Characterization of a novel splice variant of δ ENaC subunit in human lungs

Run-Zhen Zhao,1 Hong-Guang Nie,2,5 Xue-Feng Su,1 Dong-Yun Han,1 Andrew Lee,1 Yao Huang,3 Yongchang Chang,3 Sadis Matalon,4 and Hong-Long Ji1,2

1Departments of Cellular and Molecular Biology, 2Texas Lung Injury Institute, University of Texas Health Science Center at Tyler, Tyler, Texas; 3Barrow Neurological Institute, St. Joseph’s Hospital and Medical Center, Phoenix, Arizona; 4Department of Anesthesiology, University of Alabama at Birmingham, Birmingham, Alabama; 5Institute of Metabolic Disease and Drug Development, China Medical University, Shenyang Liaoning, China

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The lining fluid along airway and alveolar epithelial surface is precisely regulated by apical and basolateral ion transport systems. The epithelial sodium channels (ENaC) are the main conduits for the entry of sodium ions (Na+) from the luminal fluid into the cell interiors (12, 15, 34). Native lung amiloride-inhibitable cation channels have been functionally classified into three groups: highly selective for Na+ over other cations, moderate, and nonselective channels (12, 15, 34). The highly selective cation channels have been thought to consist of heteromultimeric αβδ ENaC subunits (6). The molecular basis of the other two groups, however, remains in dispute. Channels comprised of α ENaC alone and channels consisting of the αβγ three subunits expressed in nonpolarized epithelial cells behave as nonselective phenotypes (23, 33, 56). Recently another δ ENaC variant was detected in the human brain and was thought to act as a pore-forming subunit (44). The distribution of δ ENaC in other nonepithelial tissues, such as the heart, pancreas, sperm cells, glial cells, melanoma cells, and mesothelial cells, in humans has also been reported (1, 4, 20, 53). Even though the physiological impact of δ ENaC channels is unknown, the physical intermolecular crosstalk between δ and other three ENaC subunits indicates the potential existence of δ ENaC-containing channels in pulmonary epithelial cells (29). This is supported by a very recent finding that δ ENaC channels contributed to ~50% of amiloride-sensitive salt transport across primary human nasal epithelial cells (3). We and others (3, 29, 37) have recently shown that an additional ENaC subunit (termed δ ENaC) may account for diverse observations in the biophysical properties of native epithelial Na+ channels.

Biophysical and pharmacological studies such as those in Xenopus oocytes have shown that heteromultimeric αβδ ENaC channels exhibited high selectivity for Na+ over Li+ ions, less amiloride sensitivity than αβγ ENaC (25, 36, 44), and were regulated by changes in extracellular pH (24, 36, 53). In addition, δ ENaC, a slicing variant of the first clone (defined as δ1 ENaC here), was detected in the human lung (29) and central neuronal tissues (16, 36). δ2 ENaC encodes a protein of 704 amino acid residues, whereas a peptide of 638 proteinoic acids is encoded by the δ1 ENaC (16, 44).

Mutagenesis of the NH2-terminal domains of α ENaC revealed the presence of highly conserved motifs implicated in channel gating kinetics, ion selectivity, and exocytosis (8, 17, 43). For example, a novel splice variant of the mouse α ENaC subunit with deletion of the intracellular NH2-terminal domain, when coexpressed with the wild-type β and γ ENaC subunits in Xenopus oocytes, showed lower single-channel activity (10). Similar differences in the functional domains of the NH2 termini between δ1 and δ2 subunits may contribute differential regulation of the channel activity and/or trafficking by intracellular signals (16).

Lung ENaC expression and function are regulated by physiological stimuli (e.g., temperature, mechanical, and acid stress) and noxious challenge (including allergens, pathogens, and pollut-
ants). Up to 60% of alveolar fluid clearance is governed by ENaC (35). Herein we aimed to characterize the biophysical and pharmacological features of δ2 ENaC cloned from human lung epithelial tissues. Human β and γ ENaC subunits were complimentary coexpressed with δ2 ENaC to obtain detectable current levels (29). Our studies showed that δ2βγ channels had diverse biophysical and pharmacological properties from those of δ1βγ channels in extracellular Na\(^+\) affinity, cation selectivity, anion selectivity, responses to external pH and capsapazepine, protein trafficking, and single-channel behavior. The divergent characteristics of δ1βγ and δ2βγ channels suggest that it may result from the diversity in their NH\(_2\) termini. Coexistence of δ2βγ ENaC with αβγ and δ1βγ channels in human lung epithelial cells may contribute to heterogeneities of native epithelial cation channels described previously (12, 15, 34).

**METHODS AND MATERIALS**

**In situ hybridization.** Human lung slides of healthy subjects were provided by NIH Lung Tissue Research Consortium (LTRC). In situ hybridization (ISH) oligonucleotide probes were synthesized by Sigma and labeled with digoxigenin (DIG) or biotin following the manufacturer’s instructions (DIG oligonucleotide tailing kit; Roche Diagnostics, Indianapolis, IN). The sequences of sense and antisense for δ1 ENaC: 5’-GGACACCCGGC CAGCCCACAA GCTCCACACT CCCACCTCA GCACC-3’; antisense: 5’-GGTGCTGAGG GTGGGAGTGT GGAGCTTGGG GTCTGGCCGG TGTCC-3’; δ2 ENaC, sense: 5’-GCCAC CTGAA GGGAT GGCAG CACAG ACCCA CTCAG CACAA GGGTGC-3’; antisense: 5’-GCAGC GTTGT GC- TGA GTGGG TCTGT GCTGC CATCC CTTCA GGGTG-3. The sense probes were used as negative controls. Labelling efficiency was determined by dot-blot comparison with the standards provided by the manufacturer. ISH was performed using standard hybridization procedures with DIG-labeled probes. Briefly, lung slices were deparaffinized and rehydrated. The slides were fixed with 4% paraformaldehyde in diethyl pyrocarbonate (DEPC)-PBS at room temperature for 10 min. Following two washes with DEPC-PBS, slides were treated with Proteinase K (100 μg/ml) at 37°C for 15 min. Slides were rinsed once with DEPC-water and prehybridized at 42°C for 2 h in prehybridization solution (IsHyb ISH kit, Biochain Institute, Hayward, CA). Probes were added to the hybridization solution at 500 ng/ml, and slides were incubated at 42°C for 16 h. Posthybridization stringency washes included: 2× SSC at 45°C for 10 min, 1× SSC at 45°C for 10 min, and 0.2× SSC at 42°C for 15 min twice. The slides were then incubated with 1× blocking solution for 1 h. Alkaline-phosphatase conjugated anti-DIG antibody was added at 1:300 diluted in PBS and incubated for overnight at 4°C. Slides were washed twice in 1× alkaline phosphatase buffer. Tissues were then incubated in standard alkaline phosphatase chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium diluted in 1× alkaline phosphatase buffer. Labeling was stopped by washing slides with distilled water, and slides were mounted in ImmunoHistoMount medium (Sigma, St. Louis, MO).

**Molecular cloning of δ2 ENaC.** Total RNA was extracted from human bronchial epithelial cell line-16HBE14o using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. RNA was dissolved in RNAse-free water and stored at −80°C. RNA was treated with DNase I (Ambion, Austin, TX) to eliminate potential genomic DNA contamination and was then extracted by acidic phenol-chloroform (Ambion) to inactivate DNase I. Total RNA concentration was assessed by spectrophotometry with the ratio of 260 nm/280 nm absorbance between 1.9–2.1. cDNAs were prepared with high capacity RNA-to-cDNA kits (no. 4387406; Applied Biosystems, Carlsbad, CA) following the user manual. All of the TaqMan primers for gene expression assays were purchased from Applied Biosystems (GAPDH, Hs99999905; α ENaC, Hs00168906). Taq-Man primers for δ2 ENaC were designed to target the sequence: ATCGCCTGGG CCTGTGGCCT CCCAGACGGC TCTCCACCTG AAGGATGAGC AGCACAGAC GCTTGCCAGC ACGGTGGCC GCAAAACAGG. The qPCR reactions were performed using a MyiQ5 real-time RT-PCR detection system (Bio-Rad, Hercules, CA) with the following procedure: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles. All PCR products were run on 1% agarose gel containing 0.1% SYBR Green. The transcriptional levels were determined as the relative gene expression using the 2^(-ΔΔCt) method.

**Real-time RT-PCR.** All total RNAs were extracted from fresh collected cells (ATCC) and frozen healthy human lung tissue using RNeasy plus Mini kits (no. 7414; QIAGEN, Valencia, CA) according to the manufacturer’s instructions. The human lung tissue was provided by the NIH LTRC. The usage of human lung tissue and experimental procedures were approved by the institutional IRB of the China Medical University. cRNA concentrations were assessed by spectrophotometry with the ratio of 260 nm/280 nm absorbance between 1.9–2.1. cDNAs were prepared with high capacity RNA-to-cDNA kits (no. 4387406; Applied Biosystems, Carlsbad, CA) following the user manual. All of the TaqMan primers for gene expression assays were purchased from Applied Biosystems (GAPDH, Hs99999905; α ENaC, Hs00168906). Taq-Ma primers for δ2 ENaC were designed to target the sequence: ATCGCCTGGG CCTGTGGCCT CCCAGACGGC TCTCCACCTG AAGGATGAGC AGCACAGAC GCTTGCCAGC ACGGTGGCC GCAAAACAGG. The qPCR reactions were performed using a MyiQ5 real-time RT-PCR detection system (Bio-Rad, Hercules, CA) with the following procedure: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles. All PCR products were run on 1% agarose gel containing 0.1% SYBR Green. The transcriptional levels were determined as the relative gene expression using the 2^(-ΔΔCt) method.
CCA TGG CTT TTC CCG GGA CGT CAC-3′ and 5′ antisense primer 5′-AGG GCC AGG ATA TTC ACC GGT GGC AGC-3′. After purification by PCR purification kit (Qiagen), the PCR product was digested by HindIII and KpnI, recovered from 1% agarose gel, and purified by PCR purification kit from Qiagen. PCR product was ligated to human ENaC pcDNA3.1-D3p to generate plasmids ENaC pcDNA3.1-D1 for 62 ENaC. For the subcloning of human 62 ENaC, PCR was performed using different 5′ sense primer 5′-ACG AAG CTT TCC AGG ACG TCA ACC GGT GGC AGC-3′ and the same 5′antisense primer 5′-AGG GCC AGG ATA TCC ACA TA-3′ and repeated PCR purification, restriction enzyme digestion, gel purification, and ligation. The plasmid was designated as human ENaC pcDNA3.1-D2. The inserts were verified by automated DNA sequencing (DNA Sequencing Core, University of Alabama at Birmingham, Birmingham, AL). The sequences of human 62 ENaC cDNA as well as 81 ENaC were submitted to GenBank (accession numbers DQ898176 and DQ898175, respectively).

Oocyte expression and dual-electrode voltage clamp. Defolliculated oocytes were cytotically injected with human ENaC cRNAs (25 ng per oocyte) and incubated in half-strength L-15 medium at 18°C. The two-electrode voltage clamp technique was used to record whole-cell currents 48 h postinjection as described previously (29). Briefly, the oocytes were continuously perfused with regular ND-96 solution (in mM: 96.0 NaCl, 1.0 MgCl2, 1.8 CaCl2, 2.5 KCl, and 5.0 HEPES-Na, pH 7.5). In experiments designed to measure the ion selectivity, Na+ salt was replaced with equal molar concentrations of either Li+ or K+ ions. For measuring apparent Na+ affinity, oocytes were perfused with solutions containing different external Na+ concentrations in an order of 0, 3, 10, 30, 100, and 120 mM. The osmolarity of all solutions was balanced with an equal molarity of N-methyl-D-glucamine. Amiloride-sensitive current-voltage relationships were acquired by stepping the holding potential in 20-mV increments from −120 to +80 mV after the monitoring currents were stable, with and without the application of amiloride (100 μM). Data were sampled at the rate of 200 Hz and filtered at 500 Hz.

To assess the rate of delivery of ENaC to the plasma membrane, termed exocytosis, we employed an ENaC mutant (γГ536C), in which a cysteine is introduced and the activity can be irreversibly blocked by [2-(trimethylammonium) ethyl] methanethiosulfonate bromide (MCTB); 100 μM, 120 to 120 mV were applied to the on-cell current and the amiloride-resistant current. One-way ANOVA computed with the built-in first-order exponential function of Clampfit 10.1 for amiloride; and desensitization time constant (τd) were calculated by fitting the current traces of activation and decay with the built-in first-order exponential function of Clampfit 10.1 (Molecular Devices) as described previously (26).

The proton-activated Na+ current was computed by subtracting the basal current from the peak current in the presence of protons. The activation time constant (τa) and desensitization time constant (τd) were used. Pipettes were filled with (Molecular Devices) as described previously (26).

RESULTS

Comparison of δ2 with δ1 ENaC. By aligning the distal segments of the intracellular NH2-terminal tails of these two δ subunits (Fig. 1, top), we found that, in addition to the 22 amino acid residues in the δ1 counterpart, 66 more amino acid residues were attached to the δ2 ENaC. The NH2-terminal tail
peptide of δ2 ENaC was positively charged (carried 9 positive and 3 negative charges), but the corresponding δ1 region was electroneutral (3 positive and 3 negative charges). As predicted by ScanProsite (www.expasy.ch) and NetPhos (www.cbs.dtu.dk), three potential phosphorylation sites were recognized in the δ2 stretch: Ser 8 for p38MAPK, Ser 17 for CK2, and Thr 55 for PKC. Furthermore, two adjacent Gly 51 and Gly52 residues in the δ2 tract were potential N-myristoylation sites.

Expression pattern in human alveolar epithelial cells. ISH using single DIG-labeled probes showed the presence of δ1 and δ2 ENaC in human lungs (Fig. 1, a–d). In additional studies, we stained human tissues with antibodies against δ1 ENaC (blue) in alveolar type I (ATI) and II (ATII) cells as labeled by specific anti-aquaporin 5 (AQP5) (brown) and anti-prosurfactant protein C (Pro-SPC) antibodies (red), respectively. g and h: Expression of δ2 ENaC (blue) in ATI (g, brown) and ATII (h, red) cells. i: Coexpression of δ1 and δ2 ENaC subunits by double labeling of δ1 (blue) and δ2 ENaC (brown) subunits. Double-labeled pneumocytes are indicated by arrows. Each image was photographed with an amplification of ×20. These results were repeated with lung slices from 5 healthy subjects, and at least 3 slices were stained for each group. j: Real-time RT-PCR. The transcriptional level of δ2 ENaC relative to GAPDH was quantitatively analyzed in 16HBEo, A549, and human lung tissues (HLT). **P < 0.01 and ***P < 0.001 compared with 16HBE. One-way ANOVA test. N = 5.
between AQP5 and prosurfactant protein C (Pro-SPC) as immunogenic markers for ATI and ATII cells, respectively. Apparently, both ATI and ATII cells express δ2 ENaC to a lesser extent compared with the δ1 counterpart (Fig. 1, e–h). In some cases, δ1 and δ2 were presented in the same alveolar cells (arrows, Fig. 1f). Interestingly, δ1 ENaC was also seen in leukocytes (Fig. 1e), in which other ENaC subunits and amiloride-sensitive channels have been detected. Because of the 100% identity between δ1 and δ2 except the extended NH2-terminal tail, we cannot draw any conclusions about the relative abundance of each subunit. We previously detected the expression of δ1 and α subunits in human respiratory epithelial tissues (29). Here we detected the transcriptional level of δ2 in human lungs, airway, and alveolar epithelial cells (16HBEo and A549) using α subunit as a positive control. δ2 ENaC is transcribed in all three human lung epithelial preparations with a significant difference between normal lung tissues and immortalized epithelial cell lines (Fig. 1j). We cannot statistically compare the expression levels of δ2 and α ENaC subunits because of a lack of standard curves with defined amounts of transcripts, even though α subunit is apparently expressed to a greater extent. The lack of a specific antibody against δ2 ENaC prevented us detecting δ2 ENaC at the protein level in human lungs.

In the following experiments, we systematically characterized the biophysical and pharmacological properties, regulation by extracellular pH, and the lifespan of proteins of heterologous δ2βγ ENaC channels expressed in Xenopus oocytes.

**Apparent external Na\(^+\) affinity.** To compare the apparent external Na\(^+\) affinity of δ2 ENaC to that of δ1 channels, oocytes expressing either δ1βγ or δ2βγ channels were perfused with bath solutions containing a variety of Na\(^+\) concentrations (0, 3, 10, 30, 100, and 120 mM). Current amplitudes measured at given external Na\(^+\) concentrations were normalized to the maximal activity obtained at 120 mM Na\(^+\) ions and were plotted as a function of bath Na\(^+\) contents (Fig. 2, A and B). By fitting the plots with the Michaelis–Menten equation, the apparent half-saturation concentration \(K_m\) for external Na\(^+\) ions was 62.8 ± 5.6 mM for δ2βγ channels, which was 18% greater than that of δ1βγ (53.1 ± 2.0 mM, \(P < 0.05\)). These data suggest that the apparent Na\(^+\) affinity of the δ2βγ, the capacity to carry Na\(^+\) ions through the channel pore, is less than that of δ1βγ channels.

**Ion selectivity of δ2βγ ENaC.** We and other groups (25, 29, 44) have reported previously that δ1βγ ENaC is more permeable to Na\(^+\) over Li\(^+\) ions, which is opposite to what is seen for αβγ ENaC channels. To characterize the cation permeation of the splicing variant δ2βγ channel, we perfused oocytes with Na\(^+\)-, Li\(^+\), and K\(^+\)-rich bath solutions in the presence and absence of amiloride. The average amiloride-sensitive whole-cell currents \(I_{AS}\), which reflect ENaC activity, were plotted as current-voltage (I-V) curves in Fig. 2, C and D. Consistent with previous observations (25, 29, 44), δ1βγ had a greater Na\(^+\) current over Li\(^+\) level at each holding potential from −120 mV to +80 mV (Fig. 2C). The whole cell Na\(^+\) currents for δ1βγ and δ2βγ were −5.043 ± 748.9 nA and −8.935 ± 977.8 nA, respectively, at −120 mV (\(P < 0.05\), Fig. 2D). Similar reversal potentials for Na\(^+\), Li\(^+\), and K\(^+\) carried currents were found between δ1- and δ2-containing channels. Na\(^+\) permeabilities of δ2βγ (Na\(^+\): Li\(^+\): K\(^+\) = 1.0:64.0:0.7) relative to other cations, as computed with the Nernst equation (Eq. 1), were similar as well (Table 1).

**Single-channel properties.** Figure 3A shows the representative single-channel Na\(^+\) and Li\(^+\) currents in cell-attached patches at −100 mV and the respective current-voltage relationships. The \(\gamma_{Na}\) and \(\gamma_{Li}\) values for δ1βγ channels, as estimated by the slope of the straight line created by linear regression, were 12.0 ± 0.6 pS and 8.2 ± 0.3 pS, which are consistent with previous observations (25, 44). The corre-
Table 1. Summary of characterized biophysical and pharmacological properties as well as protein lifespan of δ1βγ and δ2βγ ENaC

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<th>δ1bg</th>
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<tr>
<td><strong>Biophysical properties</strong></td>
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<tr>
<td>Ion selectivity (Na+/Li+/K+)</td>
<td>1.06±0.07</td>
<td>1.06±0.09</td>
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<tr>
<td>Na⁺ affinity (Kₐ, mM)</td>
<td>53.1±2.0</td>
<td>62.8±5.6</td>
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<tr>
<td>Single-channel conductance γ₀, (pS)</td>
<td>12.0±0.6</td>
<td>14.5±0.5</td>
</tr>
<tr>
<td>Single-channel conductance γ₁, (pS)</td>
<td>8.2±0.3</td>
<td>9.8±0.3*</td>
</tr>
<tr>
<td>Nₚ Na⁺ (multi-channel)</td>
<td>0.74±0.11</td>
<td>1.74±0.21†</td>
</tr>
<tr>
<td>Nₚ Li⁺ (multi-channel)</td>
<td>0.67±0.21</td>
<td>0.81±0.16</td>
</tr>
<tr>
<td>P₀ Na⁺ (single channel)</td>
<td>0.56±0.09</td>
<td>0.87±0.09*</td>
</tr>
<tr>
<td>P₀ Li⁺ (single channel)</td>
<td>0.21±0.06</td>
<td>0.61±0.13*</td>
</tr>
<tr>
<td>MOT (Na⁺)</td>
<td>0.27±0.03</td>
<td>0.91±0.66*</td>
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<tr>
<td>MOT (Li⁺)</td>
<td>0.27±0.07</td>
<td>1.10±0.68*</td>
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<tr>
<td>MCT (Na⁺)</td>
<td>0.96±0.1</td>
<td>0.22±0.03†</td>
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<tr>
<td>MCT (Li⁺)</td>
<td>0.38±0.15</td>
<td>1.42±0.49*</td>
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<td><strong>Pharmacological features</strong></td>
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<tr>
<td>Amiloride (Kₐamil, μM – 100 mV)</td>
<td>2.5±0.5</td>
<td>0.7±0.1*</td>
</tr>
<tr>
<td>Capsazepine (EC₅₀, μM – 100 mV)</td>
<td>11.9±0.2</td>
<td>6.3±0.3*</td>
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<tr>
<td>External protons</td>
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<tr>
<td>Activation time τᵣ, s</td>
<td>12.0±3.0</td>
<td>2.4±0.1*</td>
</tr>
<tr>
<td>Desensitization time τᵣ, s</td>
<td>10.1±0.4</td>
<td>1.0±0.1*</td>
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<tr>
<td>Trafficking</td>
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<tr>
<td>Delivery rate</td>
<td>29.2±17.9</td>
<td>26.6±4.2</td>
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<tr>
<td>Endocytosis rate τₑ, h</td>
<td>1.66±0.04</td>
<td>1.39±0.19*</td>
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Values are expressed as means ± SE. One-way ANOVA test was used for comparisons. *P < 0.05, †P < 0.01 vs. δ1βγ epithelial sodium channels (ENaC). MOT, mean open time; MCT, mean closed time.

sponding unitary conductances of δ2βγ channels were 14.5 ± 0.5 pS for Na⁺ and 9.8 ± 0.3 pS for Li⁺ ions (P < 0.05 vs. δ1βγ). To compare the gating pattern of δ1βγ and δ2βγ channels, we calculated the MOT and MCT (Table 1). The MCT value of δ2βγ was significantly lower than that of δ1βγ (P < 0.01). These results indicate that the gating kinetics of δ2βγ differs from those of δ1βγ channels.

Subsequently, we characterized the single-channel activity (Nₚₐ) in cell-attached patches. The Nₚₐ levels for δ1βγ were 0.67 ± 0.21 for Li⁺ ions and 0.74 ± 0.11 for Na⁺ ions, whereas those for δ2βγ ENaC were 0.81 ± 0.16 and 1.74 ± 0.21 (P < 0.01 vs. δ1βγ), respectively, for Li⁺ and Na⁺ ions (Fig. 3). In addition, we computed the open probability (P₀) for both δ1βγ and δ2βγ channels in the traces containing only one open level. As shown in Fig. 3B, the estimated P₀ value for δ2βγ was greater than that of δ1βγ (0.87 ± 0.09 vs. 0.56 ± 0.09) for Na⁺ ions (P < 0.05); similar difference in P₀ values was observed for Li⁺ ions (0.61 ± 0.13 vs. 0.21 ± 0.06, P < 0.05).

Blockade by amiloride. Blockade of ENaC activity by potassium-sparing diuretic amiloride and its analogs is a well-known pharmacological hallmark. δ1βγ ENaC was less sensitive to amiloride than with an IC₅₀ value of 2.86 μM (28-fold higher than αδγ ENaC) (25). To examine the amiloride sensitivity of δ2βγ ENaC, oocytes were perfused with solutions containing a variety of amiloride concentrations (Fig. 4, A and B). The apparent dissociation constant of amiloride (Kₐamil) at various membrane potentials was estimated by fitting the related dose-response curves as previously described (25). The Kₐamil value at a membrane potential of −100 mV for the δ2βγ was 0.7 ± 0.1 μM, which was markedly less than that of δ1βγ (2.5 ± 0.5 μM, P < 0.05). To determine the voltage dependence of amiloride inhibition, the computed Kₐamil values were then plotted against the membrane potentials (Fig. 4C). The more positive (depolarization) the membrane potential, the greater the value of Kₐamil showed for both δ1βγ and δ2βγ channels. Moreover, the estimated Kₐamil at 0 mV for δ2βγ, by fitting the voltage-dependent plot with the Woodhull equation, was significantly smaller (13.2 ± 1.0 μM vs. 41.2 ± 1.3 μM for δ1βγ, P < 0.05). Collectively, these data indicate that δ2βγ is more sensitive to amiloride but less voltage dependent for amiloride blockade compared with its δ1 counterpart.

Modulation of δβγ ENaC activity by capsazepine. It has been found that capsazepine, a competitive antagonist for the transient receptor potential vanilloid subfamily 1, potentiated the activity of δ1βγ ENaC heteromultimer expressed in Xenopus oocytes (50). In our studies, capsazepine activated inward currents at −100 mV in both δ1βγ- and δ2βγ-injected oocytes in a dose-dependent manner (Fig. 4. D and E). The EC₅₀ value of capsazepine for δ2βγ channels was approximately half of that for δ1βγ channels (6.3 ± 0.3 μM vs. 11.9 ± 0.2 μM for δ1βγ, P < 0.05), as computed by fitting the dose-response curves with Hill equation (Fig. 4F). Clearly, capsazepine is a specific activator of both types of δ ENaC channels, and the δ2 variant exhibits more sensitivity.

Extracellular proton activation. In addition to other groups, we have previously reported that both homomultimeric δ1 and heteromultimeric δ1βγ ENaC channels in oocytes were activated by extracellular protons (24, 53). δ1 ENaC has been proposed to be a candidate molecule for pH sensing in human gastrointestinal, neuronal, and skin tissues (49, 51, 52). It has been suggested that δ2 variant would also be regulated by external pH (36). To compare the pH sensitivity of the splicing variant δ2βγ with δ1βγ ENaC channels, oocytes were exposed to acidic bath solution (pH 4.0) for a time sufficient to permit the current to be evoked and then returned to basal levels to estimate the activation (τᵣ) and desensitization time constants (τₑ). As shown in Fig. 5A, inward currents in δ1βγ and δ2βγ channels were activated when the cells were superfused with acidic solution. The activation rate of the δ2βγ ENaC (2.4 ± 0.1 s) was faster than that of δ1βγ ENaC (12.0 ± 3.0 s, P < 0.01). Moreover, δ2βγ channels recovered much more rapidly from an acidic challenge (10.0 ± 0.1 vs. 10.1 ± 0.4 s for δ1βγ ENaC, P < 0.01). These results suggest that the gating of δ2βγ channels by external hydrogen ions is approximately sixfold to tenfold faster than δ1βγ channels.

Exocytosis of δ2βγ ENaC. The cytosolic amino-terminal tails of ENaC are well-known to possess key domains for protein exocytosis (2, 8, 18, 31). δ2 ENaC has a longer NH₂-terminal tail with 66 more amino acids compared with its δ1 counterpart. We reasoned that the extension of NH₂-terminal tails may alter the rate of delivering newly synthesized ENaC proteins to the plasma membrane. To address this issue, we introduced a cysteine residue into the amiloride binding site of the γ subunit (G536C), which allows irreversible blockade of the existing ENaC channel activity with a thiol modifying agent termed MTSET (40). The increasing rate of benzamil-sensitive currents is then a one-time measurement of the rate of ENaC delivered to the plasma membrane (11). The benzamil-sensitive currents (Iₕₛ), which were normalized to the level (Iₕₛbasal) prior to MTSET incubation (Iₕₛ/̅Iₕₛbasal) were plotted as a function of recording time (Fig. 6A). The channel activities of both δ1βγ and δ2βγ gradually increased within 25 min. The delivery rate of δ2βγ proteins was 8.9% faster as reflected by
These results suggest that the extension of the NH2-terminal tail together with the faster rate of amiloride-sensitive currents (Golgi apparatus. In brefeldin A-treated cells, reduction in slower than that for half of the initial current levels were 1.66 progressively in 6 h. The calculated decay constants to reduce channel conductances of Na+/H+ or Li+/H+ in cell-attached patches. The closed (dashed line) and open levels (solid bars) were labeled. The scale bar for the y-axis is 5 s and 1 pA for the y-axis. n = 6. Unitary Na+ and Li+ conductances were computed by fitting the single-channel current-voltage plots (from -120 to -40 mV) with a linear fitter. The single-channel conductances of Na+(○) and Li+ ions (□) were the slope values. B: gating kinetics. Left: representative current traces with only 1 open level. Right: open probability (P_o). Membrane potential, -60 mV; cell-attached patches. *P < 0.05 vs. δβγ, n = 5.

Fig. 3. Single-channel properties of δ ENaC channels. A: representative single-channel current traces (left) at -100 mV and respective single-channel conductances (right). Either Na+ - or Li+ -rich pipette solution was used to digitize currents in cell-attached patches. The closed (dashed line) and open levels (solid bars) were labeled. The scale bar for the y-axis is 5 s and 1 pA for the y-axis. n = 6. Unitary Na+ and Li+ conductances were computed by fitting the single-channel current-voltage plots (from -120 to -40 mV) with a linear fitter. The single-channel conductances of Na+(○) and Li+ ions (□) were the slope values. B: gating kinetics. Left: representative current traces with only 1 open level. Right: open probability (P_o). Membrane potential, -60 mV; cell-attached patches. *P < 0.05 vs. δβγ, n = 5.

the slope, suggesting that the elongation of the NH2-terminal tail of δβγ facilitates the trafficking of channel proteins.

Internalization of surface δβγ ENaC proteins. We further evaluated the rate for removing δβγ proteins from the cell surface. Brefeldin A was used to interrupt the delivery of proteins to the plasma membrane via Golgi apparatus. In brefeldin A-treated cells, reduction in amiloride-sensitive currents (I_A) assuming reflects internalization of the existing channel proteins in a functional way. As shown in Fig. 6B, the channel activity of δβγ decreased progressively in 6 h. The calculated decay constants to reduce half of the initial current levels were 1.66 ± 0.04 h, relatively slower than that for δγγ (1.39 ± 0.19 h, P < 0.05). Taken together with the faster rate of δβγ for exocytotic process, these results suggest that the extension of the NH2-terminal tail may elongate the lifespan of δ2 ENaC at cell surface.

DISCUSSION

Our previous studies showed that two δ ENaC transcripts were detected in human alveolar epithelial cell line-A549, primary human alveolar type cells, and human lung protein extracts (29, 37). As revealed by our ISH and real-time RT-PCR data, δ2 ENaC was predominately expressed in alveolar and airway epithelial cells and coexpressed with δ1 in some cells. The expression profile of δ2 was similar to that of α subunit in human lung epithelial cells and tissues. δ2βγ channels displayed the diverse properties in whole cell channel activity, single-channel conductance, gating kinetics, amiloride sensitivity, responses to capsazepine, and acidic pH. There are at least three subtypes of Na+-permeable cation channels in epithelial tissues (12, 14, 34). The highly Na+-selective channels have been attributable to the channels made of α, β, and γ ENaC subunits. The molecular basis of the other two cation channels, however, has not been completely understood. Although cloned α ENaC-comprising channels overexpressed in mammalian cells and native channels in nonpolarized cells exhibited similar features to the moderate and less selective cation channels (22, 56), under physiological conditions, salt-absorptive epithelial cells are polarized and only heteromultimeric channels are believed to have physiological function. In contrast to mammalian cells, when Xenopus oocytes were used as an expression system, monomultimeric channels composed of pore-forming α or δ ENaC subunit alone displayed biophysical and pharmacological properties similar to those of corresponding heteromultimeric αβγ and δβγ channels (24, 31).

The existence of splicing variants of δ2 ENaC may partially explain the diverse phenotypes of amiloride-inhibitable channels in lung epithelial tissues. δ2 ENaC does not coexpress with δ1 counterpart in all alveolar cells, indicating that δ2 is not a simple surrogate or alternative. In addition to the highly selective Na+ channels with a conductance of 4–5 pS, amiloride-sensitive, moderately Na+-permeable channels have been described in murine and human lung epithelial cells. In particular, highly selective Na+ channels (9 pS) and moder-
ately selective Na⁺ channels (18 pS) have been identified in murine lung tissue slices (19, 32). In human H441 cells, channels with a conductance of 11 pS were reported (21). The conductances of these native channels are similar to that of ENaC channels expressed in oocytes although the relationships between these native and the heterologous channels are unknown.

Three stoichiometric models have been proposed: 3α/3β/3γ (9-subunit), 2α/1β/1γ (4-subunit), and 1α/1β/1γ (3-subunit). δ ENaC confers the biophysical properties of αβγ channels, indicating that δ ENaC may be able to substitute α subunit in these models to construct two three-subunit channels examined in this study and three four-subunit channels (1α/1β/1γ/1α/1β/1γ, 1α/1β/1γ/2α/1β/1γ/1α/1β/1γ, 1α/1β/1γ/2α/1β/2α/1β/1γ), and five nine-subunit channels (1α/2α/1β/1γ/2α/1β/1γ/2α/1β/1γ/2α/1β/1γ/2α/1β/2α/1β/1γ/2α/1β/1γ/2α/1β/2α/1β/1γ/2α/1β/2α/1β/1γ/2α/1β/2α/1β/1γ). Considering that the architecture of native ENaC channels in human lungs is still obscure, we believe it would be better to study these proposed four-subunit models.

**Fig. 4.** Blockade of δ ENaC channels by amiloride and stimulation by capsazepine. A–C: amiloride sensitivity of δ1βγ (A) and δ2βγ (B) ENaC channels. Dose-response curves were constructed as a function of amiloride concentrations (0–1,000 μM) against whole cell Na⁺ currents at −100 mV. The apparent dissociation constant of amiloride (Kᵣamil) was computed by fitting the plots with the Hill equation; n = 7. C: voltage dependence of amiloride blockade. The calculated Kᵣamil values of δ1βγ (●) and δ2βγ ENaC (○) were plotted as a function of membrane potential (Vₘ). The plot was fitted with the Woodhull equation to evaluate the voltage dependence within the electrical field. D–F: stimulatory effects of capsazepine. Whole cell current traces of δ1βγ (D) and δ2βγ (E) at −100 mV and corresponding concentration-effect plot (F).

**Fig. 5.** Activation of δ1βγ and δ2βγ channels by external protons. A: representative current traces of proton-activated Na⁺ currents at −60 mV. Application of neutral (pH 7.5) and acidic pH (4.0) bath solutions are indicated by horizontal lines. B: kinetics of activation. Activation (τₐ) and desensitization (τᵢ) time constants were estimated by fitting activated and inactivated trace segments with the first-order exponential function (n = 10). **P < 0.01 vs. δ1βγ.
Acid-sensing ion channel 1a in the central nervous system has been implicated in long-term potentiation, suggesting that minute fluxes in synaptic pH may activate a proton-sensitive channel to enhance synaptic plasticity, learning, and memory (5, 46). More recently, ENaC, widely distributed throughout the brain and activated by protons, has been proposed to act as a pH sensor and to play a role in learning and memory in the brain and activated by protons, has been proposed to act as a pH sensor and to play a role in learning and memory in the brain (53). Proton sensitivity of δENaC may be an important mechanism for integrating external ischemic signals in inflamed and hypoxic tissues (24, 41). Our results indicate that δENaC could serve as a pH sensor to respond to noxious acidic aspiration. In the role of acid sensing, the splicing variant δ2ENaC may be a more effective pH sensor based on the increased gating kinetics and enhanced channel activity. We could not, however, verify the contribution of δ2ENaC-containing channels until the crystallized structure of ENaC is available.

Consistent with the greater whole cell channel activity, the single-channel activity of this splice variant was approximately twofold that of channels consisting of δ1βγ subunits. This enhanced channel activity most likely results from the increased channel open time, larger unitary conductance, and augmented affinity for external Na⁺ ions. In addition, the lengthened lifespan for δ2βγ ENaC at the plasma membrane seems also to contribute to the increased channel activity.

In contrast to the relatively well documented α ENaC containing channels, the pharmacological profile of δ ENaC-comprising channels has been poorly investigated. Yamamura and coworkers (54) found that capsazepine was the first known activator, whereas Evans blue was a specific antagonist specific for δ1-consisting ENaC channels. We observed that the splicing variant is more sensitive to the channel modulators amiloride and capsazepine. Combined with the fast-gating kinetics by external protons and altered rates for protein trafficking and degradation, the extended amino terminal tail apparently plays a crucial role in these changes associated with the δ2βγ ENaC. Unfortunately, neither δ1βγ nor δ2βγ channels were regulated by Evans blue in a dose-dependent manner (data not shown). Besides, this dye transiently activated human αβγ ENaC only at doses >300 nM and did not depend on given concentrations (data not shown). Lack of specific inhibitors for δβγ ENaC prevents us from distinguishing native α and δ ENaC-containing channels.

Acid-sensing ion channel 1a in the central nervous system has been implicated in long-term potentiation, suggesting that minute fluxes in synaptic pH may activate a proton-sensitive channel to enhance synaptic plasticity, learning, and memory (5, 46). More recently, δ ENaC, widely distributed throughout the brain and activated by protons, has been proposed to act as a pH sensor and to play a role in learning and memory in the human brain (53). Proton sensitivity of δβγ ENaC may be an important mechanism for integrating external ischemic signals in inflamed and hypoxic tissues (24, 41). Our results indicate that δβγ ENaC could serve as a pH sensor to respond to noxious acidic aspiration. In the role of acid sensing, the splicing variant δ2βγ may be a more effective pH sensor based on the increased gating kinetics and enhanced channel activity. We could not, however, verify the contribution of δ2βγ channels in human alveolar epithelial cells because of limited pharmacological and genetic approaches. δ ENaC is still a pseudo-gene in murine, which excludes the possibility to establish deficient colonies to investigate in vivo alveolar fluid clearance mediated by δ ENaC. It awaits further studies to answer the question of whether specific siRNAs against δ1 and δ2 ENaC subunits are applicable for studying physiological relevance in primary human alveolar cells.

We previously reported that the rate of endocytosis for human αβγ ENaC in oocytes was 9.6%/h (28). The rates for both δ1βγ (9.7%/h) and δ2β γ (9.1%/h) channels are similar. The rate of degradation is apparently determined by the Nedd4-mediated ubiquitination for αβγ ENaC (13, 39). In contrast, the endocytosis of δ1βγ ENaC proteins possibly requires the binding of COMMD1 to intracellular tails of ENaC (9). The slower rate of endocytosis of δ2βγ ENaC may result from the involvement of the extended N-terminal tails in the COMMD1-mediated ubiquitination in oocytes. In addition, the extended NH₂-terminal tails of δ2 ENaC may interact with the tyrosine-based endocytosis motifs in the NH₂-terminal tails of β and γ subunits and regulate internalization (11, 38, 45, 48). On the other hand, we found that the rate of exocytosis for δ2 channels is greater than that of δ1 ENaC. These observations are consistent with a recent publication (47). We predict that the half-life time of δ2βγ is longer than δ1βγ channels. Very recently, Taruno and Marunaka (42) reported that the rate of ENaC recycling, including the rates for insertion and retrieval, depends on the abundance of total proteins. Thus, the divergent expression levels of heterologous δ1βγ and δ2β γ channels may also contribute to the difference in the trafficking when overexpressed in oocytes.

In conclusion, our study systematically characterized a new variant, δ2 ENaC, in human lung epithelial cells. Heterologous δ2βγ ENaC exhibited a number of divergent features from the δ1βγ counterpart in biophysics and pharmacology, regulation, and the lifespan of proteins. Channels comprising of this novel splice variant may contribute to the diversities of native epithelial Na⁺ channels.

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REFERENCES


