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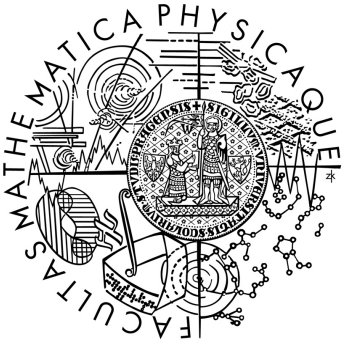
Faculty of Mathematics and Physics

DOCTORAL THESIS



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The role of charged residues in the activation and modulation of the TRPA1 ion channel

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Název práce: Význam nabitých reziduí pro aktivaci a modulaci iontového kanálu TRPA1

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Abstrakt: Důležitým receptorem pro detekci bolestivých podnětů je iontový kanál TRPA1 exprimovaný na periferních zakončeních nociceptivních neuronů, kde slouží k převodu fyzikálních a chemických signálů z okolí na signalizaci nervové soustavy. Snaha porozumět mechanizmům aktivace TRPA1 na molekulární úrovni je vedená snahou najít vhodné přístupy k léčení chronických bolestivých stavů u člověka. Naše práce se zaměřila na C-koncovou cytoplazmatickou doménu TRPA1 receptoru, kde jsme popsali možné vazebné místo pro vápník, který je nejdůležitějším modulátorem TRPA1. Díky kombinaci homologního modelování a molekulárně dynamických simulací s elektrofyziologickým měřením se nám podařilo navrhnout hypotézu, která vysvětluje molekulární podstatu dědičného bolestivého syndromu způsobeného bodovou mutací TRPA1. K objasnění principu napěťové aktivace tohoto receptoru jsme přispěli popisem aminokyselin na proximálním C-konci a v S4-S5 oblasti transmembránové domény, které se na napěťové aktivaci podílí.

Klíčová slova: Ankyrinový receptor (TRPA), C-terminální doména, strukturně-funkční vztah, napěťově závislé vrátkování, modulace vápníkem.

Title: The role of charged residues in the activation and modulation of the TRPA1 ion channel

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Abstract: Important receptor for sensing painful stimuli is ion channel TRPA1, which is expressed in peripheral endings of nociceptive neurons, where it serves as transducer of physical and chemical environmental signals to the language of the nervous system. The effort to understand the mechanisms of its activity on a molecular level is driven by the vision of progress in treatment of chronic pain in humans. Our work focused on C-terminal cytoplasmic domain of TRPA1 receptor, where we described i.a. the probable binding site for calcium, which is the most important TRPA1 modulator. Using the combination of homology modeling and molecular dynamic simulations with electrophysiological measurements we were able to explain molecular basis of familial pain syndrome caused by TRPA1 point mutation. We contributed to the understanding of the TRPA1 voltage-dependent activation mechanism by describing the amino acids in proximal C-terminus and in S4-S5 linker of transmembrane domain that are directly involved in voltage-dependent gating.

Keywords: Transient receptor potential ankyrin (TRPA), C-terminal domain, structure-function relationship, voltage-dependent gating, calcium modulation.

Contents

1 Introduction

The coincidence sometimes shows very twisted sense of humor. The TRP stands for transient receptor potential. It originated from observation that a Drosophila mutant responds to light transiently, in contrast to the sustained light-induced current of wild-type flies. In Czech the word TRP is an order to 'suffer'. For ion channels from TRP family the Czech meaning is unbelievably accurate since TRP channel often serve as primal body sensors of painful stimuli. Moreover, a malfunction of just one type can result in episodes of excruciating pain.

TRP channels from various species participate in all five of the Aristotelian senses (sight, hearing, touch, smell, and taste). They have been recognized as key transducers of sensory signals and as particularly important noxious stimuli detectors. First member of the family linked with pain and thermosensation was, in 1997, the TRPV1, that was cloned and described as both capsaicin (hot chilli pepper substance) receptor and noxious heat detector (temperatures above 43 °C) (Caterina et al. 1997). Since then, several other TRP family members participating in thermosensation and nociception were cloned and characterized: TRPV2 (Caterina et al. 1999), TRPV3 (Smith et al. 2002, Xu et al. 2002), TRPV4 (Güler et al. 2002), TRPM8 (McKemy et al. 2002, Peier et al. 2002) and TRPA1 (Story et al. 2003). Recent study addressing the epigenetic changes in DNA in individuals with various pain sensitivities detected the strongest signal in the promoter of the *trpa1* gene (Bell et al. 2014). This study suggests that the subjective pain threshold in humans is apparently dependent on topical TRPA1 expression levels. This finding is raising the likelihood that TRPA1 is indeed suitable target for pharmacological pain treatment as has been anticipated.

Despite the efforts of many groups around the world, there are still serious gaps in understanding many aspects of TRPA1 complex behaviour. This thesis focuses on elucidating the functional and structural role of TRPA1 cytoplasmic C-terminus (and spatially close structures) in relation to agonist-induced and voltage-induced gating as well as the calcium-dependent modulation.

2 Literature review

2.1 Transient receptor potential (TRP) channels

The transient receptor potential (TRP) ion channels form a large family of membrane proteins. They are mostly found in organisms with a nervous system due to their direct involvement in sensory perception. Many TRP channels are activated by various chemical and physical stimuli (including ligand binding, temperature, mechanical stimuli or voltage) and function as signal integrators. They can be grouped into seven subfamilies (see Fig. 1) based on their amino acid sequence homology: the TRPC ('Canonical'), TRPV ('Vanilloid'), TRPM ('Melastatin'), TRPP ('Polycystin'), TRPML ('Mucolipin'), the TRPA ('Ankyrin'), and TRPN ('NOMP-C', no mechanoreceptor potential C).

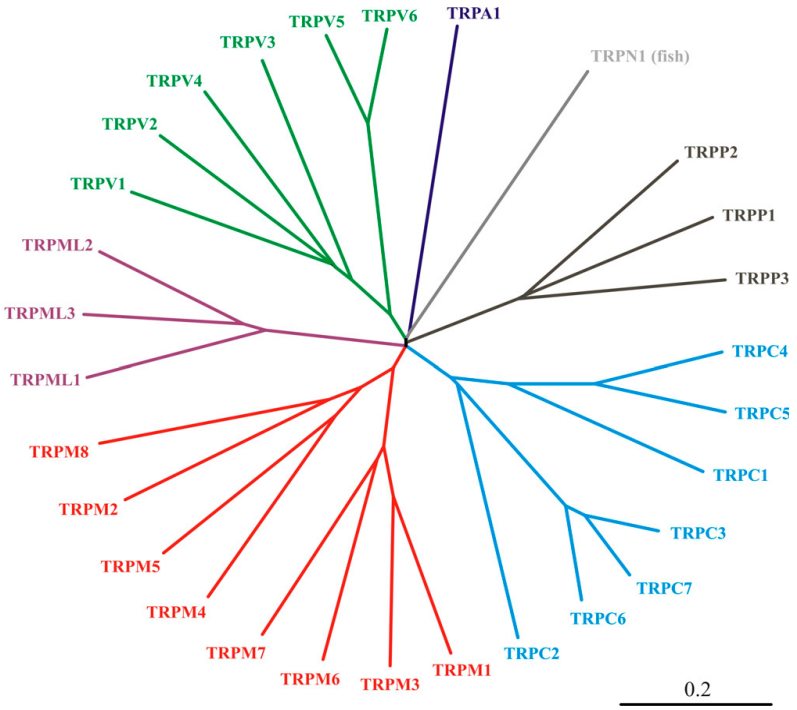


Figure 1: *The phylogenic tree of TRP channels. Note that TRPN1 is only present in lower vertebrates (e.g., zebrafish). The bar (0.2) indicates point accepted mutation units, which is the evolutionary distance between two amino acids (1 point accepted mutation unit = 1 point mutation event per 100 amino acids, which is accepted and is passed to progeny). (From Nilius and Owsianik 2011a)*

Despite the diversity of tissue and cell types where are TRP channels expressed they share several common features. They could be described as tetramers formed by subunits with six transmembrane helices (S1–S6) and containing cation-selective pores, which in several cases show high calcium permeability (Latorre et al. 2009). Interestingly TRPA1 is the third most Ca^{2+} permeable TRP channel (after TRPV5 and TRPV6) (Nilius et al. 2012) suggesting its dual role in cell signaling. The activation of TRPA1 channels on plasma membrane induce not only membrane depolarization but also substantial Ca^{2+} influx, that triggers cellular events resulting in high signal gain. Thanks to high fractional Ca^{2+} current, even low expression of TRPA1 is sufficient to signal transduction.

2.2 Pain transduction and nociception

Knife cut as well as burn or scald activates primary afferent nerve fibers in our skin. Nociception is initiated and the information is transmitted to the spinal cord and thence to the brain, where the perception of pain is generated. Even though not very popular, the pain is essential part of our lives and as in these cases, it is mostly very useful, promoting guardian of the injured area. The ability to feel pain is absolutely necessary to an individual's survival and wellbeing. Incapability of detecting painful stimuli can be life threatening. People suffering from such hereditary neuropathies can not feel piercing pain from a sharp object, heat of an open flame or even pain from broken bone (Lumpkin and Caterina 2007). As a result, they do not behave appropriately to the situation and are permanently risking their health, often maintaining unrealized infections.

Sensation of touch, temperature and pain is enabled due to specialized sensory neurons innervating the skin. The peripheral terminals of these neurons, whose cell bodies are found in trigeminal and dorsal root ganglia (DRG), encode sensory stimuli to action potentials that propagate to the central nervous system for further processing. Based on degree of myelination and the speed of action potential propagation along afferent fibres are cutaneous sensory neurons classified as Aβ-, Aδ- or C-fibres. Whereas myelinated **Aβ-fibres** with big cell bodies do not contribute to pain sensation, the smaller Aδ-fibres and C-fibres do.

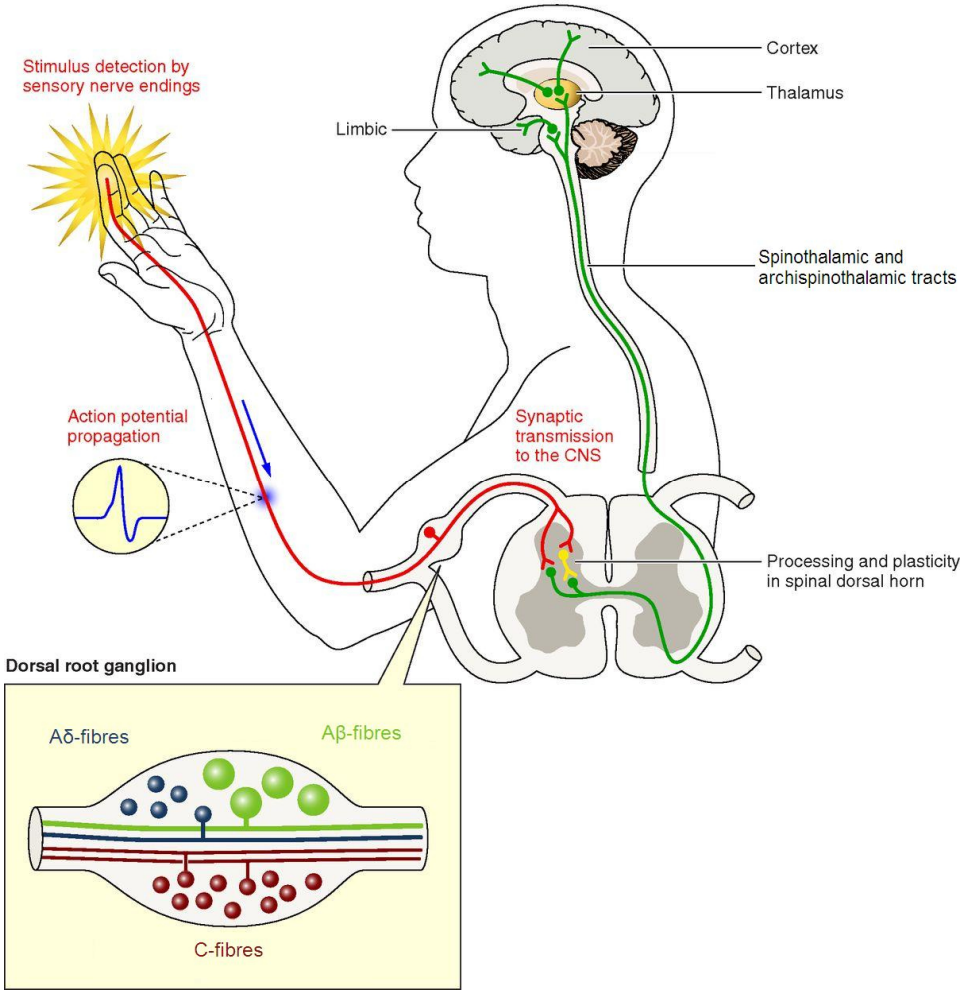


Figure 2: *Pain propagation from periphery to CNS. Pain sensing neurons have a peripheral axon innervating the distal territories (skin, visceral, etc.) where they detect painful stimuli leading to an action potential that travels along the fibres up to the cell soma in dorsal root ganglia (DRG) and then to the first relay in the dorsal spinal cord. The sensory information is processed locally in neuronal circuitry within the dorsal horn of the spinal cord before being sent to the thalamus to convey nociceptive information. Following thalamic filtering, the information is sent to the cortical structures of the pain matrix. (From Bourinet et al. 2014)*

The **Aδ-fibres** are only thinly isolated with myelin and they conduct information at 5–35 m/s. They are divided into Type I and Type II. Both types respond to intense mechanical stimulation but differ in heat responsiveness. While Type I have a threshold of ~ 53 °C the Type II have a threshold already of ~ 43 °C. This neatly corresponds with different activation thresholds of two molecular thermal sensors from TRP family of ion channels — vanilloid receptors TRPV2 and TRPV1 respectively (Lumpkin and Caterina 2007).

Unmyelinated **C-fibres** are the smallest type of primary afferent fibres. Hence they demonstrate the slowest conduction (less then 2 m/s). Most C-fibres are polymodal, responding to noxious thermal and mechanical stimuli. Others are mechanically insensitive, but respond to noxious heat (Julius and Basbaum 2001).

Sensory neurons initiating the painful sensations are known as **nociceptors**. It is estimated that 80 % of afferent Aδ-fibres and C-fibres are nociceptors. Most of these fibres can be activated by mechanical stimuli, which is considered as painful. Among them are also 'silent' or 'sleeping' nociceptors, which are responsive only when sensitized by tissue injury.

Aδ-fibres and C-fibres synapse with secondary afferent neurons in the dorsal horn of the spinal cord and the nociceptive signals progress to higher centers in the brain (Fig. 2) following one of two main pathways. They originate in different spinal cord regions and ascend to terminate in different areas in the central nervous system (CNS). The first is called the spinothalamic tract and is important for pain localization. The second is called the archispinothalamic tract and is involved in the emotional aspects of pain.

From a neurobiological perspective, tree types of pain are distinguished (Woolf 2010). **Nociceptive pain** is essential to detect and minimize contact with damaging or noxious stimuli and, as was mentioned in the first paragraph of this chapter, it is very useful. **Inflammatory pain,** that accompany inflammation has also protective role and usually resolves spontaneously due to healing. On the other hand, it needs to be reduced in cases of ongoing inflammation and severe or extensive injury. Sometimes the pain persists in the absence of injury and no longer fulfills a useful physiological function. Such case is called **pathological pain.** If it occurs after damage to the nervous system, then is called neuropathic pain.

Over the years, the study of transient receptor potential (TRP) channels has provided insight into the mechanisms of nociception and has helped to understand the detection and processing of painful stimuli. It has been shown that TRPA1 receptors, which are expressed in nociceptors, are involved in initial step of pain sensations. These molecular detectors of thermal and chemical stimuli are considered as attractive drug target to treat inflammatory disorders and neuropathic pain.

2.3 Inflammatory and neuropathic pain

The inflammation is immune response to tissue damage or infection. The early steps in inflammatory response include the release of inflammatory mediators by immune cells to infiltrate the affected area. Such mediators include ATP, bradykinin, prostagladins, histamine, proteases or glutamate (Basbaum et al. 2009). Several inflammatory mediators subsequently activate a subset of primary afferent sensory neurons (Fig. 3). The neurons release inflammatory neuropeptides like substance P, calcitonin gene-related peptide (CGRP) or neurokinin A causing extravasations of plasma proteins into the surrounding tissue (Bautista et al. 2013). It has been shown that neuropeptide release and neurogenic inflammation can be caused by direct TRPA1 activation by tissue damage release factors, 4-hydroxynonenal or 4-oxononenal (Taylor-Clark et al. 2008).

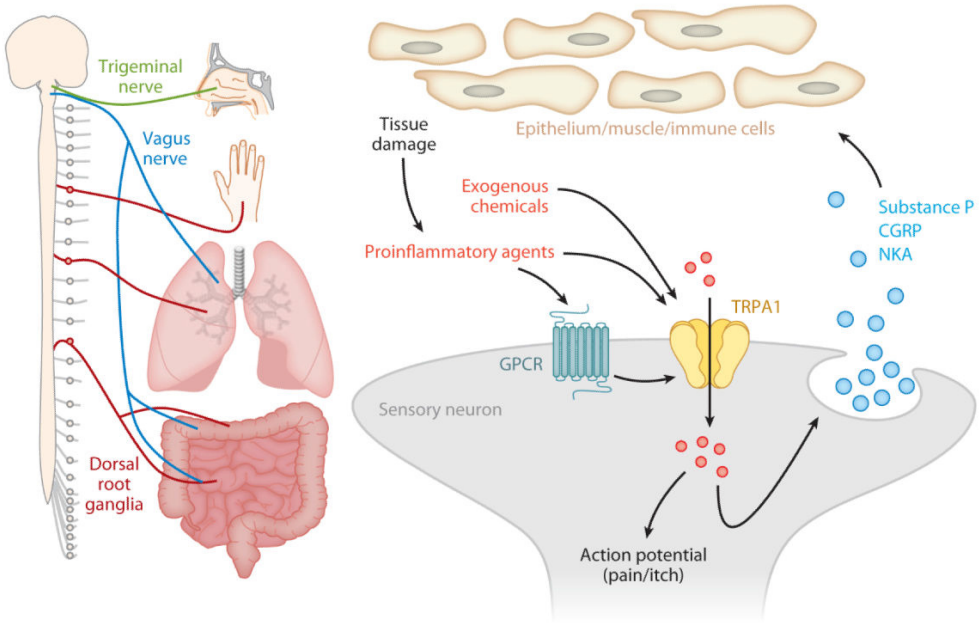


Figure 3: *Mechanisms of neurogenic inflammation. Left: TRPA1 is expressed by sensory afferents that have cell bodies in the vagus nerve (blue), trigeminal ganglia (green), and dorsal root ganglia (red). These afferents innervate peripheral targets, including the skin, the airways, and the gastrointestinal tract. Right: TRPA1 activation is required for the release of neuropeptides such as substance P, calcitonin gene-related peptide (CGRP), and neurokinin A (NKA), which promote and modulate inflammatory responses. In addition, signaling molecules downstream of G protein–coupled receptors (GPCRs) regulate TRPA1 channel activity. (From Bautista et al. 2013)*

The TRPA1 receptor plays an important role both as a target for inflammatory mediators and as a gatekeeper for the release of inflammatory neuropeptides by C-fibres (Bautista et al. 2013). Several studies independently showed that administration of TRPA1 antagonist in inflammatory pain models significantly reduces allodynia and hyperalgesia (Petrus et al. 2007, Caceres et al. 2009, Del Camino et al. 2010). Experiments with TRPA1-deficient mice showed a significant decrease of responses to proinflammatory agent bradykinin and simultaneous attenuation of pain hypersensitivity (Bautista et al. 2006). Since then, numerous papers identified the TRPA1 as a key player in various types of chronic inflammatory disorders.

Neuropathic pain, which often accompanies diabetes mellitus and renal failure, is linked with altered level of highly reactive compound methylglyoxal. This substance accumulates in all cells, in particular neurons, and leaks into plasma as an index of the severity of the disorder. It is known that cytotoxic methylglyoxal covalently bind to particular arginine, lysine and cysteine residues (Brownlee 2001). As one of the molecular methylglyoxal's target was identified the TRPA1, whose activation causes nociceptors excitation and neuropeptides release (Eberhardt et al. 2012).

Airway irritants such as cigarette smoke or chlorine has been shown to activate pulmonary sensory neurons via activation of TRPV1 and TRPA1 channels. Cough, which often follows, is a reflex which helps to clear the respiratory tract of environmental irritants, mucus and foreign particles. There are several pieces of evidence that TRPA1 participate in the generation of irritant-induced cough reflexes (Bautista et al. 2013). Among them belongs the attenuation of TRPA1 agonist evoked cough by TRPA1 inhibitors (Birrell et al. 2009). Interestingly, the genetic contribution of TRPA1 to cough remains unknown, because the only TRPA1 knockout animal bred so far are mice, which as the species do not have a cough reflex (Bautista et al. 2013). Nevertheless, mice do show alterations in breathing patterns upon exposure to airway irritants and this alternations are absent in TRPA1 knockout mice (Gu and Lin 2010, Taylor-Clark et al. 2009).

According to World health organization (WHO) the most common chronic disease among children is the asthma. An inflammatory disease characterized by recurrent attacks of breathlessness, wheezing and coughing. An asthma attack is usually triggered by airway exposures to allergens or chemical irritants. Using the

murine ovalbumin model of asthma condition was shown that TRPA1 downregulation as well as TRPA1 antagonist's administration leads to a decrease of asthmatic responses. The downregulation of TRPA1 inhibits allergen-induced leukocyte infiltration in the airways, reduces cytokine and mucus production, and almost completely abolishes airway hyperreactivity to contractile stimuli (Caceres et al. 2009, Nilius et al. 2012). The exact role of TRPA1 in asthma is not known. The participation of TRPA1 in tuning sensory neuron activity is the most likely but other possibilities can not be dismissed yet. Intriguing possibility offers presence of TRPA1 in one or more subtypes of immune cells; however the expression of TRPA1 in immune cell lines is not consistently observed (Bautista et al. 2013, Stokes et al. 2006). Anyway, TRPA1 demonstrated its potential as a promising target in the development of drugs aimed at treating the asthma, cough and other allergic inflammatory conditions (Bautista et al. 2013).

2.4 Heritable disorders of pain sensation

Altered pain sensitivity has been linked to several different genes including voltage-gated Na^+ and Ca^{2+} channels or K^+ channels (Kullmann and Hanna 2002). The studies of these disorders provided important insights into the molecular mechanisms of pain perception. Interestingly, there are two human heritable disorders of pain sensation caused by point mutation in *trpa1* gene.

The first one was identified in a family from Colombia (South America) and was described by team around Barbara Kremeyer (2010). The disorder manifested itself by episodes of severe pain localized principally to the upper body and triggered by conditions of fatigue, fasting and cold. Affected individuals reported no altered pain sensitivity outside the episodes and even their neurological examination was normal. The genome-wide linkage scan detected a single transition in *trpa1* gene resulting in the N855S mutation in the transmembrane part of TRPA1.

The N855S mutant was expressed in HEK293 cells and characterized electrophysiologically. It has been shown that the N855S mutation does not alter exogenous or endogenous ligand binding, but does increase current flow through the activated channel at negative membrane potentials. The inward current was increased 5-fold on activation at normal resting potentials. The enhance activity of the N855S

mutation is consistent with the observed pain syndrome. Specific TRPA1 antagonist inhibits the mutant channels, promising a useful therapy for this disorder.

The second pain sensation alternating mutation of TRPA1 has been identified among N-terminal ankyrin repeats. The *trpa1* gene variant resulting in E179K point mutation has been recently discovered in patients with acute pain and paradoxical heat sensation (Binder et al. 2011), which is defined as a burning heat sensation when a noxious cold stimulus is applied. Different mechanisms contribute to paradoxical heat sensation, including Aδ-fibres impairment (Susser et al. 1999) or dysfunction of thalamic cells (Craig and Bushnell 1994).

Apparently, among neuropathic pain patients, the frequency of paradoxical heat sensation is significantly lower if the patient carry E179K variant of TRPA1 (Binder et al. 2011). The clinical observation suggested a diminished sensitivity to noxious cold and subsequent in vitro study (May et al. 2012) indeed found that this mutant is not activated by cold even though it is highly expressed in both cold (4 °C) and heat (49 °C)-treated cells. Residue E179 is located on the convex surface of the ankyrin repeat 4 and is probably exposed to the solvent, where it is unlikely involved in proper protein folding or maintaining protein stability. However, gel electrophoresis experiments with E179K mutant showed diminished ability to form protein complexes upon cold stimulation. Thus the loss of cold activation was attributed to the loss of ability to interact with associated proteins or with other TRPA1 monomers during oligomerization.

Most recently, Bell et al. (2014) has discovered that disorders of human heat pain sensation can be associated also with the epigenetic changes in DNA and not only with variations in DNA. Epigenetic changes such as methylation of DNA promoter can downregulate gene expression. Utilizing the extensive genome-wide analyses of DNA methylation Bell et al. (2014) found that TRPA1 promoter is hypermethylated in individuals with lower pain thresholds. Consistently with that, they observed modest nominally significant increase in *trpa1* gene expression levels in skin biopsies of individuals with higher pain thresholds. Apparently our pain sensitivity can be significantly altered due to tuning of TRPA1 receptor, let's look closer.

2.5 Ankyrin subfamily of TRP

TRPA1 is the only known mammalian member of the ankyrin subfamily. But for example fruit fly contains 4 TRPA1 homologues and it is believed that all animals contain at least one *trpa1* gene (Nilius et al. 2012). The name ankyrin originate from multiple (14–19) ankyrin repeats at N-terminal tail. Most other TRPs have up to 8 ankyrin repeats, and the only other TRP protein with more than that is the mechanosensory channel TRPN1 (29 ankyrin repeats) in flies, nematodes, and fish, but not found in mammalian genomes (Garcia-Anoveros and Nagata 2007). Ankyrin repeat is common structure motif composed of 33 amino acids in helix-turn-helix conformation.

The ankyrin repeats seems to serve as protein–protein interaction site, they can provide elasticity and make molecular springs (Lee et al. 2006) but their exact function and purpose of their relative abundance in TRPA1 remains unclear. Experiments with truncations of ankyrin repeats revealed that intact ankyrin repeat domain is necessary for plasma membrane expression of TRPA1 (Nilius et al. 2011b). Nevertheless, study (Moparthi et al. 2014), that used purified hTRPA1 inserted into artificial lipid bilayer, showed that the N-terminal ankyrin repeat domain (hTRPA1 without ARD; Δ1–688) is not needed for activation of hTRPA1 by cold, electrophiles, and nonelectrophilic compounds or inhibition by HC030031. This study also suggested that the N-terminal ankyrin repeat domain may modify hTRPA1 channel behaviour in a voltage-dependent manner.

2.6 TRP structure

Recently the David Julius's laboratory achieved a great success, which helped to exploit advances in electron cryo-microscopy to determine the structure of a mammalian TRP channel, TRPV1, at 3.4 Å resolution (Fig. 4), breaking the side-chain resolution barrier for membrane proteins without crystallization (Liao et al. 2013, Cao et al. 2013). TRPV1 is coexpressed with TRPA1 in a subset of sensory neurons. They modulate each other's activity and they may even form functional TRPV1–TRPA1 heteromers (Fisher et al. 2014). These channels share a similar general topology. The functional channels assemble as tetramers. Each subunit has

large N- and C-terminal cytosolic domains that ‘‘hangs’’ underneath the transmembrane domain. This two-domain arrangement referred to as a ‘‘hanging gondola’’ has been observed in 3D structure of both TRPV1 and TRPA1 channels.

For TRPA1 channel, the best structural insight available during the work on this thesis originated from 16 Å resolution structure (Fig. 5a) obtained by single-particle electron microscopy (EM) (Cvetkov et al. 2011). The primary structure of TRPA1 and TRPV1 transmembrane segments share 22 % of identical residues. So at least the transmembrane core of the TRPV1 structure helped to imagine how TRPA1 channel looks like and functions.

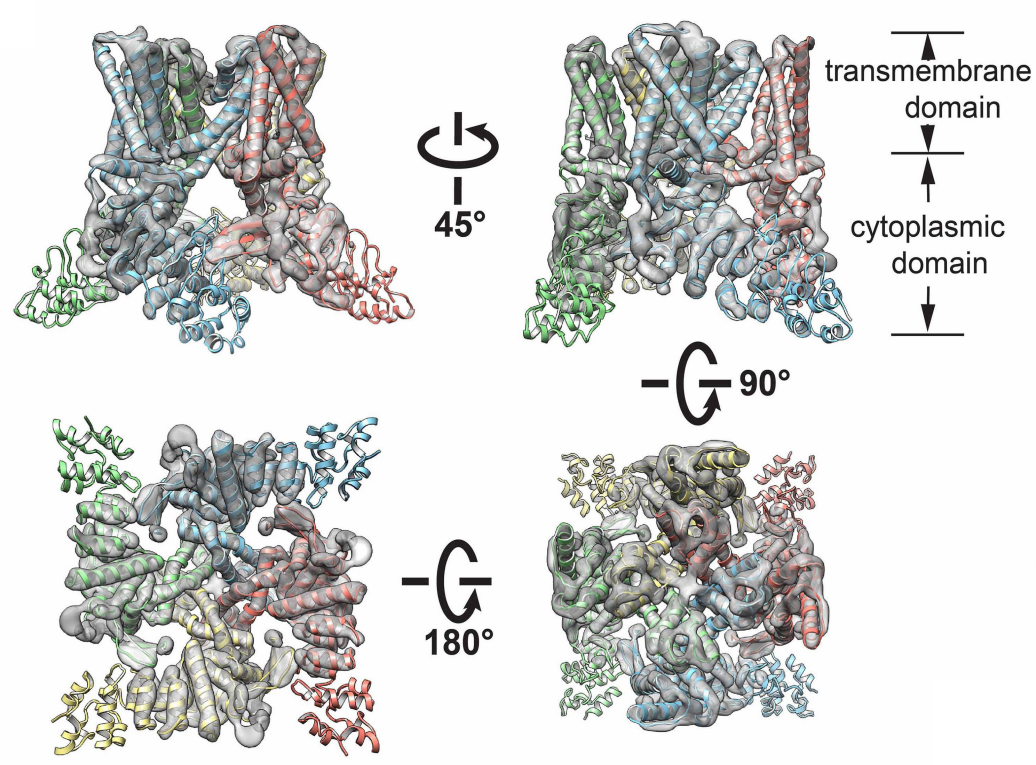


Figure 4: *Three-dimensional reconstruction of TRPV1. Different views of the three-dimensional reconstruction of TRPV1 determined by single-particle cryo-EM (resolution* 3.4 Å*) fitted with the atomic model of TRPV1 in which residues L360 to A719 were built de novo, and L111 to E359 were docked from the ankyrin repeat crystal structure. (From Liao et al. 2013)*

The transmembrane part of TRPV1 channel is organized in two distinct domains. One form the central ion-conducting pore by tetrameric assembly of S5-P-S6 segments (P stands for the re-entrant loop with a pore helix located between S5 and S6). The other contains surrounding S1–S4 voltage-sensor-like segments. These two domains are connected by a helical S4–S5 linker that runs parallel to the

membrane. Each S1–S4 domain associates with the S5-P-S6 region of an adjacent subunit, forming a pinwheel-like structure through this 'domain swap' organization (Liao et al. 2013). In contrast to voltage-gated ion channels, the S1–S4 domain of TRPV1 remains static during channel activation and provides an external surface for binding of lipophilic ligands, such as capsaicin and resiniferatoxin (Cao et al. 2013). During TRPV1 activation the S4–S5 linker moves to facilitate gating of the pore. The importance of the S4–S5 linker for proper TRPA1 channel function was exposed by the identification of a single amino acid substitution (N855S) which has been associated with familial episodic pain syndrome (Kremeyer et al. 2010).

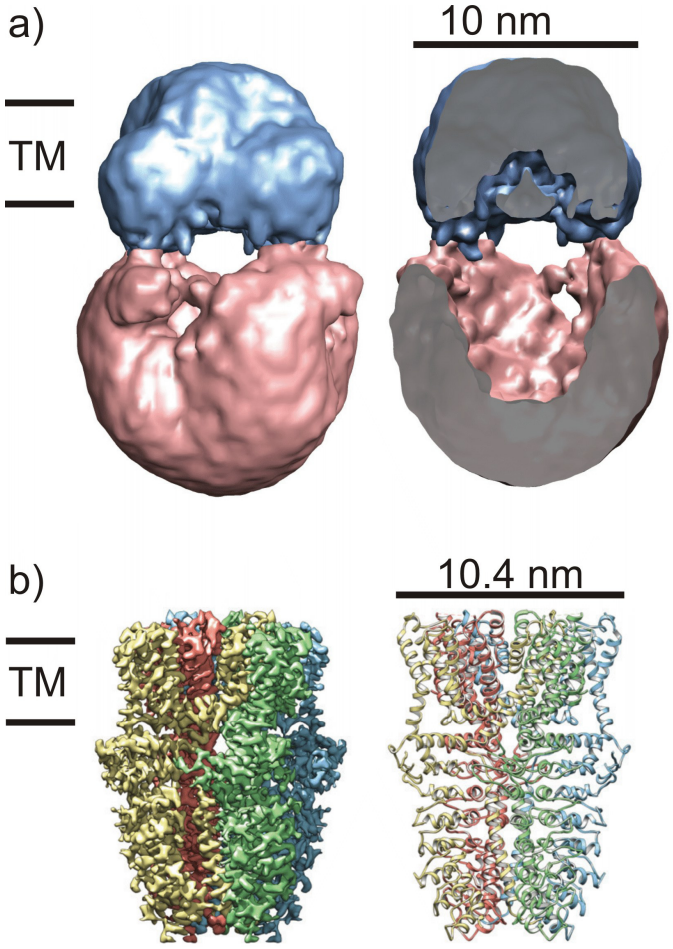


Figure 5: *Three-dimensional reconstruction of TRPA1. a) Three-dimensional reconstruction of TRPA1 determined by single-particle EM (resolution 16 Å). Blue colored transmembrane domain (TM) and pink colored cytoplasmic domain. The cut-away view shows the internal cavity in the TRPA1 electromagnetic density. (From Cvetkov et al. 2011) b) Left: Three-dimensional density map of TRPA1 from AITC-treated sample filtered to 3.5 Å resolution with each subunit colour-coded. Right: Ribbon diagram of TRPA1 atomic model for residues Lys 446–Thr 1078, including the last five ankyrin repeats. (From Paulsen et al. 2015)*

TRPV1 opening is associated with major structural rearrangements in the outer pore, including the pore helix and selectivity filter, as well as pronounced dilation of a hydrophobic constriction at the lower gate, suggesting a dual gating mechanism (see Fig. 6). Allosteric coupling between upper and lower gates may account for rich physiological modulation exhibited by TRPV1 and other TRP channels. (Cao et al. 2013)

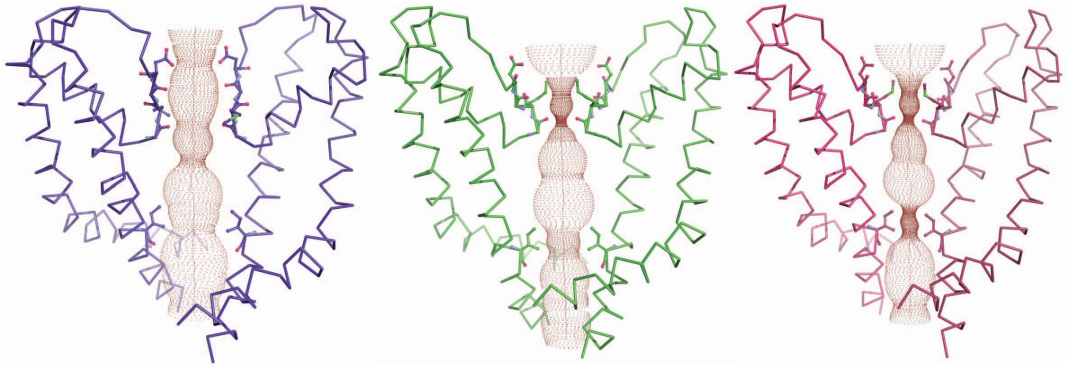


Figure 6: *The pore profiles of TRPV1 channel. Solvent-accessible pathway along the pore for fully open state (purple), lower gate open state (green) and both the selectivity filter and lower gate closed (red). (From Cao et al. 2013)*

A characteristic feature of many TRP channels is the so called TRP domain, the 23–25 amino-acid-long region located just after S6. Liao at al. (2013) showed that the TRP domain folds as α-helical structure that runs parallel to the inner leaflet of the membrane. TRP domain closely interacts with S4–S5 linker but also with pre-S1 helix serving as a structural integrator that facilitates allosteric coupling between channel domains (Cao et al. 2013, Liao et al. 2013). Although TRPA1 lacks 'true' TRP domain (TRPA1 does not share the highly conserved residues among the TRP domains), just after S6 is also predicted helical structure H1 which presumably serves a similar function as TRP domain (see Fig. 7).

Just before this thesis was submitted, the article revealing the structure of human TRPA1 (at 4 Å resolution) had been published (Paulsen et al. 2015). Using electron cryo-microscopy techniques, Paulsen and colleagues confirmed that TRPA1 resembles TRPV1 structure in transmembrane core and revealed several unexpected features, including an extensive coiled-coil assembly domain in TRPA1 C-terminus (Fig. 5b).

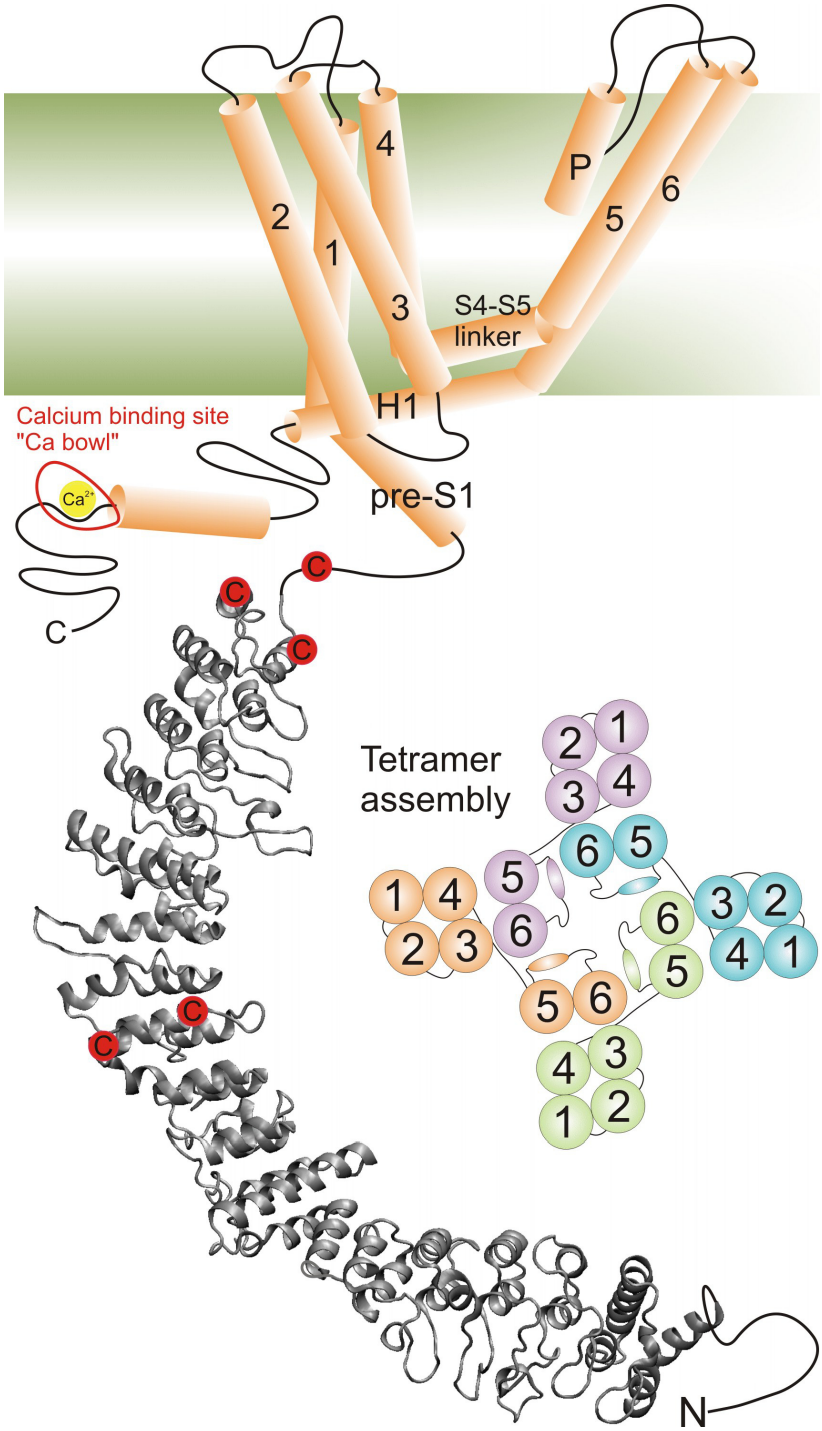


Figure 7: *The overall topology of TRPA1 subunit. The N-terminal ankyrin repeats appearance is adopted from crystal structure of TRPV1 ankyrin repeat motif (Lishko et al. 2007). Six transmembrane helixes are numbered, the P stands for the re-entrant loop with a pore helix. The predicted helical structure H1, which presumably serve the same function as TRP domain from other TRP channels, is marked. In the N-terminus are shown the cysteine residues responsible for binding electrophilic agonists. In the C-terminus, the location of predicted calcium binding site is highlighted. Top view of channel assembly is presented. With each subunit in different color, the 'domains swap' organization is neatly noticeable.*

2.7 TRPA1 expression

Each TRPA1 channel subunit is a rather large protein (1,119 amino acids in human). There is growing evidence that TRPA1 is widely expressed channel present in many organs and tissues including brain, heart, lung, inner ear, skeletal muscle, pancreas, and skin (Stokes et al. 2006, Atoyan et al. 2009). But the clearest role has this polymodal receptor for noxious stimuli detection in a subpopulation of sensory neurons – nociceptors. These special neurons are activated by potentially life-threatening events and, upon excitation, signal pain. TRPA1 expression can be found in sensory neurons of dorsal root ganglia, trigeminal ganglia and nodose ganglia (Bautista et al. 2005).

The genome-wide scan analyzed epigenetic changes of 50 identical twins discordant for heat sensitivity and in 50 further unrelated individuals (Bell et al. 2014). The results revealed nine differentially methylated regions associated with high or low pain sensitivity. The strongest signal originated from the promoter of *trpa1* gene. The subjects with higher pain thresholds exhibited significantly increased expression levels of TRPA1 in the skin, which is consistent with downregulatory effect of DNA methylation in the TRPA1 promoter. Because TRPA1 is generally considered not to be a direct heat sensor in mammals, this finding supports the idea of mutual interaction of TRPA1 with the heat-sensing channels TRPV1, TRPV2, and TRPV3. These receptors were also differentially methylated in the study, yet not significantly.

2.8 TRPA1 activators

The peculiatiry of TRPA1 function lies in its ability to be activated and also modulated by a plethora of stimuli of diverse origin. First of all, TRPA1 is strongly activated by allyl isothiocyanate, the pungent compound of mustard oil (Tab. 1). This and other electrophilic ligands covalently bind to cysteine and lysine residues in the N-terminus (Bandell et al. 2004; Jordt et al. 2004). In the pool of TRPA1 agonists belong also methyl salicylate (in wintergreen oil) (Bandell et al. 2004), cinnamaldehyde (in cinnamon) (Bandell et al. 2004), allicin and diallyl disulphide (in garlic) (Bautista et al. 2006; Macpherson et al. 2007), acrolein (an irritant in vehicle

exhaust fumes and tear gas) (Bautista et al. 2006; Andersson et al. 2008) or endogenous compounds such as H_2O_2, the alkenyl aldehydes 4-hydroxynonena, 4-oxo-nonenal, 4-hydroxyhexenal and the cyclopentenone prostaglandin, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (Trevisani et al. 2007; Andersson et al. 2008). Interestingly, TRPA1 is also a sensor for gasses like O_2 (Takahashi et al. 2011), CO_2 (Garrity 2011), H_2S (Miyamoto et al. 2011) or NO (Miyamoto et al. 2009).

Another set of chemicals activates TRPA1 by yet unknown mechanism, probably in the manner of a more common non-covalent interaction with a specific binding site. Residues S876, T877 and G878 located in the fifth transmembrane segment are crucial for the effect of menthol. This cooling agent is a good example of a non-covalent TRPA1 modulator with a bimodal effect (Karashima et al. 2007). Low concentrations cause the channel opening whereas higher concentrations cause inhibition. Similar bimodal effects are common to lots of non-electrophilic activators of TRPA1 such as the antimycotic drug clotrimazole or the psychoactive drugs nicotine and Δ9 tetra-hydrocannabinol (THC from marijuana) (Talavera et al. 2009).

Among the compounds whose therapeutic or side effects are linked to TRPA1 channel function belong anesthetic agents (propofol or lidocaine), nonsteroidal anti-inflammatory drugs or even antibacterial agents, which are often added to pharmaceuticals and cosmetics (parabens and alkyl esters of p-hydroxybenzoate) (Fujita et al. 2007). Interestingly, TRPA1 is also activated by long-wavelength ultraviolet radiation (UVR, type A), the main component of sunlight (Bellono et al. 2013).

Lots of TRPA1 activators show species–specific effects. Mice, but not TRPA1 knockout mice, show a remarkable aversion to caffeine-containing water (Nagatomo and Kubo 2008). Coffee has a spicy hot taste to mice probably resembling taste of mustard or horseradish. Apparently just one mutation of Met 268 to Pro is needed to switch caffeine activation effect to suppression (Nagatomo et al. 2010).

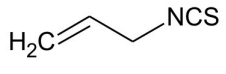
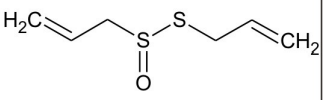
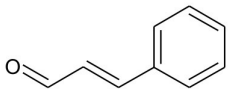
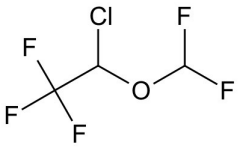
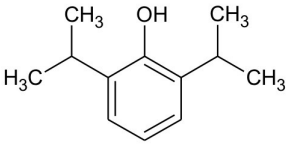
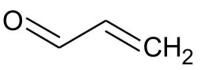
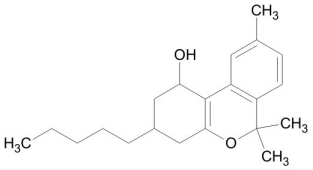
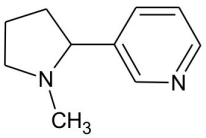
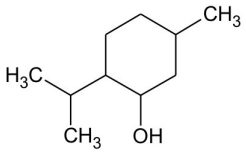
Chemical	Structure	Example of origin
Allyl isothiocyanate		Wasabi, horseradish, mustard oil
Allicin		Garlic
Cinnamaldehyde		Cinnamon
Isoflurane		Anaesthetic
Propofol		Anaesthetic
Acrolein		Vehicle exhaust fumes, tear gas
Δ9 tetrahydrocannabinol		Marijuana
Nicotine		Tobacco
Menthol		Mentha piperita

Table 1: *Selection of TRPA1 agonists.*

Beside its important role as a chemosensor, TRPA1 has been initially described as molecular sensor for noxious cold (<17 °C) (Bandell et al. 2004). Although its thermosensitivity has been over the time questioned, several papers have brought evidence for direct activation of TRPA1 by cold. Karashima et al. (2009) showed that heterologously expressed TRPA1 is activated by cold in a Ca^{2+}-independent manner. Subsequent study (Camino et al. 2010) implied that in vivo TRPA1 has minor role in acute cold sensation but it is a key mediator of cold hypersensitivity in pathological conditions. The clear evidence that the TRPA1 is intrinsically cold sensitive provided the recent study (Moparthi et al. 2014) of purified hTRPA1 inserted into artificial lipid bilayers. It moreover showed that the N-terminus ARD (1–688) is not required for activation by cold.

There is another TRPA1 particularly interesting activator, zinc. It is an essential heavy metal required for the structure or function of over 300 proteins. Despite the role of free zinc as a signaling molecule, high concentrations of zinc have cytotoxic effects and an overexposure to zinc can cause pain and inflammation through unknown mechanisms. Hu et al. (2009) showed that zinc activates TRPA1 through unique mechanism that requires zinc influx through TRPA1 channels and subsequent activation via specific intracellular cysteine and histidine residues. TRPA1 is highly sensitive to intracellular zinc, as low nanomolar concentrations activate TRPA1 and modulate its sensitivity.

2.9 TRPA1 and calcium association

Calcium ions are crucial for proper physiological function of TRPA1. Their importance was recognized together with TRPA1 identification (Story et al. 2003) back in 2003. Positively charged calcium ions and negatively charged phosphate ions are the two primary signaling elements in the cells (Clapham 2007). Whereas extracellular Ca^{2+} concentrations are around 2 mM, intracellular concentrations are kept around 100 nM and cells invest much of their energy to maintain this 20 000-fold gradient.

Although TRPA1 is often referred to as nonselective channel, its favouring of calcium is noticeable. The putative selectivity filter contains the aspartate residuum (D918) which controls Ca^{2+} permeability of the channel (Wang et al. 2008).

Combined patch-clamp and Fura-2 fluorescence measurements revealed that the fraction of TRPA1 inward current that is carried by Ca^{2+} is much higher than in case of a nonselective channel (Karashima et al. 2010). This phenomenon probably arises from a partial block (~35 %) of the monovalent cation current caused by binding of Ca^{2+} in the lumen of the pore.

The TRPA1 conductance and opening probability are also directed by Ca^{2+} concentration. The single channel conductance of TRPA1 in Ca^{2+} free solution is around 110 pS and decreases approximately to its half (60 pS) when the Ca^{2+} is added to the solution (Karashima et al. 2010). TRPA1 surface expression levels are dynamically controlled by agonist exposure. The increase of TRPA1 surface levels in the presence of agonist mustard oil is not observed in Ca^{2+} free solutions, suggesting crucial role of Ca^{2+} in TRPA1 trafficking to plasma membrane (Schmidt et al. 2009).

Calcium ions interacting with TRPA1 channels originate in two destinations. One of the sources is intracellular stores. This Ca^{2+} reaches only the parts of TRPA1 that are exposed to cytoplasm. Other calcium ions originate in extracellular space. These ions have access to extracellular part of the channel and, after passing through the channel pore, also to intracellular side.

The kinetics of TRPA1 agonist- and voltage- induced currents strongly depends on Ca^{2+} concentration. Typically Ca^{2+} first potentiates the TRPA1 current response and then causes inactivation, which is almost complete and irreversible. Wang and col. were first who showed that these processes are independent (Wang et al. 2008). The elevation of intracellular Ca^{2+} causes the Ca^{2+}-dependent potentiation. The Ca^{2+}-dependent inactivation follows only if the Ca^{2+} is also present in extracellular solution. This suggests that Ca^{2+}-dependent inactivation requires Ca^{2+} entry.

Potentiation and inactivation of TRPA1 by Ca^{2+} could be facilitated by binding of Ca^{2+} either to specific intracellular domain within TRPA1 or to other proteins regulating TRPA1. The most obvious candidate, the calmodulin, was excluded (Zurborg et al. 2007, Doerner et al. 2007) very soon. Little more complicated was the story of phosphatidylinositol 4,5-bisphosphate (PIP_2). Depletion of PIP_2, which follows the elevation of intracellular Ca^{2+}, regulates activity of many TRP channels (TRPV1, TRPM8 etc. see Rohacs and Nilius 2007). Although several studies supported the role of PIP_2 in TRPA1 modulation (Rohacs 2009) the newly developed methods for PIP_2 level manipulation revealed that agonist responses of

TRPA1 are not significantly influenced by changes in PIP_2 levels (Wang et al. 2008, Nilius et al. 2012). Therefore the PIP_2 was also ruled out as a mediator of Ca^{2+}-dependent potentiation and inactivation of TRPA1.

Other candidates for a direct calcium binding site are negatively charged residues on the intracellular side of TRPA1. The sequence motif resembling EF-hand motif is located in the N-terminus of TRPA1 (Hinman et al. 2006). Pharmacological and site-directed mutagenesis indicated that this motif is involved in Ca^{2+}-dependent activation (Doerner et al. 2007). However, further study showed that residues in a putative EF-hand neither play a critical role in Ca^{2+}-dependent potentiation nor inactivation of TRPA1. (Nilius et al. 2011b, Wang et al. 2008) Even structural predictions consider the EF-hand motif among ankyrin repeats to be strange. Structural comparisons of calmodulin EF-hand like motif and the ankyrin repeat finger suggest that these two structures are mutually exclusive (Gaudet et al. 2008). Moreover, a deletion of the whole EF-hand like domain renders the channel non-functional (Nilius et al. 2011b). Together these findings exclude the EF-hand like motif to be the calcium binding site which modulates TRPA1 directly. Consequently new search along TRPA1 protein was started.

3 Aim of the Thesis

- Functionally characterize the C-terminus of TRPA1 receptor by mapping the impact of charged neutralizing point mutations of basic residues on TRPA1 channel gating.

- Select TRPA1 C-terminus regions which are promising candidates for functionally important domains and study them by introducing further residues substitutions.

- Elucidate the function of the distal part of TRPA1 C-terminus using truncation mutants.

- Determine how the highly acidic cluster in the TRPA1 C-terminus is involved in calcium-dependent modulation of this channel.

- Use the homology modeling and molecular dynamic simulations to verify proposed interactions between the acidic cluster and a calcium ion.

- Clarify the structural basis for TRPA1 related channelopathy, caused by gain-of-function mutation N855S.

4 Materials and methods

4.1 Chemicals and solutions

All chemicals were prepared using deionised water, which was for molecular biological methods further purified by Simplicity 185 device (Millipore, USA) and sterilized. If not stated otherwise, all chemicals were purchased from Sigma-Aldrich.

The extracellular control solution (ECS)

The cells were during experiments washed by one of the following control extracellular solutions:

ECS for measuring whole-cell currents and current-voltage relationships contained: 160 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES and 10 mM glucose. The solution was adjusted to pH 7.3 with NaOH. The final osmolarity was 320 mOsm.

ECS for experiments with precise Ca^{2+} concentration contained: 150 mM NaCl and 10 mM HEPES, with an added 2 mM HEDTA for the Ca^{2+}-free solution, and 2 or 10 mM $CaCl_2$, for the Ca^{2+}-containing solutions, adjusted to pH 7.3 with NaOH, 300 mOsm.

The intracellular solution (ICS)

ICS for whole-cell patch-clamp experiments solution contained: 125 mM Cs-gluconate, 15 mM CsCl, 5 mM EGTA, 10 mM HEPES, 0.5 mM $CaCl_2$ and 2 mM MgATP. The solution was adjusted to pH 7.3 with CsOH. The final osmolarity was 286 mOsm.

For experiments with precise Ca^{2+} concentration high buffer ICS was used. It contained: 145 mM CsCl, 5 mM EGTA, 3 mM $CaCl_2$, 10 mM HEPES, 2 mM MgATP, pH 7.3, adjusted with CsOH, 320 mOsm. The pipette solution containing

100 μM free Ca^{2+} was obtained by adding 10.24 mM Ca^{2+} and 10 mM EGTA to the internal solution.

Agonists

An allyl isothiocyanate (AITC) solution was prepared from a 0.1 M stock solution in DMSO stored at 4 °C. AITC solution at concentration 200 μM or 100 μM were used for application on the cells containing 0.2 % or 0.1 % of DMSO, respectively.

Cinnamaldehyde (Cin) solution was prepared prior to use from a 0.1 M stock solution in DMSO stored at 4 °C. A 100 μM Cin solution was used for application on the cells containing 0.1 % of DMSO.

4.2 Cell cultures and transfection

Human embryonic kidney cells (HEK293T) (ATCC, USA) were used in all experiments as an expression system. The cells were cultured in Opti-MEM I medium (Invitrogen) supplemented with 5 % fetal bovine serum (PAN Biotech GmbH, Aidenbach, Germany) in incubator at 37 °C and 5 % concentration of CO_2 establishing proper pH in cultivating solution. The cells were grown in multi-well cell culture plate covered by colagen (SERVA Electrophoresis GmbH, Heidelberg, Germany) and poly-L-lysine for better adhesion of cell membranes to the surface.

Only the cells that reached 80–90 % confluency 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) using the magnet-assisted transfection (IBA GmbH, Gottingen, Germany) method. Green fluorescent protein (GFP; TaKaRa) in the amount of 300 ng was always co-transfected as a marker for transfection efficiency. After the transfection, the rest of transfection solution was washed with phosphate buffered saline (PBS) buffer containing: 137 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 4.3 mM Na_2HPO_4 adjusted to pH 7.3 with NaOH. Then the cells were passaged using 0.2 % solution of trypsin (Invitrogen, Carlsbad, CA, pH=7.3) and transferred to new plates with measuring cover slip prepared at the bottom. The cells were used for experiment 24–48 hours 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.

4.3 Site-directed mutagenesis

Constructs of hTRPA1 channel were made using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, USA). For each mutation the primer of length about 30 amino acids was designed and synthesized (by VBC-Biotech, Vienna, Austria). As the wild-type template served human TRPA1 (in the pCMV6-XL4 vector, OriGene). Only for construction of C-terminally GFP-tagged D1080A mutant was used as a template C-terminally GFP-tagged human TRPA1 (in the pCMV6-AC-GFP vector; OriGene). The polymerase chain reaction (PCR) was performed using mix of oligonucleotides (Invitrogene, Life Technologies, USA) in Thermo-cycler device (Eppendorf, Hamburg, Germany). After the PCR was completed, the template DNA was eliminated by enzymatic digestion with a restriction enzyme DpnI which is specific for methylated DNA. The PCR product was detected using horizontal electrophoresis with 1 % agarose gel (70 mV, 50 min). According to the above-mentioned kit manual, the XL10-Gold ultracompetent cells were transformed by 2 µl PCR product in heat shock protocol. The cells were then cultured on agar plates and subsequently in Lysogeny Broth (LB) medium over night at 37 °C in shaking incubator. The DNA from multiplied bacterial cells was isolated using Plasmid DNA Miniprep Kit (Life Technologies). The presence of the intended mutation was then confirmed by sequenation on ABI Prism 3100 Genetic Analyzer device (Institute of Microbiology, Academy of Sciences of the Czech Republic).

4.4 Electrophysiology

Whole-cell membrane currents were recorded from HEK293T cells using patch clamp technique. The measuring equipment consisted of an inverted microscope Eclipse TS100 (Nikon) placed on antivibration table TMC (Technical Manufacturing Co.), an Axopatch 200B amplifier (Axon Instrument, USA), a digitizer for low-noise experiments Digidata 1440A (Molecular Devices, USA) and computer with pCLAMP 10 software (Molecular Devices, USA) installed. Patch electrodes were pulled from a borosilicate glass tube with a 1.65 mm outer diameter by employing a horizontal puller P-1000 (Sutter Instrument Company, USA). The tip of the pipette

was heat-polished. Its resistance was 3–5 MΩ. The glass microelectrodes were filled with intracellular solution and placed in a holder controlled by micromanipulator MP-255 (Sutter Instrument Company). A silver/silver chloride (Ag/AgCl) reference electrode was submerged into the extracellular bath (Fig. 8). Series resistance was compensated by at least 70 % in all recordings. The experiments were performed at room temperature (23–25 °C). A system for rapid superfusion of the cultured cells was used for drug application (Dittert et al. 2006). Capillary of the application system is placed at a distance of less than 100 µm from the surface of the examined cell. For continuous measurements the membrane potential was hold at -70 mV, if it is not stated otherwise.

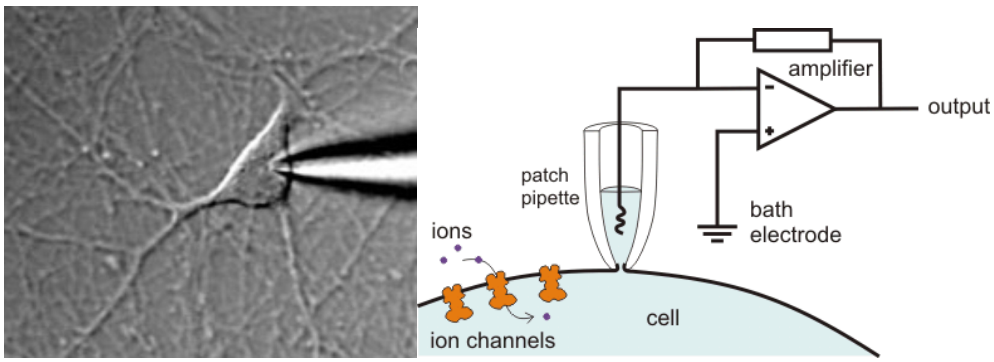


Figure 8: *Patch clamp scheme. Left: Cultured cell under whole-cell patch-clamp conditions. Right: Whole cell configuration enables to record currents through multiple channels at once, over the membrane of the entire cell.*

4.5 Fluorescence measurements

For membrane-targeting experiments, we used the C-terminally GFP-tagged mutant D1080A of hTRPA1 (wild-type in them pCMV6-AC-GFP vector; OriGene) and the cyan fluorescent protein-tagged pleckstrin homology domain of phospholipase C δ1 (kindly provided by Tamas Balla, NICHD, National Institutes of Health, Bethesda, MD). For fluorescence measurements, we used the Cellˆ R imaging system based on an Olympus IX-81 inverted microscope (Olympus) equipped with a dual emission setup (Dual-View Optical Insights), a Polychrome V polychromator (Till Photonics), and a Hamamatsu Orca ER camera (Hamamatsu Photonics). Intensity profiles were measured using the program ImageJ (National Institutes of Health).

4.6 Homology Modeling and Molecular Dynamics Simulations

The template structures for homology modeling were obtained from the Protein Data Bank. For the calcium binding motif the structure of human BK channel (code 3MT5) was used as a template (Yuan et al. 2012). A model of the transmembrane part of human TRPA1 was based on the Kv1.2-2.1 paddle chimera template structure (code 2R9R, Long et al. 2007). TRPV1–TRPA1 chimeric structure was based on high-resolution cryo-EM structure of apo TRPV1 (code 3J5P).

The sequence alignments were performed using ClustalW software (Larkin et al. 2007) and carefully manually adjusted to ensure the appropriate positions of conserved residues and functional sites. Transmembrane helices positions were predicted using TOPCONS software (Bernsel et al. 2009) or extracted using DSSP algorithm (Kabsch and Sander 1983).

Candidate homology models (10–20 structures) were built with the software package MODELLER (Eswar et al. 2006, Martí-Renom et al. 2000, Šali et al. 1995). The best structure was selected according to the MODELLER objective function, deviation of unmodified parts of the channel from the template (in case of chimeric models) and visual inspection. An all-atom structures and topology files were generated using VMD (Humphrey et al. 1996) or the program LEaP from the AMBER suite (Case et al. 2010) and TIP3P water model (Jorgensen et al. 1983). Forces were computed using the Amber99SB force field or the CHARMM 27 force field for proteins, lipid and ions (Beglov and Roux 1994, MacKerell et al. 1998, Schlenkrich et al. 1996). Molecular dynamic simulations were produced using the software package NAMD (Phillips et al. 2005) or ACEMD (Harvey et al. 2009) running on local workstations equipped with NVIDIA graphic processing units. Molecular dynamic trajectories were visualized using VMD (Humphrey et al. 1996).

4.7 Analysis of electrophysiological data

The electrophysiological data were analysed using pCLAMP 10 (Molecular Devices). Curve fitting and statistical analyses were performed using SigmaPlot 10 (Systat Software). Statistical significance was determined by Student's t-test or analysis of variance as appropriate. Differences were considered significant at $P < 0.05$, if not stated otherwise.

Voltage-induced activation

Voltage-dependent gating parameters were estimated from steady-state current-voltage (I-V) relationships obtained at the end of 60 or 100 ms voltage steps from −80 to +200 mV in increments of +20 mV (see Fig. 9) by fitting the conductance $G=I/(V-V_{rev})$ as a function of the test potential V to the Boltzmann equation:

$$G = \frac{G_{max} - G_{min}}{1 + exp[\frac{-zF}{RT}(V - V_{1/2})]} + G_{min}$$

where z is the apparent number of gating charges, $V_{1/2}$ is the half-activation voltage, G_{min} and G_{max} are the minimum and maximum whole-cell conductance, V_{rev} is the reversal potential, and F is Faraday's constant, R is the gas constant, and T is temperature.

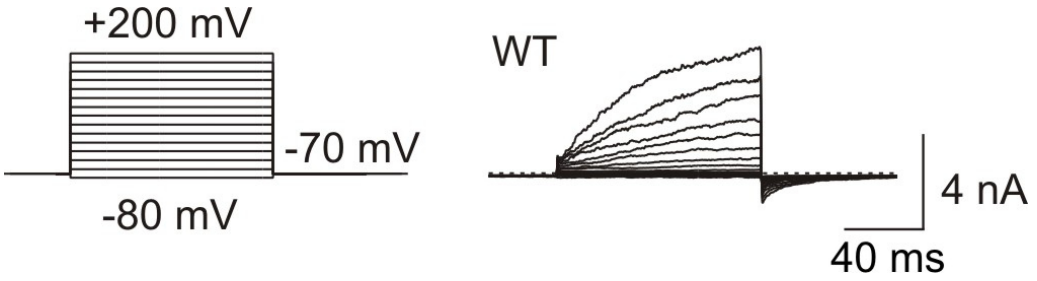


Figure 9: *Voltage stimulation protocol. Left: Scheme of voltage step protocol recorded approximately 1 minute after whole-cell formation. Right: Representative current trace of wild-type channel in response to voltage step protocol in control extracellular solution.*

The half-activation voltage $V_{1/2}$ was individually determined for every mutated channel and wild-type channel to compare apparent voltage sensitivity. The lower is the value of $V_{1/2}$ the bigger is the apparent voltage sensitivity of the mutated

channel. The shift of $V_{1/2}$ can be caused either by functional changes of a voltage sensor or by a more complex intervention in the gating mechanism of ion channel.

All data is presented as means ± the standard error of the mean. The activation time constant τ_{on} for outward currents was measured at +200 mV and obtained by fitting a monoexponential function to the current traces.

Agonist-induced activation

The current response after exposure to the supersaturating concentration of agonist (200 μM AITC) was continuously measured at holding potential -70 mV in the whole-cell configuration. The responses of wild-type TRPA1 channels are multiphasic. An initial gradually increasing phase is followed after 10–20 s by a steeper (calcium-dependent) secondary activation phase and then the current declines back to the baseline as channels inactivate. The activation was characterized by a value of maximal current amplitude at the peak, the inactivation by the time constant T_{50} representing time from peak to the point where current reaches the half of its maximum (see Fig. 10).

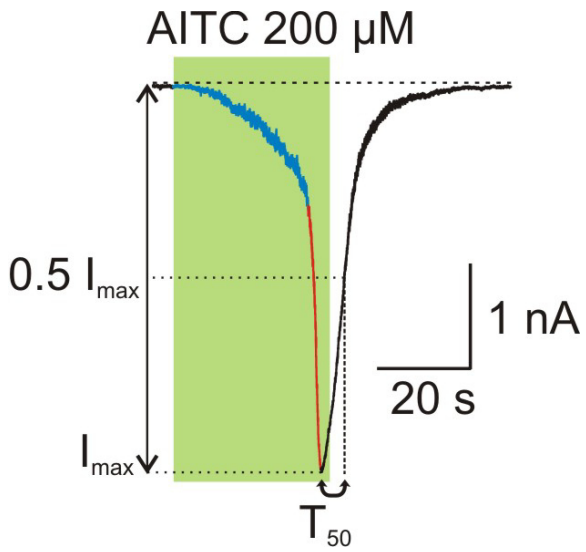


Figure 10: *Agonist-induced activation. Continuous measurement of wild-type TRPA1 channels at holding potential -70 mV in control extracellular solution containing 1 mM Ca^{2+}. Green rectangle indicates the duration of agonist (200 μM AITC) exposure. Note the multiphasic response. The first activation phase is drawn blue, the steep secondary phase is in red and the inactivation phase with marked T_{50} is drawn black.*

Calcium-induced potentiation

The TRPA1 activation characteristics are severely regulated by Ca^{2+} ions. The initial agonist activation is first by calcium presence augmented and then inhibited in the process of self-modulating feedback loop. To explore these processes, we utilized the stimulation protocol previously used by Wang el al. (2008). We measured whole-cell membrane currents in absence of extracellular Ca^{2+}. Intracellular Ca^{2+} was buffered with 5 mM EGTA in the patch pipette to assess the effects of permeating calcium ions. The membrane potential was ramped from -80 to +80 mV (1 V/s). First, the channels were activated by 40 s exposure to agonist (100 μM cinnamaldehyde or 100 μM allyl isothiocyanate), then the agonist was washed out for 10 s and Ca^{2+} at a concentration of 2 mM or 10 mM was added to the extracellular solution (see Fig. 11).

The cinnamaldehyde (a partial agonist of TRPA1) evoked slowly developing currents that slightly relaxed upon washout. Subsequent addition of the Ca^{2+} induced pronounced potentiation followed by an inactivation which was almost complete within 1 min. The calcium induced potentiation was quantified as the fold increase in the amplitude after the addition of Ca^{2+} with respect to the preceding current level. The 10–90 % rise time was also evaluated and showed to be clearly dependent on the external Ca^{2+}. The higher concentration substantially accelerates the potentiation. The Ca^{2+}-dependent inactivation was characterized using a half-decay time, relative to peak (T_{50}). Once more, higher concentration of calcium provoked faster inactivation, most likely due to concomitant reduction in the degree of Ca^{2+} induced potentiation.

On the other hand, the allyl isothiocyanate as the full TRPA1 agonist elicited rapidly developing membrane currents, which obviously reached the saturation within the given 40 s. The AITC induced activation was described by the mean time constant τ_{on}. The subsequent addition of Ca^{2+} to the bath solution induced only inactivation, characterized by percentage decline of the current at 160 s.

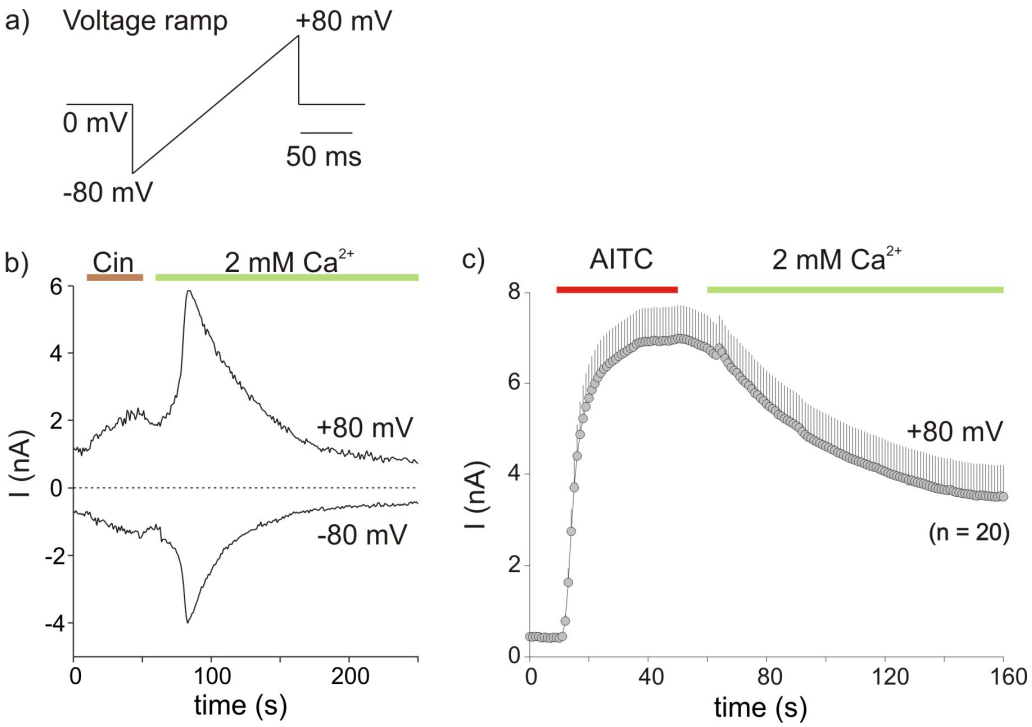


Figure 11: *Calcium-induced potentiation. a) The shape of stimulus voltage ramp that was applied every second to the tested cell. b) Time course of representative whole-cell currents through wild-type human TRPA1 measured at +80 mV and -80 mV as indicated. The application time of 100 μM Cin (partial agonist) and following addition of calcium is depicted above the graph. c) Average current at +80 mV from 20 cells expressing wild-type human TRPA1 upon activation of full agonist 100 μM AITC. The data represents the means ± S.E. Note that AITC in this case activates channels close to saturation and addition of Ca^{2+} did not produced noticeable potentiation. Instead the channels undergo inactivation, which is slow and only partial due to the covalent nature of AITC interaction with TRPA1.*

5 Results and discussion

5.1 C-terminal basic residues

TRPA1 is generally considered as a promising target for development of analgesic and anti-inflammatory drugs. Despite the intensive work on this topic in recent 10 years it seems that we are still at the beginning of the journey to fully understand the complexity of TRPA1 properties. We add a piece to the puzzle by characterization of C-terminal tail, whose functional importance was until then overlooked.

We were directed towards the C-terminal tail by a prior study (Benedikt et al. 2009) from our laboratory, which showed a great impact of mutations in the predicted inner vestibule of the TRPA1 channel on several aspects of channel functioning including changes in the voltage-dependent activation/deactivation kinetics and a significant increase in the current variance at depolarizing potentials (for summary, see Enclosure 3). Keeping in mind the unresolved question about the effect of PIP_2 on TRPA1 channel we decided for the initial screening of C-terminus with focus to basic residues. They may represent interaction partners for negatively charged ligands such as phosphoinositides or inorganic polyphosphates and might also act as a sensor for changes in transmembrane voltage. TRPA1 is weakly voltage sensitive, but its fourth transmembrane segment (S4) lacks the charged residues, that are proved to be essential for membrane voltage sensing in canonical voltage-gated channels. Thus, the location of TRPA1 voltage-sensing domain remains unknown and the charged residues along the C-terminal tail has to be considered as a potential part of such domain.

There are 27 basic residues along the C-terminal tail of human TRPA1. We used site-directed mutagenesis to individually neutralize all of them. The HEK-293T cells were transiently transfected to express these mutated TRPA1 channels. Whole-cell patch-clamp recording was used to characterize their phenotypes. We were curious whether there are any changes: In agonist-dependent gating (using the supersaturating concentration of strong TRPA1 agonist AITC) or/and in voltage-dependent gating (using the voltage step protocol from -80 mV up to +200 mV in steps of +20 mV). The electrophysiological measurements (see Enclosure 1) pointed

to a limited number of residues and two regions in C-terminal tail that are indeed critically involved in allosteric regulation of the TRPA1 channel.

- **Lysine 969**: The first basic residue at C-terminal tail K969 showed remarkable phenotype when it was replaced with alanine, isoleucine, arginine or glutamate. The agonist induced activation of wild-type TRPA1 at holding potential -70 mV is multiphasic with an initial gradually increasing phase followed by a steeper (Ca^{2+}-dependent) secondary phase that occurred 10–20 s after AITC is applied to the cell for the first time. The steep secondary phase of activation is typically followed by Ca^{2+}-dependent inactivation. The charge-swapping mutation K969E totally abolished the gradually increasing phase of activation, whereas the K969R, K969I and K969A mutations, on the contrary, substantially prolonged it. From further measurement (see Enclosure 1) we concluded that K969 plays on important structural role in TRPA1 channel gating. This finding was recently supported by 3D structure of TRPV1 channel, where the proximal C-terminal helix was identified to be an essential structural element, which probably provides a physical substrate through which stimuli allosterically affect pore conformation (Liao et al. 2013).

- **The most proximal helix (H1)**: It has been proposed that the key pore helix residue, L906, plays an essential role in responding to the voltage-dependent gating in case of TRPA1 channel (Wan et al. 2013). Mutations at this position alter the activation/inactivation responses to voltage, probably due to altered responses to voltage sensor movements. This finding directs the search for voltage sensor to the close proximity of S6 helix, which is in close contact with pore helix and can easily sense and transfer the conformational changes from voltage sensor to channel gate/s. Intriguingly, in 3D structure of TRPV1 channel there is a sharp bend after S6 and the most proximal C-terminal helix runs parallel to the inner leaflet of the membrane (Liao et al. 2013). This raises the possibility that the charged residues within the most proximal C-terminal helix are situated properly to feel changes of transmembrane voltage. In our study (Enclosure 1) we described three residues situated on the same (more hydrophobic) side of the first proximal helix R975, K988 and K989. Neutralization of these residues resulted in ‘gain-of-function’ mutants (R975A, K988A and K989A) which exhibited higher sensitivity to depolarizing voltages. Together with above mentioned latest structural findings we can nowadays

summarize that it is very likely that these residues are included in the electric field of the membrane and may directly cooperate on voltage sensing.

- **Predicted H4 helix**: A second significant region was found in the middle of C-terminal tail, where secondary structure is predicted to be helical (helix H4, see Enclosure 1). This is supported by our finding that two mutated residues K1048A and K1052A, which would be neighbours on α-helix, exhibited the same behaviour. They did not produce measurable currents in response to AITC at a holding potential of −70 mV neither exhibited voltage-dependent currents (up to +200 mV in the absence or presence of AITC). On the other hand the R1050A situated between them (and on the opposite side of predicted helix) exhibited wild-type like phenotype in all tested aspects. At the end of H4 helix, there is located K1071, whose neutralization led to disrupted agonist induced responses. The first gradually increasing phase of AITC induced response was apparently prolonged and immediately followed by inactivation. The steep secondary phase of activation never occurred, indicating a failure of calcium-dependent activation pathway. Curiously, the similar phenotype was obtained by neutralizing of residue K1092, which is separated from K1071 by negatively charged loop. These findings bring us on the track of the calcium binding site. We focused on this region of the C-terminal tail in our following work (Enclosure 2).

5.2 Truncation of the C-terminus

Once we established that C-terminus is a key modulatory domain for TRPA1 channel activation, we asked ourselves the following question: How long the C-terminus needs to be to preserve proper physiological function? The answer was found introducing the stop codons at E1094 and N1100, resulting in two C-terminal truncation mutants, TRPA1-Δ26 and TRPA1-Δ20. Whereas truncation of the C-terminus by 26 residues renders the channel non-functional (TRPA1-Δ26 was not sensitive to any of the stimuli tested), the TRPA1-Δ20 truncation mutant was functional, but with altered phenotype.

Compared to wild-type channel the TRPA1-Δ20 truncation mutant exhibited slower Ca^{2+}-dependent inactivation. For wild-type channel, after previously exposed

to agonist in Ca^{2+} free conditions, the introduction of 2 mM Ca^{2+} induced the potentiation that was followed by an inactivation, which reached the 50 % of its peak value in 31.9 ± 4.0 s at + 80 mV and in 18.9 ± 2.0 s at - 80 mV (n = 19). Whereas for TRPA1-Δ20 truncation mutant the half-decay time at + 80 mV was almost 3-fold slower (T_{50} of 89.0 ± 18.4 s, n=6), it exhibited a normal degree of Ca^{2+}-induced potentiation and a normal responsiveness to voltage and Cin (see Enclosure 2). These results further supported our presumption that negatively charged region around D1080 can serve as a high affinity Ca^{2+} binding site.

5.3 Gain-of-function mutants within Acidic cluster of TRPA1 C-terminus

Lysine 1071, the residue above described to be involved in Ca^{2+} modulation, is located at the very beginning of a region prominently rich on acidic residues. Highly conserved stretch E1077–D1082 (ETEDDD) shares substantial sequence similarity with the Ca^{2+}-binding domain found in the human bestrophin-1 (hBest1) channel (Xiao et al. 2008) or the so-called Ca^{2+} bowl domain of the superfamily of BK channels (Yuan et al. 2010). In an effort to explore the function of this acidic cluster, we individually mutated all aspartates and glutamates to alanines and lysines (Enclosure 2). In addition, we constructed mutations E1077K and E1079K. To our surprise, we detected dramatic increase of outward voltage-induced currents through the E1077A and E1077K mutant channels. We reasoned that this sensitizing effect might reflect either a gain-of-function (constitutively active) phenotype or tonic activation due to an increased expression of the mutant channels on the cell surface (see more about gain-of-function mutations in Enclosure 3).

In fact, the very similar acidic cluster was described to serve as phosphorylatable sorting motif of a large number of ion channels including channels from TRP family (e.g. TRPC3, TRPV4) (Köttgen et al. 2005). We studied this possibility using additional amino acid substitutions of S1076 and T1078, both predicted to be phosphorylated by casein kinase CK2. The alanine and aspartate mutations were used to mimic the nonphosphorylated and phosphorylated forms of the protein, respectively. We performed a series of experiments, which detected altered phenotypes, but we were able to conclude that this particular part of TRPA1

seems to be unlikely involved in trafficking to the membrane. Moreover we identified one more gain-of-function mutant T1078D whose sensitivity to membrane voltage was increased while its responsiveness to Cin remained unchanged. While T1078D is located in the primary sequence far from the membrane proximal regions it is unlikely a part of voltage sensor and the observed changes in its voltage modulation are most likely caused by altered allosteric coupling between the activation sites (voltage sensor, Ca^{2+} sensor) and movement of the gate. This makes our region of interest very likely to accommodate yet unidentified important regulatory domain.

5.4 Acidic cluster of TRPA1 C-terminus

The ability of the TRPA1 acidic cluster to form the high affinity Ca^{2+} binding site was explored by utilizing the methods of structural bioinformatics. The homology modeling was used to replace the acidic stretch in the structure of the Ca^{2+}-binding domain of BK channel with acidic cluster from human TRPA1 (Enclosure 2). Subsequent molecular dynamic simulations proved the ability of the acidic cluster from the TRPA1 to form a Ca^{2+}-binding domain and in addition illustrated the possible structural layout of calcium-binding pocket. Two residues, D1080 and D1082, are predicted to use oxygen atoms from their side chains for direct contact with the calcium ion, whereas I1074 and E1077 are in contact with the calcium ion via their main chain carbonyl oxygen atoms.

These results are in good agreement with our experimental data. We used the same stimulation protocol as is above described for truncation mutants (see chapter 5.2 and Fig. 11) also for charge-neutralized mutants and find out, that kinetics of Ca^{2+}-dependent potentiation were dramatically changed in four out of the six mutants: E1073A, D1080A, D1081A and D1082A. The most affected was the D1080A mutant. Nearly half of the D1080A-expressing cells tested (n=17) exhibited no Ca^{2+}-induced potentiation, instead, the Cin-induced currents decayed in the presence of Ca^{2+} to their initial value obtained before the agonist was applied to these cells. This seems to be not caused by an altered surface expression, which has been confirmed by the fluorescence images of the cells expressing D1080A mutant of the C-terminally GFP-tagged human TRPA1. Instead, our results suggest that this

mutation most likely has an effect on Ca^{2+} affinity. The increase of internal concentration of Ca^{2+} from 150 nM, that mimic the physiological level, to 100 μM improved the Ca^{2+}-dependent potentiation of D1080A resembling the responses of wild-type (with 150 nM Ca^{2+} internally).

Collectively, our findings identify the conserved acidic motif in the C-terminus that is actively involved in TRPA1 modulation by Ca^{2+} and may represent its long-sought Ca^{2+}-sensing domain. Interestingly, recent thorough analysis of human-rattlesnake TRPA1 chimeras suggests that a predominant locus of calcium-dependent desensitization is centered on ankyrin repeat (AR) 11 at N-terminus, utilizing the fact, that rattlesnake TRPA1 does not exhibit Ca^{2+}-dependent desensitization (Cordero-Morales et al. 2011). However, Cordero-Morales et al. ruled out the simple model in which calcium binds directly to negative charges differentially present in the region between AR 7–14 of human TRPA1. They converted all aspartate residues (8 in all), which are exclusively present in human TRPA1 and absent in rattlesnake TRPA1, to alanine and found minimal effect on AITC-evoked activation and desensitization. This finding is particularly interesting when compared to our results from the C-terminus, where single mutation of aspartates caused significant changes in Ca^{2+} modulation and the double mutation (whether E1077Q/E1079Q, E1079Q/D1081N, or D1080N/D1082N) was sufficient to render the channel non-functional. The possible explanation, why alternation in N-terminal ARs causes defect in Ca^{2+} desensitization, comes from structural insight borrowed from TRPV1 3D structure (Liao et al. 2013), where the part of AR domain from one subunit interacts with C-terminus of an adjacent subunit, packing the cytosolic part of the channel together. The Ca^{2+}-dependent desensitization thus may arise from a subunit-subunit interaction.

5.5 Inter subunit salt bridges may underlie structural bases of heritable pain syndrome - Structural modeling

Recently Kremeyer et al. (2010) provided evidence that variation in the *trpa1* gene can alter the pain perception in humans. Several members of one Colombian family suffer from episodes of severe upper body pain triggered by fasting or fatigue (for insight, see Enclosure 3). This, so far only described TRPA1 channelopathy, is

caused by single amino acid substitution of an asparagine by a serine (N855S) in the S4–S5 linker of TRPA1. Although original paper described the functional impact of this mutation, the question why such modest substitution has so dramatic impact for affected individuals remained open.

To answer it, we built a homology model of transmembrane part of human TRPA1 based on the Kv1.2-2.1 paddle chimera template and ran molecular dynamic (MD) simulations (for details, see Enclosure 4). They revealed that the directly neighbouring residuum of N855, the E854, takes a part in salt bridge with K868 of an adjacent TRPA1 subunit (Fig. 12). Therefore, we followed the staring hypothesis that the functional changes of N855S mutant may originate, at least in part, from changes in inter-subunit interactions.

Although the overall sequence identity between the transmembrane parts of TRPA1 and TRPV1 is not high (~20 %), in the S4–S5 linker region it reaches almost 50 %. In fact, in TRPV1 structure, the obviously similar inter-subunit interaction can be also observed (Liao et al. 2013). In TRPV1, the interacting residues are R579 and Q561 from adjacent subunits, which correspond to N855 and R872 in TRPA1. To investigate whether the altered phenotype of N855S mutant is caused by inter-subunit interactions involving cognate residues in TRPA1, we built another homology model based directly upon the unliganded (apo) structure of TRPV1, only with S4–S5 linker (Q560–R575) swapped for homologous region E854–K868 from TRPA1. During the MD simulations performed with this model inter-subunit salt bridges E854–K868 were not formed spontaneously, yet results based on this model supported the proximity and mutual orientation of the E854 and K868 residues.

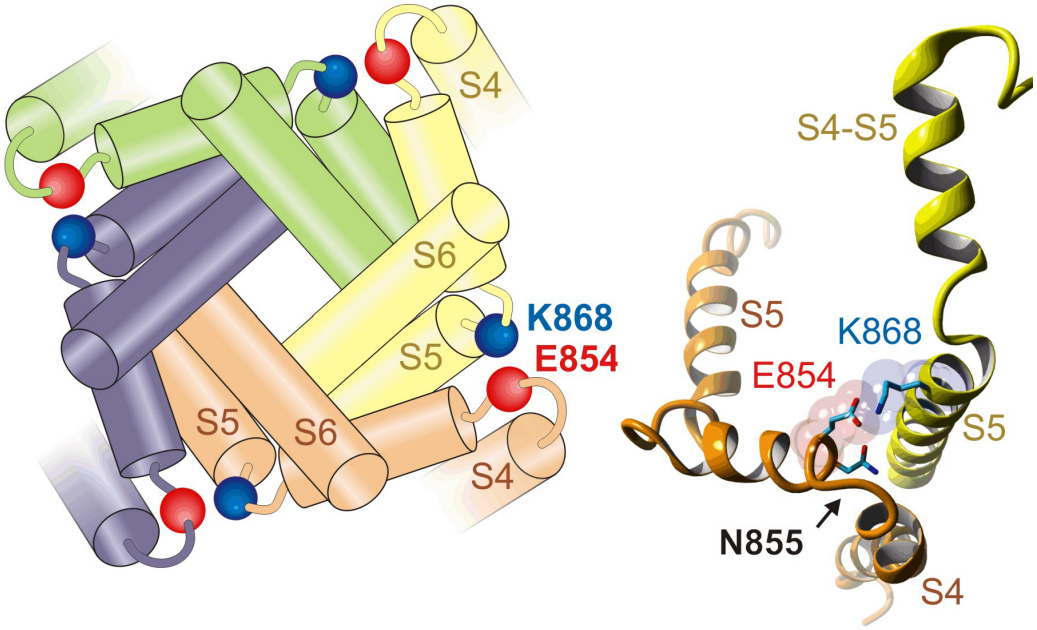


Figure 12: *Inter-subunit interactions of TRPA1 transmembrane domain. Left: Cartoon of bottom view on TRPA1 pore domain. Cylinders represent α-helixes. Each TRPA1 subunit is drawn in different colour. Blue and red balls indicate position of residues K868 and E854. Between them, inter subunit salt bridges are predicted. Right: Snapshot of homology model with depicted residues of interest. Note that N855 (mutation N855S is responsible for heritable human pain disorder) lay in immediate vicinity of predicted salt bridge between E854 and K868.*

5.6 Inter subunit salt bridges may underlie structural bases of heritable pain syndrome - Electrophysiological measurements

Parallelly to the theoretical modeling, we investigated our hypothesis using whole-cell patch clamp recordings from transiently transfected HEK293T cells (Enclosure 4). We constructed the charge-neutralizing (E854A, K868A) and the charge-reversing (E854R, K868E) mutants, and the charge-swapping double mutant (E854R/K868E) of human TRPA1 and characterized their phenotypes in order to find out whether the predicted inter-subunit interactions contribute to the TRPA1 channel functioning. The voltage-dependent gating was tested by a series of voltage steps from -80 mV to +200 mV. The chemical sensitivity was measured using the similar protocol as was described above for truncation mutants (see chapter 5.2 and Fig. 11) except this time, the full agonist allyl isothiocyanate (AITC, 100 µM for 40 s) was used for activation.

Mutations of K868 produced channels with an increased basal conductance at negative potentials and were virtually independent of the applied voltage. Agonist stimulation elicited much smaller responses indicating that the K868 is involved in coupling of different stimuli and important to stabilize the closed conformation of the channel. The single mutations of E854 also produced channels with impaired voltage- and agonist-induced gating. However, the introduction of both mutations together (double mutant E854R/K868E) significantly rescued the channel function supporting the hypothesis about spatial proximity of these two sites.

We further investigated how fragile is the electrostatic balance on the S4–S5 linker by constructing additional charge-neutralizing or charge-swapping mutants. Among them (for thorough list, see Enclosure 4), we detected mutant R852E with a rare gain-of-function phenotype. The half-maximal activation voltage was significantly shifted towards less depolarizing potentials (98 ± 2 mV versus 127 ± 2 mV for wild-type) resulting in increased basal channel activity and close-to-saturation chemical responses. Intriguingly, for R852E, Ca^{2+} did not substantially potentiate the Cin-induced responses and instead inactivated them faster then in wild-type, suggesting an exquisite role for R852 in the Ca^{2+}-dependent modulation of TRPA1.

Taken complexly, the S4–S5 linker is a key component in allosteric coupling of stimulus sensing and gate opening. We concluded that the altered phenotype of the channelopathy-causing mutation at N855 may arise from its vicinity with residues apparently involved in allosteric coupling or voltage sensing. Our results imply that functionally important is not only the precise electrostatic balance that is adjusted via spatial arrangement of several charged residues, but also the inter-subunit contacts facilitated by E854 and K868 from adjacent subunit. The exquisite changes in voltage-dependent gating observed in the R852 mutants and the selective disruption of voltage-dependent gating in the charge-swap E854R/K868E channels led us to speculate, that these residues may comprise the voltage-sensing domain itself.

5.7 Additional remarks concerning high-resolution TRPA1 structure

Just before the submission of this thesis Paulsen and co-workers (2015) published the structure of human TRPA1, at 4 Å resolution. The structure includes proximal N-terminus with last five ankyrin repeats, transmembrane core and proximal C-terminus. It has been confirmed that TRPA1 H1 helix is structurally and topologically analogous to the TRP domain in TRPV1. Moreover Paulsen and colleagues cited our paper Samad et al. 2011 (Enclosure 1) in the context of revealed tetrameric coiled-coil assembly domain (Fig. 13). Its existence was not expected for TRPA1 channel, but is in good agreement with our data (above mentioned H4 helix) and structurally explains them.

Unfortunately the resolved structure ends just before acidic cluster (Enclosure 2) of TRPA1 C-terminus, but the last resolved residue T1078 is intriguingly spatially close to predicted putative EF-hand domain. Inter subunit salt bridges that we proposed in our recent paper (Enclosure 4) could not be observed in Paulsen et al. structure, but the location of involved residues E854 and K868 matches location predicted by our model. The residues are close enough to possibly make contacts in another state of channel then the one captured by the new structure.

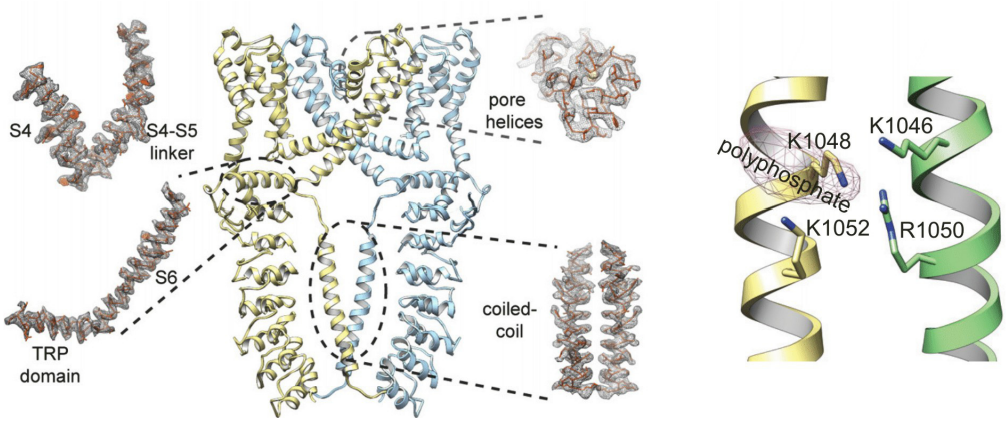


Figure 13: *Structural details of TRPA1. Left: Cryo-EM densities of the S4, S4-S5 linker, pore helices, S6, TRPlike domain and coiled-coil in longitudinal cross sections are superimposed on an atomic model. Only two diagonally opposed subunits are shown for clarity. Right: C-terminal coiled-coil mediates polyphosphate association. (From Paulsen et al. 2015)*

6 Conclusion

Presented thesis focused on the elucidation of structure-function relations of ion channel that has been recognized to be a crucial player in many physiological and pathological processes and therefore considered as important target for pharmacological applications. In fact, in summer 2014 the TRPA1 antagonist GRC 17536 finished the Phase 2 study for treating diabetic neuropathy with promising outcome (Glenmark 2014). This raised the hope that the effort to understand fundamental aspects of TRP receptors behaviour was spent reasonably.

In the initial study, we characterized the C-terminus of TRPA1 with respect to positively charged residues. The proximal part consisting of presumably α-helical structure H1 just after the last transmembrane helix showed remarkable impact on voltage-dependent and also agonist-dependent gating, revealing H1 as an important structural element. The second significant region was found centered around K1048 and K1052. The charge neutralising mutation on these sites rendered channels non-functional, suggesting their essential role in channel activation or proper plasma membrane expression. The finding that mutations around the C-terminal highly acidic cluster produce channels with severely changed gating properties led us to hypothesize that acidic cluster could be involved in TRPA1 modulation with calcium ions.

Based on our electrophysiological experiments and molecular dynamic simulations, we concluded that acidic cluster E1077–D1082 indeed substantially participates in modulation of TRPA1 by Ca^{2+} and, moreover, is able to serve as a direct calcium binding site. Surprisingly, we found that intact end (last 20 AA) of C-terminus is essential for proper calcium-dependent inactivation of the channel.

In our latest study, we have benefited from the recently revealed atomic-level structure of related TRPV1 channel and proposed the structural explanation for a *trpa1* gene related disease. Our homology model revealed and electrophysiological experiments confirmed that the open state of TRPA1 is stabilized by inter-subunit salt bridges between E854 and K868. We suppose that it is exactly disturbance of this interaction what causes the changes in channel's activation and thus episodes of pain.

Together our results contribute to our fundamental understanding of the TRPA1 receptor, an important player in pain and inflammation.

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8 List of abbreviation

AA	Amino acid
ACEMD	Accellera molecular dynamics software
AITC	Allyl isothiocyanate
AR	Ankyrin repeat
ARD	Ankyrin repeat domain
BK channel	Big potassium channel
cDNA	Complementary deoxyribonucleic acid
CGRP	Calcitonin gene-related peptide
Cin	Cinnamaldehyde
CK2	Casein kinase 2
CNS	Central nervous system
cryo-EM	Single-particle electron cryo-microscopy
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglia
ECS	Extracellular control solution
EGTA	Ethylene glycol tetraacetic acid
GFP	Green fluorescent protein
H1 and H4	The most proximal and the fourth predicted helix of C-terminus
HEDTA	N-{2-[Bis(carboxymethyl)amino]ethyl}-N-(2-hydroxyethyl)glycine
HEK	Human embryonic kidney cells
HEK293T	Human embryonic kidney cell line with T antigen
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid
hTRPA1	Human transient receptor potential ankyrin channel 1
Kv channel	Voltage-gated potassium channel
LB	Lysogeny Broth medium
MD	Molecular dynamic
NAMD	Nanoscale molecular dynamics software
NKA	Neurokinin A
PBS	Phosphate buffered saline
PCR	polymerase chain reaction

PIP_2	Phosphatidylinositol 4,5-bisphosphate
S.E.	Standard error
S1–6	Transmembrane helix 1–6
TRP	Transient receptor potential
VMD	Visual molecular dynamics software

List of amino acids

A	Ala	alanine
C	Cys	cysteine
D	Asp	aspartic acid
E	Glu	glutamic acid
F	Phe	phenylalanine
G	Gly	glycine
H	His	histidine
I	Ile	isoleucine
K	Lys	lysine
L	Leu	leucine
M	Met	methionine
N	Asn	asparagine
P	Pro	proline
Q	Gln	glutamine
R	Arg	arginine
S	Ser	serine
T	Thr	threonine
V	Val	valine
W	Trp	tryptophan
Y	Tyr	tyrosine

9 List of enclosures

Papers published in peer-reviewed journals:

1) Samad A, Surá L, Benedikt J, Ettrich R, Minofar B, Teisinger J, Vlachová V.
The C-terminal basic residues contribute to the chemical- and voltage-dependent activation of TRPA1.
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2) Surá L, Zíma V, Maršaková L, Hynková A, Barvík I, Vlachová V.
C-terminal acidic cluster is involved in Ca^{2+}-induced regulation of human transient receptor potential ankyrin 1 channel.
The Journal of Biological Chemistry, 287(22):18067-77, 2012
3) Boukalová S, Touška F, Maršaková L, Hynková A, Surá L, Chvojka S, Dittert I, Vlachová V.
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Structural modeling and patch-clamp analysis of painrelated mutation TRPA1-N855S reveals inter-subunit salt bridges stabilizing the channel open state.
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Bolest, 3: 109-114, 2010
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Ankyrin receptor – ion channel activated by psychoactive drugs: its relation to the mechanisms of nociception.
Psychiatrie, Supp. 2:5-9, 2010

Author's note: Papers till 2014 has been published under my maiden name Surá.