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Essential role for the putative S6 inner pore region in the activation gating of the human TRPA1 channel

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ABSTRACT

The ankyrin transient receptor potential channel TRPA1 is a sensory neuron-specific channel that is gated by various proalgesic agents such as allyl isothiocyanate (AITC), deep cooling or highly depolarizing voltages. How these disparate stimuli converge on the channel protein to open/close its ion-conducting pore is unknown. We identify several residues within the S6 inner pore-forming region of human TRPA1 that contribute to AITC and voltage-dependent gating. Alanine substitution in the conserved mid-S6 proline (P949A) strongly affected the activation/deactivation and ion permeation. The P949A was functionally restored by substitution with a glycine but not by the introduction of a proline at positions −1, −2 or +1, which indicates that P949 is structurally required for the normal functioning of the TRPA1 channel. Mutation N954A generated a constitutively open phenotype, suggesting a role in stabilizing the closed conformation. Alanine substitutions in the distal G*XXXG* motif decreased the relative permeability of the channel for Ca^{2+} and strongly affected its activation/deactivation properties, indicating that the distal G962 stabilizes the open conformation. G958, on the other hand, provides additional tuning leading to decreased channel activity. Together these findings provide functional support for the critical role of the putative inner pore region in controlling the conformational changes that determine the transitions between the open and close states of the TRPA1 channel.

1. Introduction

Transduction ion channels gate in response to a variety of external signals and this process is critical for the proper functioning of sensory neurons. One such transduction protein belonging to the transient receptor potential (TRP) channel superfamily of cation channels, the ankyrin TRPA1, is required for the response to various proalgesic agents such as allyl isothiocyanate (AITC), cinnamaldehyde, tear gasses, deep cooling, hypertonic and mechanical stimuli or highly depolarizing voltages [1–8]. In addition, TRPA1 is activated by calcium ions that enter through the channel and bind to its N-terminus [9,10]. Despite a wealth of pharmacological and functional data on TRPA1, little is known about the processes by which this channel undergoes conformational changes that open and close ('gate') its ion-permeable pore. The growing amount of evidence on the polymodal nature of TRPA1 indicates that such gating process must involve a multitude of interactions differentially coupling the conformational changes to gate opening induced by all of these activating stimuli (AITC-binding site, EF-hand Ca^{2+} binding domain, cold, mechanical or voltage sensor). In particular, the fact that the TRPA1 channel dynamically controls its critical properties such as unitary conductance and open channel probability depending on the permeating calcium ions [9–11] makes understanding the gating process a complex exercise.

Like other members of the TRP channel superfamily, TRPA1 is thought to be a homotetramer assembled with fourfold symmetry around a centrally located aqueous pore [12,13]. All experimental data obtained so far on various TRP channels [14–19] and the recently determined electron cryomicroscopical 19 Å structure of the vanilloid receptor TRPV1, a TRPA1-related channel [20], corroborate a general topological model in which each subunit of the channel contains six transmembrane spanning domains (S1–S6) with a pore-lining P region linking the S5 and S6 domains. The carboxyl-terminal portion of the S6 inner helix probably forms the most constricted region of the permeation pathway, and might therefore encompass an as-yet-unmapped gate of the channel. The potential role of this domain in the gating of TRPA1 channel has not been previously characterized.

Here, we used scanning mutagenesis of the residues within the putative inner pore S6 region of TRPA1 with the aim of understanding of how this channel opens and closes its pore in response

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to chemical and voltage stimuli. We provide evidence that this specific domain plays an active role in AITC and voltage-dependent channel gating and identify several residues that are critical for controlling this process.

2. Materials and methods

2.1. Expression and constructs of hTRPA1 channel

HEK293T cells were cultured in OPTI-MEM I medium (Life Technologies) supplemented with 5% FBS as described previously [18,21]. Cells were transiently co-transfected with 300–400 ng of cDNA plasmid encoding wild-type or mutant human TRPA1 (wild-type in the pCMV6-XL4 vector, OriGene) and with 300 ng of GFP plasmid (TaKaRa) per 1.6-mm dish using the Magnet-assisted Transfection (IBA GmbH) method. Cells were used 24–48 h after transfection. At least two independent transfections were used for each experimental group. The wild-type channel was regularly tested in the same batch as the mutants. The mutants were generated by PCR using the QuikChange XL Site Directed Mutagenesis Kit (Stratagene) and confirmed by DNA sequencing (ABI PRISM 3100, Applied Biosystems).

2.2. Electrophysiology

Whole-cell membrane currents were recorded by employing an Axopatch 200B amplifier and pCLAMP9 software (Molecular Devices). Patch electrodes were pulled from a glass tube with a 1.65 mm outer

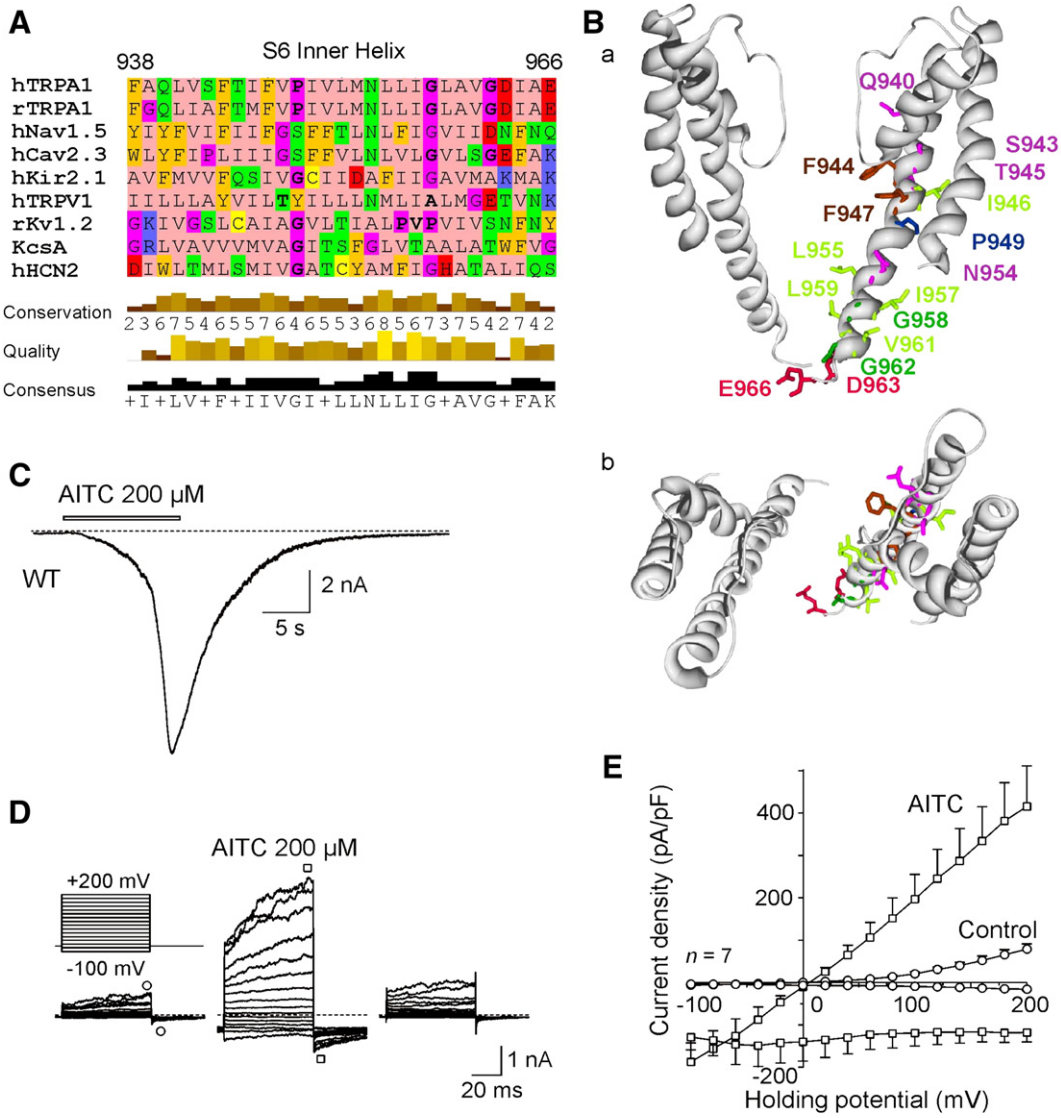

Fig. 1. Control experiments for functional screening of putative inner pore region (Q940-E966) of human TRPA1 channel. (A) Sequence comparison of the inner S6 domain of human TRPA1 (GenBank accession number NM_007332) with that of rTRPA1 (NM_207608), $hNa_V1.5$ (ABR15763), $hCa_V2.3$ (L27745), hKir2.1 (AF011904), hTRPV1 (NP_542436.2), $rK_V1.2$ (P63142.1), KcsA (P0A334) and hHCN2 (AAC28444.2). Protein sequences were aligned using MUSCLE multiple alignment software (http://www.ebi.ac.uk/Tools/muscle/) with Zappo score colors. Conserved amino acid residues proposed to serve as gating hinges in some of the channels are indicated with bold type. (B) Homology model of the S5-P-S6 region of TRPA1 based on the Kv1.2 structure after refinement by 20 ns of molecular dynamics. Two of the four channel subunits are viewed from the side (a) and from the top (b). The residues selected for mutagenesis are indicated. Aliphatic residues are in light green, small hydrophobic residues in dark green, and aromatic residues in brown form together the group of hydrophobic residues. Polar residues are in magenta and the negatively charged in red. Proline is colored blue. (C) Whole-cell current induced by application of 200 μM allyl isothiocyanate (AITC) in wild-type hTRPA1-expressing cell at −70 mV. Bar indicates duration of drug application. (D) Activation of wild-type TRPA1 by a series of voltage steps (inset; holding potential, −70 mV; voltage steps from −100 mV to +200 mV; increment, +20 mV) recorded in control extracellular solution (open circles) and in the presence of 200 μM AITC (open squares). (E) Averaged voltage–current relationships and tail current amplitudes from 7 independent recordings using the protocol shown in D. *I–V* curves determined at the end of the 60 ms voltage pulses, at the times indicated in D.