocols were used. Either current response was recorded at 100 Hz using a protocol that generated steps of 200-ms duration to voltages between −120 and 100 mV in 20-mV steps, returning to a holding potential of −40 mV for 200 ms in between ["pulse *protocol I*" (36)]. This protocol was repeated continuously to allow the monitoring of current responses of the cells to

changes in external solution. In addition, conventional voltage pulse protocols were used that recorded data at a much higher sampling rate of 5 kHz ["pulse *protocol II*" (36)]. As before, holding potential was −40 mV, and voltages ranged from −120 to 100 mV, but the step size was 10 mV. For single-channel experiments, data were sampled at 10 kHz.

Solutions and Chemicals (Patch Clamp)

Pipette solution for whole cell experiments, designated as "K-gluconate" contained (in mM) 1 KH_2PO_4, 10 HEPES, 0.8 $CaCl_2$, 0,9 $MgSO_4$, 5 EGTA, 123 K-gluconate, and 10 NaCl. Extracellular NaCl solution contained the following ion concentrations (in mM): 130 NaCl, 1 NaH_2PO_4, 5 KCl, 10 HEPES, 1.7 $CaCl_2$, and 0.9 $MgCl_2$. With the use of this basic recipe, NaCl was substituted by either choline chloride or NH_4Cl or KCl in the solutions designated by these ions. In solutions labeled "NH_4" (65 mM), "K" (65 mM), or "NH_4 + K", the chloride salts of the designated cations (each at 65 mM) were used to substitute for NaCl. Osmolarity was adjusted with choline chloride (~65 mM). In experiments in which $BaCl_2$ and TEACl were added to the solutions, osmolarity of the different solutions was adjusted by mannitol.

For inside-out single-channel experiments, standard 130 mM NH_4Cl solution was used to fill the pipette (facing the external side of the membrane), whereas calcium was reduced in the KCl bath solution facing the cytosolic side and contained (in mM) 130 KCl, 3 NaCl, 5 EGTA, 0.9 $MgSO_4$, 0.8 $CaCl_2$, 10 HEPES, and 1 KH_2PO_4. The solutions designated as NH_4Cl, NaCl, choline chloride, or K-gluconate were equal in composition but contained the respective ions instead of KCl. Liquid junction potentials were corrected according to established methods (3).

Analysis

To compare whole cell data from different cells with each other, initial inward and outward current in NaCl solution at −120 and 100 mV were assigned the value of 100%. All other currents were seen in relationship to these values. Significance testing was performed using the paired Student's *t*-test and standard software. The number *n* refers to the number cells used; care was taken to include cells from different animals.

Reversal potentials were estimated by linear regression between the zero-current values just above and just below the zero level for each cell and corrected for liquid junction potential. Relative permeability ratios (P_b/P_a) were calculated by switching the major ion in the external solution, measuring the corresponding reversal potential (E_{rev}), and using Goldman-Hodgkin-Katz theory (29): $E_{rev,b} - E_{rev,a} = RT/zF \times \ln(P_b[B]_o)/(P_a[A]_o)$.

In single-channel experiments, current-voltage relationships were fitted using the Goldman-Hodgkin-Katz equation for two ions (29): $I = E \times F^2/(R \times T) \times (P_{NH4}[NH_4]_i + P_K[K]_i - (P_{NH4}[NH_4]_o + P_K[K]_o) \times \exp\{[-E \times F/(R \times T)]/[1 - \exp(-E \times F/(R \times T)]\}$ and standard software (SigmaPlot for Windows, 8.02).

RESULTS

Effect of Increasing Luminal Ammonia Concentrations on I_{sc}

In a first series of experiments, the effect of adding different concentrations of ammonia to the luminal side of rumen epithelium was studied using the Ussing chamber technique. At the end of the equilibration period, the I_{sc} and the total tissue G_t exhibited constant values of 0.68–0.80 $\mu eq \cdot cm^{-2} \cdot h^{-1}$ and 2.13–2.68 mS/cm^2, respectively, reflecting electrogenic sodium transport (34, 36). This did not change significantly during the next 30 min in control tissues. As in the study by Bödeker and Kemkowski (5), luminal ammonia caused a significant increase of I_{sc} up to 0.45 $\mu eq \cdot cm^{-2} \cdot h^{-1}$ (Fig. 1). A new steady state of I_{sc} was obtained after 10–15 min, and the rise of I_{sc} (ΔI_{sc}) exhibited saturation kinetic properties (Fig. 1; pH 7.4).

This change of I_{sc} was pH dependent and was related to the luminal ammonia concentration. At pH 6.4, effects of an influx of NH_3 should be lowest. However, I_{sc} changed most at this pH, suggesting influx of ammonium in the ionized form.

Determination of Ammonia Flux Rates

It is impossible to distinguish between transport of NH_4^+ through a potassium channel and of Na^+ via the electrogenic pathway (34, 36) by measuring I_{sc} alone. In addition, no information on the transport of ammonia as NH_3 is obtained. Therefore, ammonia flux rates were measured directly.

Total ammonia was kept constant (30 mM), and concentration of NH_3 varied by increasing the pH from 6.4 to 6.9 and 7.4. This should induce an increasing gradient in NH_3 concentration at an almost constant concentration of NH_4^+. At pH 6.4, a steady-state ammonia flux [J_{ms} (NH_3 + NH_4^+)] of 0.82 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ could be measured from the mucosal to the serosal side. An increase in pH on the luminal side of the membrane resulted in a significant ($P < 0.05$) stimulation of ammonia flux rates (Fig. 2).

The intercept (*y*-axis, $NH_3 = 0$) represents the flux of NH_4^+ and shows that it has the same magnitude (0.7 $\mu eq \cdot cm^{-2} \cdot h^{-1}$) as the ammonia-dependent increase of I_{sc} at pH 6.4 (Fig. 1). Thus it appears that at a pH of 6.4, ammonia is predominantly transported as NH_4^+ across the apical membrane and should be seen as the major ion responsible for the increase in I_{sc}. At pH 6.9, total flux is doubled and the flux rates of NH_3 and NH_4^+ are almost equal. The total flux of ammonia is 3.41 ± 0.53 $\mu eq \cdot cm^{-2} \cdot h^{-1}$ at pH 7.4, and hence, NH_3 transport clearly exceeds that of NH_4^+. This change in ammonia transport should influence pH_i (42) and, possibly, Na transport via NHE.

Effect of Ammonia on Na and Cl Transport at pH 7.4

Increasing luminal ammonia concentrations in a CO_2/HCO_3^--free buffer at a luminal pH of 7.4 significantly ($P < 0.05$) decreased the mucosal-to-serosal flux of sodium [J_{ms} (Na)].

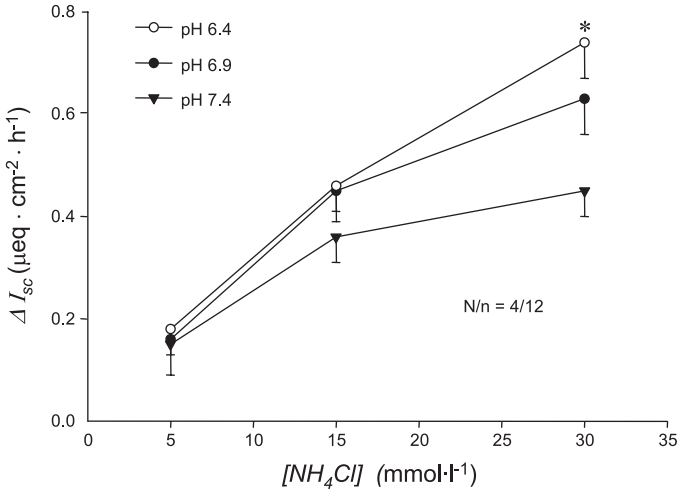

Fig. 1. Ammonia-dependent increase of short-circuit current (ΔI_{sc}) at various luminal pH. ΔI_{sc} is significantly higher at pH 6.4 compared with pH 7.4 at 30 mM ammonia (*$P < 0.05$).

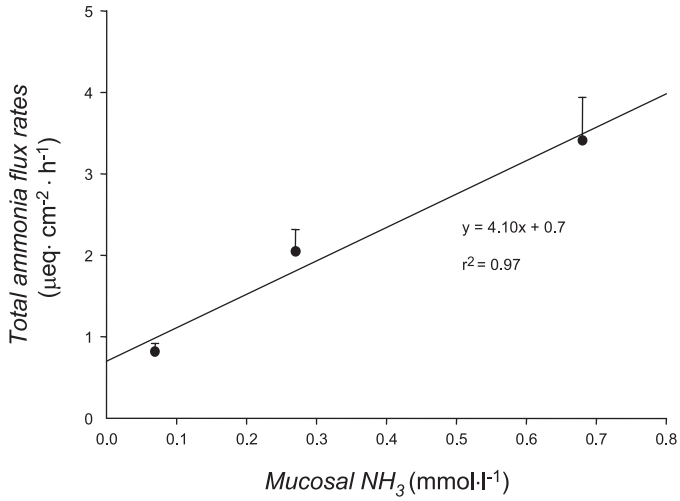

Fig. 2. Total ammonia flux in the mucosal-serosal direction at 30 mM ammonia in the luminal solution at pH 6.4 (0.07 mM NH_3), 6.9 (0.27 mM NH_3), and 7.4 (0.68 mM NH_3).

As a consequence, the net flux [J_{net} (Na)] ($P < 0.05$) decreased from 2.01 ± 0.11 (control) to 0.74 ± 0.12 μeq·cm $^{-2}$·h^{-1} at 30 mM NH_4Cl (Table 1).

This inhibitory effect of ammonia on J_{net} (Na) showed a curvilinear relationship with Michaelis-Menten-type kinetics, which allowed the calculation of K_m (8.00 mM) and of V_{max} (maximum inhibition = 1.58 μeq·cm^{-2}·h^{-1}; Fig. 3).

Ammonia (30 mM) obviously abolished electroneutral Na transport via NHE (see DISCUSSION). This is consistent with the predominant flow of NH_3 at pH 7.4. The inhibitory effect of ammonia was also observed with a CO_2/HCO_3^--containing buffer solution (Table 2).

An increase of pH_i may influence ruminal Cl transport, which is mediated via apical Cl^-/HCO_3^- exchange (32) and coupled to NHE by pH_i. Indeed, luminal ammonia (30 mM) significantly ($P < 0.05$) enhanced J_{ms} (Cl) and J_{net} (Cl)$_{net}$ from 1.39 ± 0.33 to 3.09 ± 0.30 μeq·cm $^{-2}$·h^{-1} in a CO_2/HCO_3^--containing buffer solution (Table 2).

Effect of Ammonia on Na and Cl Transport at pH 6.9 or 6.4

The above results support the preliminary conclusion that at pH 7.4, ammonia enters the cells predominantly via diffusion of the lipophilic form NH_3. Lowering the luminal pH decreases the concentration and diffusion of NH_3 and should diminish effects on pH_i. Two series of experiments (luminal pH of 6.9 and 6.4) were performed to test this hypothesis. Ammonia did not influence Na fluxes at pH 6.9 (Table 3).

In contrast, ammonia significantly ($P < 0.05$) stimulated Na transport at pH 6.4 (Table 4). J_{ms}(Na) and J_{net}(Na) increased from 6.01 ± 0.42 and 4.37 ± 0.42 to 7.90 ± 0.47 and 6.50 ± 0.50 μeq·cm^{-2}·h^{-1}, respectively. Luminal ammonia (30 mM) did not show any significant effect on Cl flux rates at pH 6.40 (Table 4).

Note the different responses of I_{sc} and G_t to ammonia. To measure I_{sc}, PD_t was clamped to zero, and an activation of voltage-dependent currents appears unlikely. I_{sc} showed an almost linear correlation with the concentration of ammonia at pH 6.4 (where influx of NH_3 is minimal; Table 4 and Fig. 1). Conversely, the nonlinear increase of G_t with the concentration of ammonia may reflect activation of voltage-dependent currents (34, 36).

Effect of PD_t

Because NH_4^+ is charged, uptake across the luminal membrane through a K channel should be modulated by the PD_a. PD_a was altered by variation of PD_t (34), and the effect on the transport of Na both in the presence and the absence of luminal ammonia (30 mM) was studied. All solutions were titrated to a pH of 6.4, so that ammonia should be present almost exclusively in the ionized form.

Imposing a PD_t of + 25 mV (serosal side positive) significantly depolarizes PD_a by 15 mV from some −50 to −35 mV (34). This PD_t is too low to induce significant stimulation of electrogenic sodium transport (34) against a high background of electroneutral sodium transport (stimulated by presence of short-chain fatty acids in the current study).

In line with this, a PD_t of +25 mV only led to a small, nonsignificant reduction of J_{ms} (Na) and J_{net} (Na) (Table 5). Likewise, serosal-to-mucosal flux (J_{sm}) (Na) was not significantly changed either by changing PD_t or by the addition of ammonia. All changes probably represent effects on paracellular, passive flow.

As before, ammonia (30 mM) stimulated J_{ms} (Na) and J_{net} (Na), both under short-circuit conditions and at +25 mV ($P <$ 0.05 vs. control).

However, in the presence of ammonia (30 mM), J_{ms} (Na) and J_{net} (Na) could be reduced significantly by increasing PD_t from 0 to +25 mV ($P < 0.05$). The potential-induced decrease in J_{net}(Na) (J_{net} at PD_t 0 mV − J_{net} at PD_t +25 mV) was significantly greater in the presence of ammonia (1.59 ± 0.27 μeq·cm^{-2}·h^{-1}) than in its absence (0.77 ± 0.28 μeq·cm^{-2}·h^{-1}). This suggests that depolarization of the apical membrane reduced the influx of NH_4^+ needed to stimulate the NHE in the presence of ammonia.

Table 1. *Effect of increasing luminal ammonia concentrations on Na flux rates, I_{sc}, and G_t at luminal pH 7.4 in the absence of CO_2/HCO_3^- (HEPES buffer)*

	Na					
NH_4Cl, mM	J_{ms}	J_{sm}	J_{net}	I_{sc}	G_t	*N/n*
0	2.84±0.09	0.82±0.08	2.01±0.11	0.61±0.13	1.99±0.12	4/5
5	2.17±0.01*	0.77±0.04	1.40±0.09*	0.81±0.05	2.00±0.09	4/6
15	1.63±0.11*	0.64±0.09	0.99±0.06*	0.87±0.07	2.01±0.22	4/6
30	1.43±0.19*	0.69±0.10	0.74±0.12*	1.02±0.11*	2.24±0.32*	4/6

Values are means ± SE; *N* is no. of animals; *n* is no. of tissues. *$P < 0.05$ for comparison with the control group (0 mM NH_4Cl); Na flux rates, CI flux rates, and short circuit current (I_{sc}) (μeq·cm^{-2}·h^{-1}); G_t, transepithelial conductance (mS/cm^2); J_{ms}, mucosal-to-serosal flux; J_{sm}, serosal-to-mucosal flux; J_{net}, net flux.

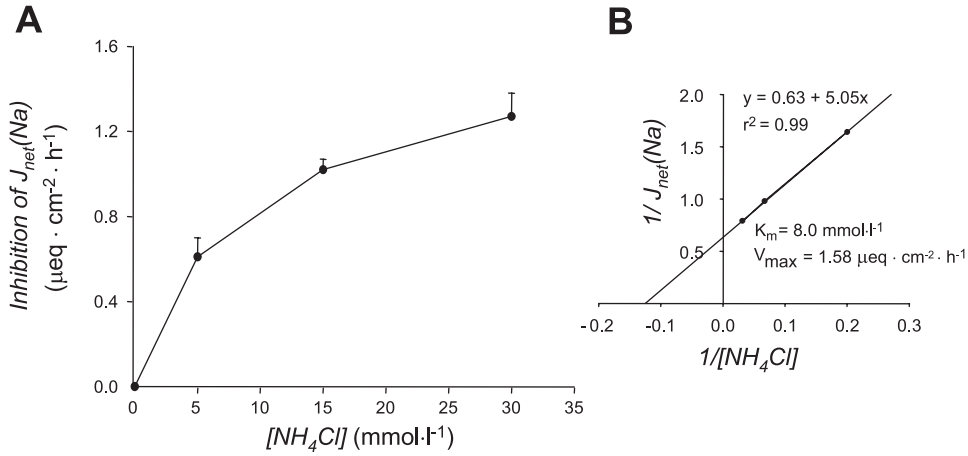

Fig. 3. *A*: inhibition of net flux (J_{net}) (Na) by increasing luminal ammonia concentrations at pH 7.4. *B*: double reciprocal plot that reveals a Michaelis-Menten constant of 8.00 mM and a maximal inhibition of 1.58 μeq·cm^{-2}·h^{-1}.

Effect of Amiloride

The assumption that the stimulatory effect of ammonia (30 mM) on Na transport at pH 6.4 is caused by enhanced NHE activity was tested by blocking this exchanger by 1 mM mucosal amiloride (38), completely blocking the effects of ammonia on net Na transport (Table 6). Note that amiloride had no effect on I_{sc}.

Effect of Mucosal K

To test for mutual inhibition of NH_4^+ and K^+ in a common pathway, we studied the modulation of Na transport by K. Luminal solutions contained 15 mM ammonia at pH 6.4. Increasing luminal K concentration from 5 (control) to 45 mM caused a significant reduction of J_{ms} (Na) from 8.70 ± 0.67 (control) to 6.01 ± 0.65 μeq·cm^{-2}·h^{-1}, respectively, with concurrent significant reduction of J_{net}(Na). J_{sm}(Na) remained unchanged (Table 7).

Patch-clamp Experiments

Patch-clamp experiments in the whole cell configuration. K-GLUCONATE PIPETTE SOLUTION: EFFECT OF NH_4Cl. In a first series of experiments, rumen epithelial cells were brought into the whole cell configuration using the K-gluconate solution described in the MATERIALS AND METHODS (Fig. 4).

Cells were superfused with high-sodium (NaCl) solution until current had stabilized, indicating replacement of the cytosolic fluid with the pipette solution. Mean inward current density at −120 mV was −12 ± 3 pA/pF, mean outward current density at +100 mV was 32 ± 8 pA/pF (n = 33), in good agreement with a previous study (36).

When NaCl was replaced by NH_4Cl (130 mM) in the external solution, inward current rose to 189 ± 17% of the original value (100%) measured in NaCl solution at a pipette potential of −120 mV ($P < 0.01$, n = 17). Outward current at +100 mV rose to a mean level of 132 ± 10% (P = 0.007, n = 17; Fig. 4*A*). After washout of NH_4Cl with NaCl, inward and outward currents recovered to 112 ± 12 and 102 ± 15%, respectively, of the original values in NaCl solution (P = 0.3 and P = 0.9, no significant difference to the value before application of ammonia). Reversal potential rose in all cells studied from a mean value of −33 ± 4 (NaCl) to −6 ± 4 mV (NH_4Cl; n = 17, $P < 0.001$) and back to −25 ± 3 mV (NaCl; $P < 0.01$).

Conversely, replacement of NaCl in the bath solution with choline chloride did not induce significant changes in either inward current (85 ± 12%, n = 7, P = 0.2), outward current (88 ± 9%, P = 0.9), or reversal potential level (−25 ± 7 mV, P = 0.7), in line with a previous study (36).

Exposure to $BaCl_2$ (5 mM), known to block potassium channels, resulted in a significant decrease in NH_4Cl-induced inward current to 70 ± 9% (n = 7, $P < 0.05$, −120 mV) of the level in NH_4Cl solution without $BaCl_2$ (100%), whereas outward current dropped to 77 ± 7% (n = 7, $P < 0.01$, +100 mV) of the original outward current level. Conversely, TEACl (10 mM) had no significant effect on the inward current induced by NH_4Cl (102 ± 20%, n = 4, P = 0.9), whereas outward current dropped slightly to 80 ± 7% (n = 4, $P < 0.05$). Reversal potential was not significantly altered by either the addition of $BaCl_2$ (3 ± 8 mV, n = 7, P = 0.1) or TEA (−10.3 ± 15 mV, n = 4, P = 0.05) to NH_4Cl bath solution.

K-GLUCONATE PIPETTE SOLUTION: EFFECT OF KCL. Similar effects were observed when NaCl bath solution was replaced with KCl bath solution (Fig. 4*B*). This induced a rise of both inward current and outward current to 344 ± 66% (n = 8, P = 0.007, −120 mV) and 218 ± 80% (n = 8, P = 0.003, +100 mV), respectively, with reversal potential rising to −10 ± 7 mV (n = 8, P = 0.01).

Table 2. *Effect of increasing luminal ammonia concentrations on CI flux rates, I_{sc} and, G_t at luminal pH 7.4 (CO_2/HCO_3^- buffer)*

	Cl					
	J_{ms}	J_{sm}	J_{net}	I_{sc}	G_t	N/n
0	3.76±0.60	2.37±0.35	1.39±0.33	1.15±0.08	3.39±0.34	4/8
30	5.31±0.29	2.22±0.32	3.09±0.30*	1.59±0.09*	3.72±0.29	4/8

Values are means ± SE. For corresponding data on Na fluxes, see Ref. 1. *$P < 0.05$.

Table 3. *Effect of increasing luminal ammonia concentrations on Na flux rates, I_{sc}, and G_t at luminal pH 6.9 (CO_2/HCO_3^- buffer)*

NH_4Cl, mM	Na			I_{sc}	G_t	N/n
	J_{ms}	J_{sm}	J_{net}			
0	5.46±0.85	1.24±0.09	4.22±0.91	0.89±0.13	1.96±0.19	4/6
5	5.73±0.86	0.95±0.09*	4.78±0.81	1.00±0.12	2.01±0.13	4/6
15	5.80±0.59	0.94±0.08*	4.86±0.53	1.24±0.17	2.23±0.18	4/6
30	5.13±0.86	0.88±0.07*	4.25±0.85	1.46±0.14*	2.38±0.15	4/6

Values are means ± SE; *$P < 0.05$ for comparison with the control group (0 mM NH_4Cl); Na flux rates and I_{sc} ($\mu eq \cdot cm^{-2} \cdot h^{-1}$); G_t (mS/cm^2).

In KCl solution, TEACl (10 mM) blocked KCl^--induced inward and outward current to values of 58 ± 20 ($n = 4$, $P < 0.05$, −120 mV) and 66 ± 18% ($n = 4$, $P < 0.01$, +100 mV), respectively. Effects of TEACl on cells in NaCl solution did not reach significance level ($n = 3$). The effects of $BaCl_2$ (5 mM) were less pronounced, with inward current dropping to 72 ± 17% ($n = 4$, $P < 0.05$) of the original level in the presence of KCl, whereas changes in outward current did not reach significance level [92 ± 7% ($n = 4$, $P < 0.05$)]. The currents were also blocked by quinidine (100 μM) with inward current sinking to 82 ± 3% ($n = 3$, $P < 0.05$), outward current to 47 ± 23% ($n = 3$, $P < 0.05$), whereas reversal potential remained the same as in KCl solution.

K-GLUCONATE PIPETTE SOLUTION: INTERACTION OF KCL AND NH_4CL AT PH 6.4. Ussing chamber experiments suggest that influx of NH_4^+ into ruminal epithelial cells is inhibited by application of K^+. To test for the hypothesis that K^+ and NH_4^+ use a common pathway, cells were exposed to solutions containing either 65 mM NH_4Cl, 65 mM KCl, or a mix of both ions (osmolarity adjusted with choline chloride). All solutions were titrated to a pH of 6.4, at which the concentration of NH_3 should be minimal (Fig. 4*C*).

Exchanging pH of external NaCl solution from 7.4 to 6.4 did not significantly alter either inward current, outward current, or reversal potential (−33 ± 2 mV, $n = 8$, $P = 0.1$). Application of NH_4Cl (65 mM) increased inward current (−120 mV) from 100 to 167 ± 19% ($n = 6$, $P = 0.01$), whereas changes in outward current did not reach significance (116 ± 8%, $P = 0.1$). Reversal potential rose from −33 ± 2 to −24 ± 2 mV ($P = 0.02$), significantly less than that obtained with 130 mM of NH_4Cl [P (unpaired) = 0.009]. Conversely, currents at −120 mV were not significantly different from those observed in solutions containing 130 mM NH_4Cl (Fig. 4*C*), suggesting effects of saturation.

The application of KCl (65 mM) increased mean inward current to 180 ± 21% [$n = 4$, P (paired) = 0.03], whereas outward current and reversal potential only rose slightly to 137 ± 16% ($P = 0.2$) and −26 ± 4 mV ($P = 0.1$), respectively. These values are not significantly different from those obtained with NH_4Cl (65 mM) solution.

If the independence principle holds (29), application of a mix of NH_4Cl (65 mM) and KCl (65 mM) should result in additive depolarization. Indeed, after application of a mix of both salts, cells depolarized to an end value of −18 ± 2 mV ($P = 0.004$ vs. NaCl) in KCl + NH_4Cl solution or by a difference of 15 ± 9 mV. This value corresponds roughly to the numeric sum of the depolarizations obtained with NH_4Cl (65 mM) and KCl (65 mM; 12 ± 5 mV, $P = 0.3$) as calculated for the individual cells. However, the current responses to NH_4Cl + KCl solution were much lower than those predicted by the independence principle. Inward current rose relative to NaCl, reaching 175 ± 27% ($n = 7$, $P = 0.03$). Relative to NH_4Cl (65 mM), inward current dropped in three cells in KCl + NH_4Cl solution (Fig. 4*C*), rose slightly in two others, and remained unaltered in one cell, with no significant net effect on the mean value. Independence theory would have predicted a rise in all cells by ~80%. A rise of this magnitude was not observed in any of the cells.

NH_4Cl + KCl solution did not induce significant changes in outward current level vs. NaCl solution (112 ± 11%, $P = 0.3$). However, a decrease versus the level in NH_4Cl (65 mM) solution could be observed, with outward current dropping visibly in five of six cells (to 85 ± 5% of the level in NH_4Cl (100%; $n = 5$, $P = 0.04$; Fig. 4*C*).

Further reduction of NH_4Cl concentration to 32.5 mM (choline replacement) resulted in a smaller, but still clearly visible, induction of inward current ($n = 2$).

Table 4. *Effect of increasing luminal ammonia concentrations on Na and CI flux rates, I_{sc}, and G_t at luminal pH 6.4 (CO_2/HCO_3^- buffer)*

NH_4Cl, mM	J_{ms}	J_{sm}	J_{net}	I_{sc}	G_t	N/n
			Na			
0	6.01±0.42	1.64±0.12	4.37±0.42	1.10±0.05	2.98±0.29	5/7
5	7.24±0.41	1.66±0.08	5.58±0.36	1.33±0.08	2.99±0.28	5/8
15	7.50±0.64*	1.58±0.16	5.92±0.67*	1.49±0.11	3.00±0.25	5/7
30	7.90±0.47*	1.40±0.12	6.50±0.50*	1.89±0.21*	3.34±0.38*	5/8
			Cl			
0	5.93±0.63	2.67±0.18	3.26±0.09	1.09±0.09	3.27±0.26	4/8
30	6.42±0.69	2.41±0.23	4.01±0.55	1.73±0.05*	3.50±0.30	4/8

Values are mean ± SE; *$P < 0.05$ for comparison with the control group (0 mM NH_4CI); Na flux rates, CI flux rates, and I_{sc} ($\mu eq \cdot cm^{-2} \cdot h^{-1}$); G_t (mS/cm^2).

Table 5. *PD-dependent effect of ammonia on Na flux rates ($\mu eq \cdot cm^{-2} \cdot h^{-1}$)*

NH_4^+Cl, mM	mV	Na		
		J_{ms}	J_{sm}	J_{net}
0	0	4.34±0.59	1.24±0.08	3.10±0.60
	+25	3.81±0.47	1.48±0.12	2.33±0.47
30	0	6.51±0.44*	1.11±0.08	5.40±0.39*
	+25	5.37±0.29*	1.56±0.20	3.81±0.25*

Values are means ± SE; *$P < 0.05$ for comparison with the control group (0 mM NH_4Cl); Na flux rates in $\mu eq \cdot cm^{-2} \cdot h^{-1}$. $N = 3$; $n = 9$.

Choline chloride pipette solution. To minimize interference between K^+ and NH_4^+, cells were filled with choline chloride pipette solution (Fig. 5, *A* and *B*).

In these cells, reversal potential was 11 ± 3 mV ($n = 7$) in NaCl bath solution, significantly higher than in the potassium gluconate-filled cells ($P < 0.01$). As before, no changes in inward or outward current could be detected when NaCl bath solution was replaced with choline chloride bath solution (100 ± 5%, $P = 0.9$, $n = 8$, −120 mV and 100 ± 7%, $P = 0.9$, $n = 8$, +100 mV; Fig. 5*A*). Reversal potential was not significantly different than in the presence of external sodium (5 ± 1 mV, $P = 0.09$). Replacement of NaCl external solution with NH_4Cl solution resulted in an increase of inward current to 221 ± 36% ($n = 7$, $P < 0.01$), whereas changes in mean outward current were not significant (140 ± 36%, $n = 7$, $P = 0.2$; Fig. 5*A*). Reversal potential in NH_4Cl bath solution [19 ± 2 mV ($n = 12$)] was significantly higher than in either NaCl ($P < 0.01$) or choline chloride solution ($P < 0.01$). Iberiotoxin (10^{-7} mM), a blocker of high-conductance calcium-activated potassium channels, had no impact on either the current induced by NH_4Cl (100 ± 8%, $P = 0.9$, $n = 4$ and 86 ± 16%, $P = 0.4$, $n = 4$) or the reversal potential (21 ± 3 mV, $P = 0.6$).

NH_4Cl pipette solution. Cells were filled with an NH_4Cl pipette solution. In NaCl bath solution, reversal potential was −14 ± 4 mV ($n = 12$), significantly lower than in choline chloride-filled cells ($P = 0.007$), reflecting efflux of NH_4^+.

When cells were superfused with NH_4Cl solution (pH 5.8, reduction of NH_3), cells depolarized to 4.1 ± 3 mV ($n = 8$, $P = 0.01$) with inward current level at 404 ± 139% of the value in NaCl solution, ($n = 8$, $P < 0.05$, −120 mV) and outward current at 213 ± 66% ($n = 8$, $P < 0.01$, +100 mV; Fig. 5, *C* and *D*). Addition of quinidine (100 μM) to NH_4Cl solution lowered inward current to 42 ± 17% of the value in NH_4Cl solution ($n = 3$, $P < 0.05$) and outward current to 43 ± 3% ($n = 3$, $P < 0.01$). Reversal potential remained the same (5 ± 4 mV, $P = 0.2$). Values recovered to 90 ± 20% ($n = 3$, $P = 0.6$, inward) and 85 ± 4% ($n = 3$, $P = 0.06$, outward) of the level at the beginning of the experiment after washout with NaCl buffer. Superfusion with KCl raised both inward and outward current, both of which were blocked by quinidine ($n = 1$). Reversal potential changed from −6 (NaCl) to 26 mV (KCl) and to 6 mV (KCl + quinidine).

Table 7. *Effect of luminal potassium on Na transport in the presence of luminal ammonia*

$[NH_4Cl]$ and $[K^+]$, mM	Na, $\mu eq \cdot cm^{-2} \cdot h^{-1}$			*N/n*
	J_{ms}	J_{sm}	J_{net}	
15 and 5	8.70±0.67*	1.80±0.10	6.90±0.68*	3/6
15 and 45	6.01±0.65†	1.81±0.17	4.20±0.60†	3/6

Values are means ± SE; different symbols in the same column indicate significantly difference at $P < 0.05$. Brackets indicate concentration.

At a concentration of 65 mM NH_4Cl, inward current of the NH_4Cl-filled cells rose to 197 ± 54% ($n = 4$, pH = 6.4), reversal potential to 2.5 ± 6. Inward current did not change significantly when KCl was given in addition to NH_4Cl (each at 65 mM; 205 ± 52%, $n = 4$), whereas reversal potential rose to 19 ± 4 mV [$n = 4$, $P < 0.01$ vs. NH_4Cl (65 mM) alone]. KCl (65 mM) hyperpolarized two of three tested cells, suggesting that in these cells, stimulation of NH_4^+ efflux (at 130 mM) exceeded the additional influx of K^+ (65 mM).

Relative permeability ratios. In a previous study (36), we were able to show that K-gluconate-filled cells were depolarized by removal of external chloride, so that in NaCl solution, P_K/P_{Na} must be $<1.8 \pm 0.5$. From these measurements, it appears that the larger part of the outward current and the reversal potential in NaCl solution are due to chloride influx and not to K efflux. A similar estimate of <1.6 can be obtained (indirectly) by comparing cells filled with Cs-methanesulfonate, CsCl, choline chloride, and K-gluconate (36).

On the other hand, superfusion of the cells with KCl solution also resulted in a depolarization of similar magnitude as that observed after removal of Cl. If P_K/P_{Na} is calculated from the reversal potentials (as described in MATERIALS AND METHODS) in NaCl solution and in KCl solution (both 130 mM), a much higher value of $P_K/P_{Na} = 5 \pm 2$ is obtained. This discrepancy can be resolved if one assumes that permeability for potassium rises with the concentration of this ion in the bath solution. This assumption is confirmed by observing the increase in (TEACl sensitive) outward current after superfusion with KCl solution.

Other permeability ratios at 130 mM were: $P_{Na}/P_{choline} = 1.0 \pm 0.1$, $P_{NH4}/P_{Na} = 2.8 \pm 0.4$, and $P_{NH4}/P_K = 0.7 \pm 0.4$.

A relative permeability ratio for NH_4^+ efflux in NaCl solution can be calculated from NH_4Cl-filled cells superfused with NaCl solution and choline-filled cells superfused with NaCl solution, yielding $P_{NH4}/P_{choline} = 2.6$ (130 mM), which is in good agreement with the value obtained for P_{NH4}/P_{Na} (130

Table 6. *Interaction between ammonia and Na flux rates after luminal application of amiloride (1 mM) at a luminal pH of 6.40*

NH_4Cl, mM	Na, $\mu eq \cdot cm^{-1} \cdot h^{-1}$			I_{sc}	*N/n*
	J_{ms}	J_{sm}	J_{net}		
0 + Amiloride	2.25±0.09	1.81±0.09[a]	0.44±0.10	0.52±0.05[a]	3/6
30 + Amiloride	2.06±0.05	1.47±0.07[b]	0.59±0.08	1.13±0.08[b]	3/6

Values are means ± SE; different letters in the same column indicate significant differences at $P < 0.05$. Na flux rates and I_{sc} in $\mu eq \cdot cm^{-2} \cdot h^{-1}$.

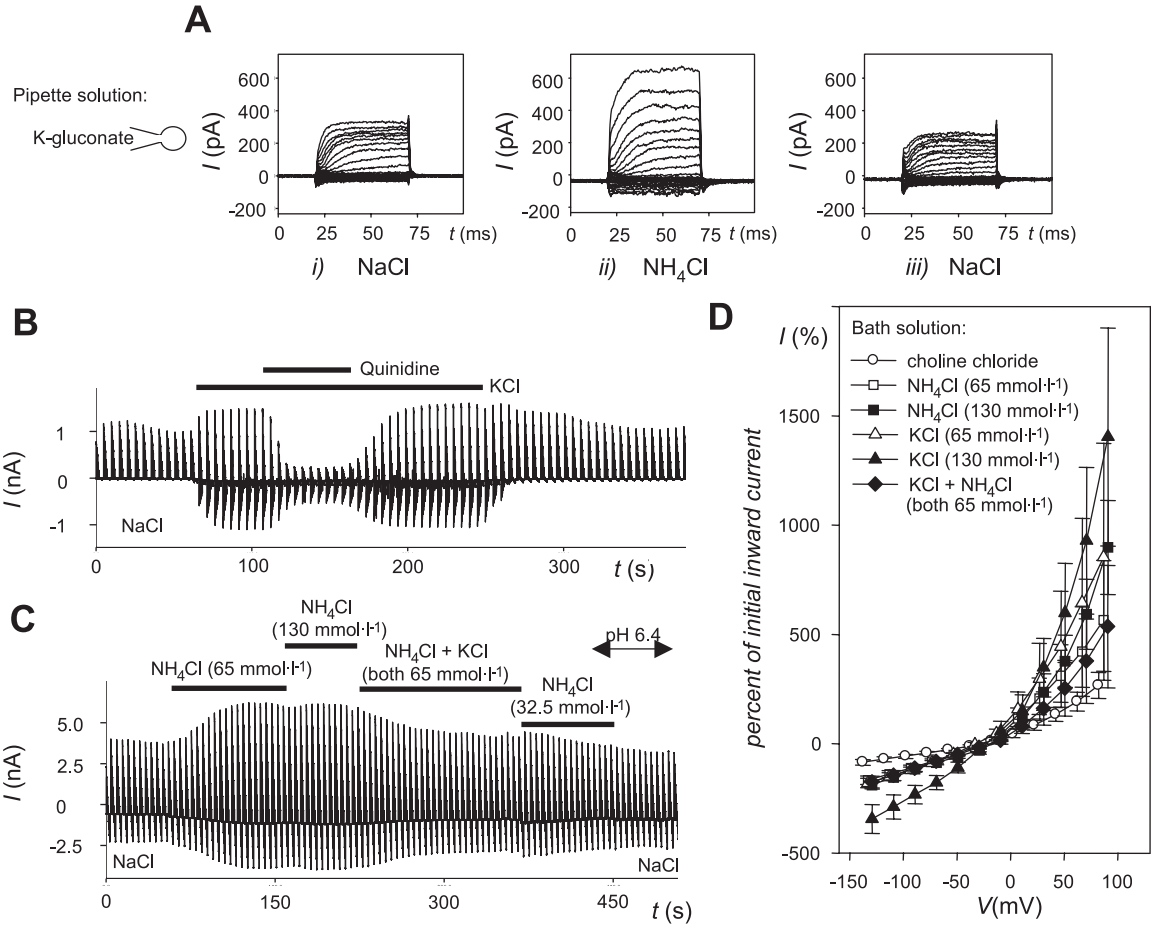

Fig. 4. *A*: current measured in ruminal epithelial cell filled with K-gluconate pipette solution in 130 mM NaCl solution (*i*), in 130 mM NH_4Cl solution (*ii*), and after washout with NaCl solution (*iii*), all using voltage *protocol II* [see MATERIALS AND METHODS and (36)]. Not only inward, but also outward current was stimulated. *B*: current measured in ruminal epithelial cell filled with K-gluconate pipette solution in response to voltage *protocol I*. Both inward and outward current increased in response to the application of KCl solution. Both the NH_4Cl and the KCl-induced currents could be blocked by quinidine (100 μM). *C*: anomalous mole fraction effects were observed when cells were exposed to a mix of KCl and NH_4Cl. In the bath, NaCl was replaced by NH_4Cl (at 65 or 130 mM) or a mix of KCl and NH_4Cl (at 65 mM each), with osmolarity adjusted in all solutions (choline chloride). pH of all solutions was titrated to 6.4. *D*: mean values of currents from all cells filled with K-gluconate solution, normalized to inward current in NaCl solution at the beginning of the experiment at a pipette potential of −120 mV (= −100%). Note induction not only of inward current, but also of outward current and the nonadditive responses to a mix of ions (see DISCUSSION).

mM) from K-gluconate-filled cells. This suggests that the permeability of ruminal cells for NH_4^+ is not as dependent on concentration as that of K^+.

Patch-clamp experiments in the single-channel configuration. Experiments in the inside-out configuration of the patch-clamp technique with symmetrical 130 mM NH_4Cl solution in pipette and bath showed a channel with a conductance level of 135 ± 12 pS (n = 5; Fig. 6, *A* and *D*).

When NH_4Cl was replaced by NaCl, downward channel openings corresponding to inward current into the pipette at negative potentials disappeared; outward openings were rare and too small for further evaluation (Fig. 6*B*). Conversely, when NH_4Cl was replaced with an equimolar amount of KCl, channel openings reappeared and could also be observed when KCl was replaced by potassium gluconate in the bath solution (Fig. 6*C*). Removal of ATP (1 mM) from the cytosolic side (n = 7) did not affect channel openings. The data were fitted by the Goldman-Hodgkin-Katz equation for the two ions of sodium and potassium (Fig. 6*E*), yielding a permeability ratio of P_{NH4}/P_K = 0.5 ± 0.2 (n = 7). Note the difference between permeability ratio as derived from the reversal potential and the ratio of absolute current values at negative potential levels (29). Such deviations from the independence principle are typically observed when ions interact with each other in the long, narrow pore of a potassium channel (29).

DISCUSSION

Very little is known about the interaction between ammonium transport and the NHE in transporting epithelia (1, 13, 14). To our knowledge, the facilitation of sodium absorption by acidification of luminal pH in the presence of ammonia has not been reported before. This is surprising in light of the high concentrations of ammonia to be found both in the rumen of sheep and cattle (25) and the human gut (55). Note that the absorption of fatty acids (short-chain fatty acids) is linked to acidification and thus to the uptake of sodium via the sodium-proton exchanger (NHE) in both tissues (24, 33).

In diarrhea, pH values of stool water >8.00 have been measured (44) with a concurrent rise in NH_3 concentration. This should elevate intracellular pH and reduce the number of

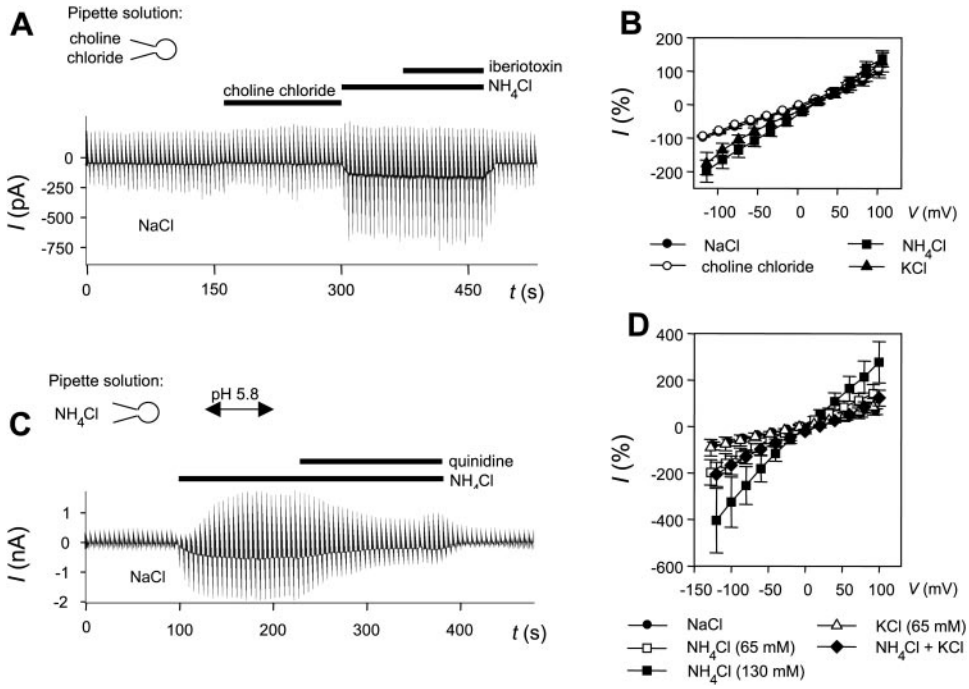

Fig. 5. *A*: current measured in response to voltage *protocol II* in ruminal epithelial cell filled with a choline chloride pipette solution. Changing from NaCl solution to choline chloride solution had no significant effect, whereas superfusion with NH_4Cl solution induced a marked increase in inward, but not in outward current. Iberiotoxin (10^{-7} mM) had no effect on this current. *B*: summary of the results from cells filled with choline chloride solution. Current was normalized to inward current in NaCl solution at −120 mV (=−100%). *C*: both inward and outward current of cells filled with NH_4Cl pipette solution increased markedly when external NaCl solution was replaced with NH_4Cl solution (pH 5.8). This increase in current could be blocked by quinidine (100 mM). *D*: summary of results from cells filled with NH_4Cl solution. Osmolarity of all solutions was adjusted with choline chloride.

protons available for extrusion by the NHE. This mechanism could help to explain the loss of sodium and water in these conditions.

In sheep and cattle, animals not given sufficient time to adapt to a diet rich in energy and protein (as in fattening) are prone to a condition known as ruminal acidosis (22, 32). Large shifts in the NH_4^+/NH_3 ratio occur, and uptake of protons not only due to the uptake of fatty acids as previously described (24) but also as NH_4^+, should be discussed. Thus a better understanding of the uptake routes for ammonia in the rumen and the interaction between ammonia and the NHE appears necessary.

The current study demonstrates that ammonia crosses the ruminal epithelium not only in the form of lipophilic NH_3 but also in its ionized form as NH_4^+. The relative transport rates of the two forms depend on ruminal pH, with an impact on the absorption rate of Na^+ by the rumen epithelium via NHE.

The suggestion of NH_4^+ uptake at pH 6.40 through a putative, quinidine-sensitive K channel by Bödeker and colleagues (5, 6) is supported by the present study. In patch-clamp experiments, we were able to show that ruminal epithelial cells express quinidine-sensitive channels of 130 pS that conduct potassium and NH_4^+, but not sodium, with interference when K^+ and NH_4^+ are applied simultaneously. Competition between NH_4^+ and K^+ for a common, electrogenic uptake pathway could also be demonstrated in Ussing chamber experiments.

The pH-dependent absorption of ammonia from the rumen has long been known (25). The increase of ammonia toxicity at a luminal pH greater than 7.30 (16) supports the assumption of an enhanced absorption of NH_3 with increasing pH. This suggestion is supported by the determination of ammonia flux rates in this study. The wide physiological variation of ruminal pH (5.4–7.4) and ammonia concentrations (up to 70 mM) only has a minimal effect of 1.8% on the concentration of NH_4^+ but great impact on free NH_3, which increases from 0.07 to 0.68 mM (at 30 mM luminal ammonia), resulting in an almost linear increase in NH_3 flux through the epithelial cells (Fig. 2). Conversely, at the pH values below 6.9, which predominate in the rumen, uptake of NH_4^+ as a source of protons must be considered. It is a well-known fact that both NH_3 and NH_4^+ affect pH_i in opposite directions (7, 42, 46) and should thus have an impact on the amount of Na^+ transported by the NHE.

Recent in vitro studies with isolated rumen epithelial cells (REC) have demonstrated that recovery from acidification of pH_i is reduced by inhibitors of NHE and that exposure of REC to 20 mM NH_4Cl induces an increase in pH_i to 7.8 (42), which inhibits NHE (2). In line with this observation, our findings indicate that in the intact rumen epithelium, electroneutral Na transport is abolished at pH 7.4. The remaining J_{net} (Na) at 30 mM ammonia represents electrogenic Na transport and accounts for I_{sc}. J_{net} (Na) and I_{sc} have the same magnitude if the measured I_{sc} (1.02 $\mu eq \cdot cm^{-2} \cdot h^{-1}$) is corrected for the NH_4^+-dependent component (some 0.4 $\mu eq \cdot cm^{-2} \cdot h^{-1}$) of I_{sc} [1.02–0.40 = 0.62 $\mu eq \cdot cm^{-2} \cdot h^{-1}$ vs. a J_{net} (Na) of 0.74 $\mu eq \cdot cm^{-2} \cdot h^{-1}$; see Table 1].

Thus it emerges that at a ruminal pH of 7.4 and at physiological ammonia concentrations, electroneutral Na transport is inhibited (Fig. 7*A*). Lowering the luminal pH from 7.4 to 6.9 does not alter Na fluxes. A further decrease of luminal pH to 6.4 causes a concentration-dependent increase in Na transport (Fig. 7*B*). The involvement of the NHE in ammonia induced enhanced Na transport is supported by the observation that mucosal amiloride (1 mM) prevents the stimulating effect of ammonia at a luminal pH of 6.40.

Our data (Fig. 2) allow an approach for calculating the permeabilities of NH_3 and NH_4^+. The slope of the regression should reflect the impact of the increase in NH_3, and its numerical value represents the flux rate of NH_3 per millimole per liter of NH_3 applied. The intercept of ammonia flux on the *y*-axis ($0.7 \cdot \mu eq \cdot cm^{-2} \cdot h^{-1}$) predominantly or solely represents NH_4^+ flux at a luminal concentration of 30 mM, from which the flux rate of NH_4^+ per millimole per liter of NH_4^+ can be derived

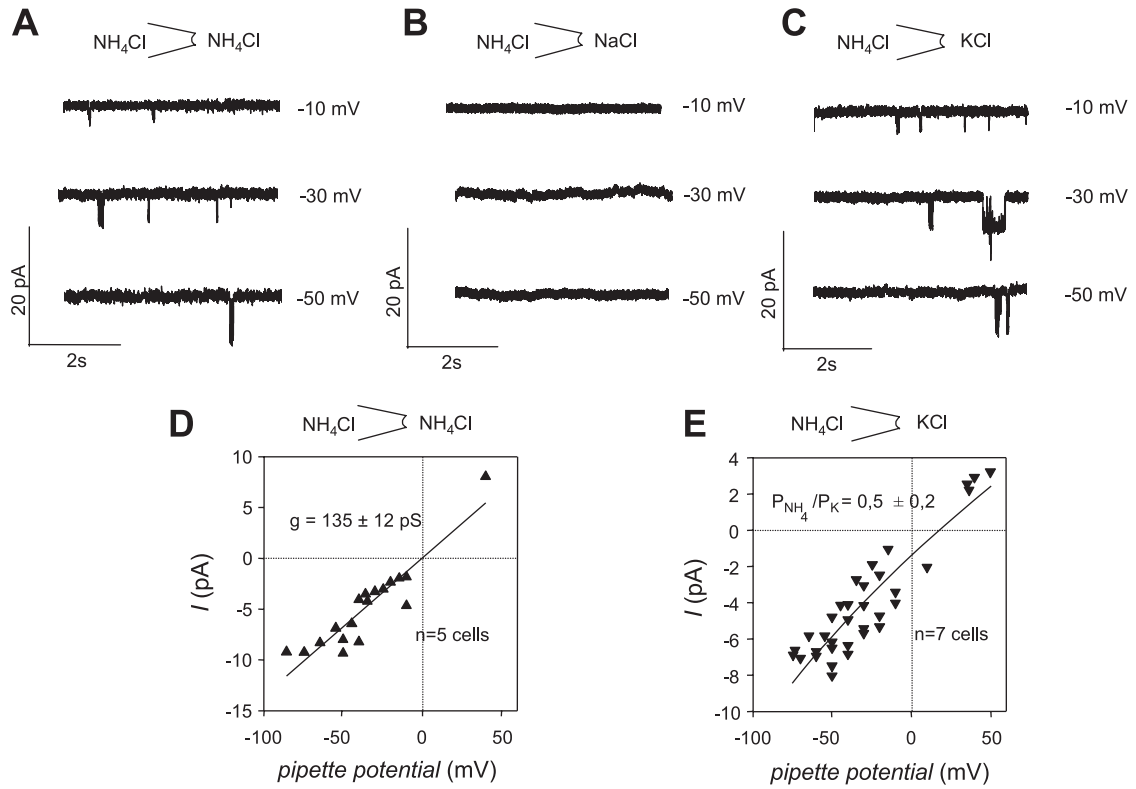

Fig. 6. *A*: current traces at 3 different pipette potentials in the inside-out mode with symmetrical 130 mM NH_4Cl solution on both sides of the membrane (with calcium high on the outside and low inside). *B*: ditto, with 130 mM NaCl solution on the cytosolic side of the membrane. *C*: ditto, with 130 mM KCl solution on the cytosolic side. *D*: plot of unitary conductance levels of the channel openings of 5 different cells in response to the pipette potential applied. In symmetrical NH_4Cl solution, data can be fitted linearly, yielding a conductance of 135 ± 12 pS. *E*: plot of unitary conductance levels against the pipette potential, this time with KCl solution facing the cytosolic side. Data were fitted using the Goldman-Hodgkin-Katz equation for 2 ions, yielding a relative conductance value of $P_{NH4}/P_K = 0.5 \pm 0.2$ (see MATERIALS AND METHODS). Note that inward currents at negative potential levels are not significantly lower in KCl solution than in NH_4Cl solution, leading to a difference between permeability ratio, as derived from the reversal potential, and the ratio of absolute current values at negative potential levels (see Fig 4*C*, DISCUSSION, and Ref. 29).

by simple division. The permeability ratio $P_{NH3}/P_{NH_4^+}$ can be obtained by dividing the slope of the regression by this value, yielding a value of $P_{NH3}/P_{NH_4^+} \approx 175$. The value that is reported for the human colon (~400) is even higher (15). Thus the effects of luminal pH on the absorption of ammonia, and Na transport via the NHE, should be considerable in both tissues.

Despite the high value of $P_{NH3}/P_{NH_4^+}$, which can lead to the incorrect assumption that transport of NH_4^+ is "negligible," the amount of ammonia absorbed in the ionic form is considerable due to the low concentration of NH_3 in relationship to NH_4^+ at physiological levels of pH. At neutral pH, and a total concentration of 30 mM ammonia, only 0.27 mM is in the form of NH_3 so that even if the permeability of NH_4^+ is 175 times lower than that of NH_3, the concentration is higher by a factor of 110. This means that for every millimole per liter crossing the membrane as NH_3, 110/175 = 0.63 mmol or ~40% of the total amount of ammonia will be transported as NH_4^+. At a slightly acidic pH of 6.4 ($[NH_3] = 0.07$ mM), conversely, >70% is absorbed as NH_4^+, whereas at a pH of 7.4 ($[NH_3] = 0.68$ mM), 80% of ammonia is absorbed as NH_3. This is in good agreement with our observations concerning the stimulation of sodium transport via the NHE at a pH of 6.4 and its inhibition at a pH of 7.4.

The patch-clamp data in this study show that ruminal epithelial cells express quinidine-sensitive potassium channels, as suggested previously (5). Whereas additional uptake of ammonia through specific transporters cannot be ruled out (31, 53, 54), these transporters cannot explain the large NH_4^+-induced currents observed in both the Ussing chamber and patch-clamp data in this study. Note that transporters from the Rh gene family are typically saturated by a few millimoles per liter of NH_4^+, and that transport is not affected by a change in membrane potential (54).

Conductance of NH_4^+ through nonselective cation channels has been reported for a number of other tissues (10). In rumen, this appears unlikely. The internal solution for patch-clamp experiments was chosen to minimize contributions of the nonselective cation channel (36), and no appreciable increase in conductance of sodium compared with choline could be observed. Note that under physiological conditions with divalent containing solution, the sodium conductance of rumen epithelium is saturated at 30 mM Na^+ (20, 47). However, exposure to NH_4^+ at pH 6.4 induced an additive increase in the transport rates of NH_4^+ and Na^+, with a corresponding increase in I_{sc}. Thus there was no sign of competition between Na^+ and NH_4^+ for a common pathway. In contrast, K^+ and NH_4^+ interfered with each other both in the patch-clamp and Ussing chamber experiments.

The question may arise if the currents observed in the patch-clamp measurements were induced by cell swelling or changes in pH_i due to influx of NH_3. However, capacitance

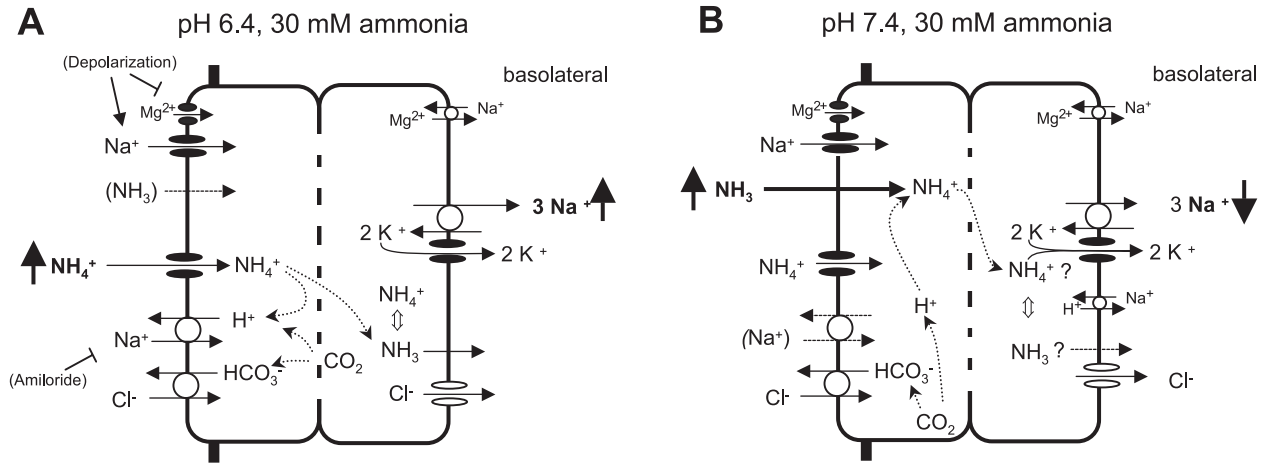

Fig. 7. Simplified putative model for interaction of the Na^+/H^+ exchanger (NHE) in ruminal epithelium with ammonia and luminal pH. *A*: at acidic pH, concentration of NH_3 is negligible, and ammonia is absorbed mostly in the form of NH_4^+. NH_4^+ rises to a higher level than maintenance of equilibrium between NH_3 and NH_4^+ in the more alkalic environment of the cytosol will allow, and NH_4^+ dissociates. The data suggest that it leaves on the basolateral side by diffusion as NH_3. The released proton is available for exchange with sodium by the NHE in the apical membrane, whereas an equimolar amount of Na^+ is excreted by the Na/K-ATPase, increasing I_{sc}. Note that the apical membrane is depolarized by the influx of NH_4^+ (with negative effects on magnesium absorption (11, 36, 48), and possible (discrete) positive effects on electrogenic Na uptake (34, 36). The ammonia-induced increase in J_{net}(Na) can be blocked by amiloride. *B*: at alkalic pH (7.4), concentration of NH_3 rises. Some ammonia permeates potassium channels as NH_4^+, but a much larger amount diffuses through the lipid membrane as NH_3, leading to a very steep increase in the cytosolic concentration of NH_3. Maintenance of the equilibrium between NH_4^+ and NH_3 requires a shift in the opposite direction, and all but a tiny amount of NH_3 is protonated to form NH_4^+. This raises pH (42) and inhibits NHE, and thus absorption of sodium by the apical NHE falls. Comparison of J_{net}(Na), J_{net}(Cl), and I_{sc} suggests that basolateral extrusion of ammonia is electrogenic. Possibly, NH_4^+ is flushed out with the stream of potassium leaving the basolateral side through a potassium channel coupled to NaK-ATPase (35). Or, NH_4^+ dissociates and leaves as NH_3, with the proton stimulating the basolateral NHE. Note that in vivo, the ruminal epithelium is a functional syncytium consisting of several layers of cells connected by gap junctions (26), which helps to understand why protonation occurs before NH_3 that enters apically can simply pass through the layer of cells and exit on the basolateral side.

remained stable, and both induction of current and depolarization could be observed at values of pH (5.8, 6.4), at which the concentration of NH_3 was negligible. Note also that stimulation of current was observed in all cases. Stimulation of potassium and/or chloride currents should induce hyperpolarization in K-gluconate-filled cells, and not the depolarization observed. Effects of ammonia persisted after the removal of either intracellular chloride (as in the K-gluconate experiments) or potassium (as when NH_4 or choline was used to replace K in the pipette solution). The experiments also show that reversal potential increases when NH_4Cl is used to replace choline chloride externally and decreases when the same is done internally, in line with our assumption.

The NH_4Cl-induced inward current was blocked by $BaCl_2$ or quinidine but not by iberiotoxin. Single-cell experiments showed a conductance of ~130 pS in symmetrical NH_4Cl solution that was permeable to both K^+ and NH_4^+ with a P_{NH4}/P_K of 0.5 ± 0.5. This permeability ratio, derived from the reversal potential (29), corresponds roughly to the slightly higher value of $P_{NH4}/P_K = 0.7 \pm 0.4$ found in whole cell experiments at a 130 mM concentration of both ions.

However, note that under conditions in which the pore is saturated, the permeability ratio as derived from the reversal potential deviates from the ratio of the absolute permeabilities, as derived by dividing the absolute current values (29). This effect can be seen clearly in Fig. 4*C*, where current is not doubled by doubling the concentration of NH_4^+, as independence theory would demand.

Permeation properties of K^+ channels are generally similar in that they must reconcile two apparently contradictory properties: a high rate of ion conductance and a high selectivity (4, 29). A single-file, multi-ion pore is the preferred model and predicts the effects that we observed in ruminal epithelial cells, such as the fact that currents induced by a mix of K^+ and NH_4^+ were lower than the sum of the currents induced by each ion alone (Fig. 4, *C* and *D*); in other words, that the two ions interfered with each other when flowing through the lumen of the pore(s). Typically, effects on reversal potential were not pronounced: at the reversal potential, the flux of ions through the pore comes to a halt.

Another seemingly paradox observation is that in the current study, we observed stimulation not only of inward, but also of outward current when cells were exposed to either K^+ or NH_4^+ from the external side.

Again, the multi-ion channel model predicts such effects. Electrostatic repulsion speeds ion flow when such channels become multiply occupied. In many cases, channel pores have to be fully occupied (with a distinct number of ions) before a current begins to flow. Another property of multi-ion channels leading to concentration-dependent permeability is that permeant ions entering the pore from one side may nudge out ions blocking the pore from the inside. Thus the channel can discriminate between currents into the cell and out of it; in other words: the channel is (inwardly or outwardly) rectifying (29).

Alternately, occupancy of an ion-selective site in the pore by a permeant ion from either the outside or the inside via the "foot-in-the-door" mechanism is known to prevent entry into the inactivated state in potassium channels with C-type inactivation (29, 56).

These basic properties of potassium channels may explain an old paradox. It is undisputed that elevation of potassium depolarizes the membrane of ruminal epithelium (20, 35, 40) and that potassium is absorbed across the ruminal wall (50),

stimulating I_{sc} (5). However, various studies have shown that in NaCl Ringer, apical secretion of potassium is minimal (21, 35) despite the activity of the basolateral Na/K-ATPase.

We suggest that permeability for potassium is, indeed, low in apical NaCl solution and may be further reduced by the presence of NH_4^+ but is stimulated by the elevation of mucosal potassium. The physiological importance of this mechanism is evident in that it prevents a drain of potassium from the serosal side into the ruminal lumen under low-potassium conditions. Note that in multi-ion channel theory, NH_4^+ ions should be able to "sweep" individual potassium ions caught in the single file of NH_4^+ streaming through the pore into the cell, even against an electrochemical gradient for potassium (29).

Beyond this, we are unable to precisely identify the potassium channel or, more likely, group of channels that conducts ammonia in ruminal epithelium. Unfortunately, data on the large family of potassium channels are incomplete (27) and depend greatly on the tissue in which the channel is expressed. Conductance of NH_4^+ by various potassium channels is well documented (27), as is the presence of potassium channels in the apical membrane of intestinal tissues (18), which may represent a pathway for NH_4^+ efflux (8) from the lumen. Large-conductance calcium-activated potassium channels have been reported to conduct NH_4^+, but these channels display only minimal open probability at hyperpolarized voltages, ruling out these channels as a possible route for NH_4^+ influx in our experiments, as does the lack of an effect of iberiotoxin.

It is difficult to resist speculation concerning the role of NH_4^+ for the pathophysiology of hypomagnesaemia in ruminants. Hypomagnesaemia (grass tetany) is linked to use of artificial fertilization techniques with high concentrations of potassium and ammonia. Inhibition of magnesium absorption not only by K^+ (11, 35, 36, 48) but also by ammonia has been known for many years (11, 23) as has the reduction of magnesium uptake via depolarization of the apical membrane (35, 48). Thus we suggest that uptake of NH_4^+ inhibits magnesium absorption via depolarization of the apical membrane, as previously described for K^+ (11, 35, 48).

Care et al. (11) also noted that inhibitory effects of NH_4^+ and K^+ on Mg^{2+} uptake were additive, which is in line with additive effects of NH_4^+ and K^+ on membrane potential as suggested by this study. Inhibition of Na transport by ammonia at a pH of ~7 was also reported, which can be attributed to the fact that at this pH, transport of NH_3 is larger than transport of NH_4^+. However, the finding that addition of K^+ results in a further decrease in Na absorption is in contrast to the effects of K^+ alone (34, 36, 50). We suggest that in Care's experiments, K^+ inhibits flux of NH_4^+ into the cells. Thus the stimulatory effect of NH_4^+ on NHE is terminated, and the inhibitory effect of NH_3 (alone) leads to a net inhibition of sodium transport by elevation of K.

In conclusion, this study presents evidence that in the rumen, electroneutral Na transport is inhibited by ammonia entering the cytosol in the form of NH_3 and is stimulated by entry of NH_4^+ through potassium channels. The luminal pH determines the predominant form of luminal ammonia uptake, NH_3 or NH_4^+ and, hence, the effect of ammonia on Na transport. Because the physiological pH of the ruminal fluid is <6.9, ammonia enhances Na absorption. This modulation of NHE activity by ammonia appears to be as important as the "classic" effect of short-chain fatty acids (24, 33). Furthermore, it is interesting to note that electrogenic uptake of ammonium increases with adaptation to a high-protein diet (1).

The basic physiological mechanism outlined in this study should be valid not only for the rumen, but also for the human colon due to the similarities between the two tissues, namely electroneutral Na transport via NHE (19, 37, 43), abundance of K channels (33, 51), and a wide variation of ammonia concentrations and pH in the ingesta (9).

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