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Epithelial Na^+ channel δ subunit mediates acid-induced ATP release in the human skin

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ABSTRACT

The amiloride-sensitive epithelial Na^+ channel (ENaC) regulates Na^+ homeostasis in cells and across epithelia. Although we described that ENaCδ is a candidate molecule for a pH sensor in the human brain, the physiological and pathological roles of ENaCδ in non-neuronal tissues are still unknown. Here we show a novel physiological function of ENaCδ in peripheral tissues in humans. Expression analyses at the level of mRNA clearly revealed that ENaCδ was abundantly expressed in human epidermis and keratinocytes. In addition, ENaCδ protein was detected in there. In cultured keratinocytes, acidic stress (pH 5.0) evoked ATP release, which was significantly reduced in the presence of 100 μM amiloride or 10 μM benzamil. In conclusion, ENaCδ may be involved in the mechanism underlying pH sensing followed by the regulation of cell viability in the human skin.

The degenerin/epithelial Na^+ channel superfamily has striking functional diversity including Na^+ absorption, acid sensing, peptide gating, acidosis-evoked nociception, and mechanotransduction. The amiloride-sensitive 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. ENaCα 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 [1,2]. Recently, we showed that ENaCδ and its variant were widely distributed throughout the brain and were activated by protons, indicating that it may contribute to pH sensation in the human brain [3,4].

The mammalian surface layer in skin has been largely attributed to monitoring environmental stresses such as temperature, pressure, and pH, and cell tolerance from painful and noxious damage. The epidermal keratinocytes therefore require multiple ion channels and receptors in response to various stressors; for example, transient receptor potential vanilloid subfamily (TRPV) 1 for thermal and acidic detectors [5,6], TRPV3 for peripheral thermal transduction [7], TRPV4 for warmth and osmotic signals [8,9], and some purinergic receptors for stressor-inducible ATP release followed by repairing and refolding the injured cells [10,11].

In this investigation, we examined whether ENaCδ is expressed in human epidermis and keratinocytes using reverse transcription-polymerase chain reaction (RT-PCR), *in situ* hybridization, immunostaining, and Western blot. Acidic-evoked ATP release through ENaCδ activation was also confirmed in cultured keratinocytes. These results showed that proton-sensitive ENaCδ was abundantly expressed in human epidermis and keratinocytes, and cultured keratinocytes released ATP by acidic stimulus though the activation of ENaCδ.

Materials and methods

Molecular biology. All experiments were approved by the Ethics Committee of Nagoya City University and were conducted in accordance with the Declaration of Helsinki. Human samples were taken from autopsies within 24 h after death with permission. The extraction of total RNA, RT-PCR, electrophoresis, cloning, and subsequent sequencing were performed as described previously [4,12]. PCR amplification was carried out for 35 cycles. Specific PCR primers for human ENaCs were designed as follows: for ENaCδ (GenBank Accession No. NM_002978), (+) 5′-TAC CGC CTC TAC CAG GAC CTG GA-3′ and (−) 5′-CTG GCC TCT GGC TTG ATG CTG GA-3′ (467 bp); for ENaCα (NM_001038), (+) 5′-TGG TGC ACG GGC AGG

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ATG AAC CT-3′ and (−) 5′-CCG AGG GCC ATC GTG AGT AAC CA-3′ (432 bp); for ENaCβ (NM_000336), (+) 5′-CAT CCA GGC CTG TCT TCG CTC CT-3′ and (−) 5′-CCA GAA GCC AAA CTG GCC ACC CA-3′ (442 bp); for ENaCγ (NM_001039), (+) 5′-CCC AGT ACA GCC AGC CTC TAC CT-3′ and (−) 5′-GGA CAT GGG GGA GCC TGT TTC CA-3′ (517 bp).

In situ hybridization. Human tissues were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 24 h at 4 °C, washed with phosphate buffer, dehydrated, and embedded in paraffin wax. Continuous paraffin-embedded sections were prepared at 5 μm thickness. These samples were hydrated, treated with proteinase K solution (50 mM Tris–HCl, 5 mM EDTA, 10 μg/ml proteinase K, pH 8.0) for 10 min, and fixed with paraformaldehyde in phosphate buffer for 5 min. After washing in phosphate buffer, they were acetylated (0.1 M triethanolamine, 0.25% anhydrous acetic acid), dehydrated, and air-dried. *In situ* hybridization with specific [^{35}S]-labeled riboprobes was performed as described previously [13], except for washout in high-stringency solution at 60 °C. The specificity of the hybridization signal was confirmed by a control study with each sense riboprobe. For light microscopic examination, some sections were stained with 0.25% thionine.

Immunohistochemistry. Paraffin-embedded sections (5 μm thickness) of the human skins were hydrated, washed in 0.01 M phosphate-buffered saline, and pretreated with 5% normal goat serum (DakoCytomation, Glostrup, Denmark) in phosphate-buffered saline for 30 min at room temperature. Cultured keratinocytes were similarly treated with normal goat serum. These samples were treated with 1 μg/ml of a polyclonal antibody of human ENaCδ (ENACd41-A; Alpha Diagnostic International, San Antonio, USA) for 12 h at 4 °C, thereafter were covered with Cy3-labeled anti-rabbit antibody solution (1:1000 dilution; Jackson Immunoresearch, West Grove, USA) for 1 h at room temperature, as described previously [13]. Staining specificity was confirmed by signal ablation with its antigenic peptide (ENACd41-P) according to the manufacturer's protocol.

Western blot. For Western blot analysis, solubilized protein of human skin, cultured keratinocytes or Chinese hamster ovary (CHO) cells transfected with either the vector or human ENaCδ gene (1–5 μg for each) [3], was loaded on a 10% acrylamide gel, transferred to an Immobilon-P transfer membrane (Millipore, Bedford, USA), and immunoblotted with 1 μg/ml of human ENaCδ antibody and anti-rabbit HRP antibody (1:1000 dilution; Promega). Control loading was carried out using an anti-actin antibody (Sigma–Aldrich, St. Louis, USA). Signals were detected using an ECL Plus Western Blotting Detection System (Amersham, Piscataway, USA). Staining specificity was confirmed by signal ablation with an antigenic peptide.

ATP release assay. A human keratinocyte cell line (PHK16-0b) was obtained from the Health Science Research Resources Bank (Osaka, Japan) and cultured in Defined Keratinocyte-SFM medium (Invitrogen, Carlsbad, USA) supplemented with 10% heat-inactivated fetal bovine serum, 20 U/ml penicillin G, and 20 μg/ml streptomycin. Keratinocytes were cultured in a 96-well plate at 37 °C to $\sim 1 \times 10^4$ cells per well. Acidic stress was induced in a 37 °C incubator for 1 h. Amiloride or benzamil was exposed for 1 h before acidic stimulus. These supernatants (100 μl) were assayed for ATP released from keratinocytes using a CellTiter-Glo Luminescent Cell Viability Assay (Promega). The cell number in a well was determined using a Cell Counting Kit-8 (Dojin, Kumamoto, Japan).

Drugs. Pharmacological reagents were obtained from Sigma–Aldrich (St. Louis, USA). Amiloride or benzamil was dissolved in dimethyl sulfoxide at a concentration of 100 mM as a stock solution.

Statistics. Pooled data are shown as means ± SEM. Statistical significance among groups was determined by Scheffé's test after one-way analysis of variance. Significant difference is expressed in the figures ($^{**}p < 0.01$).

Results

Expression of ENaCδ in human skin and keratinocytes

The expression of mRNA encoding ENaCδ in human skin and cultured keratinocytes (PHK16-0b) was examined by RT-PCR using total RNA extracted from human skin, cultured keratinocytes and specific oligonucleotide primers. RT-PCR analysis showed a clear expression of ENaCδ transcript (467 bp) in human skin and keratinocytes (Fig. 1A). Accessory subunits, β and γ (442 and 517 bp, respectively), clearly showed mRNA expression in both types of samples. Another core unit, ENaCα (432 bp), was also detected from both samples. We also confirmed that the RT-PCR fragment was in fact derived from mRNA of each ENaC subunit by sequencing the PCR products. When the RT procedure was not performed, there were no detectable expression signals. These results could be reproduced by five repeated experiments.

To determine the distribution pattern of ENaCδ, [^{35}S]-radioactive *in situ* hybridization was performed in human skin with specific riboprobes. The morphological approach by *in situ*

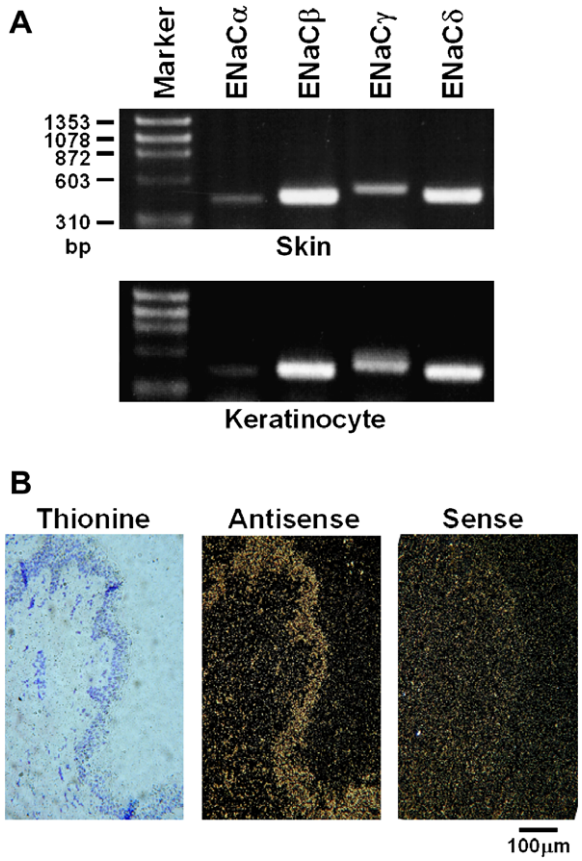

Fig. 1. Expression of ENaCδ in human skin and keratinocytes. Expression of mRNA encoding ENaCs in human skin and cultured keratinocytes (PHK16-0b) was observed using RT-PCR and *in situ* hybridization. (A) Typical transcript images of PCR product from human ENaCs after the RT procedure are presented. Clear expression of ENaCδ transcript (467 bp) was detected in human skin (upper) and cultured keratinocytes (bottom). Accessory subunits, ENaCβ (442 bp) and γ (517 bp), and another core unit, ENaCα (432 bp), also clearly identified the mRNA expression. Without the RT procedure, there were no detectable positive signals. Ready-Load φX174 RF DNA/HaeIII fragments (Invitrogen) were used as a DNA size markers. (B) A typical emulsion autoradiogram of *in situ* hybridization clearly shows the selective staining for ENaCδ throughout the surface layer of human skin with an antisense riboprobe (middle), but not with a control sense probe (right). For light microscopic images, a continuous section was stained with 0.25% thionine (left). Note that positive signals of ENaCδ mRNA were localized throughout the epidermis layer of human skin, but not in the dermis and subcutaneous regions.

hybridization clearly showed that an abundant expression of ENaCδ mRNA was observed in the human skin, but not with a control sense riboprobe ($n = 10$ for each; Fig. 1B). For light microscopic images, continuous sections were stained with 0.25% thionine. Comparison between dark and light field images revealed that positive signals of ENaCδ were localized throughout the surface layer (consisting largely of the epidermis) of human skin, but not in subsurface regions such as the dermis and subcutaneous tissue. Slight signals were also detected in sweat glands.

ENaCδ protein in human skin and keratinocytes

Further evidence of ENaCδ expression in human skin and cultured keratinocytes was provided by an immunohistochemical approach using an affinity-purified polyclonal antibody for human ENaCδ. Immunoreactivity to ENaCδ was observed in the epithelial cutaneous layer of human skin and most keratinocytes but not in the presence of the control peptide ($n = 7$ for each; Fig. 2A).

Western blotting analysis revealed that ENaCδ protein (approximately 70 kDa, compared with CHO cells transiently transfecting the ENaCδ gene) was expressed at readily detectable levels in human skin and cultured keratinocytes, but not in the presence of the control peptide ($n = 3$; Fig. 2B).

ATP release by acidic stimulus in cultured keratinocytes

It has been established that multiple stressors promote ATP release for cell tolerance, repair and refolding in different types of cells [10,11]. To examine whether acid-sensitive ENaCδ activity is involved in the functional signaling system in response to stress stimulus, an ATP release assay was performed in cultured keratinocytes (Fig. 3). Acidic stress (pH 5.0) caused ATP release from keratinocytes (3.22 ± 0.31 pmol per 10^4 cells, $n = 20$, $p < 0.01$ vs. resting level of 0.15 ± 0.05 pmol, $n = 20$). Acidosis-evoked ATP release was not affected by the pretreatment of amiloride at lower concentration of 10 μM, which causes an ENaCα-preferred inhibitory effects (3.24 ± 0.30 pmol, $n = 20$, $p > 0.05$ vs. absence) but was significantly inhibited by amiloride at higher concentration of 100 μM, which blocks ENaCδ as well as ENaCα (1.77 ± 0.15 pmol, $n = 20$, $p < 0.01$). The addition of 10 μM benzamil that inhibits both ENaCs, was also significantly reduced ATP release from cultured keratinocytes (1.55 ± 0.10 pmol, $n = 12$, $p < 0.01$).

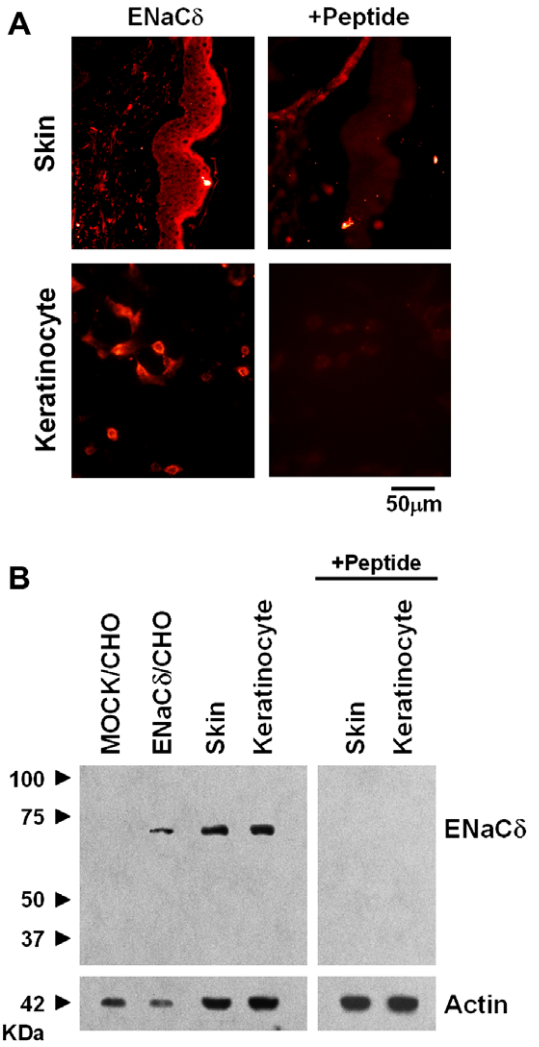

Fig. 2. ENaCδ protein in human skin and keratinocytes. Expression of ENaCδ protein in the human skin and cultured keratinocytes is shown by immunostaining and Western blotting using an affinity-purified polyclonal antibody for human ENaCδ. (A) Immunohistochemical analysis of ENaCδ protein in human skin (upper) and cultured keratinocytes (lower) without (left) or with (right) the antigenic peptide is presented. A specific signal can be detected in most keratinocytes in the epidermal cutaneous layer but not in the presence of the control peptide. Cultured keratinocytes also showed abundant ENaCδ expression by immunostaining. Fifteen to 25 keratinocytes (approximately 20 μm in diameter) are fixed per image (200×250 μm). Staining specificity was confirmed by the signal ablation with the antigenic peptide. (B) In Western blot analysis of human skin, cultured keratinocytes, and CHO cells transiently transfected either the vector alone (MOCK/CHO) or human ENaCδ (ENaCδ/CHO), and a specific signal can be detected at a molecular mass of approximately 70 kDa in the skin, cultured keratinocytes, and ENaCδ/CHO cells. Staining specificity was confirmed by signal ablation with antigenic peptide. Control loading was carried out using an anti-actin antibody.

Discussion

Amiloride-sensitive ENaCs, members of the degenerin/ENaC superfamily, regulate essential control elements for Na^+ homeostasis in cells and across epithelia. Because the ENaCαβγ complex is expressed mainly in epithelia such as the kidney, lung, and colon, playing a pathophysiological role, its physiological and pharmacological characterization has been well documented [1,2]. On the other hand, the physiological function of ENaCδ has not yet been identified. Recently, we described that ENaCδ is abundantly expressed throughout the human brain, and channel activity is constitutively enhanced at a pH lower than 6.5 in a pH-dependent manner [3]. These data suggest a possible role for ENaCδ as a key component of proton-activated currents in the human brain, while functional roles of ENaCδ in peripheral tissues are still unknown. In this study, we examined whether ENaCδ is expressed in the epidermis that can be exposed to acidic solutions under environmental conditions in health and during disease. Expression analysis showed an abundant expression of ENaCδ in the epithelial layer of human skin and keratinocytes. ENaCδ itself can induce a functional current, but the heteromultimeric channel with βγ-subunits produces a larger current [12,14], as is the case with ENaCα [15,16]. In human skin and keratinocytes, the expression of accessory ENaCβ, γ, and another core ENaCα could be detected, as reported previously [17–19].

The activity of ion channels is thought to be associated with cell differentiation for the repair of epithelial cells injured by stress factors. It has been published that epidermal skin and cultured keratinocytes possess defense mechanisms against various environmental stresses and the underlying condition is partially due to Na^+ transport by the ENaCαβγ complex [17–19]. Acidic stimulus is known to trigger esophageal epithelial proliferation [20–22]. Using rabbit cultured esophageal epithelial cells, cell proliferation increased with small pH decreases [22]. However, ENaCα alone or the ENaCαβγ complex is unlikely to be involved in the

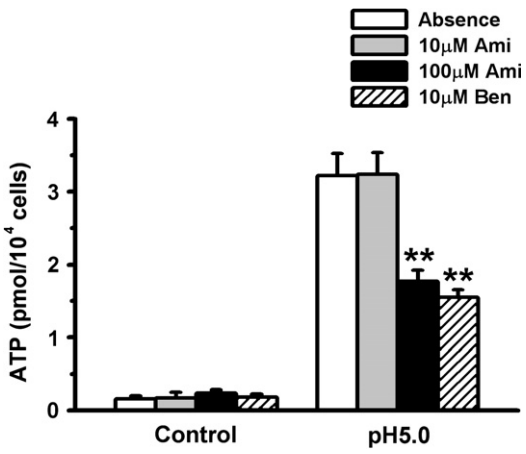

Fig. 3. ATP release by acidic stimulus in cultured keratinocytes. The concentration of ATP released by acidic stimulus was assayed in cultured human keratinocytes at 37 °C. Acidic stress (pH 5.0) for 1 h caused the release of ATP from keratinocytes (open). Amiloride was exposed for 1 h before acidic stimulus. Acidosis-induced ATP release was not affected by 10 μM amiloride (ENaCα-preferred; $p > 0.05$ vs. control; gray) but was partially inhibited by pretreatment with 100 μM amiloride (for ENaCδ and ENaCα; $p < 0.01$; closed). The addition of 10 μM benzamil (for both ENaCs) was also significantly reduced ATP release ($p < 0.01$; hatched). Experimental data were obtained from 20 to 26 wells for each. Statistical significance of the difference is expressed as $^{**}p < 0.01$ vs. absence.

mechanism underlying the acid-evoked response because of the insensitivity of ENaCα under acidic pH [23], suggesting that ENaCδ or the heteromultimeric complex forms functionally as a proton-activated channel in the epithelial layer of human skin. The mechanisms of acid-induced cell proliferation are not known at that point, but might be associated with ENaCδ, as described in this study. It is unclear whether the acidic activation of ENaCδ transfers to sensory neurons, although it has been reported that thermal or hypo-osmolarity stress releases ATP from epithelial cells and causes action potentials in nerve terminals via the $P2X_3$ receptor from the analysis of $P2X_3$- or TRPV1-null mice [24–26]. Rather than peripheral thermal or osmotic transduction as speculated on TRPV3 and TRPV4 in keratinocytes [7–9], it is possible that ENaCδ mediates cellular viability via acid-induced ATP release in keratinocytes. Some purinergic receptors expressed in epidermal keratinocytes are gated by stressor-inducible ATP release and lead to the repair and refolding of injured cells by epidermal differentiation, proliferation, and apoptosis [10,11], suggesting that the cutaneous barrier system may be mediated by an acidic stress-induced ATP signal through the pathway of ENaCδ activation in human keratinocytes.

In conclusion, we found that the transcript and protein encoding proton-sensitive ENaCδ were distributed in the epidermal layer in the skin and keratinocytes in humans. Thus, we speculate that ENaCδ may play a role in pH sensing in human skin, likely described originally as a physiological function of a proton-activated channel in the human brain [3]. This finding in our study provides a starting point for a number of exciting follow-up investigations into the physiological and pathological roles of ENaCδ in *vitro* and *in vivo* in humans. This finding also provides us with a novel candidate molecule for an acid sensor in human skin.

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References

[1] D. Alvarez de la Rosa, C.M. Canessa, G.K. Fyfe, P. Zhang, Structure and regulation of amiloride-sensitive sodium channels, Annu. Rev. Physiol. 62 (2000) 573–594.
[2] S. Kellenberger, L. Schild, Epithelial sodium channel/degenerin family of ion channels: a variety of functions for a shared structure, Physiol. Rev. 82 (2002) 735–767.
[3] H. Yamamura, S. Ugawa, T. Ueda, M. Nagao, S. Shimada, Protons activate the δ-subunit of the epithelial Na+ channel in humans, J. Biol. Chem. 279 (2004) 12529–12534.
[4] H. Yamamura, S. Ugawa, T. Ueda, M. Nagao, S. Shimada, A novel spliced variant of the epithelial Na+ channel δ-subunit in the human brain, Biochem. Biophys. Res. Commun. 349 (2006) 317–321.
[5] M. Denda, S. Fuziwara, K. Inoue, S. Denda, H. Akamatsu, A. Tomitaka, K. Matsunaga, Immunoreactivity of VR1 on epidermal keratinocyte of human skin, Biochem. Biophys. Res. Commun. 285 (2001) 1250–1252.
[6] K. Inoue, S. Koizumi, S. Fuziwara, S. Denda, K. Inoue, M. Denda, Functional vanilloid receptors in cultured normal human epidermal keratinocytes, Biochem. Biophys. Res. Commun. 291 (2002) 124–129.
[7] A.M. Peier, A.J. Reeve, D.A. Andersson, A. Moqrich, T.J. Earley, A.C. Hergarden, G.M. Story, S. Colley, J.B. Hogenesch, P. McIntyre, S. Bevan, A. Patapoutian, A heat-sensitive TRP channel expressed in keratinocytes, Science 296 (2002) 2046–2049.
[8] M.K. Chung, H. Lee, M.J. Caterina, Warm temperatures activate TRPV4 in mouse 308 keratinocytes, J. Biol. Chem. 278 (2003) 32037–32046.
[9] D. Becker, C. Blasé, J. Bereiter-Hahn, M. Jendrach, TRPV4 exhibits a functional role in cell-volume regulation, J. Cell Sci. 118 (2005) 2435–2440.
[10] I. Novak, ATP as a signaling molecule: the exocrine focus, News Physiol. Sci. 18 (2003) 12–17.
[11] E.M. Schwiebert, A. Zsembery, Extracellular ATP as a signaling molecule for epithelial cells, Biochim. Biophys. Acta 1615 (2003) 7–32.
[12] H. Yamamura, S. Ugawa, T. Ueda, M. Nagao, S. Shimada, Capsazepine is a novel activator of the δ subunit of the human epithelial Na+ channel, J. Biol. Chem. 279 (2004) 44483–44489.
[13] H. Yamamura, S. Ugawa, T. Ueda, S. Shimada, Expression analysis of the epithelial Na+ channel δ subunit in human melanoma G-361 cells, Biochem. Biophys. Res. Commun. 366 (2008) 489–492.
[14] R. Waldmann, G. Champigny, F. Bassilana, N. Voilley, M. Lazdunski, Molecular cloning and functional expression of a novel amiloride-sensitive Na+ channel, J. Biol. Chem. 270 (1995) 27411–27414.
[15] C.M. Canessa, L. Schild, G. Buell, B. Thorens, I. Gautschi, J.D. Horisberger, B.C. Rossier, Amiloride-sensitive epithelial Na+ channel is made of three homologous subunits, Nature (London) 367 (1994) 463–467.
[16] F.J. McDonald, M.P. Price, P.M. Snyder, M.J. Welsh, Cloning and expression of the β- and δ-subunits of the human epithelial sodium channel, Am. J. Physiol. 268 (1995) C1157–C1163.
[17] C. Roudier-Pujol, A. Rochat, B. Escoubet, E. Eugène, Y. Barrandon, J.P. Bonvalet, N. Farman, Differential expression of epithelial sodium channel subunit mRNAs in rat skin, J. Cell Sci. 109 (1996) 379–385.
[18] M. Brouard, M. Casado, S. Djelidi, Y. Barrandon, N. Farman, Epithelial sodium channel in human epidermal keratinocytes: expression of its subunits and relation to sodium transport and differentiation, J. Cell Sci. 112 (1999) 3343–3352.
[19] T. Mauro, M. Guitard, M. Behne, Y. Oda, D. Crumrine, L. Komuves, U. Rassner, P.M. Elias, E. Hummler, The ENaC channel is required for normal epidermal differentiation, J. Invest. Dermatol. 118 (2002) 589–594.
[20] D.R. Carpizo, A.J. Reaka, W.R. Glaws, N. Pooley, L. Schmidt, A.G. Halline, J.L. Goldstein, T.J. Layden, Acute acid exposure increases rabbit esophageal cell proliferation, J. Lab. Clin. Med. 131 (1998) 157–162.
[21] A. Lanas, Y. Royo, J. Ortego, M. Molina, R. Sáinz, Experimental esophagitis induced by acid and pepsin in rabbits mimicking human reflux esophagitis, Gastroenterology 116 (1999) 97–107.
[22] P. Jimenez, A. Lanas, E. Piazuelo, F. Esteva, Effects of extracellular pH on restitution and proliferation of rabbit oesophageal epithelial cells, Aliment. Pharmacol. Ther. 13 (1999) 545–552.
[23] M.L. Chalfant, J.S. Denton, B.K. Berdiev, I.I. Ismailov, D.J. Benos, B.A. Stanton, Intracellular H+ regulates the α-subunit of ENaC, the epithelial Na+ channel, Am. J. Physiol. 276 (1999) C477–C486.
[24] D.A. Cockayne, S.G. Hamilton, Q.M. Zhu, P.M. Dunn, Y. Zhong, S. Novakovic, A.B. Malmberg, G. Cain, A. Berson, L. Kassotakis, L. Hedley, W.G. Lachnit, G. Burnstock, S.B. McMahon, A.P. Ford, Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice, Nature (London) 407 (2000) 1011–1015.
[25] V. Souslova, P. Cesare, Y. Ding, A.N. Akopian, L. Stanfa, R. Suzuki, K. Carpenter, A. Dickenson, S. Boyce, R. Hill, D. Nebenuis-Oosthuizen, A.J. Smith, E.J. Kidd, J.N. Wood, Warm-coding deficits and aberrant inflammatory pain in mice lacking P2X3 receptors, Nature (London) 407 (2000) 1015–1017.
[26] L.A. Birder, Y. Nakamura, S. Kiss, M.L. Nealen, S. Barrick, A.J. Kanai, E. Wang, G. Ruiz, W.C. de Groat, G. Apodaca, S. Watkins, M.J. Caterina, Altered urinary bladder function in mice lacking the vanilloid receptor TRPV1, Nat. Neurosci. 5 (2002) 856–860.