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Acid-sensing ion channel 2 (ASIC2) in the intestine of adult zebrafish

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ABSTRACT

Acid-sensing ion channels (ASICs) in mammals monitor acid sensing and mechanoreception. They have a widespread expression in the central and peripheral nervous system, including the gut. The distribution of ASICs in zebrafish is known only in larvae and at the mRNA level. Here we have investigated the expression and cell distribution of ASIC2 in the gut of adult zebrafish using PCR, Western blot and immunohistochemistry. ASIC2 mRNA was detected in the gut, and a protein consistent with predicted ASIC2 (64 kDa molecular mass) was detected by Western blot. ASIC2 positivity was found in a subpopulation of myenteric neurons in the enteric nervous system, as well in enteroendocrine epithelial cells. These data demonstrate for the first time the occurrence of ASIC2 in the gut of adult zebrafish where it presumably acts as a chemosensor and a mechanosensor.

Acid-sensing ion channels (ASICs) are H^+-gated, voltage-insensitive cation channels beginning to the amiloride-sensitive degenerin/epithelial Na^+ channel (DEG/ENaC) superfamily (see for a review [5]). Structurally, ASICs consist of two transmembrane domains and a large extracellular loop [5,9,21]. In mammals, ASICs are encoded by five different genes (for references see [5,10]), and are widely expressed in both the central and peripheral nervous systems [5,7,10,14]. Similarly as in mammals, six *ASIC* genes have been cloned in zebrafish (zASICs), coding for proteins with similar predicted molecular masses (around 60 kDa) which share 60–75% of amino acid identity with rat ASICs [15]. In zebrafish larvae, the expression of ASICs mRNAs has been found restricted to the central nervous system and the retina [15].

Functionally, ASICs not only monitor moderate deviations from the physiological values of extracellular pH, but also participate in mechanoception and nociception (see [5]). Consistently with these functions, ASICs are expressed in functionally segregated populations of dorsal root ganglion (DRG) sensory neurons [7] as well as in nerves and sensory structures where nerve transduction takes place (see for references [1]). Furthermore, ASIC knockout mice show different sensory deficits (see for a review [9]).

ASICs are also involved in gastrointestinal physiology in mammals (see [4–6]). So, ASICs are expressed by DRG afferent gastrointestinal neurons [7,16], in neurons of the myenteric and submucous plexuses [22]. In the gut ASICs are relevant to the regulation of acid secretion, motility and mucosal protection (see [4–6]). Moreover, ASIC3 makes a critical positive contribution to mechanosensitivity in gut afferents (Jones et al. [8] and Page et al. [14]), and the disruption of *ASIC2* gene altered digestive activity in the whole animal by disrupting emptying patterns from the upper or lower gastrointestinal tract (see [14]). Finally, levels of ASIC3 increased in gastrointestinal disturbances associated with inflammation [22].

All together the above data strongly support a role of ASICs in the vertebrate gut, and therefore the knowledge of the expression and distribution of ASICs in the gut are important in order to understand their role in both physiological and pathological conditions. In the present study we have investigated the expression of ASIC2 in the gut of adult zebrafish, since this animal is used as a model to study different human pathologies involving the gut [2]. ASIC2 was the only ASIC protein detected in the gut of zebrafish in a preliminary screening study carried out in our laboratory, and for this reason we focused our research in ASIC2.

Adult zebrafish (*Danio rerio*; $n = 15$), 6 months old, were obtained from CISS (Centro Ittiopatologia Sperimentale Sicilia), University of Messina, Italy. The specimens were anaesthetized with MS222 (ethyl-m-amino benzoate; 0.4 g L^{-1}) and sacrificed by decapitation. The intestine from 10 animals was isolated, cleaned in cold saline

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solution, and processed for Western blot (4 animals) or PCR (6 animals). The remaining five animals were whole fixed in Bouin's fixative for 24 h then routinely processed for paraffin embedding. The pieces cut 10 μm thick in serial sections, and collected on gelatine-coated microscope slides.

Total RNAs were extracted from fresh samples using Trizol reagent (Sigma; St. Louis, MO). The integrity of RNA was checked using agarose gel electrophoresis. RNA extracted was reverse-transcribed in a final volume of 20 μL using 20 U of Superscript RNA-ase H2 Reverse Transcriptase (Gibco BRL, Gaithersburg, MD) in the manufacturer's buffer containing 2 μg RNA, 5 μM oligo (dT), 12–18 mM dNTPS, 40 U RNA-ase inhibitor (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK), 0.1 μg/μL BSA and 10 mM DTT. The reaction took place at 42 °C for 90 min. The sequences of the oligonucleotide primers were based upon the published sequences for *D. rerio* ASIC2 (GeneBank accession number NM_214788) and *D. rerio* β-actin (GeneBank accession number NM_131031), and were the following: for ASIC2 forward: 5′-TTGTTGGCCAGCTTAGTCCT-3′, reverse: 5′-GGGTCGGTAGTTAGTGAAGT-3′; for β-actin forward: 5′-CACAGATCATGTTCGAGACC-3′, reverse: 5′-GGTCAGGATCTTCATCAGGT-3′. The conditions of amplification were as follows: 2 U Taq DNA Polymerase (Promega, Madison, WI), 1 μM primers, 10 ng zebrafish brain cDNA, 0,2 mM each dNTP in 15 μL Taq DNA Polymerase buffer. The reaction was performed in a thermal cycler (Hyband Th. Cycler) with the following program: 1 min 94 °C initial denaturation, then 10 cycles of 94 °C 1 min, 65 °C 30 s and 72 °C 45 s, followed by 20 cycles of 94 °C 1 min, 61 °C 30 s, 72 °C 45 s and a 5 min final extension at 72 °C. The PCR products were visualized by ethidium bromide staining under UV light following electrophoresis on a 2% agarose gel with a marker Ready Load 1 kb plus DNA ladder (Invitrogen, Carlsband, USA).

For Western blot lysates prepared from intestine homogenates were processed following procedures previously described [3]. Briefly, the samples were pooled and homogenized (1:2, w/v) in Tris–HCl buffered saline (TBS, 0.1 M, pH 7.5) containing 1 μM leupeptin, 10 μM pepstatin and 2 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 25,000 rpm for 15 min at 4 °C and the resulting pellet dissolved in Tris–HCl 10 mM, pH 6.8, 2% SDS, 100 mM dithiothreitol, and 10% glycerol at 4 °C. The lysates were analyzed by electrophoresis in 15% discontinuous polyacrylamide SDS gels. After electrophoresis, proteins were transferred to a nitrocellulose membrane and antibody non-specific binding was blocked by immersion for 3 h in PBS containing 5% dry milk, and 0.1% Tween-20. The membranes were then incubated at 4 °C for 2 h with a rabbit polyclonal antibody against a synthetic peptide from the extracellular domain of mouse ASIC2 conjugated to an immunogenic carrier protein (Lifespan Biosciences, Seattle, WA, USA; catalogue LS-C93915). The antigen is homologous in human and rat, and share high aminoacid identity with zebrafish [15]. After incubation, the membranes were washed with TBS pH 7.6 containing 20% Tween-20, and incubated again for 1 h with the goat anti-rabbit IgG (diluted 1:100) at room temperature. Membranes were washed again and incubated with the PAP complex diluted 1:100 for 1 h at room temperature. Finally, the reaction was developed using a chemiluminescent reagent (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK) and exposed to Hyperfilm. Marker proteins were visualized by staining with Brilliant Blue.

To investigate the distribution of ASIC2 deparaffinised and rehydrated sections were processed for indirect peroxidase immunohistochemistry as follows: deparaffinised 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 nonspecific binding were blocked (3% H_2O_2 and 25% foetal calf serum, respectively) and sections were incubated overnight at 4 °C with the primary antibody, against ASIC2 described above (Lifespan Biosciences) used diluted 1:200. After, sections were rinsed in the same buffer, and incubated with goat anti-rabbit IgG (Amersham, UK) diluted 1:100 for 1 h at room temperature. Finally, sections were washed, the immunoreaction was visualized using 3–3′DAB as a chromogen, and the sections slightly counterstained with Harris haematoxylin to ascertain structural details. The specificity of the immunoreactivity developed was

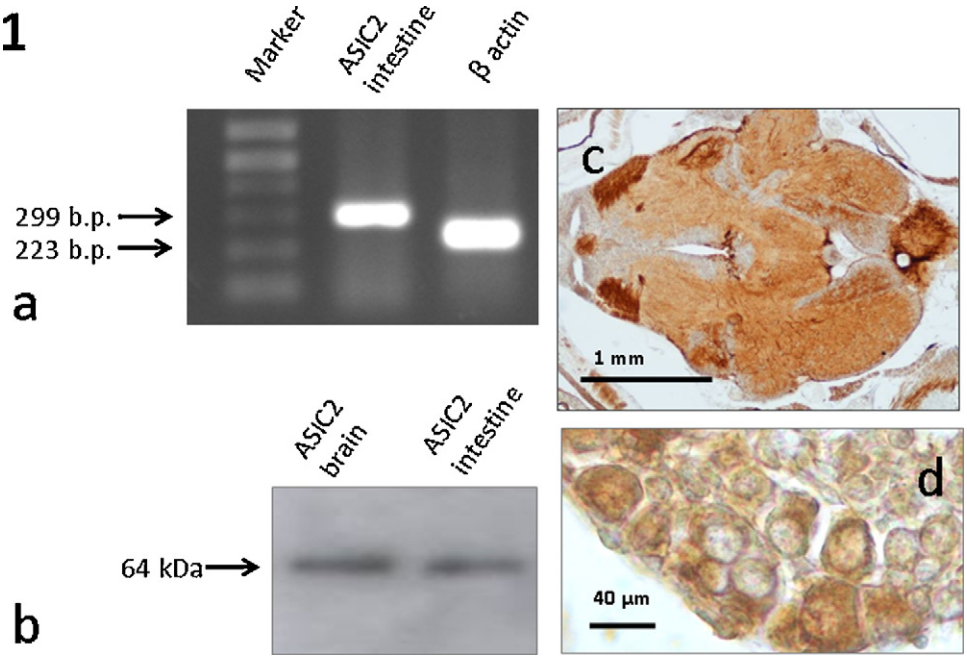

Fig. 1. (a) PCR analysis of TRPV4 and β-actin mRNA expression in the intestine of adult zebrafish. A 299 bp fragment of TRPV4 mRNA was detected. The integrity of the samples was demonstrated by the presence of a 220 bp fragment of β-actin mRNA. (b) Western blot detection of ASIC head homogenates from adult zebrafish. The anti-ASIC2 antibody used recognizes a protein band of about 64 kDa consistent with the complete ASIC2. Widespread expression of ASIC2 can be observed in the central system (c) as well as in DRG.