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A novel spliced variant of the epithelial Na^+ channel δ-subunit in the human brain

Hisao Yamamura [a,*], Shinya Ugawa [a], Takashi Ueda [a], Masataka Nagao [b], Shoichi Shimada [a]

[a] *Department of Molecular Morphology, Graduate School of Medical Sciences, Nagoya City University, Nagoya 467-8601, Japan*
[b] *Department of Forensic Medical Science, Graduate School of Medical Sciences, Nagoya City University, Nagoya 467-8601, Japan*

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Abstract

The amiloride-sensitive epithelial Na^+ channel regulates Na^+ homeostasis in cells. Recently, we described that the δ-subunit is a candidate molecule for a pH sensor in the human brain. Here, an N-terminal spliced variant of the δ-subunit is cloned from human brain, and designated as the δ2-subunit, which is expressed with the original δ-subunit (δ1-subunit) at the same level in the human brain. Functional analyses revealed that the physiological and pharmacological properties (interaction with accessory βγ-subunits, activation by acidic pH, amiloride sensitivity) of the δ2-subunit were similar to those of the δ1-subunit. In conclusion, the activities of both subunits may be involved in the mechanism underlying pH sensing in the human brain.

Keywords: Epithelial Na^+ channel; δ-subunit; Spliced variant; Brain; pH sensor; Amiloride; Human

The degenerin/epithelial Na^+ channel superfamily has striking functional diversity including Na^+ absorption, acid-sensing, peptide-gating, acidosis-evoked nociception, and mechanotransduction [1,2]. The epithelial Na^+ channel (ENaC) has four homologous subunits (α, β, γ, and δ) in mammals and is an essential control element for the Na^+ transport pathway in cells and across epithelia [3–7]. The α-subunit is expressed mainly in epithelia such as the kidney, lung, and colon, and binds with β- and γ-subunits to be involved in the control of Na^+ balance, blood volume, and blood pressure. Recently, we showed that the δ-subunit was widely distributed throughout the brain and was activated by protons, indicating that it may contribute to pH sensation in the human brain [8]. In pharmacological profiles of the δ-subunit, we described that capsazepine and icilin potentiated the channel activity whereas Evans blue acted as a specific inhibitor for this subunit [9–11].

In this investigation, we found an alternative N-terminal spliced form of the δ-subunit, or δ2-subunit, which had 88% amino acid identity to the known δ-subunit. It is possible that both δ-subunits are expressed in the human brain. The physiological and pharmacological features (interaction with accessory βγ-subunits, activation by acidic pH, amiloride sensitivity) of the δ2-subunit were analyzed using electrophysiological recording in the *Xenopus oocyte* expression system to compare with the original δ-subunit. The coexpression of these δ-subunits was analyzed in *X. oocytes*.

Materials and methods

Molecular biology. All experiments were approved by the Ethics Committee of the Nagoya City University Graduate School of Medical Sciences and were conducted in accordance with the Declaration of Helsinki. The extraction of total RNA and reverse transcription-polymerase chain reaction (RT-PCR) was performed as described previously [9]. PCR amplification was performed for 35 cycles and the products were run on a 1% agarose gel in Tris-acetate/EDTA buffer and visualized with ethidium bromide. The transcripts were recovered from the gel fragments, cloned,

* Corresponding author. Fax: +81 52 852 8887.
E-mail address: yamamura@med.nagoya-cu.ac.jp (H. Yamamura).

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and sequenced. Specific PCR primers for human ENaCδ (GenBank Accession No. U38254) were designed as follows: (+), 5′-GGA CGG GAG AAT GGA AGC AGC CA-3′, and (−), 5′-GAA CTG TCG GGC CTG GCA GTC CA-3′ (base 21–825 in the open reading frame). To isolate full-length cDNA of the δ-subunit, the following primers were designed from the ENaCδ-related expressed sequence tag database (BI520370 and AI199647): (+), 5′-gaa ttc gcc gcc acc ATG GCT GAG CAC CGA AGC ATG GAC GGG AGA-3′, or (+), 5′-gaa ttc gcc gcc acc ATG GCT TTC CTC TCC AGG ACG TCA CCG GT-3′, and (−), 5′-tct aga TCA GGT GTC CAG AGT CTC AAG GGG CTG GGG CCC AGC CCA GCT-3′ (base −104 or 1 to the stop codon, respectively). The sequences indicated in lowercase letters are *Eco*RI (gaa ttc) and *Xba*I (tct aga) recognition sites, and the Kozak sequence (gcc gcc acc), which were added to insert PCR products into vector DNA in the proper orientation and to promote effective translation, respectively.

Electrophysiological recording. Electrophysiological studies using a two-electrode voltage-clamp technique were performed in the *X. oocyte* expression system, as described previously [9]. In brief, cRNA transcript(s) (1 ng for the homomeric channel or each 0.01 ng for coexpression) was injected into oocytes. After injection, oocytes were incubated at 20 °C in a recording solution supplemented with 100 μM amiloride for 24–48 h. The recording solution had an ionic composition of 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5. Oocytes were clamped at a holding potential of −60 mV. All electrophysiological experiments were carried out at room temperature (24 ± 1 °C). Pharmacological reagents were obtained from Sigma–Aldrich (St. Louis, USA).

Statistics. Pooled data are shown as the means ± SE. Statistical significance between the two groups was determined by Student's *t* test. The data of the relationship between amiloride and the current amplitude were fitted using the following equation after normalization: relative current (%) $= 100 - (100 - C)/\{1 + (K_d/[Ami])^n\}$, where C is the component resistant, K_d is the apparent dissociation constant, [Ami] is the concentration of amiloride, and *n* is the Hill coefficient.

Results and discussion

Expression of δ-subunit in human brain

Human ENaCδ has been reported to be expressed mainly in the brain, while other subunits are distributed in non-neuronal tissues such as the kidney, lung, and colon [5–8]. Taking the expression pattern into account, we isolated the full-length clone of the δ-subunit from human brain cDNA using PCR primers, and found a transcript with a different length in a nucleotide different from the known δ-subunit. The fragments of 805 and 896 bp were easily identified by ethidium bromide staining after 35-cycle PCR amplification (Fig. 1). These sequence analyses revealed that the short form was the original δ-subunit, whereas the longer form was a novel spliced variant of the δ-subunit. Based on the band density after visualization with ethidium bromide, the ratio of these δ-subunits was almost the same. These results could be reproduced by repeated experiments ($n = 5$).

Cloning of δ2-subunit from human brain

We examined to identify the full-length sequence of the spliced variant obtained from human brain cDNA, and identified two different-length clones: a full-length clone of the known δ-subunit, and an alternative spliced variant at the N-terminal region (Fig. 2). Here, we propose that the

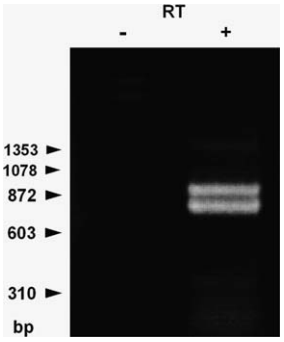

Fig. 1. Expression of δ-subunit in human brain. Expression of ENaCδ in the human brain was observed using a RT-PCR method with specific probes. A typical gel image of PCR product before (−) and after (+) RT procedure is presented. Clear expressions of two different-length transcripts were detected (805 and 896 bp), but there were no detectable bands without RT. Similar results were obtained from five independent experiments. Numbers shown at the far left indicate the base size obtained from Ready-Load ϕX174 RF DNA/*Hae*III fragments (Invitrogen, Carlsbad, USA) as a DNA size marker.

former was the δ1-subunit and the longer form was the δ2-subunit. The predicted primary structure of δ2-subunit protein revealed 703 amino acids with 88% identity to the δ1-subunit of 638 amino acids. The δ2-subunit included a 94-bp nucleotide insertion between the original exons-1 and -2 and thus shifted the coding frame for protein translation, resulting in extension of the sequence alignment of exon-1 in the direction of the N-terminal and the appearance of a novel exon with a predicted first in-frame methionine upstream of exon-1. These results clearly showed that two different-length transcripts, δ1- and δ2-subunits, were expressed at the level of mRNA in the human brain. The alternative spliced variant of mammalian ENaC has been published for the α- and β-subunits [12–17]. Interestingly, three amino acids after 94-bp insertion were different between these δ-subunits (Fig. 2B), which may originate from the substantial difference in the translation regulated by some promoter(s) or expression-modulating factor(s). This may imply that either gene is provided with endogenous compensation when the other gene lacks these functions.

Functional expression of δ2-subunit

To determine whether homomeric δ2-subunit constructed an amiloride-sensitive channel, the δ2-subunit was expressed in *X. oocytes*. At a holding potential of −60 mV, inward currents were induced in δ2-injected oocytes, and the current was mostly inhibited by 100 μM amiloride (by 113 ± 8 nA, $n = 6$, $p > 0.05$ vs. δ1 of 105 ± 6 nA, $n = 5$; Fig. 3A). In native oocytes, the application of amiloride did not induce any current (by 2 ± 1 nA, $n = 10$), because amiloride-induced currents were mediated by inhibition of the δ-subunit. It has been reported that the δ-subunit itself can induce currents when expressed in oocytes, but the heteromultimeric channel with β- and γ-

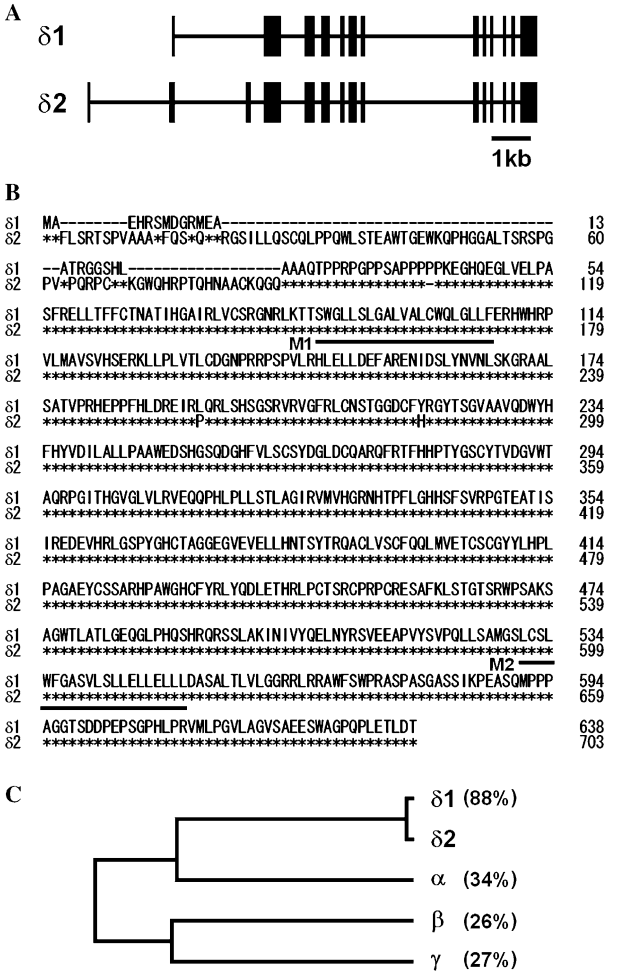

Fig. 2. Cloning of δ2-subunit from human brain. Sequence alignment coding of the known δ-subunit (δ1-subunit) and a novel N-terminal spliced variant (δ2-subunit) are shown. (A) Genomic alignments analyzed using the Human Genome Browser created by the Genome Bioinformatics Group of the University of California at Santa Cruz (http:/genome.ucsc.edu/) are illustrated. (B) Predicted primary structures of these δ-subunit genes are arranged using GENETYX-WIN software (Version 4.0; GENETYX, Tokyo, Japan). Common amino acids between alignments and predicted transmembrane domains are indicated by asterisks (*) and underlining (*M1* and *M2*), respectively. Transmembrane domains are searched with UniProt Knowledge (Swiss-Prot and TrEMBL) developed by the Swiss Institute of Bioinformatics (http://www.expasy.org/sprot/). (C) Sequence identities of the δ2-subunit to other subunits at the amino acid level are illustrated.

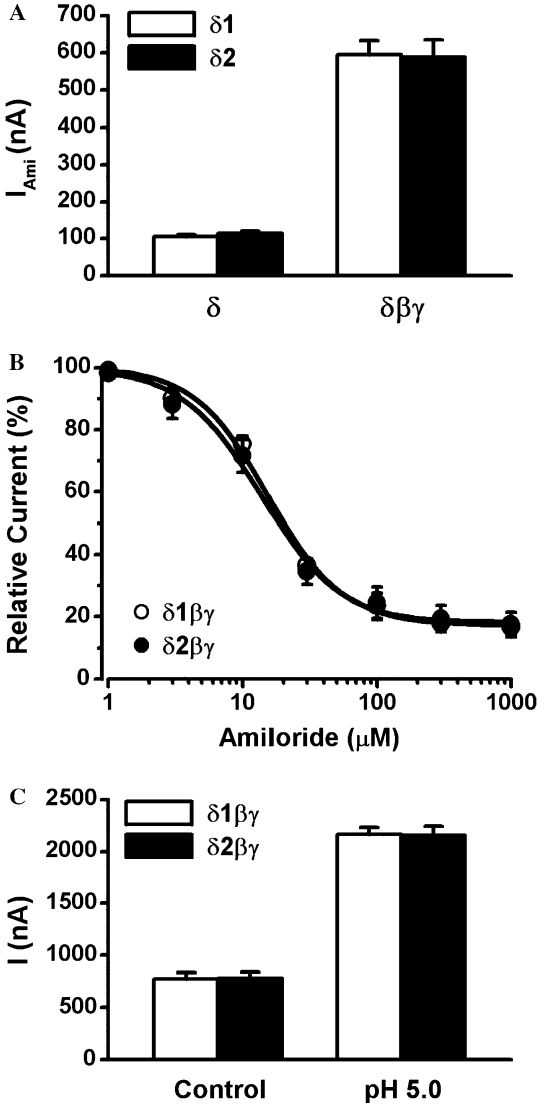

Fig. 3. Functional expression of δ2-subunit. Whole-cell currents of the δ2-subunit were recorded at a holding potential of −60 mV in the *X. oocyte* expression system using a two-electrode voltage-clamp technique. (A) Amiloride-sensitive currents of δ1- (*open*) or δ2-subunit (*closed*) in the absence and presence of accessory βγ-subunits are summarized. (B) Sensitivities to amiloride in the inward currents in δ1βγ- (*open*) or δ2βγ-expressed (*closed*) oocytes are plotted. IC_{50} values were approximately 15 μM. (C) δ1βγ (*open*) or δ2βγ (*closed*) current is potentiated during acidic pH (pH 5.0) application. Macroscopic current properties of δ2-subunit were clearly similar to those of δ1-subunit ($p > 0.05$). Experimental data were obtained from 4 to 13 oocytes.

subunits produces a larger current, as is the case with the α-subunit [4,6,7]. Therefore, we confirmed whether the N-terminal spliced variant was able to interact with the accessory βγ-subunits by coexpression in *X. oocytes*. When δ2- and βγ-subunits were coinjected in oocytes, amiloride-sensitive currents were observed (589 ± 47 nA, $n = 13$, $p > 0.05$ vs. δ1βγ of 594 ± 39 nA, $n = 13$).

In the next set of experiments, the concentration dependency of the inhibitory effects by amiloride was analyzed in δ2βγ-expressing oocytes. The inhibitory effect of heteromultimeric δ2βγ current at −60 mV by amiloride was concentration-dependent with an IC_{50} value of 14 μM and a Hill coefficient of 1.4 ($n = 4$, in δ1βγ, 15 μM, and 1.6, respectively; Fig. 3B).

We have demonstrated that protons activate δ-subunit activity [8]. Neuronal activity is well known to be associated with pH fluctuations. Acid-sensing ion channel 1 in the central nervous system has been implicated in long-term potentiation, suggesting that minute fluxes in synaptic pH may activate acid-sensitive channels to enhance synaptic plasticity, learning, and memory [18]. These findings provide a starting point for a number of exciting follow-up investigations into the role of the neuronal degenerin/epi-

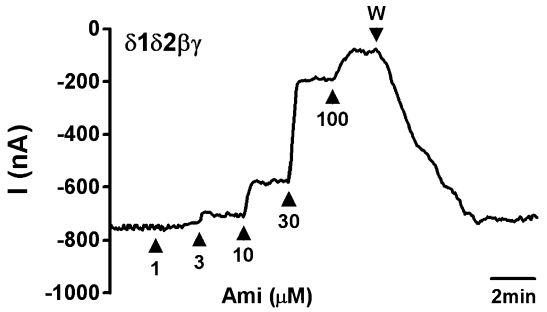

Fig. 4. Coexpression of δ1- and δ2-subunits. When δ1-, δ2-, and βγ-subunits were all injected into *X. oocytes*, the inhibitory effect of the heteromultimeric channel current by amiloride was in a concentration-dependent manner, and the inhibition was recovered by the removal of amiloride. A typical trace of inward current sensitized with amiloride (*Ami*) in a δ1δ2βγ-injected oocyte is plotted. Note that the macroscopic properties of the δ1/δ2-subunit current were similar to those of either δ-subunit alone. Similar results were obtained from five oocytes.

thelial Na^+ channel family in the brain. Therefore, we examined whether the δ2-subunit channel had the property of proton activation (Fig. 3C). The application of pH 5.0 potentiated inward currents at −60 mV in δ2βγ-injected oocytes (by 1372 ± 97 nA, $n = 10$, $p > 0.05$ vs. δ1βγ of 1391 ± 87 nA, $n = 10$).

Coexpression of δ1- and δ2-subunits

These electrophysiological studies indicate that the macroscopic properties of the δ2-subunit were clearly similar to those of the δ1-subunit, suggesting that the novel N-terminal region of the δ2-subunit is not contained in essential codes for membrane trafficking, interaction for channel complex, and binding with amiloride or proton. Therefore, the coexpression of these δ-subunits was challenged in *X. oocytes*. Although δ1-, δ2-, and βγ-subunits were all injected into oocytes, the modulation of physiological and pharmacological properties on the heteromultimeric channel could not be detected ($n = 5$; Fig. 4). In the novel N-terminal region, there are no particular sequences that can be phosphorylation targets by protein kinase A or C, or interact with known proteins. Further studies may identify unknown protein which is able to recognize a novel region for modulating channel activity, as that we described previously that 14-3-3 protein modulates the expression of ENaCs by phosphorylation-dependent interaction with Nedd4-2 ubiquitin ligase at the C-terminal region [19,20].

Conclusion

We found that transcripts encoding the δ-subunit of ENaC (δ1-subunit) and a novel spliced variant (δ2-subunit) were expressed in the human brain, and both possessed fundamental channel functions. Thus, we speculate that the alternative spliced variant may play a role in pH sensing in the human brain, described as a physiological function of the original δ-subunit.

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