Protons Activate the δ-Subunit of the Epithelial Na⁺ Channel in Humans*

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The amiloride-sensitive epithelial Na⁺ channel (ENaC) controls Na⁺ transport into cells and across epithelia. So far, four homologous subunits of mammalian ENaC have been isolated and are denoted as α, β, γ, and δ. ENaCδ can associate with β and γ subunits and generate a constitutive current that is 2 orders of magnitude larger than that of homomeric ENaCα. However, the distribution pattern of ENaCδ is not consistent with that of the β and γ subunits. ENaCδ is expressed mainly in the brain in contrast to β and γ subunits, which are expressed in non-neuronal tissues. To explain this discrepancy, we searched for novel functional properties of homomeric ENaCδ and investigated the detailed tissue distribution in humans. When human ENaC was expressed in Xenopus oocytes and Chinese hamster ovary cells, an electrogenic acid-induced current, half-maximal pH for an activation of 5.0, and the acid-induced current was abolished by amiloride. The most striking finding was that the desensitization of the acid-evoked current was much slower (by ~10% 120 s later), dissociating from the kinetics of acid-sensing ion channels in the degenerin/epithelial Na⁺ channel family, which were rapidly desensitized during acidification. RNA dot-blot analyses showed that ENaCδ mRNA was widely distributed throughout the brain and was also expressed in the heart, kidney, and pancreas in humans. Northern blotting confirmed that ENaCδ was expressed in the cerebellum and the hippocampus. In conclusion, human ENaCδ activity is regulated by protons, indicating that it may contribute to the pH sensation and/or pH regulation in the human brain.

Four homologous epithelial Na⁺ channel (ENaC)† subunits ( α, β, γ, and δ), members of the degenerin/epithelial Na⁺ channel superfamily, have been cloned in mammals (1–5). There is an overall ~37% amino acid identity between the α, β, γ, and δ subunits. The δ subunit of ENaC was originally described as mainly being expressed in the human brain (5). ENaCδ can associate with β and γ subunits to form a heteromeric channel because the coexpression of these three subunits increases the Na⁺ current (5). The tissue distribution pattern of ENaCδ, however, is quite different from that of β and γ subunits. ENaCδ is expressed mainly in the brain, pancreas, testis, and ovary (5), whereas β and γ subunits are expressed mainly in the kidney, lung, and colon (3, 4). In addition, the expressed sequence tag data base shows that an ENaCδ gene has been found in humans and chimpanzees (GenBank™ accession numbers U38254 and O46547, respectively), but for now, there is no evidence for the orthologues in rats and mice. This suggests that ENaCδ associates with unknown subunits or that homomeric ENaCδ has its own unknown physiological function in humans. Our goal was to identify the novel functional properties of human ENaCδ (hENaCδ) using electrophysiological techniques and to investigate the more detailed tissue distribution of ENaCδ in humans by Northern blot and RNA dot-blot analyses.

Here we describe how ENaCδ is expressed widely in the human brain, such as in the cerebellum and hippocampus, and how the channel activity is enhanced by external protons in both Xenopus oocyte and Chinese hamster ovary (CHO-K1) cell expression systems. These results provide the novel profile that ENaCδ responds to acidification in humans.

EXPERIMENTAL PROCEDURES

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. Two expressed sequence tag clones (GenBank™ accession numbers BI520370 andAI199647) were obtained from the Medical Research Council Geneservice (Babraham, UK) to prepare a full-length hENaC containing a 1,914-nucleotide open reading frame. Rat acid-sensing ion channel 2a (rASIC2a; U53211) was isolated as described previously (6).

Xenopus Oocyte Electrophysiology—The cDNA sequence of hENaCδ or rASIC2a was subcloned into a plu/messcript vector (Stratagene, La Jolla, CA) with 5'- and 3'-untranslated regions of Xenopus β-globin added at the multicloning site to promote stable mRNA expression in Xenopus oocytes. Using an mMESSAGE mMACHINE T3 kit (Ambion, Austin, TX), cRNAs were synthesized and 5 ng was injected into Xeno-
pus oocytes. The control oocytes were injected with an equal volume of diethyl dicarbonate-treated water and are described as native oocytes throughout. Electrophysiological recordings were taken 48–72 h after injection using a two-electrode voltage clamp technique with a CEZ-1200 amplifier (Nihon Kohden, Tokyo, Japan) and a MacLab A/D converter (ADInstruments, Colorado Springs, CO). The recording solution had an ionic composition of 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES. The pH of the solution was adjusted to 7.5 with NaOH. The recording solution for pH experiments was prepared with the equivalent MES (pKₐ = 6.15) instead of HEPES (pKₐ = 7.55) and adjusted to a suitable pH with either NaOH or HCl. The recording chamber was continuously perfused with solution at a flow rate of 5 ml/min. All electrophysiological experiments were carried out at room temperature (24 ± 1 °C).

CHO-K1 Cell Electrophysiology—The coding sequence of hENaCδ
FIG. 1. Activation of hENaCδ current by protons in Xenopus oocytes. Whole-cell currents were recorded at a holding potential of −60 mV in Xenopus oocytes. A, a hENaCδ-expressed oocyte possessed a larger inward current than a native oocyte. The larger current in a hENaCδ oocyte was mostly inhibited by 100 μM amiloride (Ami) but not the minimum current in a native oocyte. B, in a hENaCδ oocyte, the application of pH 5.0 caused constitutive enhancement of the inward current, and the current increase was influenced by 100 μM amiloride. Neither the application of pH 5.0 nor amiloride induced any current in a native oocyte. The current-voltage relationships before (pH 7.5; a) and during acidification (pH 5.0) in the absence (b) and presence (c) of 100 μM amiloride are shown (inset). The acidic stimulus enhanced the hENaCδ activity at all voltages examined. C, the effects of pH 5.0 in native (open) or hENaCδ (closed) oocytes are summarized. D, to analyze the desensitization of a pH 5.0-evoked current in hENaCδ oocytes, these currents were normalized with the peak amplitude in the presence of an acidic medium and compared with those in rASIC2a-expressed oocytes. Five and three typical traces of the pH 5.0-evoked current in hENaCδ and rASIC2a oocytes were plotted, respectively. E, the desensitization of the pH 5.0-evoked current in hENaCδ (closed) and rASIC2a (hatched) oocytes was evaluated as the I/I_max value 120 s later. The number of oocytes used is in parentheses. The statistical significance of the difference is expressed as *p < 0.01 versus control (***) and p < 0.01 versus pH 5.0 (###).
was subcloned in a pTracer-CMV2 vector (Invitrogen, Carlsbad, CA) and transiently transfected into CHO-K1 cells using LipofectAMINE 2000 (Invitrogen). The control cells were transfected with the vector alone, described as native cells throughout. Experiments were carried out 48–72 h later using the whole-cell patch clamp technique with an Axopatch 200B amplifier and pCLAMP 8 software (Axon Instruments, Foster City, CA). The transiently transfected cells were identified from green fluorescence protein expression. The extracellular solution had an ionic composition of 137 mM NaCl, 5.9 mM KCl, 2.2 mM CaCl2, 1.2 mM MgCl2, 14 mM glucose, and 10 mM MES. The pH of the solution was adjusted to 7.4 with NaOH. The pipette solution contained 140 mM KCl, 1 mM MgCl2, 10 mM MES, 2 mM Na2ATP, and 5 mM EGTA. The pH was adjusted to 7.2 with KOH.

**RNA Dot-Blot—**A human multiple tissue expression array (version 2) was purchased from Clontech (Palo Alto, CA). A 32P-labeled hENaC6 probe was prepared from the full-length hENaC6 cDNA fragment using a Ready-To-Go DNA labeling beads (-dCTP) kit (Amersham Biosciences). The membrane was prehybridized in a modified Church’s buffer (0.5 mM NaHPO4, pH 7.2, 10 mM EDTA, and 7% SDS) for 6 h at 65 °C and then hybridized in a buffer containing 1.5 × 106 cpm/ml of a 32P-labeled probe for 12 h at 65 °C. After hybridization, the membrane was repeatedly washed in 2× SSC and 1% SDS for 20 min at 55 °C, and exposed to Kodak X-AR film for 1 week at ~80 °C.

**Northern Blot—**Human samples free from neurological disorders were taken within 24 h after death with permission of the deceased persons’ families. (Ethical principles and considerations were observed regarding forensic and related research using human organs and fluids obtained from autopsies.) Human cerebellums and hippocampi were rapidly frozen on dry ice immediately after their removal at autopsy and were kept at ~80 °C until use. From freshly frozen tissues, 2 μg of poly(A)+ RNAs were obtained using a FastTrack 2.0 kit (Invitrogen). The membrane was prehybridized in a hybridization buffer (5× saline/sodium phosphate/EDTA, 5× Denhardt’s solution, 50% formamide, 20 μg/ml salmon sperm DNA, and 1% SDS) for 6 h at 42 °C and hybridized in a buffer containing 2.0 × 106 cpm/ml of the 32P-labeled hENaC6 probe for 12 h at 42 °C. After hybridization, the membrane was washed twice in 2× SSC at room temperature followed by two additional washes with a solution containing 1× SSC and 0.5% SDS for 20 min at 55 °C, and exposed to Kodak X-AR film for 1 week at ~80 °C.

**Statistics—**Pooled data are shown as the mean ± S.E. Statistical significance between the two groups and among groups was determined by Student’s t test and Scheffe’s test after one way analysis of variance, respectively. Significant difference is expressed in the figures (*, p < 0.05; ** or ‡ , p < 0.01). The data on the relationships between proton concentrations and current responses were fitted using the following equation (see Fig. 2B): \( I_{\text{max}} = I_{\text{norm}} \times \left[ 1 + \frac{K_d}{[\text{H}^+]_{\text{norm}}} \right] + C \), where \( I_{\text{norm}} \) is the maximum amplitude of the acid-evoked current, \( K_d \) is the apparent dissociation constant of protons, \([\text{H}^+]_{\text{norm}}\) is the concentration of extracellular protons, \( n \) is the Hill coefficient, and \( C \) is the constant.

**RESULTS AND DISCUSSION**

**Characterization of hENaC6 Current in Xenopus Oocytes—**When hENaC6 was expressed in Xenopus oocytes, an inward current was induced at a holding potential of ~60 mV, and the current was mostly inhibited by 100 μM amiloride (Fig. 1A).

The mean amplitude of the amiloride-sensitive current in hENaC6-expressed oocytes was 160 ± 9 nA at ~60 mV (n = 27, p < 0.01 versus native of 4 ± 1 nA, n = 22). The macroscopic properties of the hENaC6 current in our experimental system were similar to those reported previously (5). It has been reported that hENaC6 itself can induce some currents when expressed in Xenopus oocytes, but the heteromultimeric channel with β and γ subunits produces a larger current (5), as is the case with ENaCα2. However, unlike ENaCα2, which plays a pathophysiological role as an ENaCβγ complex in epithelia such as the kidney, lung, and colon (3, 4, 6, 7), it is unclear whether ENaC6 associates with other subunits in vitro to play a role in physiological functions. It has been reported that hENaC6 has been expressed mainly in the brain, pancreas, testis, and ovary (5), whereas β and γ subunits are expressed mainly in the kidney, lung, and colon (3, 4). Therefore we focused on homomeric hENaC6 in this study.

**Proton Activation of hENaC6 Current in Xenopus Oocytes—**Interestingly, the activity of hENaC6 showed pH dependence in the Xenopus oocyte expression system. At a holding potential of ~60 mV, the application of pH 5.0 induced an inward current in hENaC6-injected oocytes (514 ± 41 nA, n = 17, p < 0.01 versus native of 62 ± 4 nA, n = 10; Fig. 1, B and C). The current-voltage relationship showed that the acidic pH stimuli enhanced the channel activity at all voltages examined in hENaC6 oocytes. The addition of 100 μM amiloride dramatically blocked the acid-evoked current (87 ± 2% decrease, n = 17, p < 0.01) and, moreover, significantly inhibited the current (to 62 ± 8 nA, n = 17, p < 0.01 versus the initial resting current of 175 ± 6 nA). During a sustained acidic pulse at pH 5.0, there was a slight decline in the amplitude of the acid-evoked current in hENaC6 oocytes (6 ± 1% decrease at 120 s, n = 17). In sharp contrast, rASIC2α was activated by extracellular acidification and rapidly desensitized even as the pH remained steady (90 ± 1% reduction, n = 3; Fig. 1, D and E). The activity of the acid-induced hENaC6 current was constitutive in the presence of an acidic medium, with clear distinction from the major properties of acid-sensitive
degenerin/epithelial Na⁺ channels such as rASIC2a expressed in the brain (9, 10), which was rapidly activated by protons and desensitized even during acidic pH (Fig. 1, D and E, and Refs. 6, 10, and 11). The kinetics of desensitization during acidification on ASIC1a and ASIC2a are faster; the pH 6.0-evoked ASIC1a current is desensitized to the resting level within 30 s (12, 13), and the pH 5.0-induced ASIC2a current is reduced to 30% of peak amplitude within 30 s (9, 11, 14). On the other hand, pH-sensitive ASIC3 current possesses unique biphasic kinetics or a transient larger current followed by a sustained current of smaller amplitude (13, 15). The desensitization of the acid-evoked hENaC current was much slower than that of ASICs, and no significant desensitization was observed during acidification on an acid-elicited hENaC current.

In hENaCΔ-expressed oocytes, the gradual decrease in pH from 7.5 to 3.0 showed that the hENaCΔ current was increased by acidic pH below 6.5 in a proton concentration-dependent manner (704 ± 75 nA at pH 3.0, n = 13, p < 0.01) and was mostly saturated at pH 4.0 (Fig. 2). The half-maximal pH for activation of the hENaCΔ current was 5.0, and the Hill coefficient was 0.6. The proton-induced currents were from the hENaCΔ channel because in control oocytes minimal acid-evoked currents were observed at lower than pH 4.0 (122 ± 56 nA at pH 3.0, n = 7) and the currents were insensitive to 100 µM amiloride (n = 5).

Acid-evoked hENaCΔ Currents in CHO-K1 Cells—Further evidence for the acid-evoked activation of hENaCΔ was provided by whole-cell voltage clamp studies of CHO-K1 cells transiently expressing hENaCΔ (Fig. 3). At a holding potential of −60 mV, 100 µM amiloride-sensitive inward currents were observed in hENaCΔ-transfected cells (193 ± 15 pA, n = 9, p < 0.01 versus native) but not in native cells (6 ± 2 pA, n = 9). Extracellular acidification induced larger amiloride-sensitive currents in an acidic pH-dependent manner in hENaCΔ cells (504 ± 58 pA at pH 5.0, n = 9, p < 0.01) than any observed in native cells (47 ± 10 pA, n = 9). The pH response profile in CHO-K1 cells was very similar to that obtained in the oocytes.

Although both ENaC and ASIC families belong to the degenerin/epithelial Na⁺ channel superfamily, the functional properties of ENaCs have been thought to differ from those of ASICs. ENaCs are constitutively active channels, whereas ASICs require acidic stimulation for activation (7, 8, 10). The members of the ASIC family have been implicated as transducers of pH stimuli because these channels are activated by acidification in an extracellular compartment (8, 10). The profile of pH responsiveness on ASICs varies with these...
channels (8, 10); the half-maximal pH values for the activation of heterologously expressed ASIC1a, ASIC2a, and ASIC3 are 6.2–6.4 (12, 13), 4.35 (11, 14), and 6.5–6.7/3.5 (for peak/sustained current) (13, 15), respectively. On the other hand, ENaC is blocked by acidic pH (16–18). In this investigation, using two different expression systems, we found that the activity of hENaC was clearly enhanced by extracellular acidification and the response was influenced by amiloride.

**Expression of ENaC in Human Tissues**—The ENaC distribution was analyzed using a human RNA dot-blot array. The RNA dot-blot analyses demonstrated that ENaC mRNAs were widely expressed in various human brain regions such as the cerebellum, cerebral cortex, hippocampus, caudate nucleus, and putamen (Fig. 4A). Moreover, strong blotting signals were also found in the heart, kidney, and pancreas as well as in the fetal brain, heart, and kidney. Next we performed Northern blot analyses (Fig. 4B) using the poly(A)⁺ RNAs obtained from freshly frozen human cerebellum and hippocampus, where an intense signal was shown among brain in RNA dot-blot analysis. The expression of ~5.5-kb mRNA was observed in the cerebellum. Moreover, a faint signal of ~5.5 kb was detected in the hippocampus.

Both RNA dot-blot and Northern blot analyses showed that ENaC mRNA was expressed in human brain tissue such as the cerebellum and hippocampus. Neuronal activity is well known to be associated with pH fluctuations (19). In this investigation, hENaC started to open at pH 6.5 and induced larger amiloride-sensitive currents with more acidic pH. More recently, ASIC1a in the central nervous system has been implicated in long term potentiation, suggesting that minute fluxes in synaptic pH may activate ASICs to enhance synaptic plasticity, learning, and memory (20, 21). These findings (21) provide a starting point for a number of exciting follow up investigations into the role of the neuronal degenerin/epithelial Na⁺ channel family in the brain. Preliminary experiments into acquiring an ENaC clone from the neuronal tissues of other mammals, such as rats and mice, were unsuccessful. A further search of the expressed sequence tag data base showed that not even a partial fragment of ENaC has been found yet except for in humans and chimpanzees (GenBank™ accession numbers...
U38254 and O46547, respectively). Nevertheless, other ENaC subunits (α, β, and γ) have been isolated not only from mammals but also from amphibians and birds. The corresponding genomic assignments of ENaCα were identified on human chromosome 1p36.3-p36.2 (22). ENaCα may be a unique gene constructed on the genome of only primates in the process of evolution.

In this investigation, we found that hENaCα was abundantly expressed throughout the human brain, such as in the cerebellum and hippocampus, and the channel activity was constitutively enhanced at a pH lower than 6.5 in a pH-dependent manner when expressed in *Xenopus* oocytes and CHO-K1 cells. Taken together, these data suggest a possible role for ENaCα as a key component of proton-activated currents in the human brain.

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**REFERENCES**

1. Canessa, C. M., Horisberger, J. D., and Rossier, B. C. (1993) *Nature* **361**, 467–470
2. McDonald, F. J., Snyder, P. M., McCray, P. B., Jr., and Welsh, M. J. (1994) *Am. J. Physiol.* **266**, L728–L734
3. Canessa, C. M., Schild, L., Buell, G., Thorens, B., Gautschi, I., Horisberger, J. D., and Rossier, B. C. (1994) *Nature* **367**, 463–467
4. McDonald, P. J., Price, M. P., Snyder, P. M., and Welsh, M. J. (1995) *Am. J. Physiol.* **268**, C1157–C1163
5. Waldmann, R., Champigny, G., Bassilana, F., Voilley, N., and Lazdunski, M. (1995) *J. Biol. Chem.* **270**, 27411–27414
6. Ugawa, S., Minami, Y., Guo, W., Saishin, Y., Takatsuji, K., Yamamoto, T., Tohyama, M., and Shimada, S. (1998) *Nature* **395**, 555–556
7. Alvarez de la Rosa, D., Canessa, C. M., Fyfe, G. K., and Zhang, P. (2000) *Annu. Rev. Physiol.* **62**, 573–594
8. Kellenberger, S., and Schild, L. (2002) *Physiol. Rev.* **82**, 735–767
9. Price, M. P., Snyder, P. M., and Welsh, M. J. (1996) *J. Biol. Chem.* **271**, 7879–7882
10. Waldmann, R., and Lazdunski, M. (1998) *Curr. Opin. Neurobiol.* **8**, 418–424
11. Ugawa, S., Ueda, T., Minami, Y., Horimoto, M., and Shimada, S. (2001) *Neuroreport* **12**, 2141–2145
12. Waldmann, R., Champigny, G., Bassilana, F., Heurteaux, C., and Lazdunski, M. (1997) *Nature* **386**, 173–177
13. Sutherland, S. P., Benson, C. J., Adelman, J. P., and McCleskey, E. W. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 711–716
14. Champigny, G., Voilley, N., Waldmann, R., and Lazdunski, M. (1998) *J. Biol. Chem.* **273**, 15418–15422
15. Waldmann, R., Bassilana, F., de Weille, J., Champigny, G., Heurteaux, C., and Lazdunski, M. (1997) *J. Biol. Chem.* **272**, 26975–26978
16. Chalfant, M. L., Denton, J. S., Berdiev, B. K., Ismailov, I. I., Benos, D. J., and Stanton, B. A. (1999) *Am. J. Physiol.* **276**, C477–C486
17. Zhang, P., Fyfe, G. K., Grichtchenko, I. I., and Canessa, C. M. (1999) *Biophys. J.* **77**, 3043–3051
18. Konstas, A. A., Mavrellos, D., and Korbmacher, C. (2000) *Pflügers Arch. Eur. J. Physiol.* **441**, 341–350
19. Chester, M., and Kula, K. (1992) *Trends Neurosci.* **15**, 396–402
20. Bianchi, L., and Driscoll, M. (2002) *Neuron* **34**, 337–340
21. Wemmie, J. A., Chen, J., Askwith, C. C., Hruska-Hageman, A. M., Price, M. P., Nolan, B. C., Yoder, P. G., Lamani, E., Hoshi, T., Freeman, J. H., Jr., and Welsh, M. J. (2002) *Neuron* **34**, 463–477
22. Waldmann, R., Bassilana, F., Voilley, N., Lazdunski, M., and Mattei, M. (1996) *Genomics* **34**, 262–263