Annals of Anatomy 207 (2016) 27–31

Contents lists available at ScienceDirect

Annals of Anatomy

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

Acid-sensing ion channel immunoreactivities in the cephalic neuromasts of adult zebrafish

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ARTICLE INFO

Article history:
Received 21 December 2015
Received in revised form 21 June 2016
Accepted 22 June 2016

Keywords:
Acid-sensing ion channels
Cephalic lateral line
Neuromasts
Zebrafish
Immunohistochemistry

ABSTRACT

The neuromasts are the morphofunctional unit of the lateral line system serving as mechanosensors for water flow and movement. The mechanisms underlying the detection of the mechanical stimuli in the vertebrate mechanosensory cells remain poorly understood at the molecular level, and no information is available on neuromasts. Mechanotransduction is the conversion of a mechanical stimulus into an electrical signal via activation of ion channels. The acid-sensing ion channels (ASICs) are presumably involved in mechanosensation, and therefore are expected to be expressed in the mechanoreceptors. Here we used immunohistochemistry to investigate the occurrence and distribution of ASICs in the cephalic neuromasts of the adult zebrafish. Specific immunoreactivity for ASIC1 and ASIC4 was detected in the hair cells while ASIC2 was restricted to the nerves supplying neuromasts. Moreover, supporting and mantle cells; i.e., the non-sensory cells of the neuromasts, also displayed ASIC4. For the first time, these results demonstrate the presence of the putative mechanoproteins ASIC1, ASIC2 and ASIC4 in neuromasts, suggesting a role for these ion channels in mechanosensation.

1. Introduction

The neuromasts are the sensory units of the lateral line system of the teleosts. They contain specific hair cells that sense water flow and movement (Cernuda-Cernuda and García-Fernández, 1996; Bang et al., 2001). Thus, neuromast sensory cells are functionally mechanoreceptors in which mechanotransduction; i.e., the conversion of mechanical stimuli into electro-chemical signals, takes place (Ghysen and Dambly-Chaudière, 2007). This process occurs because of the presence of mechanotransducer ion channels on sensory nerve endings and specialized sensory cells, which generate an ion flux in response to mechanical stimuli (see for a review Gillespie and Walker, 2001; Lumpkin and Caterina, 2007; Arnadottir and Chalfie, 2010; Delmas and Coste, 2013; Ranade et al., 2015).

The mechanotransducer channel must satisfy several criteria (Ernstrom and Chalfie, 2002; Christensen and Corey, 2007), and, although different eukaryotic ion channels have been proposed in this regard, at present, the unique ion channel which fulfills these conditions appears to be Piezo2 (Ranade et al., 2014; Woo et al., 2015). Other ion channels which may be required or necessary for mechanosensing, but not directly for mechanotransduction, include members of the acid-sensing ion channels (ASICs) family. ASICs belong to the Degenerin/Epithelial Na channels (DEG/ENaC) superfamily, and are Na^+-selective voltage-insensitive, amiloride-sensitive cation channels (Waldmann et al., 1997; Lingueglia, 2007; Lumpkin and Caterina, 2007; Baron and Lingueglia, 2015) that monitor moderate deviations in extracellular pH. They also intervene in mechanosensation and nociception (Wemmie et al., 2006; Sherwood et al., 2012; Zha, 2013). At present, six ASIC proteins (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4) have been identified in mammals (Krishtal, 2015) and the zebrafish orthologs and paralogs (zASICs: zASIC1.1, zASIC1.2, zASIC1.3, zASIC2, zASIC4.1, and zASIC4.2) have been also identified (Paukert et al., 2004), while there is no evidence of ASIC3 occurrence in zebrafish.

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http://dx.doi.org/10.1016/j.aanat.2016.06.007
0940-9602/

In mammals, ASICs are widely expressed in neurons of both central and peripheral nervous system but they have also been detected in non-neuronal tissues (Lingueglia, 2007; Holzer, 2009, 2011). In zebrafish, ASICs have been found in different sensory organs like retina (Paukert et al., 2004; Viña et al., 2015b), olfactory rosette (Viña et al., 2015a) and taste buds (Viña et al., 2013). As far as we know, the occurrence of ASICs has not been previously investigated in the neuromasts from this species. The present research was designed to investigate the occurrence of ASIC immunoreactivities (IRs) in the neuromasts of adult zebrafish to provide information about their distribution and possible involvement in mechanosensation.

To be a reasonable candidate for transducing or, at least, sensing a mechanosensory stimulus, an ion channel must be expressed at a proper time and in the right place. Thus, the hair cells of the neuromasts are thought to express ion channels that can act as mechanodetectors and/or mechanotransducers, as in the case of ASICs. The present research was designed to investigate the occurrence of ASICs in neuromasts of adult zebrafish to provide information about the distribution of these proteins in the sensory organs of adult zebrafish, and their possible involvement in mechanosensation.

2. Material and methods

2.1. Zebrafish breeding and tissue treatment

Six month-old Zebrafish (*Danio rerio*; $n = 10$) were used in this study. Animals were obtained from CISS (Experimental Center of Ichthyopathology of Sicily; University of Messina, Italy) where had been reared at a constant temperature of 28.5 °C and fed twice a day. The fish were anaesthetized with MS222 (ethyl-*m*-amino benzoate; 0.4 g L^{-1}) and sacrificed by decapitation. The heads were fixed in Bouin's solution for 24 h, routinely embedded in paraffin wax, cut at 10 μm thick serial sections, and collected on gelatin-coated microscope slides to be processed for immunocytochemistry. A preliminary attempt to localize ASICs in the neuromasts of zebrafish larvae was unsuccessful. For this reason, the study was conducted exclusively on adult animals.

2.2. ASICs detection using indirect peroxidase immunohistochemistry

Deparaffinized and rehydrated sections were rinsed in Tris–HCl buffer (0.05 M, pH 7.5) containing 0.1% bovine serum albumin and 0.2% Triton-X 100. The endogenous peroxidase activity and non-specific binding were blocked (3% H_2O_2 and 25% foetal calf serum, respectively) and sections were incubated overnight at 4 °C with the primary antibodies listed in Table 1. These antibodies had been tested in zebrafish for immunohistochemistry in paraffin-embedded tissues in previous studies (Viña et al., 2013, 2015b). Thereafter, sections were rinsed in the same buffer, and incubated with an HRP-conjugated goat anti-rabbit IgG secondary antibody (Amersham, Buckingamshire, UK) diluted 1:100 for 1 h at room temperature. Finally, sections were washed and the immunoreaction was visualized using 3,3′ Diaminobenzidine (DAB) as a chromogen. The specificity of immunoreactivity (IR) was tested by incubating sections with specifically preabsorbed antisera instead of the primary antibodies (5 μg of the blocking peptide in 1 mL of the working solution). Under these conditions, no positive immunostaining was observed (data not shown).

2.3. Double immunofluorescence

Sections were incubated for 30 min in a solution of 1% bovine serum albumin in TRIS buffer solution (TBS) to avoid non-specific binding, followed by incubation with the primary antibodies. Incubation was carried out overnight at 4 °C in a humid chamber, with a 1:1 mixture of anti-S100 antibody and anti-ASIC1, anti-ASIC2, or anti-ASIC4 antibodies at the dilutions given in Table 1. After rinsing in TBS, the sections were incubated for 1 h with Alexa fluor 488-conjugated goat anti-mouse IgGs (Serotec, Oxford, UK), diluted 1:1000, then rinsed again and incubated for another hour with CyTM3-conjugated donkey anti-rabbit IgGs (Jackson-ImmunoResearch, Baltimore, MD, USA) diluted 1:50 in TBS.

Both steps were performed at room temperature in a dark humid chamber. Finally, to ascertain structural details, sections were counterstained and mounted with DAPI diluted in glycerol medium (10 ng/mL) then washed, dehydrated and mounted with Entellan®. Triple fluorescence was detected using a Leica DMR-XA automatic fluorescence microscope (Service of Image Analysis, University of Oviedo) coupled with a Leica Confocal Software, version 2.5 (Leica Microsystems, Heidelberg GmbH, Germany) and the images captured were processed using the NIH freeware Image J software.

To provide negative controls, representative sections were incubated with specifically preabsorbed antisera as described above. Under these conditions, no positive immunostaining was observed (data not shown).

Table 1
Primary antibodies used in the study.

Antigen	Origin	Dilution	Supplier
ASIC1	Rabbit	1:200	Abcam pcl[a]
ASIC2	Rabbit	1:200	Lifespan Biosciences[b]
ASIC3	Rabbit	1:200	Abcam pcl[a]
ASIC4	Rabbit	1:200	Lifespan Biosciences[b]
ASIC4.2	Rabbit	1:250	21st Century Biochemicals[c]
β-Tubulin	Mouse	1:100	Sigma[c]
S100 protein	Mouse	1:200	Thermo Scientific[d]

ASIC: acid-sensing ion channels 1, 2, 3 and 4.
Anti-ASIC1: Raised against a synthetic peptide to an extracellular domain epitope of human ASIC1, conjugated to immunogenic carrier protein; recognizes both subtype1 and subtype2. ab65698. Blocking peptide.
Anti-ASIC2: Antibody raised against a synthetic peptide from the extracellular domain of mouse ASIC2 conjugated to an immunogenic carrier protein. LS-B156/12883. Blocking peptide: LS-PB156.
Anti-ASIC3: Antibody raised against a synthetic peptide to an internal sequence of human ASIC3 conjugated to immunogenic carrier protein. Ab65697. Blocking peptide: LS-E12503.
Anti-ASIC4: Antibody raised against a synthetic peptide from the cytoplasmic domain of human ASIC4 conjugated to immunogenic carrier protein used as an immunogen. LS-B920. Blocking peptide: LS-PC 94536.
Anti-ASIC4.2 antibody raised against two peptides corresponding to regions near the NH_2 terminus (aa 146–160) and the COOH terminus (aa 519–533); of zASIC4.2 protein (Dymowska et al., 2015).
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3. Results

Although different cephalic neuromasts were studied, the images illustrating the results were taken from the neuromasts of the supraorbital canal (Fig. 1a and c). In the sagittal sections of the head, the sensory epithelium of the olfactory rosette served as an internal positive control for ASIC IRs (Viña et al., 2015a). Furthermore, to selectively label the hair cells, the S100 protein was used as a marker (Abbate et al., 2002; Germanà et al., 2004). ASIC IRs displayed a segregated distribution within the neuromast. ASIC1 was selectively detected in the hair cells (Fig. 2a), ASIC2 IR was restricted to the nerves supplying the neuromast (Figs. 1 b and 2 b), and ASIC4 was detected in all types of neuromast cells, although the intensity

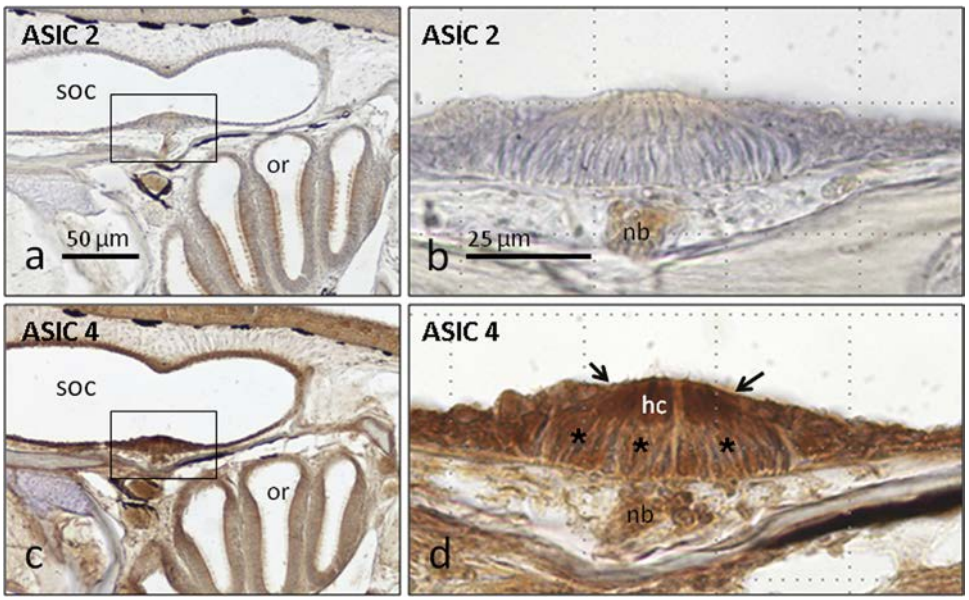

Fig. 1. Parasagittal serial sections of the head of one adult zebrafish passing through the supraorbital canal (soc) processed for the immunohistochemical detection of ASIC2 (a) and ASIC4 (c). Positive ASIC2 was observed in the nerves (nb) supplying neuromasts (b), while ASIC4 immunostaining was detected in all cell types of the neuromasts: hair cells (hc), support cells (asterisks) and mantle cells (arrows), as well as in the nerves (nb) (d). In the olfactory rosette (or) the cilia of the non-sensory olfactory epithelium were ASIC2 positive while all the cytoplasm and cilia of the sensory and non-sensory olfactory epithelium displayed a faint but specific ASIC4 immunostaining. Images in (b) and (d) are magnified view of squares in (a) and (c).

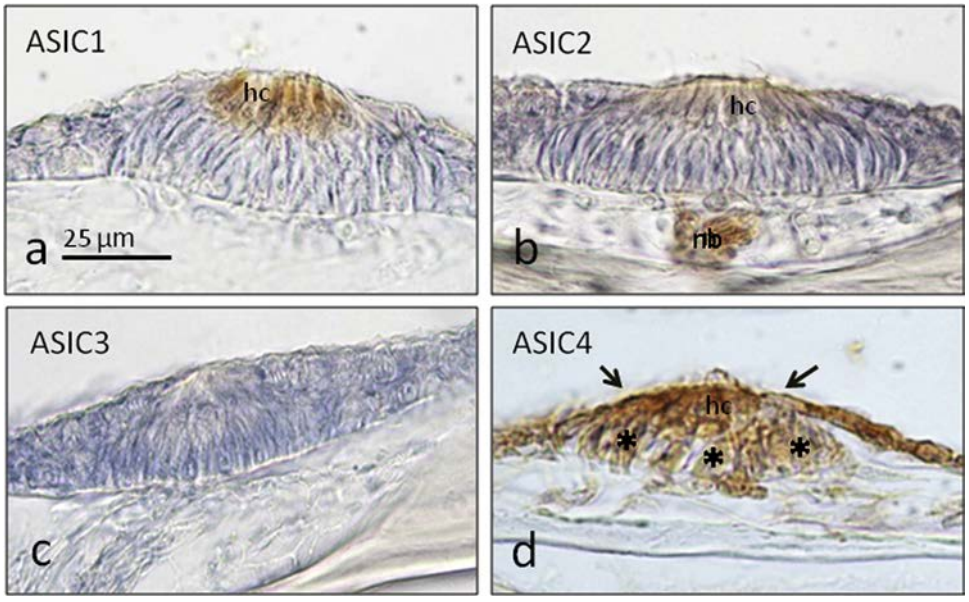

Fig. 2. Approximate serial sections of a neuromast processed for the immunohistochemical detection of ASIC ion channels. ASIC1 was detected in hair cells (hc) (a), ASIC2 in the nerves (nb) supplying the neuromasts (b), ASIC3 was absent (c), and ASIC4 occurred in hair cells, support cells (asterisks) and mantle cells (arrows) (d).

of immunostaining was apparently stronger in the hair cells than in the basal or support cells (Figs. 1 d and 2 d). As expected, ASIC3 IR was absent in the neuromasts as well as in other cells (Fig. 2c).

To ascertain the cell localization of ASICs, double immunofluorescence associated to laser confocal microscopy was performed. Hair cells in the neuromasts (Fig. 3a, d and g) and the nerves supplying them (Fig. 3g) displayed an intense S100 protein IR. In double labeling experiments (Fig. 3b, e and h), it was observed that ASIC2 IR never co-localized with S100 protein in the hair cells (Fig. 3c) while both were present in nerves (Fig. 3i). Conversely, ASIC1 (not shown) and ASIC4 (Fig. 3e) were co-localized in hair cells (Fig. 3f).

4. Discussion

In the last two decades, the study of the molecular basis of mechanic stimuli sensation has become of great interest (see Delmas and Coste, 2013; Ranade et al., 2015). Few studies, however, have been performed in the neuromasts of zebrafish, a common excellent animal model used to study the mammalian and human inner ear (Olt et al., 2014) in normal and pathological conditions (Whitfield, 2002). Among the candidate molecules to play a role in mechanosensing and/or mechanotransduction are the ion channels belonging to the ASIC family (Chen and Wong, 2013; Omerbašić

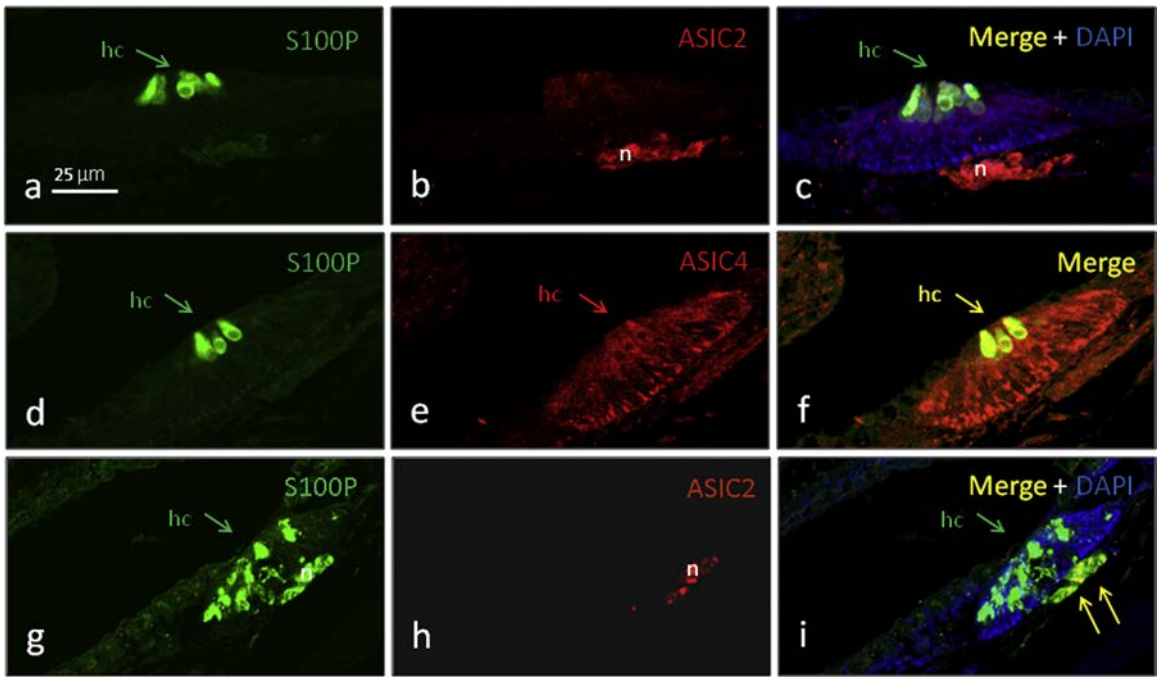

Fig. 3. Confocal laser-scanning images of ASIC2 (red, b and h), ASIC4 (red, e) and S100 protein (green, a, d and g) in adult zebrafish neuromasts demonstrating that ASIC2 and S100 protein co-localize in nerves (n) (i, yellow arrows) but not in hair cells (hc) (c and i, green arrow). ASIC4 was detected in the hair cells (f, yellow arrow) but also in the support and mantle cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

et al., 2015), but their role in mechanosensing is still controversial (see for a review Chen and Wong, 2013). ASICs have been localized in the hair cells of the mammalian inner ear (Mercado et al., 2006; Corey et al., 2004; Ugawa et al., 2006; Kikuchi et al., 2008; Vega et al., 2009) as well as cutaneous mechanoreceptors and mechanosensory neurons (Del Valle et al., 2012; Cabo et al., 2015), but no information is available about the occurrence of ASICs in the mechanosensory system of fish. In the present investigation, immunohistochemistry has been used to analyze the occurrence of ASIC IRs in the adult zebrafish neuromasts, using specific antibodies (Viña et al., 2013), and appropriate controls. Absence of cross-reactivity between antibodies was confirmed by the differential distribution of IRs within specific populations of neuromast cells. ASIC1 and ASIC4 IRs were detected in the hair cells, as both ASIC IRs co-localized with S100-IR (Abbate et al., 2002; Germanà et al., 2004). Moreover, widespread ASIC4 IR was detected in the basal and mantle cells. ASIC2 IR was instead restricted to the neuromast nerves and ASIC3 IR was always absent. Results on ASIC1 and ASIC4 IRs are absolutely new in neuromasts, while those for ASIC2 IR are in full agreement with previous results on taste buds (Viña et al., 2013) or nerve fibers and neurons of the myenteric plexus (Levanti et al., 2011).

The absence of ASIC3 IR is in total agreement with previously reported observations on the absence of this ASIC isoform in zebrafish (Paukert et al., 2004). Nevertheless, ASIC3 is present in the hair cells of the mammalian cochlea (Maubaret et al., 2002; Hildebrand et al., 2004), and all ASIC isoforms have been detected in the vestibular ganglion neurons in the rat (Mercado et al., 2006). Interestingly, and in contrast to the present results, ASIC2 has been detected in other stereociliated cells of zebrafish, such as the epithelial cells of the non-olfactory epithelium (Viña et al., 2015b). Despite the potential importance of the present findings, the precise role of ASICs in neuromast is still unknown. Our results represent a starting point for the study of ASICs in neuromast, but further studies will be necessary to elucidate the role of ASICs in these sensory organs.

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