Molecular Cloning of a Non-inactivating Proton-gated Na\(^+\) Channel Subunit Specific for Sensory Neurons*

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We have cloned and expressed a novel proton-gated Na\(^+\) channel subunit that is specific for sensory neurons. In COS cells, it forms a Na\(^+\) channel that responds to a drop of the extracellular pH with both a rapidly inactivating and a sustained Na\(^+\) current. This biphasic kinetic closely resembles that of the H\(^+\)-gated current described in sensory neurons of dorsal root ganglia (1). Both the abundance of this novel H\(^+\)-gated Na\(^+\) channel subunit in sensory neurons and the kinetics of the channel suggest that it is part of the channel complex responsible for the sustained H\(^+\)-activated cation current in sensory neurons that is thought to be important for the prolonged perception of pain that accompanies tissue acidosis (1, 2).

Many painful inflammatory and ischemic conditions are accompanied by a decrease of the extracellular pH (2, 3). H\(^+\)-gated cation channels are present in sensory neurons (1, 4–6), accompanied by a decrease of the extracellular pH (2, 3). H\(^+\)-gated cation channels are present in sensory neurons (1, 4–6), tissues where ASIC is well expressed (7). However, rapidly inactivating H\(^+\)-gated cation channels (ASIC) are not the only channels that can respond to a drop of extracellular pH. Other channels that can respond to a drop of extracellular pH with a sustained Na\(^+\) current are the transient receptor potential (TRP) channels and the acid-sensitive channel (ASC) channels. The ASC channels are activated by acids (1). Here we describe the cloning of a novel H\(^+\)-gated Na\(^+\) channel specific for sensory neurons that has both a rapidly inactivating and a sustained component.

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\(^{1}\) The abbreviations used are: ASIC, acid-sensing ion channel; ASC, acid-sensing channel; ASC, acid-sensing channel; dorsal root ganglia; DRG, dorsal root ganglia; MESS, 4-morpholinoethanesulfonic acid; DIG, digoxigenin.

Materials and Methods

Cloning of DRASIC—We used an anchored PCR approach to identify the sequences upstream and downstream of the expressed sequence tag (W62694). A double stranded adapter (anchor) was prepared by annealing the oligonucleotides GATTTAGGTGACACTATAGAATCGACGAGGTAA and PO\(_4\)-GAATTCGTCGA-CTG-NH\(_2\). The shorter oligonucleotide was protected with a 3’ NH\(_2\) group to avoid extension during the PCR reaction. This adapter was ligated to double stranded rat brain cDNA resulting in a cDNA with known sequences (the anchor) on both extremities. The so prepared anchored cDNA was used to amplify the 5’ and the 3’ end of the coding sequence by PCR. This was done using either the primer GATTAGG-GTGACACTATAGAA or TAGAATGGTGGAGGCGTATAT, which are identical to parts of the longer of the two adapter oligonucleotides together with either the sense primer (CAGAATTCCTCTAGCTATGCAAGG, for amplification of the 3’ end) or the antisense primer (CCCGAG-CAACGAGCACTCTC), for amplification of the 5’ end. The PCR products were cloned into Bluescript, and five clones each for the 5’ and for the 3’ PCR were sequenced. The anchored PCR allowed us to identify the sequences upstream of the first ATG codon and downstream of the stop codon. However all clones isolated from brain contained introns with in frame stop codons and code for truncated proteins lacking the second transmembrane domain (8). An anchor was found to be essential for channel function (10). Analysis of the tissue distribution showed that high levels of the mRNA are only found in DRG. Primers flanking the coding sequence (sense: ACGAATTCCTCTAGCTATGCAAGG; antisense: CTCGAGCGATGAGGCGTATAT) that contained an EcoRI site (sense) or an XhoI site (antisense) were used to amplify the full-length coding sequence from DRG cDNA. The PCR product was digested with EcoRI and XhoI and subcloned into the SmaI-digested PCI expression vector. One clone was sequenced on both strands, and two independent clones were sequenced on one strand using an Applied Biosystems sequencer. Unlike in brain, the three clones isolated from DRG code for full-length proteins.

Cloning of the ASIC Subunits and Electrophysiology—COS7 cells were co-transfected with DRASIC cDNA in the PCI expression vector and a vector containing the CDS receptor cDNA using DEAE-dextran. 3 days later, cells binding CD8 antibody-coated beads (11) were used for experiments. Ion currents were recorded using either the whole cell or the patch-clamp technique. The pipette solution contained (in mM): KCl 120, NaCl 30, MgCl\(_2\) 2, EGTA 5, HEPES 10 (pH 7.2). For the "0 sodium" solution NaCl was replaced by KCl. The bath solution contained in mM: NaCl 140, KCl 5, MgCl\(_2\) 2, CaCl\(_2\) 2, HEPES 10 (pH 7.2). Rapid changes in extracellular pH were introduced by opening an outlet of a microperfusion system at a distance of ~50 μm from the cell. Test solutions having a pH of less than 6 were buffered with 10 mM MES rather than HEPES. Experiments were carried out at room temperature (20–24 °C).

Northern Blot and in Situ Hybridization—4 μg of total RNA from dorsal root ganglia of 7-day-old rats and 4 μg of poly(A\(^+\)) RNA from adult rat brain were separated on a 1% formaldehyde-agarose gel and subsequently transferred to nylon membranes. The blots were hybridized with a random prime \(^{32}P\)-labeled fragment of the DRASIC cDNA corresponding to nucleotide 141–1145 in 6 × SSC, 10 × Denhardt’s solution, 0.1% SDS, 100 μg/ml herring sperm DNA, washed with 0.1 × SSC, 0.1% SDS at 70 °C, and subsequently exposed to a Fuji phosphorimager screen. For the in situ hybridizations on frozen fixed 10-μm brain sections from adult Wistar rats, we used a \(^{32}P\)-random prime-labeled fragment of DRASIC corresponding to nucleotide 141–1145. Brain sections from adult rats were hybridized with the \(^{32}P\)-end-labeled probes overnight at 37 °C in 50% formamide, 2 × SSC, and subsequently washed at room temperature in 1 × SSC, Sections (6 μm) and primary cultures of rat dorsal root ganglia were hybridized with double-stranded DNA fragments labeled with PCR with DIG-dUTP (sections), or fluorescein-dUTP (primary cultures). The probes used correspond to nucleotide 141–1145. Probe labeling, sample preparation, hybridization, and visualization of DIG nucleic acids with alkaline phosphatase-conjugated anti-DIG antibodies was carried out following the protocols from Boehringer Mannheim. Primary cultures of DRG neurons from...
RESULTS AND DISCUSSION

Comparison of the ASIC protein sequence with the data base of expressed sequence tags identified one novel member of this family of ion channels. We used anchored PCR to clone the complete coding sequence from rat DRG. The DRASIC cDNA has an open reading frame of 1599 base pairs preceded by stop codons and codes for a protein of 533 amino acids. DRASIC belongs to the amiloride-sensitive Na⁺ channel (12–18)/degenerin (19–21) family of ion channels and shares 53% sequence identity with its closest relative ASIC (Fig. 1). A DRASIC transcript of ~2.6 kilobases was detected in total RNA of DRG (Fig. 2a). In brain poly(A⁺) RNA where ASIC mRNA is abundant (7), no DRASIC transcript was detectable. Furthermore a mouse multitissue Northern blot (CLONTECH) with poly(A⁺) RNA from brain, heart, spleen, lung, liver, skeletal muscle kidney, and testis did not give any signal (not shown) with the probe that labeled the DRASIC mRNA in total RNA from DRG, indicating that DRASIC is specific for sensory neurons. In situ hybridization confirmed those results (Fig. 2, b–d). DRASIC is expressed in DRG neurons and absent in brain. The small sensory neurons are thought to carry the nociceptive signals from polymodal sensory nerve endings and interestingly small neurons are intensely labeled. The specific expression in sensory neurons suggests that the DRASIC channel has properties required for a specific function of this type of neuron.

Expression of DRASIC in COS cells induced a H⁺-gated cation channel with properties clearly distinct from those of ASIC (7). A rapid decrease of the extracellular pH from pH 7.4 to pH 4 induces a fast rising, rapidly inactivating current followed by a much slower activating sustained inward current (Fig. 3a). Surprisingly, expression of DRASIC can induce both a rapidly and a slowly activating current. The kinetics of the DRASIC current very closely resemble the biphasic H⁺-gated cation current described in sensory neurons (1). Both the transient and the sustained DRASIC current reverse at ~32 ± 3 mV (n = 5), which is close to the Na⁺ equilibrium potential of ~40 mV in the experimental conditions concerned (Fig. 3b). This indicates that the two components are highly selective for Na⁺ (gNa⁺/gK⁺ = 13.5). Unitary currents were recorded from outside-out patches in the absence of Na⁺ in the pipette (Fig. 3, c and d). The slope conductance of DRASIC is with 12.6 ± 0.2 picosiemens (n = 3) (Fig. 3d), close to that reported for ASIC (14.3 picosiemens) (7). The unitary current has a reversal potential of +62 mV (Fig. 3d), indicating an 11.5-fold higher selectivity of the channel for Na⁺ over K⁺. Amiloride inhibits the transient current with an IC₅₀ of 63 ± 2 μM (Fig. 3, e and f). The effect of amiloride on the sustained DRASIC current is complex. In the presence of 200 μM amiloride where the transient current is inhibited by 68 ± 5% (Fig. 3, e and f), the sustained current is higher than in the absence of amiloride (Fig. 3c). A closer examination of the pH dependence of the DRASIC current shows that the transient and the sustained phase can be clearly separated (Fig. 3, g–i). The transient current is activated when the pH drops only slightly (half-maximal activation at pH 6.5 when stepping from pH 7.3; Fig. 3h) but requires an initial pH above 7 for full activation (Fig. 3i). On the contrary, the sustained current needs more important acidification (below pH 4) for activity (Fig. 3h) but may still be activated if the resting pH is far below pH 7 (Fig. 3i).

The situation is similar in sensory neurons (1) where a slight acidification activates only the transient current, while both the transient and the sustained current are activated after more important drops of the extracellular pH. A H⁺-gated cation channel capable of mediating a prolonged sensation of pain during tissue acidosis should not only be activated when the pH drops rapidly but also when the pH decreases slowly, since this is likely to happen during more chronic tissue acidosis. Unlike ASIC, this requires a rapid (<1 s) drop of the pH (not shown). DRASIC responds to slow decreases of the pH (Fig. 3j). If the extracellular pH is decreased gradually by approaching the cell slowly with the perfusion outlet, the first transient current disappears, while the sustained component still develops to its full size (Fig. 3j). The kinetics of the DRASIC channel and the fact that DRASIC mRNA is only present in sensory neurons, where it is abundant, suggest that DRASIC

FIG. 1. Comparison of DRASIC with other members of this ion channel family. Residues identical or similar to the corresponding amino acid in DRASIC are printed white on black (FaNaC) or black on gray (ENaC channel family). We used anchored PCR to clone the complete coding sequence from rat DRG. The DRASIC cDNA has an open reading frame of 1599 base pairs preceded by stop codons and codes for a protein of 533 amino acids. DRASIC belongs to the amiloride-sensitive Na⁺ channel (12–18)/degenerin (19–21) family of ion channels and shares 53% sequence identity with its closest relative ASIC (Fig. 1). A DRASIC transcript of ~2.6 kilobases was detected in total RNA of DRG (Fig. 2a). In brain poly(A⁺) RNA where ASIC mRNA is abundant (7), no DRASIC transcript was detectable. Furthermore a mouse multitissue Northern blot (CLONTECH) with poly(A⁺) RNA from brain, heart, spleen, lung, liver, skeletal muscle kidney, and testis did not give any signal (not shown) with the probe that labeled the DRASIC mRNA in total RNA from DRG, indicating that DRASIC is specific for sensory neurons. In situ hybridization confirmed those results (Fig. 2, b–d). DRASIC is expressed in DRG neurons and absent in brain. The small sensory neurons are thought to carry the nociceptive signals from polymodal sensory nerve endings and interestingly small neurons are intensely labeled. The specific expression in sensory neurons suggests that the DRASIC channel has properties required for a specific function of this type of neuron. Expression of DRASIC in COS cells induced a H⁺-gated cation channel with properties clearly distinct from those of ASIC (7). A rapid decrease of the extracellular pH from pH 7.4 to pH 4 induces a fast rising, rapidly inactivating current followed by a much slower activating sustained inward current (Fig. 3a). Surprisingly, expression of DRASIC can induce both a rapidly and a slowly activating current. The kinetics of the DRASIC current very closely resemble the biphasic H⁺-gated cation current described in sensory neurons (1). Both the transient and the sustained DRASIC current reverse at ~32 ± 3 mV (n = 5), which is close to the Na⁺ equilibrium potential of ~40 mV in the experimental conditions concerned (Fig. 3b). This indicates that the two components are highly selective for Na⁺ (gNa⁺/gK⁺ = 13.5). Unitary currents were recorded from outside-out patches in the absence of Na⁺ in the pipette (Fig. 3, c and d). The slope conductance of DRASIC is with 12.6 ± 0.2 picosiemens (n = 3) (Fig. 3d), close to that reported for ASIC (14.3 picosiemens) (7). The unitary current has a reversal potential of +62 mV (Fig. 3d), indicating an 11.5-fold higher selectivity of the channel for Na⁺ over K⁺. Amiloride inhibits the transient current with an IC₅₀ of 63 ± 2 μM (Fig. 3, e and f). The effect of amiloride on the sustained DRASIC current is complex. In the presence of 200 μM amiloride where the transient current is inhibited by 68 ± 5% (Fig. 3, e and f), the sustained current is higher than in the absence of amiloride (Fig. 3c). A closer examination of the pH dependence of the DRASIC current shows that the transient and the sustained phase can be clearly separated (Fig. 3, g–i). The transient current is activated when the pH drops only slightly (half-maximal activation at pH 6.5 when stepping from pH 7.3; Fig. 3h) but requires an initial pH above 7 for full activation (Fig. 3i). On the contrary, the sustained current needs more important acidification (below pH 4) for activity (Fig. 3h) but may still be activated if the resting pH is far below pH 7 (Fig. 3i).

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is part of the channel complex responsible for the sustained H⁺-gated current in sensory neurons. However, there are important differences between the non-inactivating DRASIC current and the sustained current described in sensory neurons (1). To activate the sustained DRASIC current, the pH has to become very acidic (pH 4; Fig. 3h), while a tonic response in sensory neurons is already obtained at pH 6 (1). Furthermore, relatives of DRASIC, the amiloride-sensitive cation channel (13, 15, 16, 18) and the degenerins of Caenorhabditis elegans (19), even require several homologous subunits for correct function, and it would be surprising if this would not be the case for the H⁺-gated cation channels. ASIC, that is also expressed in sensory neurons (7), is not the missing partner of
DRASIC since co-expression of both subunits yields currents that can be explained by two independent channels (not shown).

A diversity of H$^+$-gated cation channels is described in both sensory neurons (1, 4–6) and in neurons of the central nervous system (6–9). It is therefore likely that new members of this ion channel family will be discovered in the near future. The localization of their mRNAs and proteins should allow studies about the interaction of different H$^+$-gated cation channel subunits expressed in the same type of neuron and might lead to the identification of subunit combinations with properties identical to the native H$^+$-gated channels. The identification of subunits that associate with DRASIC is of particular interest because of the potential importance of sustained H$^+$-gated cation currents for the prolonged sensation of pain caused by acids. The development of blockers that are selective for a H$^+$-gated cation channel specific for sensory neurons, such as DRASIC, might lead to the discovery of new non-addictive analgesics.

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