Identification, functional expression and chromosomal localisation of a sustained human proton-gated cation channel

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Abstract Non-inactivating or slowly inactivating proton-gated cation channels are thought to play an important role in the perception of pain that accompanies tissue acidosis. We have identified a novel human proton-gated cation channel subunit that has biphasic desensitisation kinetics with both a rapidly inactivating Na^+-selective and a sustained component. The protein shares 84% sequence identity with the proton-gated cation channel rASIC3 (rDRASIC) from rat sensory neurones. The biphasic desensitisation kinetics and the sequence homology suggest that this novel clone (hASIC3) is the human orthologue of rASIC3 (rDRASIC). While rASIC3 (rDRASIC) requires very acidic pH (pH < 4.5) for activation of the sustained current, the non-inactivating hASIC3 current starts to be activated when the pH decreases to below pH 6. hASIC3 is an acid sensor and might play an important role in the detection of lasting pH changes in human. We localised the hASIC3 gene to the human chromosome 7q35, 6.4 cRad telomeric from the microsatellite AFMA082XC9.

Key words: Proton-gated cation channel; Nociception; Amiloride; Chromosomal localisation

1. Introduction

Tissue acidosis accompanies many painful ischaemic and inflammatory conditions. In inflamed tissues pH 5.4 was reported and in fracture-related haematomas even pH 4.7 can be attained (for review see [1]). Furthermore experimental acidification of peripheral tissues provokes pain in human [2,3] and increased C-fibre activity in rat nerve-skin preparations [4]. These observations suggest a causal relation between tissue acidosis and pain. The perception of pain during tissue acidosis is thought to be caused by the opening of proton-gated cation channels present in sensory neurones [3,5,6]. Recently, the first members of the proton-gated cation channel family were cloned (ASIC1 [7], ASIC2 [MDEG1] [8], and ASIC3 [rDRASIC] [9]) and it was demonstrated that hetero-multimeric assembly of different subunits further increases the variety of H^+-gated cation channels ([8,10] reviewed in [11]). ASIC1 and ASIC2 (MDEG1) are expressed in neurones of the CNS and in sensory neurones. Both channels activate only transiently when the extracellular pH becomes acidic. However, the pain that accompanies tissue acidosis persists until the pH returns to neutral [3]. In rat [5] and in human [12] sensory neurones, a H^+-gated cation channel with both a rapidly inactivating and a sustained component was described. This channel was proposed to be responsible for the lasting perception of pain during tissue acidosis [3,5]. A H^+-gated cation channel with just this biphasic kinetics (ASIC3; previously called DRASIC) was cloned from rat dorsal root ganglia [9]. The kinetics of the ASIC3 (rDRASIC) channel and the abundance of the mRNA in sensory neurones suggest that ASIC3 (rDRASIC) is part of the H^+-gated cation channel complex that is responsible for the lasting excitation of sensory neurones during tissue acidosis.

We report here the identification, functional expression and chromosomal localisation of a human H^+-gated cation channel that has biphasic kinetics similar to that of the rat ASIC3 (rDRASIC) clone. The 'acid sensing ion channel' we identified might play an important role in nociception or where sensing lasting pH changes is important in human.

2. Materials and methods

2.1. Identification of hASIC3

Comparison of the rat DRASIC protein sequence with the data base of expressed sequence tags (EST) identified two partial cDNA sequences from human total foetus (GenBank accession numbers AA449579 and AA449322). Both sequences originate from the same clone (IMAGE ID 785700), which we obtained from the UK HGMP Resource Centre. Sequencing both strands using an Applied Biosystems automatic sequencer showed that the clone contains the entire coding sequence.

2.2. Chromosomal localisation

The human ASIC3 gene was mapped by PCR on the Genebridge 4 Radiation Hybrid DNA panel with the primers CGATTGCAGTTCAGCATCTCT (sense) and ACCATTCGGCAGCCGCACTT (antisense) at an annealing temperature of 65°C. The PCR products were analysed on 2% agarose gels. Samples were considered positive when a strong amplification of a 159 bp fragment was detected (code 1), ambiguous when a faint amplification of this fragment was detected (code 2) and negative when no amplification around 160 bp was visible (code 0). The positive control (human genomic DNA) was positive and the negative control (hamster genomic DNA) was negative. The following code sequence for the 83 radiation hybrids was obtained and entered into the RHMAPPER program of the Whitehead Institute (http://www-genome.wi.mit.edu) with a Lod score cut-off of 21: 00000 00100 00001 00021 00100 12010 00000 12112 21000 00001 10120 00010 00102 11010 00010 00212 11011 00001 100.

2.3. Expression in COS cells

The vector containing the hASIC3 coding sequence was linearised with *Not*I and blunt-ended with T4 DNA polymerase. After inactivation of the T4 DNA polymerase, the hASIC3 coding sequence was excised with *Eco*RI and subsequently subcloned into the *Eco*RI/*Sal*I (blunt) digested PCI expression vector (Promega). COS cells, at a density of 20 000 cells per 35 mm diameter petri dish, were transfected with a mix of CD8 and hASIC3-PCI (1:5) using the DEAE-dextran method. Cells were used for electrophysiological measurements 1–3 days after transfection. Successfully transfected cells were recognised by their ability to fix CD8-antibody-coated beads [13].

2.4. Electrophysiology

Ion currents were recorded using either the whole cell or outside-

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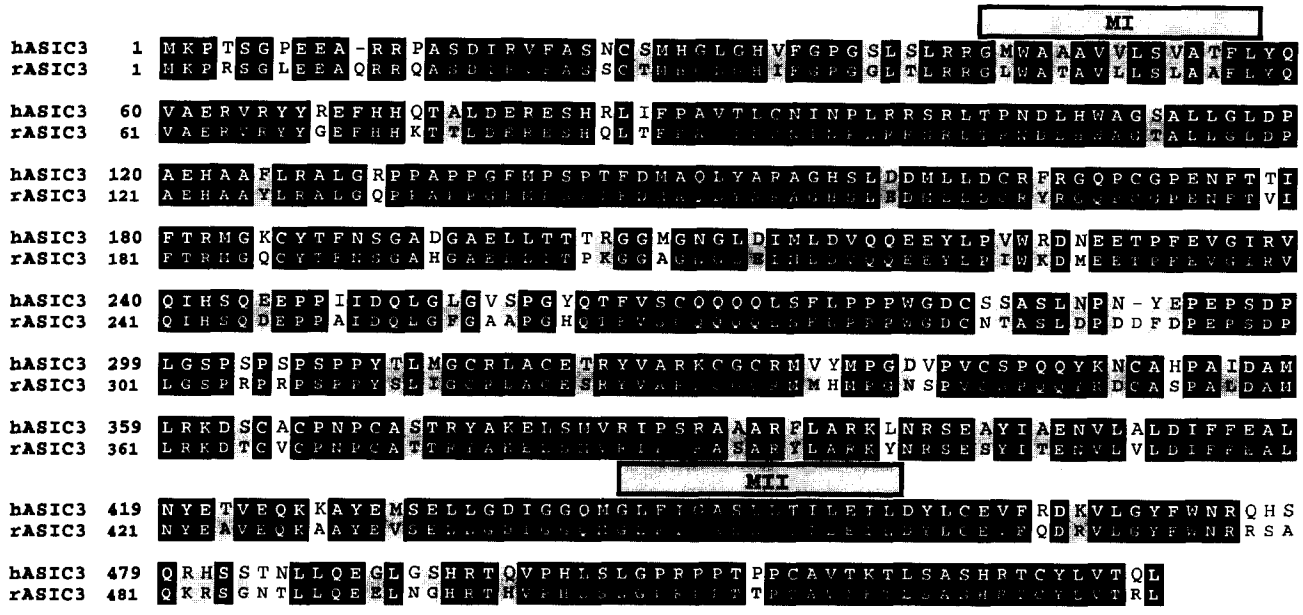

Fig. 1. Alignment of the deduced protein sequences of hASIC3 and rASIC3 (rDRASIC). Amino acids that are identical or similar in both sequences are printed white on black or black on grey background respectively. The two putative hydrophobic transmembrane domains are labelled with boxes. Sequences were aligned with the Pileup program (Genetic Computer Group, Wisconsin).

out patch-clamp technique. The pipette solution contained (in mM): KCl 120, NaCl 30, $MgCl_2$ 2, EGTA 5, HEPES 10 (pH 7.2). The bath solution contained (in mM): NaCl 140, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2, HEPES 10 (pH 7.3). Changes in extracellular pH were induced by opening one out of six outlets of a microperfusion system in front of the cell or patch. Test solutions having a pH of less than 6 were buffered with 10 mM MES rather than HEPES but were identical to the control solution in all other respects. Experiments were carried out at room temperature (20–24°C).

3. Results

We compared the rat ASIC3 (rDRASIC) sequence with the data base of expressed sequence tags and identified a novel human member of this ion channel family. This novel clone from a total human embryo library codes for a protein of 531 amino acids that shares the closest homology (84% identity, 87% homology) with rat ASIC3 (rDRASIC) (Fig. 1).

The cloning of a nearly identical cDNA from human testis (hTNaC1), although without functional expression, was reported recently [14].

Expression of the novel ASIC clone we identified in COS cells induced a H^+-gated cation current with kinetics very similar to that of rat ASIC3 (rDRASIC). When the pH is decreased rapidly from pH 7.3 to pH 5, a biphasic current is observed. A rapidly inactivating component is followed by a sustained current (Fig. 2A). These very peculiar kinetics that are also found with the rat ASIC3 (rDRASIC) [9] channel together with the sequence homology (84% amino acid, 82% nucleic acid identity) with rat ASIC3 (rDRASIC) suggest that this novel clone is the human ASIC3. We therefore call it hASIC3 (human acid sensing ion channel 3).

The pH dependence of the transient hASIC3 current ($pH_{0.5} = 6.2$, Fig. 2A) is almost identical to that reported for rASIC3 (rDRASIC) ($pH_{0.5} = 6.5$) [9]. However, the pH dependences of the sustained rASIC3 (rDRASIC) and hASIC3 currents are clearly different. While rASIC3 (rDRASIC) re-

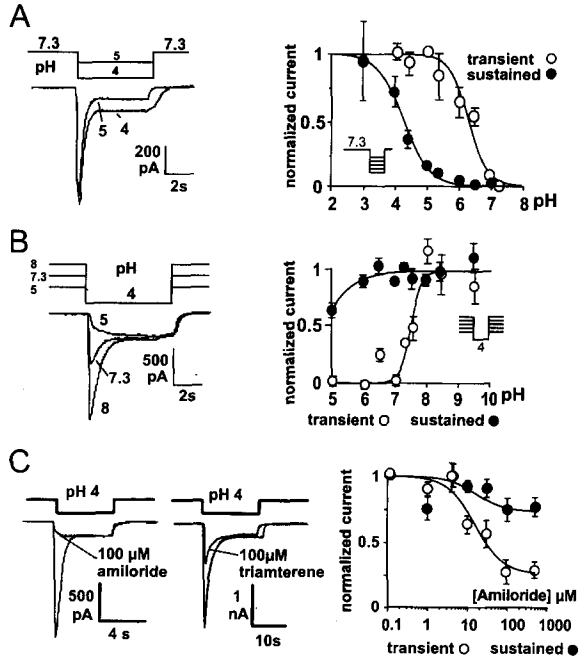

Fig. 2. pH dependence and pharmacology of hASIC3. Proton-induced membrane currents were recorded from hASIC3-transfected COS cells using the whole-cell suction-pipette technique. A: pH dependence of the hASIC3 current. H^+-gated currents were induced by decreasing the extracellular pH rapidly from pH 7.3 to the pH values indicated. The pH required for half-maximal activation was pH 6.2 for the transient current and pH 4.3 for the sustained current. B: H^+-induced hASIC3 currents depend on the resting pH. The extracellular pH was decreased rapidly from the indicated resting pH to pH 4. The currents in A and B are shown as the fraction of the saturation level of the Boltzmann fit. C: Inhibition of hASIC3 by the diuretics amiloride and triamterene. In the dose-response curve for amiloride ($K_{0.5} = 15.9$ µM), currents are expressed as fractions of the mean current in the absence of drug. Data points (○ transient current; ● sustained current) represent the average ± S.E.M. of at least five experiments. Macroscopic currents were recorded from cells clamped at −60 mV using the whole-cell suction-pipette technique.

quires very acidic pH values (pH < 4.5) [9] for activation of the sustained current, the sustained hASIC3 current starts to activate when the extracellular pH decreases to below pH 6 and reaches half-maximal activity at pH 4.3 (Fig. 2A). The channel activity of hASIC3 depends, just as that of the rASIC3 (rDRASIC) channel, on the resting pH (Fig. 2B). The maximal activity of the transient hASIC3 current was observed when the resting pH was above pH 8, indicating that a fraction of the transiently activating H^+-gated cation channels is inactivated at physiological pH. Half-maximal activation of the transient current was observed at pH 7.5, a slightly more alkaline pH than that reported for the rASIC3 (rDRASIC) clone (pH 6.5) [9]. When the resting pH was below pH 7, only activation of the sustained current could be observed after acidification of the bath medium (Fig. 2B). The sustained hASIC3 current can, just as the sustained rASIC3 (rDRASIC) channel, still be activated when the initial pH is quite acidic (pH 5) (Fig. 2B).

All members of the ASIC family cloned so far are sensitive to the diuretic amiloride. The hASIC3 channel is no exception. The effect of amiloride on the hASIC3 current is similar to that reported for rASIC3 (rDRASIC) [9]. The transient current is inhibited by amiloride (K_D=15.9 μM; Fig. 2C) as well as by triamterene (Fig. 2C), while the sustained hASIC3 current is virtually not affected by those diuretics.

The transient hASIC3 current reverses at 37.6 mV, close to the Na^+ reversal potential, indicating a high selectivity for Na^+ vs K^+ (Fig. 3A). Conversely, the sustained current discriminates much less between Na^+ and K^+ (selectivity ratio gNa^+/gK^+=1.62) as it reverses at 10.1 mV (Fig. 3A). The low selectivity for Na^+ vs K^+ of the sustained hASIC3 current clearly distinguishes the hASIC3 channel from the rASIC3 (rDRASIC) channel which is highly selective for Na^+ [9].

Proton-induced unitary currents were recorded from excised outside-out patches (Fig. 3B–D). In a narrow pH window around pH 7.3, spontaneous channel activity can be observed

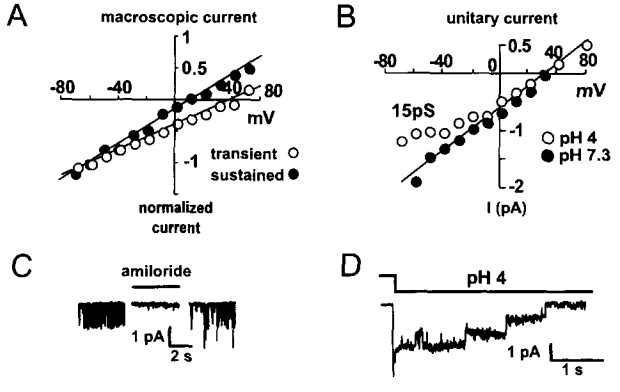

Fig. 3. Selectivity and single channel properties of hASIC3. A: Voltage dependence of the transient and sustained whole-cell current. The transient current reverses at 37.6 mV, the sustained current reverses at 10.1 mV. B: The voltage dependence of the unitary currents of spontaneously active channels at pH 7.3 or of channels activated by a step to pH 4. Slope conductance between −10 and +40 mV for both conditions is 15.0±0.6 pS. V_{rev} = 30.2 mV. The Na^+ equilibrium potential is at 40.1 mV. Examples of spontaneous channel activity at the resting pH of 7.3 (C) or activity evoked by a drop to pH 4 (D). The channel activity recorded at pH 7.3 was inhibited by 100 μM amiloride (C). Single-channel currents were recorded at −60 mV from outside-out membrane patches excised from hASIC3-transfected COS cells.

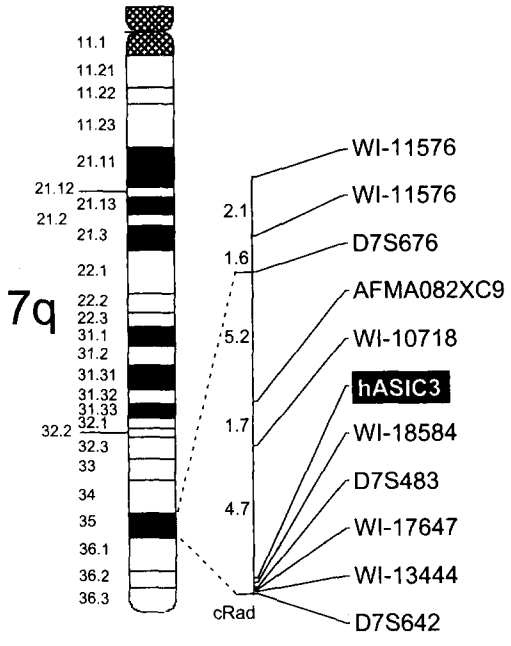

Fig. 4. Human chromosomal localisation of the hASIC3 gene. The human ASIC3 gene is localised 6.4 cRad telomeric to the framework marker AFMA082XC9 on chromosome 7 (Lod score >21). The position of hASIC3 relative to several microsatellites is shown in the right part of the figure. The relative positions of the markers and their distances (in cRad) are the output of the RHMAPPER program. The microsatellites D7S676 and D7S642 are localised on band q35 of chromosome 7 (data from http://www.ncbi.nlm.nih.gov). The cytogenic localisation of those two markers is indicated with dashed lines.

(Fig. 3C), which disappears upon an increase in pH to 8.0, a decrease in pH to 6.0 (not shown) or in the presence of 100 μM amiloride (Fig. 3C). This basal current is mainly carried by Na^+, since it reverses at 30.2 mV (Fig. 3B). When the pH on the extracellular face of an outside-out patch is decreased from pH 7.3 to pH 4, unitary currents are induced (Fig. 3D) that reverse at the same membrane potential as the spontaneously active channel (Fig. 3B). The unitary conductance of the hASIC3 channel for Na^+ is 15±0.6 pS, close to that reported for rat ASIC3 (12.6 pS) [9]. While the sustained non-selective H^+-activated hASIC3 current could be easily detected in whole cell recordings, no sustained or non-selective current could be recorded on outside-out patches. One possible explanation is that soluble factors might be necessary that are lost during excision of the patch.

The human chromosomal localisation of the hASIC3 gene was determined by PCR on a human-hamster radiation hybrid DNA panel. The hASIC3 gene is localised on the human chromosome 7q35, 6.4 cRad telomeric from the microsatellite AFMA082XC9 (Lod score >21) (Fig. 4). To our knowledge, no hereditary diseases with symptoms that are consistent with an altered function of a H^+-gated cation channel were mapped to this region of the human genome.

4. Conclusions

The hASIC3 channel subunit forms a sustained H^+-gated cation channel that has properties similar to those reported for the rat ASIC3 (rDRASIC) channel. However, very important differences exist. Most importantly, the sustained hASIC3

current requires less acidic pH for activation than rASIC3 [9]. In this respect the properties of the hASIC3 channel match better the physiological and electrophysiological data from sensory neurones than those of rat ASIC3. Subcutaneous perfusion of human volunteers with acidic buffer causes pain. At pH 5.2, the pain was rated 20% on a scale ranging from 0 to 100% (unbearable pain) [2]. Furthermore, a subpopulation of polymodal C-fibres in rat nerve-skin preparations can be excited by acidic pH [4]. The threshold for activation lies between pH 6.9 and pH 6.1, maximal stimulation is reached at pH 5.2. The endogenous H^+-gated cation channel recorded in rat sensory neurones starts to activate below pH 6.6 [5]. The pH dependence of the sustained human ASIC3 current matches closely those physiological data, while rASIC3 has a pH dependence that is shifted two pH units towards more acidic pH values [9]. One possible explanation for the differences between physiological data and the pH dependence of the sustained ASIC3 channel (especially the rat ASIC3) might be the participation of as yet unknown subunits in the formation of the native channel. Heteromultimeric assembly was previously demonstrated for the rat ASIC3 channel [9]. rASIC3 can associate with rASIC2b (MDEG2) resulting in an altered selectivity of the channel. While rASIC3 is completely Na^+-selective, the sustained current of the heteromultimeric rASIC3/rASIC2b channel does not discriminate between Na^+ and K^+. The H^+-gated cation channel recorded in rat sensory neurones does not discriminate between Na^+ and K^+ either [5], suggesting that both ASIC3 and ASIC2b participate in the formation of this ion channel in rat sensory neurones. In contrast with the rASIC3 channel, hASIC3 does not require coexpression of other subunits to generate a non-selective sustained current. The ion selectivity of sustained human H^+-gated cation channels is not known yet. A more detailed electrophysiological characterisation of human sustained H^+-gated cation channels will be necessary to allow a comparison of the properties of the native channel with those of the hASIC3 channel.

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