Ammonium interaction with the epithelial sodium channel

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Received 26 February 2001; accepted in final form 7 May 2001

Am J Physiol Renal Physiol 281: F493–F502, 2001.—The purpose of this study was to investigate the direct effect of NH4Cl on mouse epithelial Na+ channels (mENaC) expressed in Xenopus oocytes. Two-electrode voltage-clamp and ion-selective microelectrodes were used to measure the Na+ current, intracellular pH (pH_i), and ion activities in oocytes expressing mENaC. In oocytes expressing mENaC, removal of external Na+ reversibly hyperpolarized membrane potential by 129 ± 5.3 mV in the absence of 20 mM NH4Cl but only by 10 ± 7.8 mV in its presence. Amiloride completely inhibited the changes in membrane potential. In oocytes expressing mENaC, removal of external Na+ resulted in an outward current of 3.7 ± 0.9 μA when Na+ was removed in the presence of NH4Cl. In oocytes expressing mENaC, NH4Cl also caused a decrease in whole cell conductance at negative potential and an outward current at positive potential. In the presence of amiloride, steady-state current and the change in current caused by removal of Na+ were not different from zero. These results indicate that NH4Cl inhibits Na+ transport when mENaC is expressed in oocytes. The inhibition of voltage changes is not due to intracellular acidification caused by NH4Cl. Permeability and selectivity of ENaC to NH4 may play a role.

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In the renal distal nephron, NH$_4^+$ transport is critical for acid-base balance. Increases in NH$_3$/NH$_4^+$ and/or its effect on acid-base balance can have direct and indirect consequences on transport in general. For example, NH$_4^+$ has been shown to inhibit transport across the tight epithelium of the toad bladder (20). In another study on the rabbit cortical collecting duct, NH$_4$Cl was shown to decrease transepithelial voltage ($V_{te}$) and inhibit Na$^+$ reabsorption (22). In this latter study, the effect of NH$_4$Cl on $V_{te}$ was not evident when Na$^+$ reabsorption was inhibited by amiloride. This effect of NH$_4$Cl could be due to one or several causes, including voltage-dependent changes in Na$^+$ transport, pH$_i$-mediated effect on Na$^+$ conductance, and/or direct inhibition of the ENaC. The effect of NH$_4^+$ may be physiologically relevant to the increased urinary Na$^+$ observed with metabolic acidosis in vivo. This study was conducted to investigate the effect of NH$_4^+$ on Na$^+$ transport through ENaC expressed in *Xenopus* oocytes.

**METHODS**

Reagents and solutions. All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. The standard bathing solution was ND96 medium containing (in mM) 100 NaCl, 2 KCl, and 1.8 CaCl$_2$ and buffered with 5 mM HEPES to pH 7.5. The NH$_3$/NH$_4^+$ solution contained 20 mM NH$_4$Cl (replacing NaCl) at pH 7.5. In 80 mM Na$^+$ solutions, 20 mM N-methyl-d-glucamine (NMDG$^+$) replaced 20 mM Na$^+$, and in 0 Na$^+$ solutions, all Na$^+$ was replaced with NMDG$^+$. Osmolarity of all solutions was ~200 mosmol/l. OR3 medium (Leibovitz media, GIBCO BRL) contained glutamate and 500 U each of penicillin and streptomycin, with pH adjusted to 7.5 and osmolality adjusted to ~200 mosmol/l.

Isolation of oocytes. We harvested oocytes in stage 5/6 from female *Xenopus laevis*. Briefly, this was done by anesthetizing the frog by mild hypothermia in water containing 0.2% tricaine (3-aminobenzoic acid ethyl ester; Sigma, St. Louis, MO). A 1-cm incision was made in the abdominal wall, and one lobe of the ovary was externalized and the distal portion was cut. The wound was closed by a few stitches (5-0 catgut) in the muscular plane of the peritoneum followed by two to three stitches (6-0 silk) in the abdominal skin. The excised piece of ovary containing oocytes was rinsed several times with Ca$^{2+}$-free ND96 solution until the solution was clear. The tissue was then agitated in ~15 ml of sterile-filtered Ca$^{2+}$-free solution containing collagenase type 1A (Sigma) for 30–40 min. Free oocytes were rinsed several times with sterile OR3 medium, sorted, and then stored at 4°C.

Preparation of cRNA. Plasmid containing the appropriate template DNA of mouse ENaC (all a generous gift of Dr. Ton Kleyman) was purified by Wizard Plus Miniprep DNA Purification System (Promega, Madison, WI). The plasmid was then digested with an appropriate restriction enzyme that has a cleavage site downstream of the insert to produce a linear template and then with proteinase K (1 mg/ml). DNA was then extracted twice with phenol-chloroform, extracted with chloroform, and precipitated with ethanol. cDNA was transcribed in vitro with T7 RNA polymerase. The in vitro synthesis of capped RNA (cRNA) transcripts was then accomplished using mCAP RNA Capping Kit (Stratagene, La Jolla, CA). The concentration of cRNA was determined by ultraviolet absorbance, and its quality was assessed by formaldehyde-MOPS-1% agarose gel electrophoresis (37).

Injection of oocytes. Oocytes in OR3 medium were visualized with a dissecting microscope and injected with 50 nl of full-length cRNA mix of mENaC containing cRNAs for the α-, β-, and γ-subunits. At 0.05 μg/μl concentration for each subunit, each oocyte was injected with 2.5 ng/subunit. Control oocytes were injected with 50 nl of sterile H$_2$O. The sterile pipettes had tip diameters of 20–30 μm. They were backfilled with paraffin oil and were connected to a Nanoject displacement pipette (Drummond Scientific). Injected oocytes were used 2–5 days after injection with cRNA.

Electrophysiological measurements in frog oocytes. The pH microelectrodes were made from the liquid ion-exchanger type, and the resin (H$^+$ ionophore 1, cocktail B) was obtained from Fluka Chemical (Ronkonkoma, NY). Single-barreled microelectrodes were manufactured as described previously (31, 36). Briefly, alumina-silicate glass tubing (1.5 mm OD × 0.86 mm ID; Frederick Haer, Brunswick, MD) were pulled to a tip of ~0.2 μm and dried in an oven at 200°C for 2 h. The electrodes were vapor silanized with bis(dimethylamino)diethylsilane in a closed vessel (300°C). The exchanger was then introduced into the tip of the electrodes by means of a very fine glass capillary. pH electrodes were backfilled with a buffer solution (3). The electrodes were fitted with a holder with an Ag-AgCl pellet attached to a high-impedance probe of a WPI FD-223 electrometer. The pH electrodes were calibrated in standard solutions of pH 6 and 8. Only electrodes with a slope >58 mV/pH were used in our studies.

The oocyte, visualized with a dissecting microscope, was held on a nylon mesh in a special chamber where solutions flow continuously at a rate of ~4 ml/min. Solutions (6 possible) were switched by a combination of a six- and a four-way valve system, which was activated pneumatically. Very little dead space was present, and complete solution changes in the chamber occurred in ~6–8 s.

Two-electrode voltage clamp. Whole cell currents were recorded using two-electrode voltage clamp (model OC-725, Warner Instruments, Hamden, CT). For these experiments, electrodes were pulled from borosilicate glass capillaries (OD 1.5 mm; Fredrick Haer) using a vertical puller (model 700C, David Kopf Instruments). Electrodes were filled with 3 M KCl solution and had resistances of 1–4 MΩ. Bath electrodes were also filled with 3 M KCl and were directly immersed in the chamber. In most cases, oocytes were clamped at ~60 mV, and long-term readings of current were sampled at a rate of once per second. For current-voltage (I-V) relationships, oocytes were clamped at 0 mV and stepped from −80 to +60 mV in 20-mV steps (sampled at 10 times/s for 1 s at each step). Slope conductances were calculated from the slope of the I-V line between −60 and +40 mV. Inward flow of cations is defined by convention as inward (negative) current.

Statistics and data analysis. Values are means ± SE; n is the number of observations. Statistical significance was judged primarily from two-tailed Student’s t-tests. Whenever feasible, measurements were determined under control and test conditions in the same cell, and each cell served as its own control (paired data). Results are considered statistically significant if P < 0.05.

**RESULTS**

Intracellular ionic compositions in control (water-injected) oocytes and oocytes expressing mENaC. The oocyte expression system has been extensively used to study the characteristics of expressed proteins, many of which are membrane transporters and ionic channels. The high level of expression usually achieved, the lack of substantial native transport in the oocyte, and
the relative ease of measurements in this preparation make the oocyte a valuable system for study. A concern that has been inadequately addressed is that expressing an exogenous transporter may alter the cellular environment of the oocyte. An altered cellular environment may, by itself, be a factor affecting the function of the transporter. Because expressing an ion channel may cause such significant effects, we first characterized the cellular changes induced by expressing mENaC under steady-state conditions. As shown in Table 1, intracellular composition of the important monovalent ions was drastically and significantly altered. As expected, intracellular Na+ activity increased from 6.6 ± 0.6 to 30.0 ± 1.8 mM and steady-state membrane potential (Vm) was positive (+4.5 ± 1.6 mV). This was accompanied by substantial decrease in intracellular K+ activity (aK) from 74.3 ± 2.7 to 40.0 ± 0.5 mM, whereas intracellular Cl− activity (aCl) increased from 25.0 ± 0.3 to 75.3 ± 1.9 mM. pH increased slightly from 7.34 ± 0.02 to 7.5 ± 0.02, but the difference was not statistically significant (P > 0.05).

Because the ENaC is usually overexpressed, it is conceivable that acute changes in external Na+ may induce large changes in intracellular Na+ activity (aNa). To investigate this possibility, we measured the changes in aNa by Na+-selective microelectrodes when external Na+ was removed (replaced by NMDG−). In control oocytes, removal of external Na+ (for ~10 min) hardly caused any detectable decrease in aNa (ΔaNa = 0.9 ± 0.1 mM, n = 4). Unexpectedly, in mENaC oocytes, removal of external Na+ also caused a very small change in aNa (ΔaNa = 1.8 ± 0.4 mM, n = 4). These experiments indicate that intracellular Na+ in the iso- toes is not readily exchangeable, and no acute large changes in aNa are expected to occur in response to Na+ removal, even when mENaC is expressed.

Effect of NH3/NH4+ and removal of Na+ on water-injected oocytes. To study the effect of NH4+ on Na+ transport, we first characterized the changes caused by addition of NH4+ or removal of external Na+ in water-injected oocytes. As shown in Fig. 1, exposing control oocytes to NH4+ induced a hyperpolarization from 129 ± 5.3 mV in the absence of NH3/NH4+ and −100 ± 7.8 mV in the presence of NH3/NH4+ (P < 0.001). These experiments indicate that NH3/NH4+ may inhibit Na+ transport in oocytes expressing mENaC.

In these experiments, NH3/NH4+ solutions contain 20 mM NH4Cl obtained by replacing 20 mM NaCl from the control ND96 solution. Because Na+-induced voltage changes in oocytes expressing mENaC are substantial (Fig. 2A), it is conceivable that this partial removal of Na+ could be responsible in part for the reduced hyperpolarization induced by removal of Na+.

Table 1. pH, cellular ionic activities, and steady-state Vm in control oocytes and oocytes expressing mENaC

<table>
<thead>
<tr>
<th>pH</th>
<th>aNa</th>
<th>aK</th>
<th>aCl</th>
<th>Vm</th>
<th>mENaC</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>7.34 ± 0.04</td>
<td>6.6 ± 0.6</td>
<td>74.3 ± 2.7</td>
<td>25 ± 0.3</td>
<td>−70 ± 2.9</td>
</tr>
<tr>
<td>(8)</td>
<td>(8)</td>
<td>(8)</td>
<td>(8)</td>
<td>(24)</td>
<td></td>
</tr>
<tr>
<td>mENaC</td>
<td>7.5 ± 0.02</td>
<td>30 ± 1.8</td>
<td>40 ± 0.5</td>
<td>75.3 ± 1.9</td>
<td>+4.5 ± 1.6</td>
</tr>
<tr>
<td>(5)</td>
<td>(10)</td>
<td>(6)</td>
<td>(6)</td>
<td>(28)</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. Number of observations is in parentheses. Control oocytes were injected with H2O. Oocytes were bathed with a HEPES-Ringer solution (pH 7.5) in the absence of CO2/HCO3−. Measurements were obtained using single-barreled ion selective microelectrodes. pH, intracellular pH; aNa, aK, and aCl, intracellular Na+, K+, and Cl− activities, respectively; Vm, membrane potential; mENaC, mouse epithelial Na+ channel.
Effect of amiloride on $V_m$. Inhibition of the ENaC by amiloride is one of the main properties that characterize this channel. The next set of experiments was designed to investigate this possibility. As shown in Fig. 2B, removal of external Na$^+$ (from 100 to 0 mM in the bath) caused the usual large and reversible hyperpolarization observed earlier (segments ab and bc). At point c, 20 mM Na$^+$ was removed from the bath (replaced by NMDG$^+$), resulting in a small hyperpolarization of $-4 \text{ mV}$ (segment cd). On switching to NH$_3$/NH$_4^+$ solution at point d, no Na$^+$ was replaced, and the external solution still contained 80 mM Na$^+$. This maneuver caused a small depolarization (segment de). Removal of Na$^+$ in the presence of NH$_3$/NH$_4^+$ caused hyperpolarization (segment ef) that was substantially smaller than in the absence of NH$_3$/NH$_4^+$. Readdition of Na$^+$ to the bath caused $V_m$ to recover (segment fg). At point g, the bath solution was switched to a solution free of NH$_3$/NH$_4^+$ but containing 80 mM Na$^+$, which caused a small hyperpolarization (segment gh). Removal of bath Na$^+$ again (from 80 to 0 mM Na$^+$) caused hyperpolarization of $V_m$ similar to the first pulse (from 100 to 0 mM Na$^+$), which was significantly larger than the hyperpolarization of $V_m$ in the presence of NH$_3$/NH$_4^+$ (segment hi). In three paired experiments, $\Delta V_m$ (from 80 to 0 mM Na$^+$) was $-120 \pm 4 \text{ mV}$ in the absence of NH$_3$/NH$_4^+$ and only $-61 \pm 10.9 \text{ mV}$ in the presence of NH$_3$/NH$_4^+$. These experiments further indicate that the presence of NH$_3$/NH$_4^+$ is affecting the 0 Na$^+$ response in oocytes expressing mENaC.

Effect of amiloride on $V_m$. Removal at low $p\text{H}_i$ or in the presence of NH$_3$/NH$_4^+$ in oocytes expressing mENaC. In contrast to most other cells, where exposure to NH$_3$/NH$_4^+$ causes an initial increase in $p\text{H}_i$ [NH$_4^+$ prepulse (9)], in the oocytes, NH$_3$/NH$_4^+$ causes a significant sustained decrease in $p\text{H}_i$ (11, 38). One possibility is, therefore, that the low $p\text{H}_i$ is responsible for the inhibition of the Na$^+$-induced change in $V_m$ in the presence of NH$_3$/NH$_4^+$. To investigate this possibility, we decreased $p\text{H}_i$ in the presence of NH$_3$/NH$_4^+$.
of oocytes expressing mENaC, independently of NH$_3$/NH$_4^+$, and measured the $V_m$ changes induced by removal of bath Na$^+$. As shown in Fig. 3, removal of external Na$^+$ caused no change in pH$_i$ (segment $ab$) except the usual large hyperpolarization, which readily recovered on readdition of Na$^+$ (segment $bc$). At point $c$, butyrate (20 mM) was added to the bath, causing a significant decrease in pH$_i$ (segment $cd$) and very little change in $V_m$. At low pH$_i$, Na$^+$ was again removed from the bath, which did not cause a significant further change in pH$_i$, but $V_m$ became more negative (segment $de$). In five paired experiments, the hyperpolarization caused by removal of Na$^+$ was 146 ± 2.7 mV in the absence of butyrate, a value not significantly different from 149 ± 5.4 mV in the presence of butyrate ($P > 0.05$). All these changes were completely reversible: $V_m$ depolarized again on readdition of Na$^+$ to the bath (segment $ef$), and pH$_i$ fully recovered on removal of butyrate (segment $fg$). At point $g$, the oocytes were exposed to NH$_3$/NH$_4^+$, which caused a small change in $V_m$, but pH$_i$ decreased substantially (segment $gh$). When bath Na$^+$ was removed again, pH$_i$ decreased a little further and the cell hyperpolarized, as shown previously (segment $hi$). The pH$_i$ decrease caused by NH$_3$/NH$_4^+$ (0.47 ± 0.12) was not statistically different from the pH$_i$ decrease of 0.43 ± 0.07 caused by butyrate ($P > 0.05$). However, the cell hyperpolarization caused by removal of Na$^+$ in the presence of butyrate (149 ± 5.4 mV) was significantly larger than that in the presence of NH$_3$/NH$_4^+$ (109 ± 5.5 mV, $P < 0.05$). These experiments are consistent with a significant effect of

Fig. 2. A: effect of Na$^+$ removal in the presence and absence of NH$_3$/NH$_4^+$ in mouse oocytes expressing the epithelial Na$^+$ channel (ENaC). Trace represents 10 experiments showing that removal of Na$^+$ hyperpolarized the oocyte by 129 ± 5.3 mV (segment $ab$), which was readily reversed when Na$^+$ was returned to normal (segment $bc$). Exposing oocytes expressing mENaC to NH$_3$/NH$_4^+$ (segment $cd$) caused a slight hyperpolarization ($\Delta V_m = -4.3 ± 1.4$ mV). Removal of Na$^+$ in the presence of NH$_3$/NH$_4^+$ hyperpolarized the oocyte by 100 ± 7.8 mV (segment $de$), which was significantly less than the hyperpolarization in the absence of NH$_3$/NH$_4^+$ ($P < 0.05$). Hyperpolarization was reversed on readdition of Na$^+$ to the bath (segment $ef$). Removal of NH$_3$/NH$_4^+$ from the bath caused a small depolarization (segment $fg$). Removal of Na$^+$ again in the absence of NH$_3$/NH$_4^+$ caused a larger hyperpolarization (segment $gh$) as seen in the first pulse (compare with segment $ab$). Readdition of Na$^+$ fully reversed this effect on $V_m$ (segment $hi$). B: Na$^+$ removal in the presence and absence of NH$_3$/NH$_4^+$ in oocytes expressing mENaC. When bath Na$^+$ at 100 mM was removed (from 100 to 0 mM), $V_m$ hyperpolarized (from 100 to 100 mV) significantly (segment $ab$) as seen in A and was reversed on restoration of bath Na$^+$ to 100 mM (segment $bc$). Replacing 20 mM Na$^+$ with N-methyl-D-glucamine (NMDG, 80 mM Na$^+$) caused a small hyperpolarization (segment $cd$). Subsequent exposure to NH$_3$/NH$_4^+$ (at 80 mM Na$^+$) caused a small depolarization (segment $de$). Removal of Na$^+$ in the presence of NH$_3$/NH$_4^+$ caused a smaller hyperpolarization (segment $ef$) than that observed with Na$^+$ removal in the absence of NH$_3$/NH$_4^+$ (compare with segment $ab$). Readdition of 80 mM Na$^+$ to the bath reversed this effect on $V_m$ (segment $fg$). Removal of bath NH$_3$/NH$_4^+$ caused a small depolarization (segment $gh$). When bath Na$^+$ at 80 mM was removed again (going from 80 mM to 0 mM), $V_m$ hyperpolarized (segment $hi$) to the same extent as in the first pulse (compare with segment $ab$). C: effect of amiloride on $V_m$ in oocytes expressing mENaC. Exposing oocytes to amiloride (100 μM) caused hyperpolarization of 77 ± 4.1 mV (segment $ab$). Addition of NH$_3$/NH$_4^+$ in the presence of amiloride caused a transient hyperpolarization (segment $bc$) followed by sustained depolarization of 23 ± 8.7 mV (segment $cd$). Removal of Na$^+$ in the presence of NH$_3$/NH$_4^+$ and amiloride caused a small but statistically insignificant ($P > 0.05$) depolarization of $V_m$ (segment $de$). All changes were completely reversible: readdition of Na$^+$ in the presence of amiloride hyperpolarized $V_m$ slightly (segment $ef$) and removal of NH$_3$/NH$_4^+$ hyperpolarized $V_m$ even further (segment $fg$).
Fig. 3. Na\(^+\) removal at low pH, in oocytes expressing mENaC. Because NH\(_3/\text{NH}_4^+\) causes substantial intra-cellular acidification, this experiment examined the effect of low pH on changes caused by Na\(^+\) removal. Na\(^+\) removal at steady-state pH\(_i\) caused the usual large hyperpolarization of V\(_m\) but had no effect on pH\(_i\) (segment ab). Readdition of bath Na\(^+\) completely reversed this effect (segment bc). pH\(_i\) was then acidiﬁed by exposing the oocyte to 20 mM butyrate (segment cd), and V\(_m\) was not affected. At low pH\(_i\) (point d), removal of Na\(^+\) (in the continued presence of butyrate) hyperpolarized the oocyte to a value similar to that at high pH\(_i\) in the absence of butyrate (segment de). On readdition of Na\(^+\) in the presence of butyrate, V\(_m\) recovered, but pH\(_i\) was not affected (segment ef). On removal of butyrate, pH\(_i\) recovered, but V\(_m\) was not affected (segment fg). Exposing the oocyte to NH\(_3/\text{NH}_4^+\) caused a substantial decrease in pH\(_i\) comparable to that caused by butyrate and slightly depolarized V\(_m\) (segment gh). Subsequent removal of Na\(^+\) in the presence of NH\(_3/\text{NH}_4^+\) caused hyperpolarization (segment hi) that was signiﬁcantly less than that caused by removal of Na\(^+\) in the presence of butyrate (compare with segment de).

NH\(_3/\text{NH}_4^+\) on Na\(^+\)-induced V\(_m\) changes, which are independent of pH\(_i\).

Current changes in response to Na\(^+\) removal in the presence and absence of NH\(_3/\text{NH}_4^+\) in oocytes expressing mENaC. To further study the effect of NH\(_3/\text{NH}_4^+\) on Na\(^+\) transport in oocytes expressing mENaC, we measured the changes in whole cell current in response to removal of Na\(^+\). As can be seen in Fig. 4, removal of bath Na\(^+\) caused an outward deflection in whole cell current (I\(_m\); oocyte clamped at -60 mV, segment ab) that was readily reversed when Na\(^+\) was returned to the bath (segment bc). Exposing the oocyte to 20 mM total NH\(_4^+\) caused an inward change in I\(_m\) of 383 ± 5.1 nA (segment cd). This current is supposedly caused by NH\(_4^+\) entry through a nonselective cationic channel as observed in water-injected oocytes (10, 11, 36). In the presence of NH\(_3/\text{NH}_4^+\), removal of bath Na\(^+\) again resulted in outward deflection of I\(_m\) (segment de), which was reversed when Na\(^+\) was restored to the bath (segment ef). In nine paired experiments, the outward deflection in the absence of NH\(_3/\text{NH}_4^+\) was 3.7 ± 0.8 nA but was only 2.7 ± 0.7 nA in its presence (P < 0.01). These experiments are consistent with the original observation that NH\(_3/\text{NH}_4^+\) inhibits Na\(^+\) transport in the oocyte when mENaC is expressed.

Amiloride inhibition of Na\(^+\) and NH\(_4^+\) whole cell currents in oocytes expressing mENaC. The data presented so far suggest that, in the presence of NH\(_3/\text{NH}_4^+\), Na\(^+\) transport through the epithelial Na\(^+\) channel expressed in oocytes is partially inhibited. Because mENaC is blocked by amiloride, we investigated whether inhibiting mENaC would abolish the effect of NH\(_3/\text{NH}_4^+\) on Na\(^+\)-induced changes in I\(_m\). As shown in Fig. 5, exposing oocytes expressing mENaC (clamped...
at −60 mV) to amiloride completely blocked the inward current (segment abc). In the presence of amiloride, no significant change in $I_m$ occurred when Na$^+$ was removed (segment cd) or readded (segment de) to the bath. The small changes in $I_m$ in response to Na$^+$ removal or addition in the bath were much different from those in the absence of amiloride and resemble the Na$^+$-induced effects on $I_m$ that occur in water-injected oocytes. In the continued presence of amiloride, addition of NH$_3$/NH$_4^+$ (segment ef) or its removal (segment fg) did not cause any significant changes in the current. The oocyte was clamped at −60 mV.

increase in $I_m$ (segment cd) that settled to a value more positive than that before addition of NH$_3^+$, indicating an outward current (segment de). This also indicates that NH$_3^+$ entry through a conductive pathway probably did not occur. When bath Na$^+$ was removed in the presence of NH$_3$/NH$_4^+$, there was the usual positive shift in $I_m$ (segment ef), and readdition of Na$^+$ to the bath reversed this effect with an inward shift of $I_m$ to its original value (segment gh). Removal of bath NH$_3$/NH$_4^+$ (80 Na$^+$) caused $I_m$ to become more negative (segment gh), reaching a value not significantly different from the original value (at point a) before addition of NH$_3$/NH$_4^+$. In four paired experiments, the average change in $I_m$ caused by removal of bath Na$^+$ in the absence of NH$_3$/NH$_4^+$ was 3.4 ± 0.72 μA but was only 1.7 ± 0.23 μA in the presence of NH$_3$/NH$_4^+$ ($P < 0.05$). These experiments indicate that even when NH$_4^+$ influx is limited, the presence of NH$_3^+$ still reduces the Na$^+$ response in oocytes expressing mENaC.

Whole cell conductance in the presence and absence of NH$_3$/NH$_4^+$. The last set of experiments was conducted to examine the effect of NH$_3$/NH$_4^+$ on whole cell conductance of oocytes expressing mENaC. To do so, we plotted the whole cell currents in relation to test potentials used to clamp the oocyte between −80 and +40 mV in steps of 20 mV. Figure 7 shows the I-V relationship in the presence and absence of NH$_3$/NH$_4^+$. The results of this and similar experiments indicate that in the presence of NH$_3$/NH$_4^+$ the slope of the curve (i.e., conductance) is smaller at negative potentials. However, the current at positive potentials was significantly higher in the presence of NH$_3$/NH$_4^+$, indicating the possible activation of an outward current. The nature of the increased outward current at positive potentials in the presence of NH$_3$/NH$_4^+$ is not known. In eight experiments, the whole cell conductance calcu-

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Fig. 5. Amiloride inhibition of Na$^+$ and NH$_3^+$ currents in mENaC oocytes. Amiloride (100 μM) completely inhibited the inward current in oocytes expressing mENaC (segment abc). In the presence of amiloride, Na$^+$ removal (segment cd) or addition of NH$_3$/NH$_4^+$ (segment ef) did not cause any significant changes in the current. The oocyte was clamped at −60 mV.

Effect of NH$_3$/NH$_4^+$ on Na$^+$ current at positive potential. NH$_3$/NH$_4^+$ application to oocytes results in two main effects: 1) a significant intracellular acidification and 2) a huge depolarization of $V_m$. Both effects are attributed to the influx of NH$_3^+$ and a relatively low permeability of NH$_3$. In oocytes expressing mENaC, NH$_3$/NH$_4^+$ could influence Na$^+$ transport by several mechanisms: 1) NH$_3^+$ permeation into the cell results in a pH decrease, which in turn inhibits the Na$^+$ channel. 2) NH$_3^+$ could be permeating through ENaC and, therefore, affecting Na$^+$ transport through the channel. 3) Extracellular NH$_3^+$ modulates mENaC. In the next set of experiments, we investigated whether limiting NH$_3^+$ entry could influence the Na$^+$-induced response in oocytes expressing mENaC. To do so, we clamped the oocyte at positive potential (+10 mV) and measured the whole cell current in response to Na$^+$. As shown in Fig. 6, clamping the oocyte at +10 mV, the whole cell current was positive. Removal of bath Na$^+$ (from 80 to 0 mM) caused an outward deflection in $I_m$ (segment ab) that readily recovered when bath Na$^+$ was restored to control (segment bc). At point c, exposing the oocyte to NH$_3$/NH$_4^+$ caused a sharp transient increase in $I_m$ (segment cd) that settled to a value more positive than that before addition of NH$_3^+$, indicating an outward current (segment de). This also indicates that NH$_3^+$ entry through a conductive pathway probably did not occur. When bath Na$^+$ was removed in the presence of NH$_3$/NH$_4^+$, there was the usual positive shift in $I_m$ (segment ef), and readdition of Na$^+$ to the bath reversed this effect with an inward shift of $I_m$ to its original value (segment gh). Removal of bath NH$_3$/NH$_4^+$ (80 Na$^+$) caused $I_m$ to become more negative (segment gh), reaching a value not significantly different from the original value (at point a) before addition of NH$_3$/NH$_4^+$. In four paired experiments, the average change in $I_m$ caused by removal of bath Na$^+$ in the absence of NH$_3$/NH$_4^+$ was 3.4 ± 0.72 μA but was only 1.7 ± 0.23 μA in the presence of NH$_3$/NH$_4^+$ ($P < 0.05$). These experiments indicate that even when NH$_4^+$ influx is limited, the presence of NH$_3^+$ still reduces the Na$^+$ response in oocytes expressing mENaC.

Whole cell conductance in the presence and absence of NH$_3$/NH$_4^+$. The last set of experiments was conducted to examine the effect of NH$_3$/NH$_4^+$ on whole cell conductance of oocytes expressing mENaC. To do so, we plotted the whole cell currents in relation to test potentials used to clamp the oocyte between −80 and +40 mV in steps of 20 mV. Figure 7 shows the I-V relationship in the presence and absence of NH$_3$/NH$_4^+$. The results of this and similar experiments indicate that in the presence of NH$_3$/NH$_4^+$ the slope of the curve (i.e., conductance) is smaller at negative potentials. However, the current at positive potentials was significantly higher in the presence of NH$_3$/NH$_4^+$, indicating the possible activation of an outward current. The nature of the increased outward current at positive potentials in the presence of NH$_3$/NH$_4^+$ is not known. In eight experiments, the whole cell conductance calcu-
The \( \text{Na}^+ \) transport and \( \text{mENaC} \) activity is shown in Table 1. This value is almost five times more than the \( \text{NH}_4^+ \) potential and a significant outward current in the presence of \( \text{NH}_3/\text{NH}_4^+ \). In such studies, it has been argued that the high \( \text{ENaC} \) activity at the tip of the channel (6). Although some compartmentalization may occur, it is very likely that the contribution of other ions (and their respective membrane permeabilities) to the reversal potential is underestimated in oocytes expressing \( \text{ENaC} \). A third point to note is that, in our experiments, complete removal of external \( \text{Na}^+ \) resulted in only small changes in \( a_{i\text{Na}}^+ (\Delta a_{i\text{Na}}^+ = 1.8 \text{ mM}) \).

Although the volume of the oocyte is large, this was unexpected and indicates that even though \( \text{ENaC} \) is usually overexpressed, total intracellular \( \text{Na}^+ \) is not readily exchangeable through the channel. In support of this observation, we calculated the change in intracellular \( \text{Na}^+ \) from the change in current caused by removal of external \( \text{Na}^+ \) (Fig. 4). For a change in current of 3.7 \( \mu \text{A} \) over a period of \( \sim 100 \text{ s} \), the number of coulombs (\( Q \)) presumably carried by movement of \( \text{Na}^+ \) can be calculated as

\[
Q = I \times t
= 3.7 \times 10^{-6} \times 100
= 370 \times 10^{-6} \text{ coulombs}
\]

where current is measured in amperes and time in seconds. If \( \text{Na}^+ \) transfer is responsible for all this change, then the number of moles of \( \text{Na}^+ \) that would result in this change can be calculated from dividing by Faraday’s constant, which results in

\[
370 \times 10^{-6}/96,500 \text{ mol Na}^+
\]

If the oocyte volume is \( \sim 0.9 \text{ \mu l} \) (with the assumption of a spherical volume with a diameter of 1.2 mm), then the calculated change in intracellular \( \text{Na}^+ \) concentration is

\[
(370 \times 10^{-6}/96,500) \times (1/0.9)
\times (10^6) \mu \text{mol/\mu l (M) = 4.2 mM}
\]

This value is much smaller than the presumed large changes in \( a_{i\text{Na}}^+ \) and within the small range of intracellular \( \text{Na}^+ \) change measured with microelectrodes.

**Inhibition of \( \text{Na}^+ \) transport with \( \text{NH}_3/\text{NH}_4^+ \).** The present study suggests that \( \text{NH}_3/\text{NH}_4^+ \) inhibits \( \text{Na}^+ \) transport via \( \text{ENaC} \). Urinary excretion of \( \text{NH}_3/\text{NH}_4^+ \) is very important for acid-base homeostasis. Two-thirds of net acid excretion in the urine is via \( \text{NH}_4^+ \), and in systemic acidosis intratubular \( \text{NH}_4^+ \) concentrations and renal cortical ammonia are increased (10). Under these conditions, urinary levels of total ammonia can easily exceed 50 mmol/l. In the cortical collecting duct, peritubular \( \text{NH}_4\text{Cl} \) was shown to inhibit transepithelial \( \text{Na}^+ \) transport (22, 23). In other tight epithelia, \( \text{NH}_4\text{Cl} \) was also reported to influence \( \text{Na}^+ \) transport (20). This effect of \( \text{NH}_3/\text{NH}_4^+ \) on \( \text{Na}^+ \) transport and its importance in regulation of acid-base homeostasis raise the possibility that \( \text{NH}_3/\text{NH}_4^+ \) may directly or indirectly affect transport through \( \text{ENaC} \).

The first evidence for the inhibitory effect on \( \text{ENaC} \) by \( \text{NH}_4^+ \) was obtained from measurements of voltage changes induced by \( \text{Na}^+ \) removal in the presence and absence of \( \text{NH}_4^+ \). The studies showed that \( I \) in the presence of \( \text{NH}_4^+ \), the hyperpolarization caused by re-

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**Fig. 7.** Current-voltage \((I-V)\) relationship for \( \text{mENaC} \) in the presence and absence of \( \text{NH}_3/\text{NH}_4^+ \). Representative whole cell recordings show \( I-V \) relationships of oocytes expressing \( \text{mENaC} \) in the presence and absence of \( \text{NH}_3/\text{NH}_4^+ \). Control oocytes are oocytes expressing \( \text{mENaC} \) bathed in standard solution containing 80 mM Na\(^+\). The same oocyte was then exposed to a solution containing 20 mM total \( \text{NH}_4^+ \) and 80 mM \( \text{Na}^+ \). The experiment indicates a small negative shift in reversal potential and a significant outward current in the presence of \( \text{NH}_3/\text{NH}_4^+ \) at positive potentials.

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**DISCUSSION**

The \( \text{ENaC} \) plays a pivotal role in regulation of \( \text{Na}^+ \) balance, which in turn is critical for regulation of extracellular fluid volume and blood pressure. Regulation of \( \text{ENaC} \) is accomplished by a complex interaction of several mechanisms, including hormonal factors such as aldosterone and vasopressin and nonhormonal factors such as \( \text{pHi}, \text{Na}^+, \) and \( \text{Ca}^{2+} \) (8, 19, 35).

**Ionic activities.** In this study we report the first measurements of the changes in intracellular ionic activities that occur as a result of expressing \( \text{ENaC} \) in oocytes. As shown in Table 1, these changes are very significant. Three important observations are evident from these results. First, although an increase in \( a_{i\text{Na}}^+ \) is expected when \( \text{ENaC} \) is expressed, the decrease in \( a_{i\text{K}}^- \) and the increase in \( a_{i\text{Cl}}^- \) were not known or measured previously. Because the oocyte has a significant permeability to \( \text{K}^+ \) and \( \text{Cl}^- \), ignoring these changes in studies involving measurements in the intact oocyte could result in significant errors. For instance, one such possibility is an erroneous estimation of the \( \text{Na}^+ \) reversal potential, which will vary depending on the \( \text{Cl}^- \) content of the bathing medium. The second important observation is the value of \( a_{i\text{Na}}^+ \) of 30 ± 1.8 mM shown in Table 1. This value is almost five times more than that of control oocytes; however, it is much smaller than the value (50–60 mM) usually calculated from reversal potential of \( \text{Na}^+ \) in oocytes expressing \( \text{ENaC} \). In such studies, it has been argued that the high \( a_{i\text{Na}}^+ \) (calculated from the reversal potential) represents the microsopic value of \( \text{Na}^+ \) activity at the tip of the channel (6). Although some compartmentalization may occur, it is very likely that the contribution of other ions (and their respective membrane permeabilities) to the reversal potential is underestimated in oocytes expressing \( \text{ENaC} \).
moval of bath Na$^+$ was significantly inhibited, 2) the voltage inhibition by NH$_4^+$ is not due to intracellular acidification caused by NH$_4^+$, and 3) the effect of NH$_4^+$ on Na$^+$ removal was completely blocked by amiloride, demonstrating that the inhibitory effect of NH$_4^+$ is probably through an effect on ENaC. The effects of NH$_4^+$ are not due to significant changes in $a_{i}^{\text{Na}}$. Evidence for this comes from the experiment of Fig. 2B, where NH$_4^+$ inhibited Na$^+$-induced V$_m$ changes irrespective of whether initial external Na$^+$ was 100 or 80 mM. This is further supported by our previous observation that complete removal of external Na$^+$ caused only a small change in $a_{i}^{\text{Na}}$, measured with microelectrodes.

Measurement of current in response to Na$^+$ removal further demonstrated the inhibition of ENaC by NH$_4^+$. Again, in the presence of 100 or 80 mM external Na$^+$, NH$_4^+$ inhibited the outward current caused by removal of bath Na$^+$. In the presence of amiloride, neither Na$^+$ removal nor NH$_4^+$ resulted in significant changes in current. The voltage and current data indicate that NH$_4^+$ inhibits ENaC.

Many studies (7, 19, 33, 42) have addressed the role of pH in regulating ENaC activity. As reviewed by Lyall et al. (28), decreases in extracellular pH and/or pH$_i$ have usually been reported to decrease Na$^+$ transport in tight epithelia. The direct studies of Chalfant et al. (13) on oocytes expressing ENaC recently reported that a decrease in pH$_i$, but not extracellular pH$_i$, reduced single-channel open probability and open time without altering single-channel conductance. This study further indicated that the $\alpha$-subunit of ENaC is directly regulated by pH$_i$. In contrast, however, Awada et al. (7) reported that luminal acidosis over a period of minutes stimulates Na$^+$ current in A6 cells. On the other hand, more prolonged changes in pH$_i$, such as in vivo systemic acidosis, may have complicated effects. For example, Kim et al. (26) reported that long-term acid loading in vivo by NH$_4$Cl produced a large decrease in the abundance of $\beta$- and $\gamma$-subunits of ENaC but had no effect on the $\alpha$-subunit.

In our study, the presence of NH$_4^+$ clearly affects ENaC in a manner distinct from a pH$_i$ inhibition of the channel. Although NH$_4^+$ inhibited the whole cell conductance (consistent with a pH$_i$ effect), a decrease in pH$_i$ is unlikely to account for all the inhibitory effect of NH$_4^+$ on the basis of the studies with butyrate in Fig. 3 and the studies with voltage clamp at positive potential in Fig. 6. These latter experiments demonstrated that NH$_4^+$ still inhibited the Na$^+$ current, even though there was no apparent conductive NH$_4^+$ influx as evidenced from the absence of inward current on exposure to NH$_4^+$. This raises the likelihood that external NH$_4^+$, and not necessarily intracellular acidification, is responsible for inhibiting the Na$^+$ current.

At positive potentials, the presence of NH$_4^+$ caused a significant activation of an outward current not seen in control oocytes. This raises the possibility of NH$_4^+$ interacting with ENaC in a manner more complicated than simple inhibition. The nature of this voltage-dependent outward current is not clear and needs to be pursued further.

Although the interaction of several cations, such as Li$^+$ and K$^+$, with ENaC has been investigated, that of NH$_4^+$ has not been studied to our knowledge. A complicating factor in addressing this issue is the significant increase in pH$_i$ caused by NH$_4^+$.

Possible physiological implications. The effects of NH$_4^+$ are particularly relevant in states of systemic acidosis during which cortical and medullary interstitial ammonia concentrations rise. Acidosis is well known to be associated with natriuresis and diuresis. Although this has been attributed in part to decreases in proximal tubular reabsorption, effects of acidosis on distal nephron transport are also reported. Metabolic acidosis has been shown to inhibit K$^+$ secretion and Na$^+$ reabsorption in the distal tubule and collecting duct (30, 41). As discussed above, low pH$_i$ has been shown to inhibit Na$^+$ transport (presumably via ENaC inhibition) in a variety of tight epithelia (reviewed in Ref. 28). Long-term acidosis has recently been reported to decrease the distal tubule Na-Cl cotransporter and $\beta$- and $\gamma$-subunits of ENaC (26). Low pH$_i$ has also been shown to inhibit Na$^+$-K$^+$-ATPase (16). In addition to the effects of pH$_i$ and systemic acidosis, the present studies and some prior work suggest that the increased levels of NH$_3$/NH$_4^+$ that occur with acidosis may have additive effects to inhibit Na$^+$ transport. We previously demonstrated that NH$_4^+$ inhibited transepithelial Na$^+$ and K$^+$ transport in cortical collecting ducts in vitro. The present studies extend these findings to demonstrate that NH$_4^+$ inhibits Na$^+$ transport via ENaC. Therefore, acidosis inhibits renal Na$^+$ transport via a variety of mechanisms, including the effects of NH$_3$/NH$_4^+$, acting in concert. Recently, Frank et al. (17) suggested that renal ammonia content can act as a type of extracellular signaling molecule in integrating the response to systemic acidosis; the present studies would be consistent with such a role.

In conclusion, the results of this study demonstrate that NH$_4^+$ inhibits Na$^+$ transport when ENaC is expressed in the oocyte. Although some pH$_i$ effect cannot be ruled out, it is likely that a decrease in pH$_i$ is not the major cause of this inhibition. These results are important for understanding the role of NH$_4^+$ in modulating Na$^+$ transport in vivo in states of systemic acidosis.

We thank Dr. Emile L. Boulpaep for important suggestions and valuable discussions, Dr. Keith Elmslie for critical comments on the data, and Stephanie Palmer for secretarial and technical help.

This work was supported by American Heart Association Grant 0050547N, National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-54952, the Department of Veterans Affairs, and by DCI, Inc.

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