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Sodium/Bicarbonate Cotransporter NBCn1/Slc4a7 Inhibits NH_4Cl-mediated Inward Current in *Xenopus* Oocytes

Soojung Lee and **Inyeong Choi**

Department of Physiology, Emory University School of Medicine, Atlanta, GA 30322, USA

Abstract

The electroneutral Na/HCO_3 cotransporter NBCn1 (SLC4A7) contributes to intracellular pH maintenance and transepithelial HCO_3^- movement. In this study, we expressed NBCn1 in *Xenopus* oocytes and examined the effect of NBCn1 on oocyte NH_4^+ transport by analyzing changes in membrane potential, current, and intracellular pH mediated by NH_4Cl. In the presence of HCO_3^-/CO_2, applying NH_4Cl (20 mM) produced intracellular acidification of oocytes. The acidification was faster in oocytes expressing NBCn1 than in control oocytes injected with water. However, NH_4Cl-mediated membrane depolarization was smaller in oocytes expressing NBCn1. In HCO_3^-/CO_2-free solution, NH_4Cl produced a smaller inward current in NBCn1-expressing oocytes (56% inhibition by 20 mM NH_4Cl; measured at −60 mV), while minimally affecting intracellular acidification. The inhibition of the current by NBCn1 was unaffected when $BaCl_2$ replaced KCl. Current-voltage relationships showed a positive and nearly linear relationship between NH_4Cl-mediated current and voltage, which was markedly reduced by NBCn1. Large basal currents (before NH_4Cl exposure) were produced in NBCn1-expressing oocytes due to the previously characterized channel-like activity of NBCn1. Inhibiting this channel-like activity by Na^+ removal abolished NBCn1's inhibitory effect on NH_4Cl-mediated currents. The currents were progressively reduced over 72–120 h after NBCn1 cRNA injection, during which the channel-like activity was high. These results indicate that NBCn1 by its Na/HCO_3 cotransport activity stimulates NH_4^+ transport, while reducing NH_4^+ conductance by its channel-like activity.

Keywords

bicarbonate; *Xenopus*; voltage-clamp

INTRODUCTION

The kidney regulates blood pH by reclaiming filtered acid and base equivalents or by releasing them into urine. Two major acid-base components that are regulated by the kidney are HCO_3^- and NH_4^+. HCO_3^- is filtered in the glomerulus, and filtered HCO_3^- is then reabsorbed in the proximal tubule (70–80%), thick ascending limb (10–15%), and cortical collecting duct (~5%) (Boron 1992). NH_4^+, which is the main component of urinary net acid excretion, is synthesized and secreted in the proximal tubule, reabsorbed in the thick ascending limb, and is secreted again into the lumen in the collecting duct (Knepper *et al.* 1989). The kidney makes adaptive changes in HCO_3^- and NH_4^+ absorption in response to acid-base disturbance, particularly to a low blood pH (Wagner 2007;Kraut & Kurtz 2005). Chronic metabolic acidosis stimulates HCO_3^- absorptive capacity and NH_4^+ production/

Address correspondence: Inyeong Choi, Ph.D. Department of Physiology, Emory University, 615 Michael Street, Atlanta, GA 30322, USA. Phone 404-712-2092; Fax 404-727-2648; ichoi@emory.edu.

secretion in the proximal tubule and increases the net HCO_3^- and NH_4^+ absorption in the thick ascending limb.

The absorption of HCO_3^- or NH_4^+ is primarily governed by ion transporters/channels that move these ions across the plasma membrane (Weiner & Verlander 2011;Cordat & Casey 2009;Sindic *et al.* 2007;Pushkin & Kurtz 2006). In particular, the electroneutral Na/HCO_3 cotransporter NBCn1 (SLC4A7) plays an important role in transepithelial HCO_3^- movement in epithelia (Aalkjaer *et al.* 2004;Romero *et al.* 2004;Boron *et al.* 2009). NBCn1, which is a member of SLC4A bicarbonate transporters, normally moves HCO_3^- into the cytosol down the Na^+ gradient in many different cells. Thus, the primary function of this transporter is to extrude acid (Pushkin *et al.* 1999;Lauritzen *et al.* 2010;Riihonen *et al.* 2010). In addition, the transporter has associated ionic conductance that is primarily governed by Na^+ (Choi *et al.* 2000;Cooper *et al.* 2005). This channel-like activity can affect membrane potential and intracellular Na^+ levels in heterologous expression systems (Choi *et al.* 2000;Cooper *et al.* 2005). The physiological role of this channel-like activity is unclear.

We recently reported that NBCn1 stimulates radiolabeled methylammonium uptake in *Xenopus* oocytes (Lee *et al.* 2010). Oocyte membranes have been considered to be less permeable to NH_3 than to NH_4^+ (Burckhardt & Frömter 1992;Nakhoul *et al.* 2005). In response to NH_4Cl, oocytes exhibit membrane depolarization and intracellular acidification without an initial rapid alkalinization (Cougnon *et al.* 1996;Burckhardt & Burckhardt 1997;Nakhoul *et al.* 2010). On the other hand, NH_4^+ currents are independent of NH_4Cl concentrations at constant NH_3 (Boldt *et al.* 2003), suggesting that oocytes possess two different pathways: an NH_3 entry and an NH_4^+ conductance. It is thus unclear by what mechanism NBCn1 affects NH_4^+ transport and/or conductance in oocytes.

In this study, we examined the effect of NBCn1 activity and expression on oocyte NH_4^+ transport. We measured intracellular pH (pH_i), membrane potential (V_m), and current (I) during NH_4Cl application in both NBCn1-expressing oocytes and water-injected control oocytes and compared them. Our data show that NBCn1 inhibits oocyte NH_4^+ conductance by the Na/HCO_3-*independent* mechanism, while stimulating NH_4^+-mediated pH_i change by the intracellular buffering mechanism. A model for the effects of NBCn1 on oocyte NH_4^+ transport and conductance is proposed in the Discussion.

METHODS

Ethical Approval

All experiments in this study were conducted under the NIH guidelines for research on animals, and experimental protocols were approved by the Institutional Animal Care and Use Committee at Emory University.

NBCn1 expression in oocytes

Female frogs (*Xenopus laevis*) were purchased from Xenopus Express (Brooksville, FL, USA). A frog was anesthetized with fresh 0.1% 3-aminobenzoic acid ethyl ester for 30 min or until the animal was fully anesthetized. An incision in the abdomen, lateral to the midline, was made and oocytes were collected. After suture, the frog was returned to a recovery tank containing 0.1 M NaCl. Oocytes were agitated in Ca^{2+}-free ND96 solution (in mM; 96 NaCl, 2 KCl, 1 $MgCl_2$, and 10 Hepes, pH 7.5) five times for 20 min each. Follicles were removed by enzymatic digestion with collagenase type I (Sigma-Aldrich; St. Louis, MO, USA) twice for 20 min each at the concentration of 2 mg/ml. Oocytes were then washed with normal ND96 solution containing 1.8 mM $CaCl_2$ for 20 min. Oocytes at stages V-VI were sorted and stored in OR3 medium (Chang *et al.* 2008) at 18°C overnight. For cRNA injection, rat splice variant NBCn1-E (Cooper *et al.* 2005) was *in vitro* transcribed with T7

RNA polymerase using the mMessage/mMachine transcription kit (Ambion; Austin, TX, USA). The injection was done with 25 ng RNA (in 46 nl). The control was injection with sterile water. Injected oocytes were maintained at 18°C for 3–5 days before use.

Two-electrode voltage clamp

Two glass capillaries with 1 mm outer diameter (World Precision Instruments; Sarasota, FL, USA) were filled with 3 M KCl and had a tip resistance of ~1 MΩ. One electrode was connected to the voltage headstage and the other to the current cable. The two probes were connected to the voltage-clamp amplifier OC-725C (Warner; Hamden, CT, USA). Input offset voltage was adjusted by placing electrodes in the chamber containing the nominally HCO_3^-/CO_2-free recording solution (mM; 76 NaCl, 20 mM NMDG-Cl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 Hepes, pH 7.4; 195–200 mOsm/kg). An oocyte was impaled with current and voltage electrodes and superfused with the recording solution. Signals from voltage and current electrodes were sampled by the digitizer Digidata 1322A (Molecular Devices; Sunnyvale, CA, USA) interfaced to a computer, and data were acquired using pClamp 8 (Molecular Devices). The membrane voltage of the oocyte was monitored before and after applying 10 and 20 mM NH_4Cl, which replaced NMDG-Cl in the recording solution. For recording NH_4Cl-mediated current, the oocyte was clamped at −60 mV using the OC-725C. Current-voltage relations were determined by commanding voltages from −120 mV to +60 mV with 20 mV increments for 100 msec before and after NH_4Cl application. The voltage commands were imposed once the current value reached steady-state. For ionic conductance associated with NBCn1, current values were calculated by subtracting current values in control oocytes from values in NBCn1-expressing oocytes. Experiments were performed at room temperature.

Measurement of pH_i

An oocyte was impaled with three microelectrodes: a pH electrode, a voltage electrode, and a current electrode. The pH electrode was baked at 200°C, silanized with bis-(methylamino)dimethylsilane (Sigma-Aldrich), and filled with the proton ionophore 1 cocktail B (Sigma-Aldrich). The electrode was then back-filled with the phosphate buffer at pH 7.0. The pH electrode was connected to the high-impedance electrometer FD-223 (World Precision Instruments; Sarasota, FL, USA) and the signal was then routed to a custom-made subtraction amplifier. The voltage and current electrodes filled with 3 M KCl were connected to the OC-725C. Signals from pH, voltage, and current electrodes were sampled by the Digidata 1322A, and data were acquired using pClamp 8. The signal in the voltage electrode was subtracted from the signal in the pH electrode to calculate the voltage for pH. The slope of voltage to pH was calibrated by placing all three electrodes in the chamber filled with pH 6.0 and 8.0 standards (Fisher Scientific; Pittsburgh, PA, USA). The slope was typically at the range of 53 ± 3 mV/pH. For recording in the presence of HCO_3^-/CO_2, an oocyte clamped at −60 mV was superfused with a solution buffered with 25 mM HCO_3^-, 5% CO_2 (pH 7.4) and then with 20 mM NH_4Cl in the continued presence of HCO_3^-/CO_2. NaCl was replaced mole for mole with NH_4Cl. NH_4Cl was applied ~2 min after pH_i reached the lowest point. LiCl or N-methyl D-glucamine+($NMDG^+$) was used to replace NaCl in the Na^+-free experiments, and $BaCl_2$ was used to replace KCl in an effort to inhibit K^+ channels. Oocyte NH_4^+ transport activity was measured from the rate of acidification after NH_4Cl application. The rate of pH_i change (i.e., dpH_i/dt) was calculated by linear regression from the initial pH_i value over the first 2 min after NH_4Cl exposure. All experiments were performed at room temperature.

Statistical analysis

Data were reported as means ± standard error. The level of significance was assessed using the unpaired, 2-tailed Student *t*-test for comparison of dpH_i/dt, V_m, and I between control oocytes and NBCn1-expressing oocytes. The one-way ANOVA with Bonferroni post test was used to analyze daily *I* values for 5 days. The *p* value of less than 0.05 was considered significant.

RESULTS

NBCn1 cotransport activity stimulates NH_4Cl-mediated intracellular acidification in oocytes

To assess the effect of NBCn1 cotransport activity on NH_4^+ transport, we expressed the rat renal splice variant NBCn1-E (hereafter, NBCn1) in *Xenopus* oocytes and simultaneously measured pH_i and V_m during NH_4Cl application in the presence of 25 mM HCO_3^-, 5% CO_2 using the proton-selective pH microelectrode and the voltage electrode. Fig. 1A shows representative pH_i and V_m traces in a water-injected control oocyte. The oocyte was bathed initially in the HEPES-buffered (HCO_3^-/CO_2-free) solution to maintain stable pH_i. Applying HCO_3^-/CO_2-buffered medium caused pH_i to decrease as CO_2 entered the oocyte, hydrated, and produced H^+. The pH_i then reached steady-state as intracellular CO_2 rose to equal extracellular CO_2. A slight increase in pH_i was often observed (*a* in the figure), possibly due to endogenous Na/H exchange (Busch 1997). Under this condition, subsequent exposure to 20 mM NH_4Cl induced intracellular acidification (*b* in the figure). NH_4Cl also caused the oocyte membrane to be depolarized. These responses to NH_4Cl (i.e., intracellular acidification and membrane depolarization) are comparable to the previous observations by others (Cougnon *et al.* 1996;Burckhardt & Burckhardt 1997;Nakhoul *et al.* 2010).

In an NBCn1-expressing oocyte (Fig. 1B), the application of HCO_3^-/CO_2 also caused pH_i to decrease. However, the pH_i recovered from an initial CO_2-induced acidification (*a'*) as HCO_3^- transported via NBCn1 associated with intracellular H^+. Applying NH_4Cl in the continued presence of HCO_3^-/CO_2 induced a rapid acidification (*b*'). NH_4Cl-dependent intracellular acidification was evaluated by subtracting dpH_i/dt values before NH_4Cl application from values after NH_4Cl application (i.e., *b* – *a* or *b' – a'*). The acidification rate was $-10.33 \pm 0.47 \times 10^{-4}$/sec ($n = 6$) for NBCn1-expressing oocytes, which was 2.8-fold higher than $-3.73 \pm 0.49 \times 10^{-4}$/sec ($n = 7$) for controls ($p < 0.05$). Nonetheless, a smaller depolarization mediated by NH_4Cl was observed in NBCn1-expressing oocytes ($\Delta V_m = 13.4 \pm 1.2$ mV for controls and 10.3 ± 2.4 mV for NBCn1-expressing oocytes). pH_i change rates and other values are summarized in Table 1.

NBCn1 reduces NH_4Cl-mediated inward current in oocytes

To further assess the effect of NBCn1 on NH_4^+ transport, we then applied NH_4Cl to oocytes in the absence of HCO_3^-/CO_2. For this analysis, oocytes were clamped at −60 mV, close to the mean resting potential of −61 mV for uninjected oocytes (Yang *et al.* 2009b), and pH_i and *I* were simultaneously recorded. Fig. 2A shows a representative pH_i trace in a control oocyte. A gradual intracellular acidification was observed in response to NH_4Cl, reflecting that NH_4^+ transport is more favorable than NH_3 entry in oocytes (Nakhoul *et al.* 2001). Consistent with this, there was no immediate pH_i rise upon NH_4Cl exposure. The pH_i recovery after NH_4Cl removal was relatively slow probably due to low levels of accumulated NH_3 in the cells. An inward current was generated, which was abolished after NH_4Cl removal.

Fig. 2B shows a representative pH_i trace in an NBCn1-expressing oocyte. A gradual acidification in response to NH_4Cl was observed without an immediate pH_i rise. The mean dpH_i/dt during NH_4Cl application was similar between controls and NBCn1-expressing oocytes (Fig. 2C). The intracellular buffering power of *Xenopus* oocytes is reported to be similar at pH_i ranging between 7.2 and 7.5 and estimated to be 24 mM/pH unit (Cougnon *et* *al.* 2002). Thus, the rate of NH_4^+ influx, which is equal to dpH_i/dt × buffering power, was estimated to be similar between the two groups of oocytes. Nonetheless, the NBCn1-expressing oocyte produced a smaller current in response to NH_4Cl. The mean *I* in these oocytes was −125.2 ± 25.8 nA, significantly smaller than −278.2 ± 16.7 nA for controls ($p < 0.05$) (Fig. 2D). NBCn1-expressing oocytes produced large holding currents before NH_4Cl application due to channel-like activity of NBCn1.

NH_4^+ generally moves across the cell membrane by binding to the K^+ site in K^+ channels/transporters (Weiner & Hamm 2007). To test whether reduced NH_4Cl-mediated current by NBCn1 involves K^+, we repeated experiments in a solution containing $BaCl_2$ instead of KCl (Fig. 2E and F). NBCn1-expressing oocytes had a slightly higher mean dpH_i/dt ($p < 0.05$; $n = 5$ for each) probably due to higher initial pH_i in these oocytes. Nonetheless, NBCn1-expressing oocytes had substantially smaller current mediated by NH_4Cl ($p < 0.05$). The reduction was 57%, comparable to 55% in the presence of KCl (Fig. 2D). Thus, barium has a negligible effect on the transporter's ability to inhibit NH_4Cl-mediated current.

The inhibition of NH_4Cl-mediated current by NBCn1 was further examined using two-electrode voltage clamp. Fig. 3A shows representative recordings of the current caused by 10 mM NH_4Cl in oocytes (at −60 mV). NH_4Cl-mediated current was induced in both a control oocyte and an NBCn1-expressing oocyte, but the current amplitude was smaller in the NBCn1-expressing oocyte. This difference was more evident at 20 mM NH_4Cl (Fig. 3B), indicating that the inhibition depends on the amount of NH_4Cl. As summarized in Fig. 3C, the inhibition was 33% at 10 mM NH_4Cl and 56% at 20 mM NH_4Cl ($p < 0.05$ for each). In other experiments, we measured V_m during NH_4Cl application under the non-voltage clamp condition (Fig. 3D). NH_4Cl-mediated membrane depolarization was smaller in NBCn1-expressing oocytes, measured on the same day (3–4 days after cRNA injection). The reduction was 72% ($p < 0.05$; Fig. 3E)

NH_4Cl-mediated current response to voltage is reduced by NBCn1

To analyze the NH_4Cl-mediated current response to voltage, we determined current-voltage (*I–V*) relations by imposing voltage steps from −120 to +60 mV before and after NH_4Cl exposure. The voltage command after exposure was made when the current reached steady-state (~2 min). In control oocytes (Fig. 4A), NH_4Cl caused the *I–V* plot to shift down with a progressive increase in the inward direction at more negative voltages. The difference before and after NH_4Cl application is a good estimate of the conductance for NH_4Cl-mediated current (G_{NH4Cl}; *red line* in the figure). The G_{NH4Cl} was positive and almost linear with a reversal potential of 33.2 ± 2.2 mV ($n = 5$). In NBCn1-expressing oocytes (Fig. 4B), the *I–V* plot before NH_4Cl application was already steeper. Upon the exposure to NH_4Cl, the *I–V* plot shifted down. However, the change was small and almost negligible at more positive voltages. Thus, the reversal potential shifted positively. Fig. 4C shows that the G_{NH4} was reduced by 52% in NBCn1-expressing oocytes (measured between −80 and −40 mV; $n = 5$ for each).

Channel-like activity of NBCn1 reduces oocyte NH_4^+ currents

In the above data, NBCn1 caused the oocyte resting V_m to shift positively, producing a high holding current. NBCn1 also caused a steeper slope in the *I–V* plot before NH_4Cl application. These are hallmarks for channel-like activity of NBCn1 that occurs in the Na/HCO_3-*in*dependent manner (Choi *et al.* 2000;Cooper *et al.* 2005). Fig. 5A shows another example of the channel-like activity. Inward currents at negative potentials and outward currents at positive potentials were elicited in NBCn1-expressing oocytes. The zero-current voltage shifted positively by ~25 mV and the slope measured at the zero-current voltage was 6.96 ± 2.3 μS, larger than 1.13 ± 0.06 μS for controls ($n = 5$ for each). This channel-like activity is primarily caused by a Na^+ current and secondarily by outwardly rectifying anion currents (Choi *et al.* 2000;Cooper *et al.* 2005). Consistent with this, NBCn1-expressing oocytes produced Na^+ currents (Fig. 5B).

To determine whether this Na^+ current component affects NH_4Cl-mediated current, we performed experiments, in which oocytes were preincubated in Na^+-free media for 20 min and subjected to the above *I–V* protocol. Fig. 5C shows the results. The two *I–V* plots corresponded to NH_4Cl-mediated currents at different voltages in control oocytes and NBCn1-expressing oocytes. The two plots were nearly superimposed after preincubation in Na^+-free media. The current was not reversed at more positive voltages in NBCn1-expressing oocytes, probably due to incomplete washout of intracellular Na^+ after 20 min preincubation. Nevertheless, the values at the resting membrane potentials were similar between the two groups of oocytes ($p > 0.05$). Parallel experiments were then performed to examine pH_i changes in Na^+-free solution (Fig. 5D). The dpH_i/dt values were similar before and after Na^+ removal ($p > 0.05$).

In other experiments, the time course of NH_4Cl-mediated current at −60 mV was measured daily after water or NBCn1 cRNA injection (Fig. 6A). The current appeared to increase over the initial 48 h after injection, the reason for which is unclear. Nonetheless, no significant difference was observed between controls and NBCn1-expressing oocytes during this period ($p > 0.05$). At 72 h, the current began to decrease in NBCn1-expressing oocytes ($p < 0.05$). The decrease was more profound at 96–120 h. We then determined the channel-like activity of NBCn1 during these time periods by subtracting the slope in the *I–V* plot for control oocytes from the slope for NBCn1-expressing oocytes (Fig. 6B). The channel-like activity was negligible over the initial 48 h, but significantly increased at 72–120 h ($p < 0.05$; one-way ANOVA with Bonferroni post test; 4–6 oocytes for each group at different time points). Values were similar at these time points, comparable to the earlier report (Yang *et al.* 2009a) that NBCn1 expression in oocyte membranes is saturated after 72 h. These data demonstrate that there is an inverse relationship between channel-like activity and NH_4Cl-mediated current in oocytes expressing NBCn1.

DISCUSSION

In our recent report (Lee *et al.* 2010), we demonstrated that NBCn1 increases HCO_3^-/CO_2-dependent ^{14}C-methylammonium uptake in oocytes. The goal of the present study was to further investigate the effect of NBCn1 on NH_4^+ transport by electrophysiological measurements of V_m, I, and pH_i of oocytes. The major findings from this study are the following: 1) NBCn1 Na/HCO_3 cotransport activity enhances intracellular acidification mediated by NH_4Cl in oocytes; 2) In the absence of HCO_3^-/CO_2, NBCn1 inhibits inward current mediated by NH_4Cl, while producing a negligible effect on intracellular acidification; 3) NBCn1-dependent inhibition of NH_4Cl current is unaffected by barium; 4) The inhibition is abolished by Na^+ removal; and 5) NBCn1 channel-like activity appears to be responsible for inhibiting NH_4Cl-mediated current. These findings are novel and provide new information on the effect of NBCn1 on oocyte NH_4^+ transport. In addition, the findings provide the first evidence for the functional significance of NBCn1's channel-like activity. We propose that NBCn1 may play a key role in regulating NH_4^+ transport and conductance in cells, where HCO_3^- and NH_4^+ transport processes are tightly coupled.

In this study, we demonstrate that NBCn1 differentially affects pH_i and I (or V_m) caused by NH_4Cl in oocytes. The transporter stimulates intracellular acidification caused by NH_4Cl in the presence of CO_2/HCO_3^- (Fig. 1). NBCn1 moves Na^+ and HCO_3^- into oocytes and causes pH_i to recover from a CO_2-induced acidification. NH_4^+ transport is stimulated in this condition because HCO_3^- moved via NBCn1 compensates intracellular H^+ load. Thus, the intracellular buffering mechanism can account for increased rates of acidification during NH_4Cl application in the presence of HCO_3^-/CO_2. On the other hand, we observe a decrease in membrane depolarization under the same condition. Furthermore, NBCn1 inhibits NH_4Cl-mediated current without significantly altering dpH_i/dt in the absence of HCO_3^-/CO_2 (Fig. 2 and Fig. 3). Therefore, the inhibition occurs regardless of Na/HCO_3 cotransport activity of NBCn1.

The finding that NBCn1 differentially affects pH_i and *I* leads us to revisit the mechanism of NH_4^+ transport in *Xenopus* oocytes. Our data show that NH_4Cl induces membrane depolarization and intracellular acidification, consistent with previous reports by others (Cougnon *et al.* 1996;Burckhardt & Burckhardt 1997;Nakhoul *et al.* 2010). Given the reports that oocyte membranes are less permeable to NH_3 than to NH_4^+ (Burckhardt & Frömter 1992), the changes in membrane depolarization and intracellular acidification during NH_4Cl application have been considered to be due to carrier-mediated NH_4^+ transport. Upon NH_4Cl application, NH_4^+ is preferentially transported into the oocyte cytosol and dissociates into NH_3 and H^+. The dissociation results in intracellular acidification. Thus, the movement of charged NH_4^+ into oocytes induces a depolarizing inward current. NH_4^+ has been proposed to enter the oocyte cytosol via a nonselective cation channel (Burckhardt & Burckhardt 1997) or a mechanism that exhibits a linear relationship between pH_i change rate and NH_4^+ concentration (Nakhoul *et al.* 2010). Thus, according to this paradigm, the two events following NH_4Cl application (i.e., intracellular acidification and inward current) are tightly coupled to each other.

In contrast, our data show that the cotransport activity of NBCn1 preferentially stimulates NH_4^+-mediated intracellular acidification, while reducing inward current. Thus, the two events can be separated from each other. Our data also show that NBCn1 can reduce the current during NH_4Cl application, without altering intracellular acidification. This implies that oocyte NH_4^+ transport does not necessarily have to be electrogenic. The crystal structure of the bacterial ammonium transporter AmtB suggests translocation of NH_3, but not NH_4^+, via the transporter (Zheng *et al.* 2004). Mammalian NH_4^+ transporter Rh glycoproteins have been proposed to be electroneutral (Weiner & Hamm 2007). Nonetheless, we are also aware that the Rh glycoproteins exhibit electrogenic NH_4^+ transport (Nakhoul *et al.* 2005;Nakhoul *et al.* 2010). In this sense, it is also interesting to mention that we often observe negligible electrogenicity of NH_4^+ transport in some preparations of oocytes, as shown in our previous report (Lee *et al.* 2010). The exact reason for this lack of electrogenicity in some preparations is unclear although oocytes are known to exhibit seasonal variances.

Our data show that NBCn1 exhibits channel-like activity (Fig. 4 and Fig. 5), consistent with previous reports (Choi *et al.* 2000;Cooper *et al.* 2005). Other HCO_3^- transporters also display channel-like conductance. The Cl/HCO_3 exchanger AE1 in erythrocytes mediates a conductive anion flux. The DIDS-insensitive component of net ion efflux increases with increasing membrane hyperpolarization (Freedman & Novak 1997). The Cl/HCO_3 exchangers Slc26a3 (DRA, CLD) and Slc26a6 (CFEX, PAT-1) also show channel-like activity, which mediates large NO_3^- and SCN^- currents that are uncoupled to OH^- or HCO_3^- transport (Ko *et al.* 2002;Shcheynikov *et al.* 2008). The channel-like activity of NBCn1 is known to increase intracellular Na^+ levels and depolarize the membrane by 20–30 mV in *Xenopus* oocytes and HEK 293 cells (Choi *et al.* 2000;Cooper *et al.* 2005). Thus, Na^+ influx via NBCn1 may lower the electrochemical Na^+ gradient across the cell membrane, subsequently reducing the driving force for non-selective cation channels. Our finding that Na^+ removal abolishes the capability of NBCn1 to inhibit NH_4Cl current support this idea. Whether the channel-like activity affects nonselective cation channels needs further investigation.

Based on the data obtained from this study, we propose a model for the effect of NBCn1 on oocyte NH_4^+ transport (Fig. 7). NH_4^+ moves to the cytosol via endogenous NH_4^+ transport and dissociates intracellularly into NH_3 and H^+. This process is stimulated by NBCn1 as HCO_3^- compensates H^+. Intracellular NH_4^+ or NH_3 then causes the oocytes to produce an inward current that can be affected by Na^+ in the cytosol. The inward current can thus be inhibited by NBCn1 channel-like activity, which raises intracellular Na^+. Our model assumes that oocyte NH_4^+ transport is mainly electroneutral because NBCn1 reduces NH_4Cl-mediated current without significantly altering dpH_i/dt in the absence of HCO_3^-/CO_2.

What would be the physiological implication of the effect of NBCn1 on NH_4^+ transport? In the thick ascending limb, where NBCn1 is localized to the basolateral side of the cells, NH_4^+ is transported from the lumen mainly via the apical NKCC2 (Russell 2000;Kinne *et al.* 1986;Weiner & Hamm 2007). NH_4^+ translocates through the K^+ binding site in NKCC2 and is inhibited by bumetanide (Kinne *et al.* 1986). In addition, the thick ascending limb has amiloride-sensitive NH_4^+ conductance (Attmane-Elakeb *et al.* 2001;Amlal *et al.* 1994). Together with verapamil-sensitive K^+/NH_4^+ exchange, the NH_4^+ conductance contributes to apical NH_4^+ transport by 35–50% (Attmane-Elakeb *et al.* 2001). Leipziger's groups (Jakobsen *et al.* 2004;Odgaard *et al.* 2004) reported that the basolateral NBCn1 affects the apical NH_4^+ transport by moving HCO_3^- into the cells and compensating H^+ load. Our finding of NBCn1-mediated stimulation of intracellular acidification during NH_4Cl application is in good agreement with this paradigm. Nonetheless, it is unclear what physiological significance there is in the inhibition of NH_4^+ conductance by NBCn1. One explanation is that the inhibition may help protect the tubules from membrane depolarization, which is deleterious to cells. This inhibition may be critical for cell function particularly during chronic metabolic acidosis, when NH_4^+ and HCO_3^- absorptive capacity significantly increases (Wagner 2007).

In summary, our study shows that NBCn1 inhibits oocyte NH_4Cl-mediated current, while enhancing NH_4^+ uptake by the compensation of intracellular H^+ load. We envision that NBCn1 not only serves as an acid extruder to maintain intracellular pH within the physiological range, but it also plays a critical role in regulating HCO_3^- and NH_4^+ transport. It will be interesting and important to test whether NBCn1 also inhibits NH_4^+ conductance in the thick ascending limb cells.

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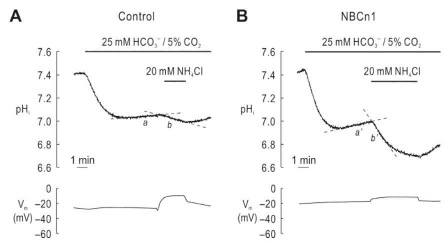

Fig. 1.
pH_i and V_m during NH_4Cl application in the presence of HCO_3^-/CO_2. **A**) Representative pH_i and V_m traces in a control oocyte. The oocyte was superfused with a HEPES-buffered HCO_3^-/CO_2-free solution and then exposed to a HEPES-free solution containing 25 mM HCO_3^-, 5% CO_2. After pH_i reached steady state, the oocyte was exposed to 20 mM NH_4Cl in the continued presence of HCO_3^-/CO_2. Intracellular acidification and membrane depolarization are shown. An initial rapid peak in V_m upon exposure to NH_4Cl is a solution delivery artifact. One of 7 oocytes is shown. **B**) Representative pH_i and V_m trances in an NBCn1-expressing oocyte. One of 6 oocytes is shown. dpH_i/dt due to NH_4^+ transport was calculated by subtracting dpH_i/dt in region *b* (or *b'*) from dpH_i/dt in region *a* (or *a'*). dpH_i/dt values and other values are summarized in Table 1.

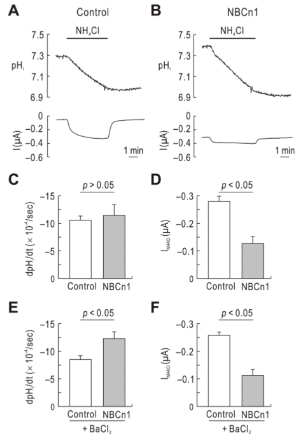

Fig. 2.

pH_i and *I* during NH_4Cl application in the absence of HCO_3^-/CO_2. **A**) Representative pH_i and *I* in a control oocyte. Oocytes were exposed to 20 mM NH_4Cl at the holding potential of −60 mV, and pH_i and *I* were simultaneously measured using microelectrodes. One of 5 controls is shown. **B**) Representative pH_i and *I* in an NBCn1-expressing oocyte. One of 6 NBCn1 oocytes is shown. **C**) Summary of pH_i change rate. The pH_i change rate (dpH_i/dt) was calculated by linear regression analysis from the initial pH_i over 2 min after NH_4Cl application. **D**) Summary of *I*. NH_4Cl-mediated current (I_{NH4Cl}) was obtained by subtracting *I* before NH_4Cl application from *I* after NH_4Cl application. **E**) Summary of dpH_i/dt in the presence of $BaCl_2$. Experiments were done in the presence of $BaCl_2$, which replaced KCl mole for mole ($n = 5$ for each). **F**) Summary of I_{NH4Cl} in the presence of $BaCl_2$. Measurements were done as described in **D** ($n = 9$ controls and 5 NBCn1).

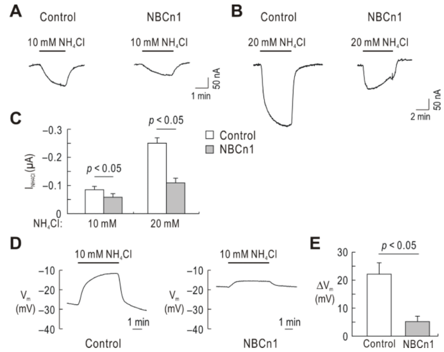

Fig. 3.

Inhibition of NH_4Cl-mediated current by NBCn1. **A**) Representative inward currents during 10 mM NH_4Cl application (clamped at −60 mV). Oocytes were superfused with HCO_3^-/ CO_2-free solution and then exposed to NH_4Cl. Measurements were done on the same days after water or NBCn1 cRNA injection. One of 6 controls and one of 5 NBCn1-expressing oocytes are shown. **B**) Representative inward currents during 20 mM NH_4Cl application. One of 6 controls and one of 8 NBCn1 oocytes are shown. **C**) Comparison of currents induced by 10 mM and 20 mM NH_4Cl. **D**) Representative V_m traces during 10 mM NH_4Cl application in a water-injected control oocyte and an NBCn1-expressing oocyte. One of 6 controls and one of 8 NBCn1-expressing oocytes are shown. **E**) Summary of NH_4Cl-induced membrane depolarization (ΔV_m).

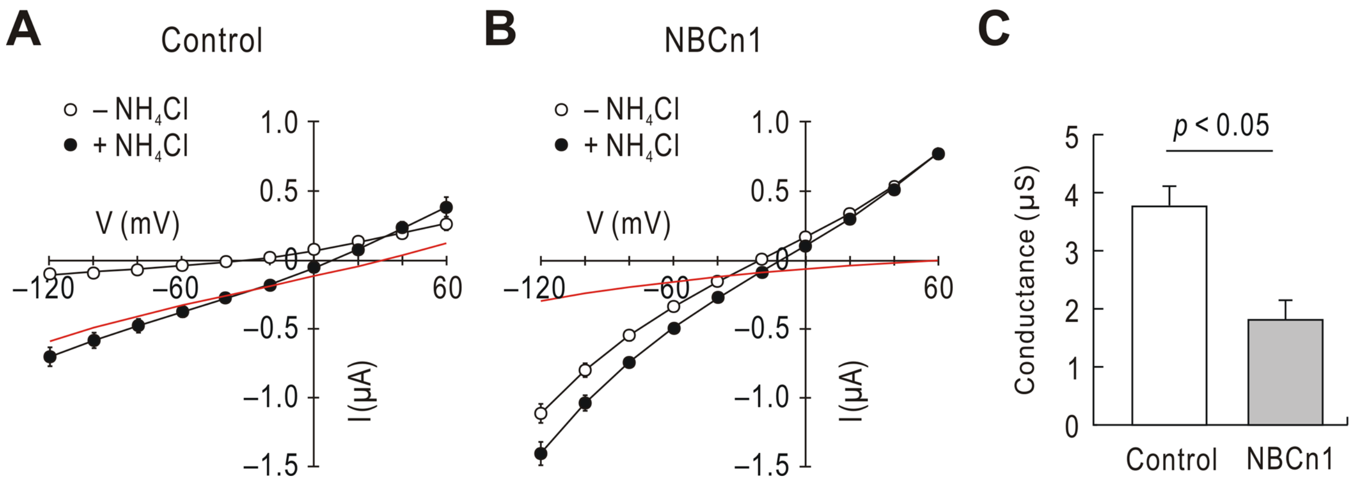

Fig. 4.

NH_4Cl-mediated currents at different voltages. **A**) Current–voltage (*I–V*) relationships before and after NH_4Cl application in control oocytes (n = 5). Oocytes were clamped at −60 mV and voltages were stepped from −120 mV to +60 mM with 20 mV increments before (*open circles*) and 2 min after NH_4Cl application (*closed circles*). The *red* line is the mean difference between the two *I–V* plots. **B**) *I–V* relationships in NBCn1-expressing oocytes (n = 5). Measurements were done using the protocol in **A**. Large basal currents before NH_4Cl application (*open circles*) are due to NBCn1 channel-like activity. **C**) Average conductances for NH_4Cl-mediated current under the two conditions. The slope conductance (G_{NH4Cl}) was measured between −80 and −40 mV using the relationships between NH4Cl-mediated current and voltage

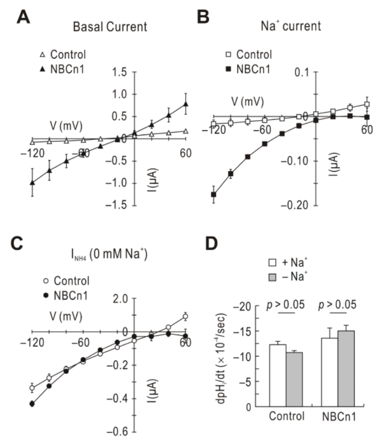

Fig. 5.

Channel-like activity of NBCn1 and its effect on NH_4Cl-mediated current. **A**) *I–V* relationships in HCO_3^-/CO_2-free solution containing 96 mM Na^+. Oocytes held at −60 mV were subjected to a step-voltage command from −120 to +60 mV. A large slope and a positive shift in the zero-current voltage are hallmarks for the channel-like activity of NBCn1 (n = 5 for each). **B**) Na^+ component of NBCn1 channel-like activity. The Na^+ component was measured by subtracting I in nominally Na^+-free solution from I in 96 mM Na^+ media (n = 4 controls and 5 NBCn1). $NMDG^+$ was substituted for Na^+. **C**) NH_4Cl-mediated currents at different voltages in Na^+-free condition. Currents were calculated using the protocol in Fig. 4 and plotted *versus* the voltage (n = 4 controls and 5 NBCn1). **D**) NH_4Cl-mediated pH_i changes mediated by NH_4Cl in Na^+-free solution. The dpH_i/dt was determined in oocytes exposed to 76 mM Na^+ (n = 4 for each) and in other oocytes exposed to 0 mM Na^+ (n = 5 for each).

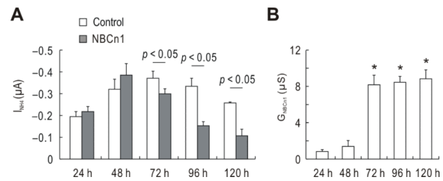

Fig. 6.

Time course of NH_4Cl-mediated current and NBCn1 channel-like activity. **A**) Time course of NH_4Cl-mediated currents after water or NBCn1 cRNA injection. Currents were daily measured in 4–6 oocytes from each group of oocytes. Measurements were done at −60 mV. **B**) Time course of NBCn1 channel-like activity. For this analysis, mean currents in control oocytes were first subtracted from currents of NBCn1-expressing oocytes at each voltage (n = 4–6 oocytes for each group at each time point). The subtracted values were then used to plot *I*–*V* relationships, from which the slope was calculated between −80 and −40 mV. The one-way ANOVA with Bonferroni post test was used to analyze the level of significance. Asterisks (*) represent $p < 0.05$ for comparison to the value at 48 h.

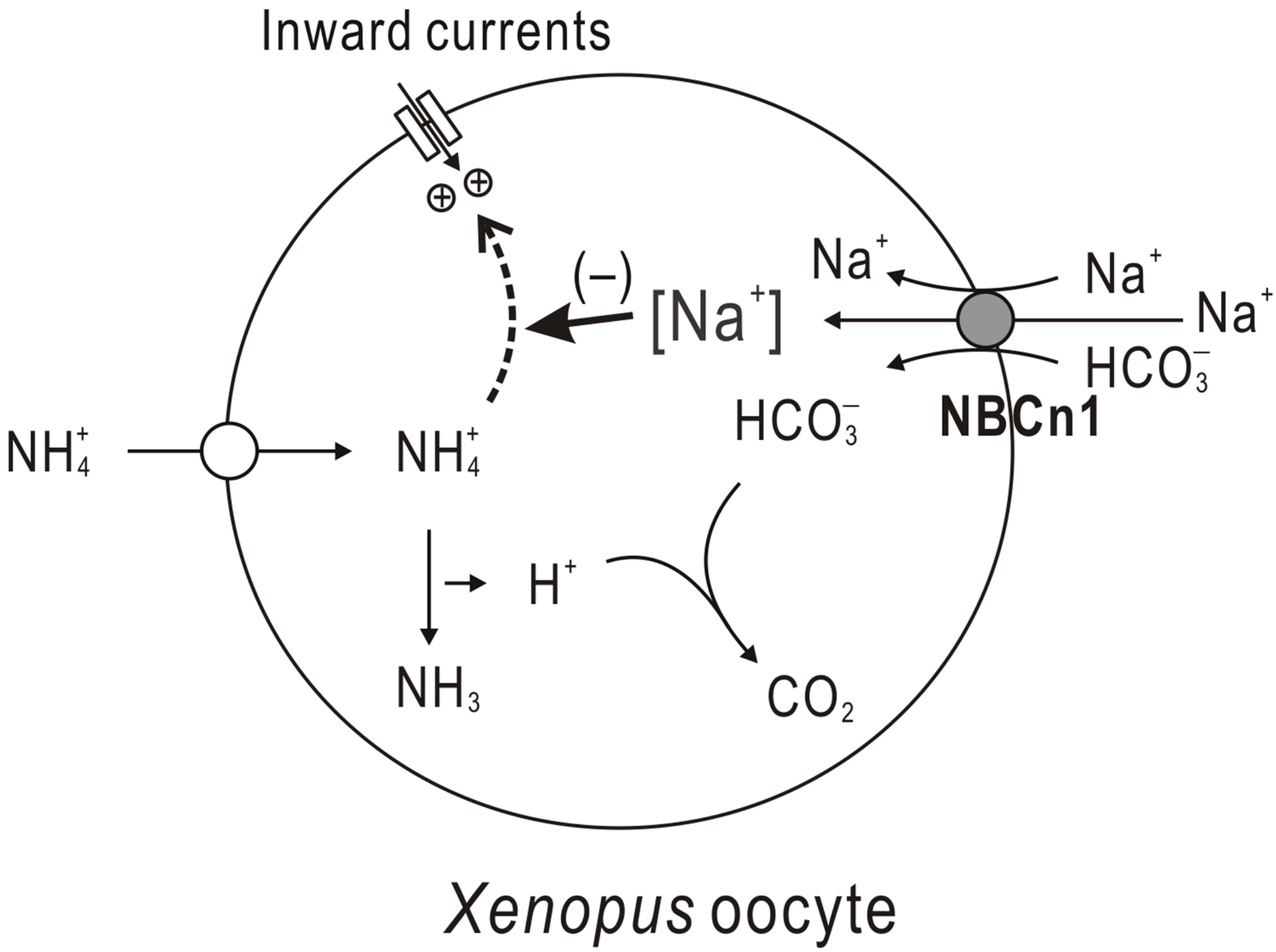

Fig. 7.

A model for the effect of NBCn1 on oocyte NH_4^+ transport. NH_4^+ enters into the cytosol via the endogenous NH_4^+ transporter and dissociates into NH_3 and H^+. Intracellular NH_4^+ or NH_3 secondarily causes the oocyte to induce an inward current that is affected by intracellular Na^+ (*dashed line*). The Na/HCO_3 cotransport activity of NBCn1 stimulates NH_4^+ transport by buffering H^+ dissociated from NH_4^+. On the other hand, the channel-like activity of NBCn1 inhibits NH_4^+ (or NH_3)-induced inward current by raising intracellular Na^+ in the Na/HCO_3-*in*dependent manner. The molecule responsible for the inward current is possibly a non-selective cation channel. The model assumes that oocyte NH_4^+ transport is mainly electroneutral.

Table 1

pH_i and V_m measurements of control oocytes and NBCn1-expressing oocytes. Recordings were performed in the presence of 20 mM NH_4Cl, 25 mM HCO_3^-, 5% CO_2.

	Control	NBCn1
Resting pH_i	7.36 ± 0.01	7.41 ± 0.03
pH_i in CO_2/HCO_3^-	6.94 ± 0.03	6.99 ± 0.02
dpH_i/dt before NH_4Cl	0.51 ± 0.12	$1.11 \pm 0.11^*$
dpH_i/dt after NH_4Cl	-3.22 ± 0.45	$-9.22 \pm 0.37^*$
dpH_i/dt due to NH_4^+ transport†	-3.73 ± 0.49	$-10.33 \pm 0.47^*$
Resting V_m	-28.7 ± 1.6	$-22.4 \pm 2.1^*$
ΔV_m	13.4 ± 1.2	10.3 ± 2.4
n	7	6

†Calculated by subtracting dpH_i/dt before NH_4Cl from dpH_i/dt after NH_4Cl.

*$p < 0.05$.