Neuroscience Letters 488 (2011) 173–177

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Detection of acid-sensing ion channel 3 (ASIC3) in periodontal Ruffini endings of mouse incisors☆

Farhana Rahman[a,b], Fumiko Harada[a,b], Isao Saito[b], Akiko Suzuki[a], Yoshiro Kawano[a], Kenji Izumi[a], Kayoko Nozawa-Inoue[a], Takeyasu Maeda[a,*]

[a] Division of Oral Anatomy, Niigata University Graduate School of Medical and Dental Sciences, 2-5274 Gakkocho-dori, Chuo-ku, Niigata 951-8514, Japan
[b] Division of Orthodontics, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

ARTICLE INFO

Article history:
Received 9 October 2010
Received in revised form 3 November 2010
Accepted 3 November 2010

Keywords:
ASIC3
Ruffini ending
Periodontal ligament
Trigeminal ganglion
Mechanoreceptor
Mechanosensation

ABSTRACT

The acid-sensing ion channel 3 (ASIC3), a member of the epithelial sodium channel/degenerin (ENaC/DEG) superfamily, has been reported to participate in acid sensing, mechanosensation, and nociception. However, no information is available regarding the precise localization and function of this molecule in the periodontal ligament, which contains abundant sensory nerves originating from the trigeminal ganglion. The present study examined the expression of ASIC3 in the lingual periodontal ligament of mouse incisors by immunohistochemistry. Furthermore, the expression of ASIC3 in the trigeminal ganglion – which innervates the periodontal ligament – was investigated at protein (immunohistochemistry and quantitative analysis) and mRNA levels (RT-PCR technique and *in situ* hybridization histochemistry). Immunohistochemistry for ASIC3 was able to demonstrate dendritic profiles of the periodontal Ruffini endings in the mouse incisors. No thin fibers terminating as nociceptive free nerve endings exhibited ASIC3 immunoreactivity. Double immunofluorescent staining revealed ASIC3 immunoreaction in the axoplasm but not in the ordinary Schwann cells – including the associated terminal Schwann cells. Observation of the trigeminal ganglia showed variously sized neurons expressing ASIC3 immunoreaction; the most intense immunopositivity was found in the small and medium-sized neurons, as confirmed by *in situ* hybridization histochemistry using a specific cRNA probe. Quantitative analysis on trigeminal ganglion neurons showed that 38.0% of ASIC3 neurons could be categorized as medium-sized neurons which mediate mechanotransduction. These findings suggest that ASIC3 functions as a molecule for mechanosensation in the periodontal Ruffini endings.

Acid-sensing ion channels (ASICs), or neuronal voltage-insensitive amiloride-blockable cation channels, belong to the epithelial sodium channel/degenerin (ENaC/DEG) superfamily [5,25]. Previous biochemical studies in mammalian organisms have identified seven different ASIC subunits: ASIC1a, ASIC1b, ASIC1b2, ASIC2a, ASIC2b, ASIC3, and ASIC4 [1,7,8,23,24]. ASICs have been reported to be distributed throughout the mammalian central and peripheral nervous systems [2,3,11,20]. To date, neuronal ASICs have been proposed to be involved in acid sensing as well as mechanoreceptive and nociceptive functions. Indeed, previous studies have confirmed ASIC1, 2, and 3 in the mechanoreceptive neurons of the dorsal root ganglia [11,12] and ASIC2a and ASIC3 in cutaneous mechanoreceptors including Meissner corpuscles, Merkel nerve endings, and palisades of lanceolate nerve endings [9,20]. In particular, ASIC3 has been regarded as an essential channel for mechanosensation due to its predominant expression in the sensory neurons [9,15,20].

The periodontal ligament, a dense connective tissue between the tooth and alveolar bone, contains abundant mechanoreceptors [6,16]. Our recent study demonstrated the immuno-expression of ENaCβ in the axon terminals of the Ruffini ending – a primary mechanoreceptor in the periodontal ligament [16], suggesting the involvement of this molecule in the transduction and modulation of mechanosensation [10]. The expression of ENaCβ in the periodontal Ruffini endings readily leads us to suppose that they possess ASIC3. In our preliminary report, many ASIC3 positive structures with dendritic ramifications existed in the periodontal ligament, but the precise localization of ASIC3 remains unclear. Thus, the present study was undertaken to examine the expression of ASIC3 in the lingual periodontal ligament of the mouse incisors by immunohistochemistry. The expression of ASIC3 in the trigeminal ganglion – which innervates the periodontal ligament – was also surveyed at protein and gene levels. Furthermore, we attempted a quantitative analysis of the size distribution of ASIC3 positive trigeminal ganglion neurons.

☆ This study was supported by grants from the Japan Society for the Promotion of Science (JSPS) (Nos. 21792064 to F.H. and 20390464 to T.M.).
* Corresponding author. Tel.: +81 25 227 2815; fax: +81 25 223 6499.
E-mail address: maedat@dent.niigata-u.ac.jp (T. Maeda).

0304-3940/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2010.11.023

Table 1
Sequence of primers for RT-PCR.

Gene	Primer sequence	Size	Accession no
ASIC1	forward: 5′-GCCTATGAGATCGCAGGG-3′ reverse: 5′-AAAGTCCTCAAACGTGCCTC-3′	305 bp	XM_128133
ASIC2	forward: 5′-GAAGAGGAAGGGAGCCATGAT-3′ reverse: 5′-GGCAGAAGTTCGCAATGTGT-3′	275 bp	NM_007384
ASIC3	forward: 5′-CCCAGCTCTGGACGCTATG-3′ reverse: 5′-TCTTCCTGGAGCAGAGTGTTG-3′	414 bp	NM_173135
ASIC4	forward: 5′-GAATGTGCCGACCACACACT-3′ reverse: 5′-GCAAGCAAAGTCTTCAAAGAGG-3′	563 bp	BC046481

Table 2
Antibody used for double staining.

	Primary antibody	Fluorescent dye
ASIC3	Rabbit anti-human ASIC3 antibody (1:100, Alomone Labs., Jerusalem, Israel)	Texas red (1:100, Vector Lab.)
PGP 9.5	Mouse anti-human PGP 9.5 monoclonal antibody (1:5,000, Ultraclone, Cambridge, UK)	FITC (1:100, Vector Lab.)
S-100 protein	Mouse anti-human S-100 protein monoclonal antibody (1:1000, Sigma–Aldrich, Inc., St. Louis, MO)	FITC (1:100, Vector Lab.)

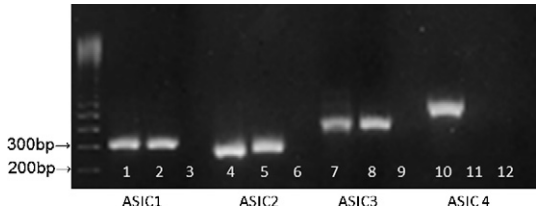

Fig. 1. RT-PCR analysis of the brain (1, 4, 7, 10) and trigeminal ganglion (2, 5, 8, 11) for ASIC1, 2, 3, and 4. PCR bands for ASIC1, 2, and 3 are detected in the trigeminal ganglion. Negative controls using DNase treated RNA do not show any band of PCR products (3, 6, 9, 12).

Male ICR mice, 8 weeks of age and weighing 25–30 g, were used in this study. All experiments were approved and performed according to the guidelines of the Niigata University Intramural Animal Use and Care Committee (approval number # 48).

Four mice were decapitated under deep anesthesia by an intraperitoneal injection of chloral hydrate (400 mg/kg). Total RNAs from the removed trigeminal ganglia and brains were isolated using TRIzol (Gibco-BRL, MD, USA), and reverse transcription was performed using a PrimeScript™ RT reagent Kit (TaKaRa, Ohtsu, Japan). The PCR reaction was carried out in the presence of gene-specific ASIC1, 2, 3, 4 primers which were chosen based on the nucleotide sequence of the cDNA of ASICs (Table 1). Isolated RNA without reverse transcription obtained from the mouse brain was used as a negative control.

An additional 10 mice were perfused transcardially *via* the left ventricle with a fixative containing 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4). Cryostat sections of the trigeminal ganglia and the upper jaws decalcified with 10% EDTA-2Na were cut at respective thicknesses of 8 μm and 16 μm. For *in situ* hybridization histochemistry, some trigeminal ganglia were embedded in paraffin, and were cut at a thickness of 4.5 μm.

The cryostat sections were reacted with a rabbit antiserum for ASIC3 (Alomone Labs., Jerusalem, Israel), and then incubated with a biotinylated goat anti-rabbit IgG (1:100; Vector Lab., Burlingame, CA), followed with horseradish peroxidase-conjugated avidin (ABC Kit, Vector Lab.). Final visualization used 3,3′-diaminobenzidine (0.04%) and hydrogen peroxide (0.003%) in a 0.05 M Tris–HCl buffer at pH 7.6. The immunostained sections were counter-stained with 0.03% methylene blue.

We employed a double immunostaining with ASIC3 together with either protein gene product 9.5 (PGP 9.5) or S-100 protein in the periodontal ligament. Information on the antibodies used is given in Table 2. For the use of mouse monoclonal antibodies, sections were blocked using an M.O.M immunodetection Kit (Vector Lab.) according to the manufacturer's instructions.

With the PCR fragment as a template, digoxigenin (DIG)-labeled RNA probes were synthesized using a DIG RNA labeling kit (Roche Diagnostics, Basel, Switzerland). Hybridization signals were then detected with a horseradish peroxidase-conjugated antidigoxigenin antibody (Roche Diagnostics), and developed using the BlueMapKit (Ventana Medical Systems, Tucson, AZ).

The cell numbers and cross-sectional areas of ASIC3 positive neurons in the trigeminal ganglion showing the nucleolus profiles were calculated from 4 sections per animal ($n = 4$; $N = 3$) using the NIH image (http://rsb.info.nih.gov/ij/download.html). We measured the relative ratio (%) of ASIC3-positive to all trigem-

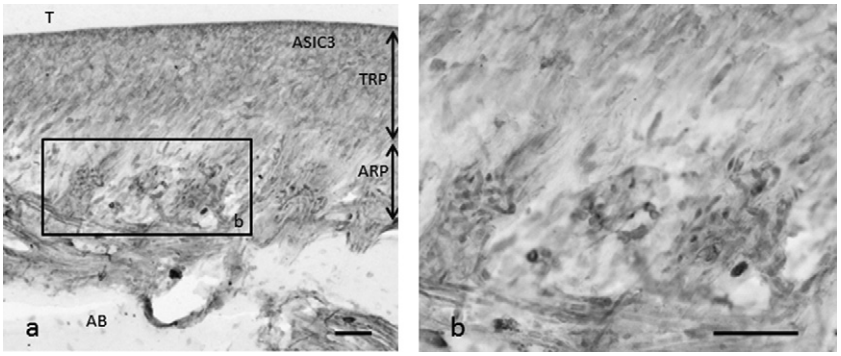

Fig. 2. Immunoreactions for ASIC3 in the upper periodontal ligament of a mouse incisor. (a) Numerous immunoreactive elements are found in the periodontal ligament, being restricted to the alveolus-related part (ARP). (b) A higher magnification of the boxed area in (a). ASIC3-immunopositive structures ramify in a dendritic fashion to form the periodontal Ruffini endings. AB: alveolar bone, T: tooth, TRP: tooth-related part. Scale bars = 100 μm.