Transient Receptor Potential Ankyrin 1 Channel: An Evolutionarily Tuned Thermosensor

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Summary

The discovery of the role of the transient receptor potential ankyrin 1 (TRPA1) channel as a polymodal detector of cold and pain-producing stimuli almost two decades ago catalyzed the consequent identification of various vertebrate and invertebrate orthologues. In different species, the role of TRPA1 has been implicated in numerous physiological functions, indicating that the molecular structure of the channel exhibits evolutionary flexibility. Until very recently, information about the critical elements of the temperature-sensing molecular machinery of thermosensitive ion channels such as TRPA1 had lagged far behind information obtained from mutational and functional analysis. Current developments in single-particle cryo-electron microscopy are revealing precisely how the thermosensitive channels operate, how they might be targeted with drugs, and at which sites they can be critically regulated by membrane lipids. This means that it is now possible to resolve a huge number of very important pharmacological, biophysical and physiological questions in a way we have never had before. In this review, we aim at providing some of the recent knowledge on the molecular mechanisms underlying the temperature sensitivity of TRPA1. We also demonstrate how the search for differences in temperature and chemical sensitivity between human and mouse TRPA1 orthologues can be a useful approach to identifying important domains with a key role in channel activation.

Introduction

The transient receptor potential (TRP) superfamily of receptors belongs to a large group of tetrameric nonselective cation channels. According to their amino acid sequence homology, the TRP channels are divided into seven subfamilies: TRPC (Canonical), TRPV (Vanilloid), TRPM (Melastatin), TRPP (Polycystin, also known as PKD), TRPML (Mucolipin), TRPA (Ankyrin), and TRPN (No mechanoreceptor potential C, also known as NOMPC). The TRPA1 channel is expressed in various organs and tissues, where it serves mainly as a universal, nociception-mediating cellular sensor activated by various environmental irritants, potentially harmful physical modalities and endogenous mediators of pathophysiological processes (reviewed in (Dai 2016, Talavera *et al.* 2020)). TRPA1 transduces these stimuli into the opening of the channel and influx of cations into the cell. In free nerve endings, such a cation influx locally depolarizes the membrane and triggers the resting sodium channels, evoking the action potential. In addition, TRPA1 channels permeate calcium ions that serve as intracellular messengers and modulators, triggering cellular pathways and modulating the activity of the channel. TRPA1 is involved in the processes of inflammation (Bandell *et al.* 2004, Bautista *et al.* 2006), migraine (Marone *et al.* 2018), diabetes (Wei *et al.* 2010), neuropathic pain (Nativi *et al.* 2013), itching (Wilson *et al.* 2013) and mechanic allodynia in anti-cancer drug treatment (Nassini *et al.* 2011), therefore it is a tempting target for novel analgesic and anti-inflammatory molecules. However, the polymodal and allosteric nature of TRPA1 should be kept in mind in order to specifically target the unwanted properties of TRPA1 modulation (e.g. cold allodynia and hyperalgesia as a side-effect of anti-cancer drugs), while preserving the useful ones (e.g. sensory response to irritant compounds and noxious cold).

The properties of TRPA1 are strongly species-specific and diverse. Therefore, some TRPA1 orthologues exhibit activation by both cold and heat (reviewed in (Hoffstaetter *et al.*

2018)). The mechanisms underlying such bimodal effects of temperature are not yet fully understood. In addition, temperature activation is modulated by voltage and various chemical stimuli, indicating an allosteric nature of TRPA1 gating. This review aims to summarize the findings about the temperature modulation of TRPA1 across the TRPA1 orthologues of various species and the related thermosensitive TRP channels. These findings will be combined with the insights into the structural and molecular context to elucidate the allosteric nature and the mechanisms of the polymodal regulation of TRPA1.

The structure of the TRPA1 channel

So far, four studies have been published that reveal the structure of the TRPA1 channel with the use of cryo-electron microscopy (cryo-EM) at a resolution below 5 Å (Paulsen *et al.* 2015, Liu *et al.* 2020, Suo *et al.* 2020, Zhao *et al.* 2020). TRPA1 shares the topology of the transmembrane domain (TMD) with all other members of the TRP ion channel family - it is composed of the voltage sensing-like domain (VSLD) assembled from helices S1-S4, C-terminal TRP-like domain (TRPL), and a pore domain (PD) containing helices S5-S6 and the pore loop with the selective filter and two pore helices (Fig. 1). The VSLD and PD are connected by the S4-S5 linker. The N-terminus is characterized by a feature shared with TRPV, TRPN and TRPC channels - the presence of ankyrin repeats (ARs), 33-residue motifs consisting of pairs of antiparallel α-helices connected by β-hairpin motifs. The ankyrin repeat domain (ARD) of TRPA1 consists of a tandem array of 16 ARs. The N-terminus is connected to the TMD through the coupling domain (CD) that contains the TRPA1-specific binding site for pungent electrophilic compounds. The C-terminus includes similar structural elements to the TRPM and TRPC channels – the coiled-coil (CC) domain and the interfacial helix (IFH), and similar to TRPV channels – a three-stranded β-sheet. To date, in total 28 mammalian TRP channels have been identified and around 136 cryo-electron microscopy TRP structures have been determined at high resolution (Huffer *et al.* 2020),

enabling us to discover the general mechanisms of TRP activation and regulation, as well as the specific features of each channel.

Species differences in the chemical sensitivity of TRPA1

Phylogenetic analyses of TRPA1 orthologues show that the TRPA1 clade diversified from the TRPA branch 500 million years ago as a chemosensory receptor for electrophilic compounds that evoke pungent and irritating sensations (Kang *et al.* 2010). The electrophilic agonists also include intracellular mediators of oxidative stress and inflammation, highlighting the TRPA1 channel as an important component of the defensive cellular system (Viana 2016). Therefore, the activation of TRPA1 by electrophiles is present in all animal classes, including insects, fish, amphibians, reptilians, avians, and mammals (as reviewed in (Chen and Kym 2009, Bianchi *et al.* 2012, Laursen *et al.* 2014)). On the other hand, non-covalent modulators mostly exhibit species-dependent bimodal action, i.e. activation at lower concentrations, but also inhibition at higher concentrations. This includes divergent effects of non-covalent modulators such as caffeine (Nagatomo and Kubo 2008), menthol (Xiao *et al.* 2008) or nicotine (Talavera *et al.* 2009) on TRPA1 orthologues. Typical non-covalent activators are natural odorants and repellents from essential oils, therefore the shift of the non-covalent modulators may be strongly related to the environmental adaptation according to the food intake and its accessibility (Startek *et al.* 2019b). The cause of this evolutionary adaptation lies in the primary structure of TRPA1 orthologues and, consequently, in alterations of their non-covalent binding sites of TRPA1 that finely tune TRPA1's properties. The comparison of TRPA1 orthologues together with the functional characterization of wild types and chimeric constructs resulted in the localization of the binding sites for caffeine (Nagatomo and Kubo 2008) and menthol (Karashima *et al.* 2007), but also for the synthesized selective TRPA1 inhibitors HC-030031 (Gupta *et al.* 2016) and A-967079 (Chen *et al.* 2011).

TRPA1-mediated detection can be attenuated by cellular mechanisms that affect the excitability of the nociceptive neurons. For example, Highveld naked mole-rats developed a complete loss of TRPA1-mediated pain responses in the presence of the electrophilic agonist allyl isothiocyanate (AITC) due to the overexpression of a voltage- insensitive leak channel (NALCN) (Smith *et al.* 2020). It is believed that in this way, the Highveld mole-rats specifically were able to overcome the presence of the aggressive Natal droptail ants, whose toxic venom evokes strong pain-related behavior by the blockage of NALCN channels (Eigenbrod *et al.* 2019).

Species differences in the temperature sensitivity of TRPA1

In addition to chemosensitivity, TRPA1 orthologues can also serve as sensors of noxious or warm temperatures (reviewed in (Saito and Tominaga 2017, Hoffstaetter *et al.* 2018, Talavera *et al.* 2020)). Species tune the temperature sensor properties of TRPA1 to utilize them in the detection of noxious temperature (Saito *et al.* 2012), maintenance of optimal core and environmental temperatures (Saito *et al.* 2019) and in the detection of infrared radiation (Gracheva *et al.* 2011). In *Drosophila melanogaster*, the TRPA1 channel was first distinguished as a receptor for electrophilic compounds and heat above 26°C (Hamada *et al.* 2008, Kang *et al.* 2010). The properties of the *Drosophila* TRPA1 orthologues (dTRPA1) are tuned by the alternative tissue-specific splicing, resulting in four TRPA1 isoforms (dTRPA1-A, B, C, D) (Kang *et al.* 2011, Zhong *et al.* 2012). Isoforms dTRPA1-A and dTRPA1-B are expressed in central brain neurons and detect heat at low thresholds and at rapid rates (Kang *et al.* 2011, Luo *et al.* 2017), triggering precautious behavior and maintaining optimal temperature (Hamada *et al.* 2008). The dTRPA1-A isoform is also expressed in the chemosensors of a proboscis, where it acts as a chemosensor thanks to its reduced thermosensitivity (Kang *et al.* 2011). The other two isoforms, dTRPA1-C and dTRPA1-D, are expressed in polymodal nociceptors as receptors of noxious conditions

(Zhong *et al.* 2012). Although both isoforms are activated by electrophiles and increased temperature, their *in-vivo* role is split: dTRPA1-C is a UV-nociceptor and detector of electrophiles, whereas dTRPA1-D acts as a noxious thermosensor (Gu *et al.* 2019). Apart from TRPA1, *Drosophila* also has other members of the TRPA family, Painless and Pyrexia, with their own isoforms. Both channels are involved in the detection of noxious heat (Hwang *et al.* 2012). Mosquitos have also tuned the properties of their TRPA1 isoforms (AgTRPA1) to match their needs. The AgTRPA1-A isoform is used for host-seeking thermotaxis, whereas AgTRPA1-B with similar electrophilic affinity but a higher temperature threshold is used for the detection of noxious temperatures (Kang *et al.* 2011). The role of the TRPA1 channel in the maintenance of optimal temperature is also considered in eusocial insects, such as honeybees (*Apis Mellifera).* The honeybee TRPA1 channels have a heat threshold of 34°C in order to maintain the proper temperature of the nest for the larvae and to induce nest-cooling behavior in time (Kohno *et al.* 2010). The approach of tuning the effect of TRPA1 channels by the presence of several forms was also evolutionarily selected in zebrafish (*Danio Rerio*) with two TRPA1 paralogs, zTRPA1a and zTRPA1b. Experiments on zebrafish with knocked-down *Trpa1a* and/or *Trpa1b* genes retained its behavioral response to both heat and cold thermal stimuli, suggesting that zTRPA1 paralogues do not contribute to the nociception *in vivo* and that there are some other channels that adopt the thermal nociception (Prober *et al.* 2008). The related TRPV1 channel that accompanies zTRPA1b in the peripheral nerve endings is used for heat nociception (Gau *et al.* 2013). In addition, zebrafish lack the TRPM8 channel, which is widely used across species in the detection of noxious cold (Kastenhuber *et al.* 2013). Potentially, the loss of zTRPA1 channels might be compensated by the presence of another cold-activated channels, such as TRPC5 channel (Von Niederhausern *et al.* 2013). The electrophysiological measurements on *Xenopus* oocytes transiently expressing one of the zTRPA1 paralogues showed the separation of their functions – zTRPA1a acts as the chemical

sensor, while zTRPA1b covers activation by warming above 25°C with no clear threshold and, surprisingly, activation by cooling below 10°C (Oda *et al.* 2016). These bimodal thermal properties were also found for a TRPA1 orthologue of the pufferfish (Oda *et al.* 2018), but not for the medaka fish orthologue, which only exhibited the heat activation (Oda *et al.* 2017).

In the western clawed frog and the green anole, the heat thresholds of TRPA1 orthologues (39.7°C and 34°C, respectively) match the thermal preferences of the species (Saito *et al.* 2012). Adaptation to the different thermal conditions in two *Xenopus* species is accomplished by functional shifts of the thermal thresholds of TRPA1 and TRPV1 channels (Saito *et al.* 2016). A comparison of the functional properties of TRPA1 orthologues of four closely related *Xenopus* species living in different temperature niches showed that the changes in the TRPA1 activity rather than sensitivity to heat contributes to the evolutionary thermal adaptation (Saito *et al.* 2019). Likewise, the sun-dwelling anole lizard species have a significantly increased temperature threshold of their behavioral and TRPA1-mediated responses than the shade-dwelling species (Akashi *et al.* 2018). These findings highlight the importance of thermal sensors in environmental adaptation.

Radiation-detecting snakes have evolved a pit organ that contains a pit membrane innervated with trigeminal ganglion (TG) neurons that serves as a passive antenna for radiant heat. The pit-bearing snakes (rattlesnake, python, boa) have evolutionarily selected the TRPA1 channel to transduce the heating from the pit-organ into neural activity (Gracheva *et al.* 2010, Geng *et al.* 2011). TRPA1 orthologues of these pit-bearing snakes exhibit robust and steep responses to heat (with a threshold of 27.6°C, 32.7°C and 29.6°C, respectively) (Gracheva *et al.* 2010). A positive correlation between the thermal sensitivity and the current peak amplitude was observed in the rattlesnake and boa snake, where the relationship was steeper in the rattlesnake orthologue (Kang 2016). This relationship could be explained by the influx of cytosolic calcium ions that potentiate both TRPA1 orthologues to a different extent

(Du and Kang 2020). A total number of 47 candidate genes related to the infrared perception in snakes were identified; the molecular mechanisms underlying their infrared perception might soon be elucidated (Tu *et al.* 2020).

TRPA1 as a noxious heat and chemical sensor is expressed in vertebrates together with TRPV1 (Story *et al.* 2003). The diversity in TRPA1-positive neuronal sensitization is also accounted for by coexpression and heteromerization with TRPV1 (Patil *et al.* 2020). The conservation of TRPV1 heat sensitivity throughout vertebrate evolution could have changed functional constraints on TRPA1 and influenced the functional evolution of TRPA1 in terms of temperature sensitivity, while conserving its noxious chemical sensitivity (Saito *et al.* 2012, Saito and Tominaga 2017). While TRPA1 is a radiant heat sensor of snakes possessing pit organs (Gracheva *et al.* 2010), the ganglion-specific splicing of TRPV1 has an analogous function in the infrared sensing of vampire bats (Gracheva *et al.* 2011).

Temperature activation of mammalian TRPA1 channels

In mammals, there is only a single gene encoding the TRPA1 channel. The mouse TRPA1 channel exists in dorsal root ganglia (DRG) in two isoforms, TRPA1a and TRPA1b. However, the TRPA1b isoform lacks the transmembrane region and is nonfunctional as a channel, and was shown to interact with TRPA1a to enhance its surface expression (Zhou *et al.* 2013). TRPA1 was first recognized in 2003 as a cold receptor in mammals (Story *et al.* 2003). Its activation by cold temperatures is still a matter of debate, as the recent studies provide evidence either supporting (Story *et al.* 2003, Sawada *et al.* 2007, Karashima *et al.* 2009, del Camino *et al.* 2010) or denying (Jordt *et al.* 2004, Knowlton *et al.* 2010, Cordero-Morales *et al.* 2011, Chen *et al.* 2013) the activation of TRPA1 by cold or its involvement in cold perception. Rodent TRPA1 is activated by lowering the temperature to 10°C, both when transiently expressed and in DRG neuronal culture (Bandell *et al.* 2004, del Camino *et al.* 2010). Cold increases the channel's open probability, slightly decreases the unitary

conductance and creates a leftward shift of the half-maximal activation voltage V_{50} (Sawada *et al.* 2007, Karashima *et al.* 2009, Chen *et al.* 2013). However, *Trpa1*-deficient mice were indistinguishable from wild-type littermates when examined for behavioral responses to cold temperatures (Bautista *et al.* 2007, Knowlton *et al.* 2010), probably because of the presence of other cold-activated channels. In line with these findings, an additional lack of TRPA1 decreases cold avoidance in *Trpm8*$^{-/-}$ mice in a temperature gradient assay (Winter *et al.* 2017). Surprisingly, rodent TRPA1 has been also found to mediate a crucial physiological role in the detection of noxious heat (Hoffmann *et al.* 2013, Vandewauw *et al.* 2018), similarly to other animal TRPA1 orthologues.

The thermal activation of human TRPA1 was for a long time a matter of debate. Human TRPA1 (hTRPA1) was found to be activated by 12°C cooling, although to a lesser extent than mouse TRPA1 (mTRPA1) (Kremeyer *et al.* 2010, Wang *et al.* 2013, Moparthi *et al.* 2014, Moparthi *et al.* 2016, Sinica *et al.* 2019). However, other groups did not observe any hTRPA1 cold-evoked currents upon cooling (Jordt *et al.* 2004, Cordero-Morales *et al.* 2011, Chen *et al.* 2013). The single residue G878 in mTRPA1 (V875 in hTRPA1) accounts for differences in the cold sensitivity of hTRPA1 and mTRPA1 (Chen *et al.* 2013), and for the temperature-dependent kinetics of voltage activation (Sinica *et al.* 2019). The introduction of part of the rattlesnake TRPA1 or dTRPA1 N-terminus to hTRPA1 was sufficient to confer heat sensitivity to the human TRPA1, emphasizing the role of the N-terminus in heat activation (Cordero-Morales *et al.* 2011). However, human and mouse TRPA1 exhibited activation upon noxious heat stimulation above 50°C (Hynkova *et al.* 2016, Sinica *et al.* 2019), potentially explaining why other authors have not seen such a high noxious heat threshold. Furthermore, the depolarizing voltage synergically increased the responses to heat (Sinica *et al.* 2019).

Modulation of temperature activation by TRPA1 agonists

Interestingly, the noxious-heat avoidance behavior of *Drosophila* with impaired dTRPA1 can be rescued by the expression of human or heat-insensitive planarian TRPA1 (Arenas *et al.* 2017). The authors suggest that TRPA1 activation is mediated by H_2O_2 and ROS, early markers of tissue damage that are rapidly produced as a result of heat exposure. Although warm temperature suppresses the activation effects of agonists such as AITC or menthol on rat and human TRPA1 orthologues (Wang *et al.* 2012), responses to H_2O_2 are potentiated by heat in Chinese hamster ovary (CHO) cells expressing mTRPA1 (Vandewauw *et al.* 2018). The presence of reducing agents in the bath solution inhibited both the cold and warm responses of hTRPA1 reconstituted in an artificial membrane (Moparthi *et al.* 2016), implying the importance of the TRPA1 redox state in heat activation. On the other hand, mild cooling markedly increases currents evoked by electrophiles and carvacrol in rat and human TRPA1, suggesting that TRPA1 is a key mediator of cold hypersensitivity (del Camino *et al.* 2010, Moparthi *et al.* 2016, Zimova *et al.* 2020). The TRPA1-positive neurons only respond to cold in the presence of some agonists, suggesting that TRPA1 is important in pathological conditions with an elevated level of proinflammatory activators, but likely plays a comparatively minor role in acute cold sensation (del Camino *et al.* 2010). TRPA1 has been also linked with mechanical and cold allodynia accompanying nerve injury (Chen *et al.* 2011), inflammation (Yamaki *et al.* 2020), or anti-cancer treatment with oxaliplatin (Nassini *et al.* 2011, Park *et al.* 2015). Acute hypersensitivity to cold temperatures induced by oxaliplatin is mediated by human TRPA1 sensitization to ROS *via* mechanisms that are, in a dose-dependent manner, governed by the inhibition of propyl hydroxylases (Miyake *et al.* 2016). Thus, the products of oxidative stress resulting from the presence of noxious heat and cold temperatures might be the driving force that sensitizes the TRPA1 channels to be activated by temperature.

In order to see how the presence of agonist modulates the temperature sensitivity of the human and mouse TRPA1 channel, we used repeated linear voltage pulses from -80 mV to +80 mV with the combination of temperature changes and application of the non-covalent agonist carvacrol at a concentration of 50 μM (Fig. 2A). The cells were held at 25°C and then exposed to 20-s episodes of cooling or warming by 10°C, first in the extracellular solution (ECS) and then in the presence of carvacrol. From the average currents obtained at the end of the episode, we calculated the potentiation effect (Fig. 2B). While the mouse TRPA1 was clearly activated by cold even in the absence of carvacrol, cold activation in human TRPA1 was not clearly visible because of the low basal currents. This result suggests that mouse TRPA1 seems to be less susceptible to modulation by temperature when the voltage sensor is activated, compared with the human orthologue. Surprisingly, both hTRPA1 and mTRPA1 are potentiated by cold at negative potentials even in the presence of carvacrol, as opposed to what (Moparthi *et al.* 2016) observed. This indicates that both channels are intrinsically activated by heat and that carvacrol uncovers the cold activation by shifting the equilibrium towards the open state of the channel. In addition, while warming increases the currents in the ECS, warmer temperatures of 35°C somewhat suppress the effect of carvacrol at negative potentials, further supporting the activation of both orthologues by cold. However, stimulation by a rather short and fast voltage protocol may result in measuring the currents far from the equilibrium. A longer interval of voltage and temperature stimulation might be beneficial to elucidate the intrinsic properties of the TRPA1 channel.

Bimodality of temperature activation and the induction of cold activation

All these findings lead to the assumption that human and mouse TRPA1 channels exhibit a bimodal activation by temperature, exhibiting condition-dependent properties of both cold and heat channels. Single-channel recordings of hTRPA1 activity in the artificial membrane and measurements of intrinsic tryptophan fluorescence consolidate hTRPA1 as an

intrinsic bidirectional thermosensor activated by both cold and heat. The authors found that hTRPA1 activation exhibits no single channel activity at a temperature around 22°C (Moparthi *et al.* 2016). In agreement with this, our study (Sinica *et al.* 2019) shows that both the heating to 35°C and cooling to 15°C of human and mouse TRPA1 channels expressed in HEK293T cells increases the membrane currents, compared to 25°C. The fact that mouse TRPA1 is markedly activated by cold probably reflects its shifted gating equilibrium towards the open state and not a steep temperature dependence of its kinetics of deactivation. Figure 3A and B shows that both TRPA1 orthologues exhibited "U-shaped" outward currents at depolarizing voltage with a saddle point around room temperature. An increase in the currents produced by cold can be observed in both TRPA1 orthologues, while the increment of the currents above 25°C may also reflect the temperature dependence of the channel's unitary conductance (Chen *et al.* 2013, Moparthi *et al.* 2016). Nevertheless, at even higher temperatures (>45°C), TRPA1 could exhibit the properties of a heat activated channel. The bidirectional effect of temperature can be even better seen when the channel is stimulated by temperature together with changes in the membrane voltage (Fig. 3C, D). Upon the exposure to noxious heat (~ 60°C), specific heat-induced currents can be observed with a steep temperature dependence over the temperature above 55°C, providing the evidence to the bimodal activation of TRPA1 channels by noxious temperature. When the noxious temperature was above 60°C, the responses to depolarizing voltage of +80 mV were strongly reduced; the reduction correlated with the maximum temperature applied, suggesting that excessive heat irreversibly impedes either the activation of the voltage-sensor or its effective coupling to the gate. Our finding is in line with (Sanchez-Moreno *et al.* 2018), where the authors also observed the irreversible action of noxious heat above 50°C at the TRPV1 channel, suppressing its subsequent activation by heat or agonist.

Most interestingly, when the channels were, after concurrent stimulation by heat and depolarizing voltage first cooled down to 5°C and then repolarized back to -70 mV, highly pronounced inward currents were induced (Fig. 3E, F). The effect of cold-sensitized inward currents was not dependent on the order of the applied stimuli, but it was not seen when the reverse repolarization occurred in the presence of noxious heat. This 'heat-induced cold activation' was present in both mouse and human TRPA1, and in both the environment of HEK293T cells and in the neuronal F11 cell line. These striking findings indicate that noxious heat above 60°C together with depolarizing voltage induces substantial structural rearrangements within the channel, leading to strong inward rectification at cold temperatures. The channel becomes inward-rectifying, being opened by negative membrane potential. Interestingly, in experiments described by (Moparthi *et al.* 2016), the cold potentiation of carvacrol-induced currents in hTRPA1 was only observed when channels were pre-exposed to warm temperatures. Thus, various sources of energy, such as those derived from agonist binding, or depolarization could drive the channel opening by cooling.

The allosteric model and the existence of temperature sensors of the TRPA1 channel

An allosteric model is capable of explaining the sensitization of an agonist-induced activation by temperature (del Camino *et al.* 2010), or, on the other hand, a loss of sensitivity to other stimuli at high concentrations of agonist (Matta and Ahern 2007). An allosteric model implies that the channel has a gate, described by an equilibrium constant *L*, and also independent voltage and thermal sensors, both described by their own constants *J* and *K* of the temperature- and voltage-dependent transitions. The sensors allosterically couple to the channel's gate and to each other according to the coupling energy terms *C*, *D* and *E*, respectively (Salazar et al., 2011). Furthermore, the introduction of temperature-dependent coupling even gives rise to channels that respond to both cooling and heating (Jara-Oseguera and Islas 2013). An eight-state allosteric model with temperature-dependent coupling of the

cold sensor to the gate was successfully used to model the bimodal properties of the TRPA1 channel in Fig. 3C-F (Sinica *et al.* 2019). The concurrent activation of the voltage and heat sensors can induce a conformational switch that leads to an increase in their energetic crosstalk *E*, an increase in the gating equilibrium constant *L*, and a drastic (~30,000-fold) decrease in the coupling of the voltage sensor to the channel gate *D*. The channel also becomes inward-rectifying, being opened by hyperpolarized voltage.

How could these changes in allosteric properties be implemented into the structural context? The gate of the channel consists of two restrictions: the upper gate involves diagonal interactions between opposite D915 residues at the selective filter, and the lower gate of two hydrophobic seals formed by residues I957 and V961 at the S6 helix (Paulsen *et al.* 2015). The residue L906 is extremely sensitive to substitutions, producing a strongly inward-rectifying phenotype with reduced sensitivity to TRPA1 inhibitor HC-030031 (Wan *et al.* 2014). Moreover, the introduction of a mosquito-to-human point mutation in the S6 helix and pore helix of dTRPA1 reverses the heat sensitivity to activation by cold (Wang *et al.* 2013). Thus, the concurrent application of excessive heat and voltage could potentially modulate the TRPA1 properties through the rearrangement of the pore helix.

The candidates for temperature sensors

The allosteric model suggests that the channel possesses independently operating voltage and temperature sensors. The existence of such sensors in the TRPA1 channel is still a matter of debate. In terms of a voltage sensor, TRP channels are only mildly activated by depolarizing voltage due to a low gating charge, as opposed to K^+ channels, where a voltage sensor contains several positive arginine residues (Jiang *et al.* 2003). However, the small gating charge of TRP channels is a crucial factor for the large voltage shifts induced by various stimuli (Nilius *et al.* 2005), and also an amplifier of thermal sensitivity (Chowdhury *et*

al. 2014). A well-defined region of the TRPA1 channel involved in activation by heat is the tandemic assembly of 16 ankyrin repeat domains (ARD), structurally divided into the proximal part of AR12-AR16 stabilized by contacts with the coiled-coil domain of the C-terminus, and the unresolved distal part of AR1-AR11 that forms a crescent-like structure heading upward towards the plasma membrane (Paulsen *et al.* 2015) (Figure 1B). Chimeric channel studies conducted on human, rattlesnake and *Drosophila* TRPA1 orthologues (hTRPA1, rsTRPA1 and dTRPA1) demonstrated that both parts of the ARD include two spatially distinct thermosensory modules – the primary module (AR10 - AR15) and the enhancer module (AR3 – AR8) (Cordero-Morales *et al.* 2011). Each module exhibits different average temperature coefficients of activation; thus together they modulate the overall thermal response properties of the native rsTRPA1 channel. The transfer of any of the rsTRPA1 modules and the primary module of dTRPA1 into the human TRPA1 orthologue is sufficient to introduce activation by temperature at 42°C (Cordero-Morales *et al.* 2011). The comparison of the primary sequences of TRPA1 orthologues of snakes with and without thermosensory properties led to the identification of 11 amino acid sites localized in the N-terminus that may be involved in the evolutionary adaptation of TRPA1 (Geng *et al.* 2011). In accordance with this finding, three single-point mutations at the AR6 of mTRPA1 are individually sufficient to make the channel activated by 40°C, while leaving the sensitivity to chemicals unaffected (Jabba *et al.* 2014). Thus, the perturbations inside the ARDs may shift the threshold of the temperature sensor. However, the mosquito (AgTRPA1) orthologue reconstituted in artificial membrane retains its thermosensitivity toward 35°C even if its N-terminus is deleted, suggesting that the N-terminal domain may tune the response but is not required for the activation by these stimuli (Survery *et al.* 2016).

In TRP channels, the thermal opening of the channel involves a large change in conformational standard-state enthalpy. The authors (Clapham and Miller 2011) proposed that

the gating of the TRP channels may be connected with folding/unfolding or wetting/dewetting processes associated with the changes in molar heat capacity. As a result, the channel can have a temperature sensor delocalized into many regions across the whole channel. Point mutations in specific regions of the channel may then influence the temperature activation by shifting the melting temperature T_m along the temperature axis. In addition, unfolding processes can be induced by both hot and cold temperature. Therefore, the heat and cold activation can involve the same mechanism in cases where both the heat and cold denaturation temperatures are experimentally (or physiologically) accessible. To date, however, researchers have only experimentally observed heat denaturation in connection with the gating of TRP channels (Diaz-Franulic *et al.* 2020).

The folding/unfolding processes have to take part in regions that are allosterically coupled to the channel's gate. A shining example of such coupling is the C-terminal coiled-coil of the bacterial voltage-gated sodium channel ($BacNa_v$) and TRPM8 channel: while the $BacNa_v$ channel is heat-activated by partial coiled-coil unfolding (Arrigoni *et al.* 2016); in TRPM8, the activation occurs after coiled-coil stabilization upon cooling down to 15°C (Diaz-Franulic *et al.* 2020). The coiled-coil domain of the TRPA1 channel is distinct from canonical coiled-coils due to two polar residues inside the structure and a rather hydrophobic exposed surface that interacts with ARD (Paulsen *et al.* 2015). A partial unfolding of coiled-coil helices occurs upon increasing the temperature to 42°C, although the multimerization remained unaffected (Martinez and Gordon 2019). Under the condition of positive coupling into the gate, the unfolding process of the coiled-coil domain might explain the remainder of the thermosensitivity in the absence of the N-terminus (Survery *et al.* 2016).

The transmembrane domain and the crucial role of annular lipids

The structural changes at the periphery have to be transduced into the TMD to further allosterically influence the structural segments that are functionally related to the upper and lower gates of the channel. According to the recently published structure of the open state of the TRPA1 channel in the presence of an electrophilic agonist, the transition from the closed to the open state involves the rotation of the entire TMD and also the S1-S4 domain, upwards translation of the pore helices and the straightening of the S4-S5 linker and the S5 segment into a single helix (Zhao *et al.* 2020). The allosteric coupling between the VSLD and PD is further supported by the evolutionary analysis of the primary sequences of TRPV1 and TRPM8 (Hilton *et al.* 2019). The sequential connection between the VSLD and PD is established by the S4-S5 linker. Increased attention is paid to the role of membrane lipids in the activation mechanisms of TRP channels by ligands and temperature (Fig. 4A). In the structurally related TRPV1, the displacement of a phosphatidylinositol molecule from the position between the VSLD, the S4-S5 linker, and the S5 helix was proposed as a mechanism of activation by heat and vanilloids (Gao *et al.* 2016). A molecule of phosphatidylcholine resides at an analogous position in the cryo-EM structure of the TRPA1 channel, establishing salt bridges with E864 and K868 from the S4-S5 linker and D802 from the S3 helix (Fig. 4B) (Suo *et al.* 2020). Displacement of this lipid with the specific non-covalent agonist GNE511 or by the introduction of bulky tryptophan mutation E864W into its binding site renders the channel open, suggesting its involvement in the mechanisms of gating (Liu *et al.* 2020). The activation by GNE511 is insensitive to TRPA1 antagonists and does not show signs of desensitization. The charge-swap mutation K868E produced a constitutively open channel with impaired voltage and electrophilic activation, as well as Ca^{2+}-dependent potentiation and inactivation (Zima *et al.* 2015), potentially indicating the destabilization of the lipid from its binding site. On the other hand, the neutralization of D802 slowed the activation and deactivation kinetics and specifically affected the activation by electrophiles (Zimova *et al.*

2018). Intriguingly, a human-to-mouse substitution of the nearby serine S804N as well as S804D mutation also severely suppressed the kinetics and created a cold-activated channel, while a neutralizing mutation S804A in the S3 helix produced a non-functional channel (Sinica *et al.* 2019). The residue S804 is around 5 Å away from the intersubunit lipid, it may also face inside the VSLD cavity. The mutation S804N may affect the gating-related movements of the channel or the binding of the lipids, potentially revealing the main difference between the gating equilibrium of human and mouse TRPA1 orthologues.

Intracellular cavities as an integrative allosteric site of TRPA1 modulation

The S804 residue is inside an intracellular, solvent-accessible cavity formed by four helical segments S1-S4 of VSLD and the TRPL helix. The VSLD intracellular cavity is a conserved regulatory element across TRP channels. Our results from (Zimova *et al.* 2018) show the importance of polar and charged residues inside the cavity as crucial determinants of the electrophilic, voltage and calcium sensitivity of TRPA1 (Fig. 4C). The results from molecular docking experiments suggest that the intracellular cavity might be a binding site of terpene agonists including carvacrol, thymol and β-myrcene, which is in agreement with the presence of icilin and menthol inside the cavity in the related TRPM8 channel (Yin *et al.* 2019). In addition, the docking simulations indicate the possibility of a lipid binding inside the VSLD cavity, specifically the negatively charged head group of phosphatidylinositol 4,5-bisphosphate (PIP_2)(Zimova *et al.* 2018) and cholesterol (Startek *et al.* 2019a). The possibility of a lipid binding into the intracellular cavity further narrows the localization of phosphatidylcholine in the cryo-EM structure of the TRPV1 channel (Gao *et al.* 2016). Neutralization of the positively charged residues contacting the inositol head group of PIP_2 impaired the voltage- and agonist-driven activation of the TRPA1 channel, while the neutralization of negatively charged E788 and E808 resulted in gain-of-function mutations (Zimova *et al.* 2018). These modulation effects may be attributed to changes in the

electrostatic potential inside the cavity and altered binding of PIP_2. Depletion of the membrane PIP_2 downregulates the wild-type TRPA1 channels and transformed the gain-of-function mutant E808A back to the wild-type level (Zimova *et al.* 2018). Importantly, the effects of PIP_2 deletions were absent in the presence of extracellular calcium, which is in line with the finding that four negatively charged residues (including E788 and E808 in hTRPA1) directly bind Ca^{2+} ions and mediate the Ca^{2+}-dependent modulation of TRPA1 (Zimova *et al.* 2018, Zhao *et al.* 2020) and other members of TRPC (Duan *et al.* 2018, Duan *et al.* 2019) and TRPM channels (Autzen *et al.* 2018, Zhang *et al.* 2018, Diver *et al.* 2019).

Another important allosteric site of the modulation of the TRPA1 channel is an interfacial cavity formed by S4, the S4-S5 linker, pre-S1, S1, and the C-terminal interfacial helix (Fig. 4D). The results from the web server-based method for predicting the interaction between peptides and anionic lipids (Lata *et al.* 2007) suggested that the IFH is capable of binding lipids that may potentially regulate the channel's activity (Witschas *et al.* 2015). With the use of biophysical methods, our team has proved that the peptide T1003-P1034 covering the IFH only interacts with the lipid membrane in the presence of PIP_2 (Macikova *et al.* 2019). A molecule of an annular lipid was localized in the cryo-EM structure of the TRPA1-C621S channel with a benzyl isothiocyanate (BITC) molecule bound to its binding site in the coupling domain. The lipid is not present in cryo-EM structures of TRPA1-C621S without an agonist (PDB ID: 6PQQ) and with the larger agonist JT010 (PDB ID: 6PQO), because of the closer position of the IFH toward the VSLD and consequently reduced cavity size (Suo *et al.* 2020). In support of this finding, the substitution of the conserved phenylalanine F1020 in the IFH helix with glycine suppressed the currents evoked by the electrophilic agonist cinnamaldehyde (Cin), but only at negative potentials. The Ca^{2+}-dependent potentiation and inactivation were unaffected (Macikova *et al.* 2019). Furthermore, it has been shown that from nanomolar concentrations of intracellular Ca^{2+}, the region next to the IFH acts as a

binding site for the Ca^{2+}/calmodulin complex that potentiates the TRPA1 channel under resting conditions (Hasan *et al.* 2017). The inclusion of the T1003-P1034 peptide in the intracellular solution prevented Ca^{2+}-dependent potentiation, similarly as in (Hasan *et al.* 2017), suggesting that the Ca^{2+}/calmodulin complex may compete for the same or overlapping binding site with PIP_2 (Macikova *et al.* 2019).

The other part of the cavity recognized as the region determining the sensitivity to several activation stimuli is composed of the S5 helix and the S4-S5 linker of the neighboring subunit. At the S4-S5 linker there is a cysteine residue C856 that was recognized with the use of calcium imaging as being one of the main targets of O_2 in hyperoxia, because the C856S mutant exhibited suppressed hyperoxia-induced currents at basal potential (Takahashi *et al.* 2011). We wanted to also see the involvement of this residue in the activation by electrophiles. Figure 5 shows the currents measured from HEK293T cells expressing the wild-type of hTRPA1 or the C856S or the C856A mutant by the repeated protocol as in (Macikova *et al.* 2019). Surprisingly, both mutant channels were already strongly suppressed at negative membrane potentials before the application of electrophiles (Fig. 5A). The impairment of the channel's function at these negative potentials continued in the presence of Cin and the subsequent application of Ca^{2+} ions (Fig. 5B). The stronger agonist AITC was able to activate both mutants to the level of the wild type, although the impairment at negative potentials was still visible (Fig. 5C). These findings imply that the C856S mutation probably impaired the coupling of the voltage sensor into the gate more pronouncedly than in the F1020G mutant (Macikova *et al.* 2019), suggesting the involvement of the interfacial cavity in the allosteric coupling of the voltage sensor and the gating of the channel.

In the S4-S5 linker, next to the C856S, there is the human channelopathy-related residue N855, the mutation of which induced an ~5-fold increase in inward currents upon activation by cold compared with wild-type hTRPA1 (Kremeyer *et al.* 2010). Also, the

mouse-to-human mutation G878V in S5 accounts for the differences in the cold sensitivity of hTRPA1 and mTRPA1 by changing the temperature dependence of their deactivation kinetics (Chen *et al.* 2013, Sinica *et al.* 2019). The same region also acts as a binding site for menthol (Xiao *et al.* 2008), which potentially explains the synergic effects of menthol and structurally related carvacrol on cold activation. In addition, the human-to-mouse mutation H1018R in the IFH exhibited significantly increased carvacrol responses upon cooling to 15°C (Zimova *et al.* 2020). On the other hand, the coexpression of the human TRPA1 channel with the voltage-sensitive protease from *Danio Rerio* (Dr-VSP), which depletes the acute levels of PIP_2, reduced the cold responses to 15°C while leaving the carvacrol-cold synergy unaffected (Zimova *et al.* 2020).

Concluding remarks

In this review, we have covered and counted various approaches to regulating the thermosensitivity of TRPA1 channels. The TRPA1 channels are evolutionarily designed to be polymodal channels with the ability to effect the interaction of several modulation stimuli. Their functional adaptations to the environmental stimuli, specific expression patterns, splicing variants, evolutionary pressure, cooperation with other proteins (TRPV1 channel, Ca^{2+}/calmodulin complex and others) substantially aid the profiling of the channels to suit their roles as polymodal sensors. The growing number of structural and functional findings in the field related to TRP channels allows us to integrate the known knowledge and make progress on the molecular mechanisms of the polymodal regulation, mechanisms of bimodal temperature activation and the channel gating. Furthermore, the recent structural findings of the role of membrane lipids may help to find a specific mode of action of the TRPA1 channel, which could be used to specifically target the allosteric effects of temperature, providing aid for pain-related TRPA1-mediated pathophysiological conditions.

Conflict of Interest

There is no conflict of interest.

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Figure legends

Figure 1: The topology overview of the human TRPA1 channel. A, The overall architecture of the TRPA1 tetramer (Protein Databank, PDB, ID: 6PQO) represented as pipes and planks. Four subunits colored by chain are shown from the side and the top. TMD, transmembrane domain, CD, coupling domain, ARD, ankyrin repeat domain. **B**, A single TRPA1 protomer with indicated domains: ARD formed by ankyrin repeats AR1-AR16. Two functionally distinct modules of ARD are indicated. Transmembrane segments S1-S6, voltage sensor-like domain formed by S1-S4, the pore domain (S5-S6 region) with the upper gate formed by D915, and the lower gate formed by hydrophobic residues I957 and V961. The TRP-like domain is a conserved site of allosteric modulation. Within the coupling domain (CD), two reactive cysteine residues (C621 and C665) important for electrophile activation are indicated.

Figure 2: Modulation of the voltage and temperature properties of the human and mouse TRPA1 channels by carvacrol. A, Representative time courses of whole-cell currents from the human and mouse TRPA1 channels expressed in HEK293T cells, measured at -80 mV and +80 mV, induced by repeated stimulation by a linear ramp protocol indicated in the inset. Cells held at 25°C and were stimulated by 20-s temperature changes to 15°C or 35°C, first, in the extracellular solution (ECS), and then in the presence of noncovalent agonist carvacrol (Carv). Last five current responses of each cooling/heating episode and also the preceding episode at 25°C were averaged and transformed to conductances for further analysis. **B,** Ratios of the conductances at the end of the cooling/heating episode, normalized by the conductances of the preceding currents at 25°C, shown as vertical scatter plots and bar charts representing mean + S.E.M. (n = 9 - 18). Significant differences of $P < 0.05$ are highlighted with the asterisks.

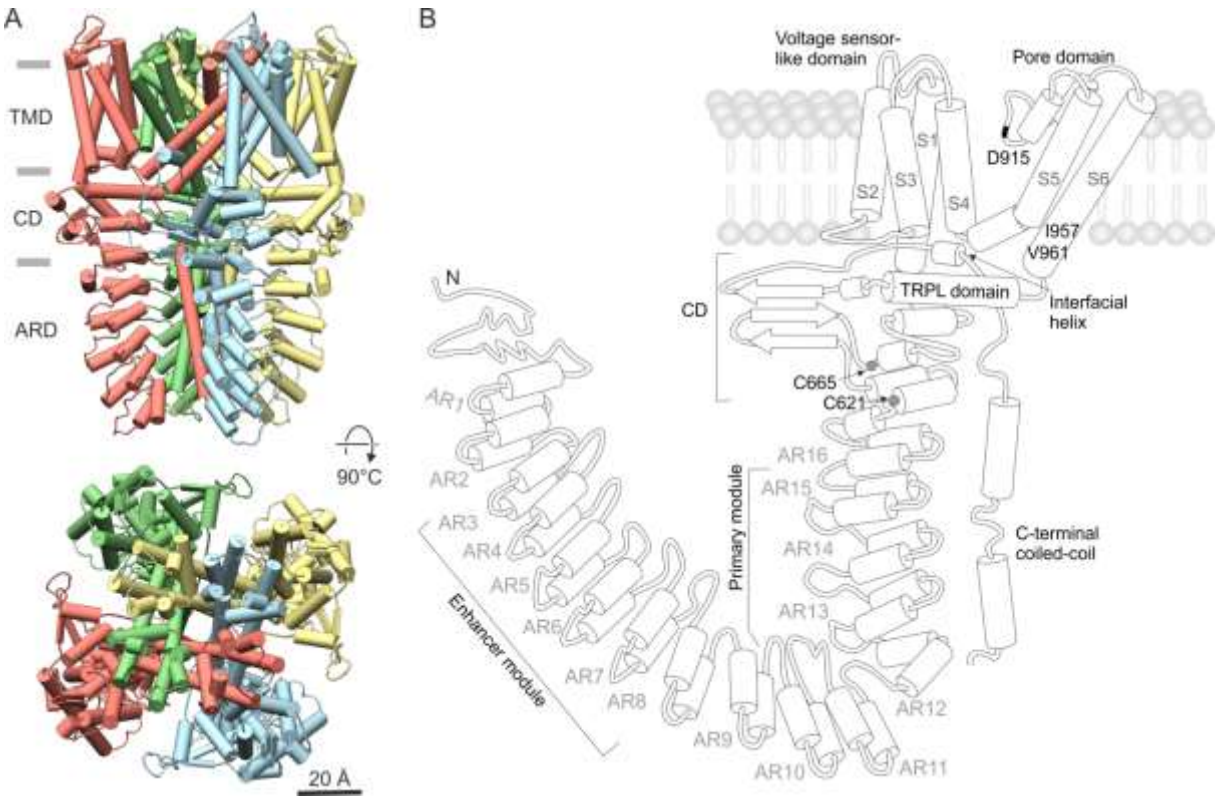
Figure 3: Activation and modulation of TRPA1 by excessive heat. A-B, Representative whole-cell currents of hTRPA1 and mTRPA1 channels transiently expressed in HEK293T cells, elicited by temperature steps ranging from ~11 to ~52°C. The cells were held at constant depolarizing voltage +80 mV. Note the „U-shaped“ of the evoked currents **C-D,** Representative whole-cell currents of hTRPA1 and mTRPA1 channels. The cells were held at -70 mV and ~ 8-10°C, during the experiment, depolarizing pulses (+80 mV; gray area) and temperature above 60°C (pink area) was applied. In both orthologues, stimulation by excessive heat suppressed the concurrent activation by depolarization voltage. For illustration purposes, the region in D was omitted (double slashes). **E-F,** The second part of the experiment from C-D. Concurrent stimulation of hTRPA1 and mTRPA1 by +80 mV and >60°C turned the channel into the cold-activated mode upon following relaxation at -70 mV. Additional heat stimulation released the channels back to their initial state. Adapted from (Sinica *et al.* 2019).

Figure 4: Lipid-binding sites in TRPA1. A, Three functionally important lipid-binding sites are shown in the structure of the transmembrane region of unliganded human TRPA1 subunit (Protein Databank, PDB, ID: 6PQQ). VSLD, voltage sensor-like domain; PD, pore domain; TRPL, TRP-like domain. **B-D**, A close-up view of the lipids shaded by colors in A. The main interacting amino acids are depicted using their one-letter codes.

Figure 5: Mutations of C856 in the S4-S5 linker impair the channel opening at resting potentials. A, Average conductance-voltage relationships of humanTRPA1 wild-type (WT) and indicated mutations expressed in HEK293T cells, measured at the end of the included voltage-step protocol from (Zimova *et al.* 2018). **B-C,** Time course of the average currents of

the TRPA1 wild-type (black line) and indicated mutants, measured by the indicated ramp protocol applied every second, at -80 mV and +80 mV. The cells were first exposed to electrophilic agonists, cinnamaldehyde (Cin) or allyl isothiocyanate (AITC), and then to 2 mM of Ca^{2+} ions. Note the suppressed current of indicated mutants at negative potentials. Data are shown as mean ± (or +/-) S.E.M., n is indicated in brackets. Bath solution and pipette solution was used as described in (Zimova *et al.* 2018).

Figure 1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TMD
CD
ARD
90°C
20 Å
B
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Pore domain
S1
S2
S3
S4
S5
S6
D915
I957
V961
N
CD
TRPL domain
Interfacial helix
C665
C621
AR1
AR2
AR3
AR4
AR5
AR6
AR7
AR8
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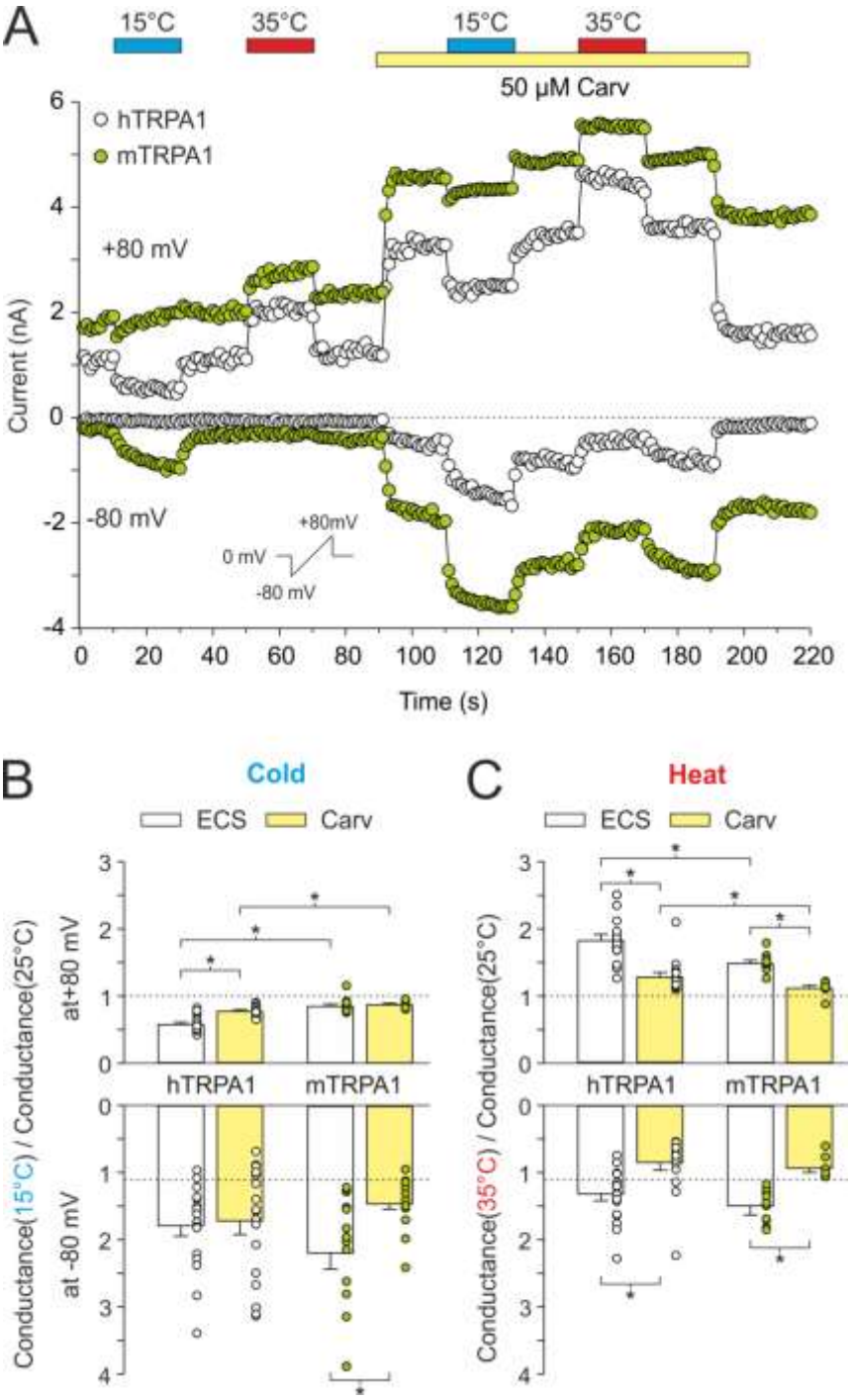
AR16
Primary module
Enhancer module
C-terminal coiled-coil

Figure 2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15°C
35°C
15°C
35°C
50 μM Carv
hTRPA1
mTRPA1
+80 mV
-80 mV
+80mV
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-80 mV
Current (nA)
Time (s)
B
Cold
ECS
Carv
Conductance(15°C) / Conductance(25°C)
at+80 mV
at -80 mV
hTRPA1
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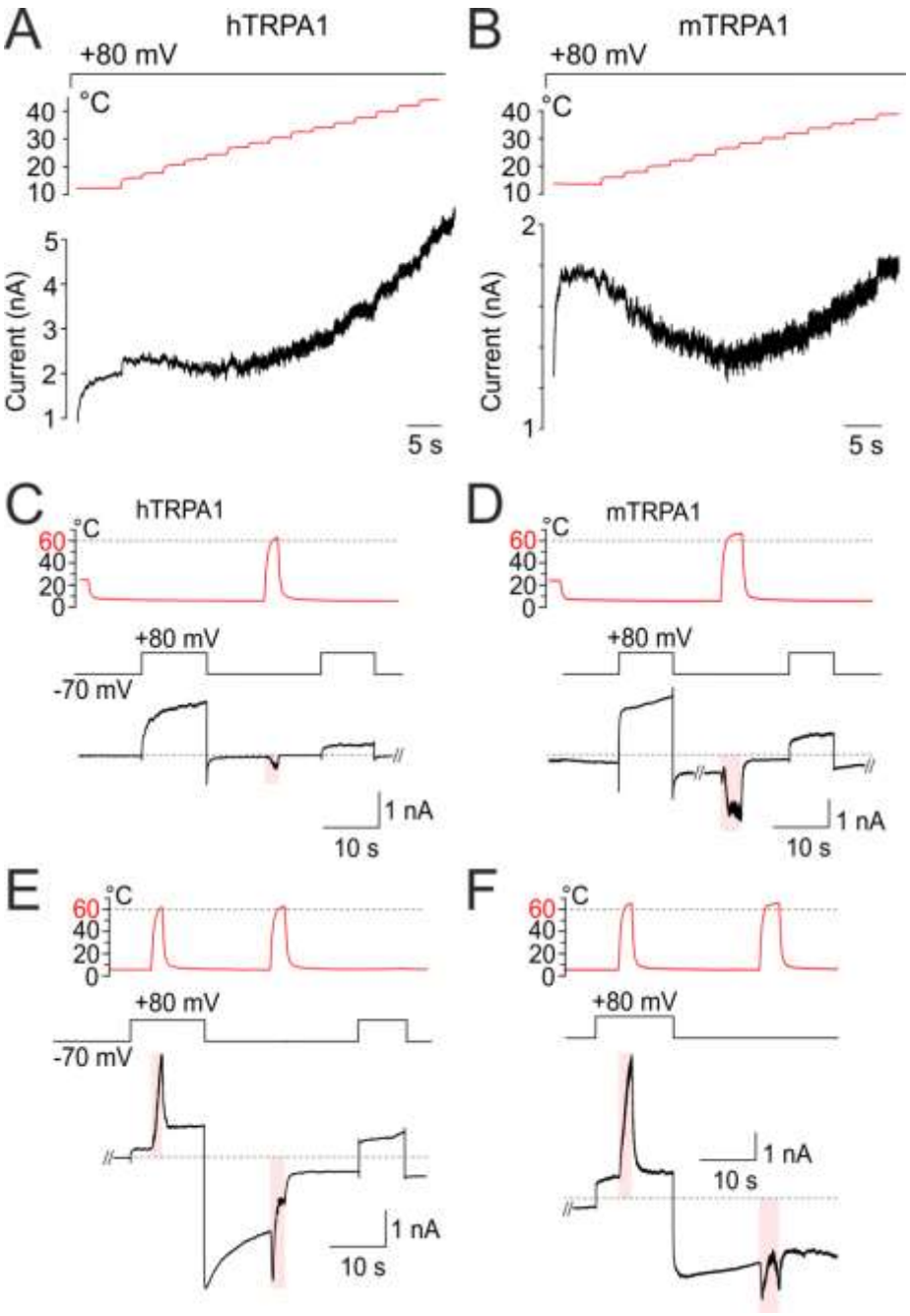
C
Heat
ECS
Carv
Conductance(35°C) / Conductance(25°C)
hTRPA1
mTRPA1

Figure 3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°C
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Current (nA)
5 s
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mTRPA1
+80 mV
°C
40
30
20
10
2
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Current (nA)
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hTRPA1
°C
60
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+80 mV
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Figure 4

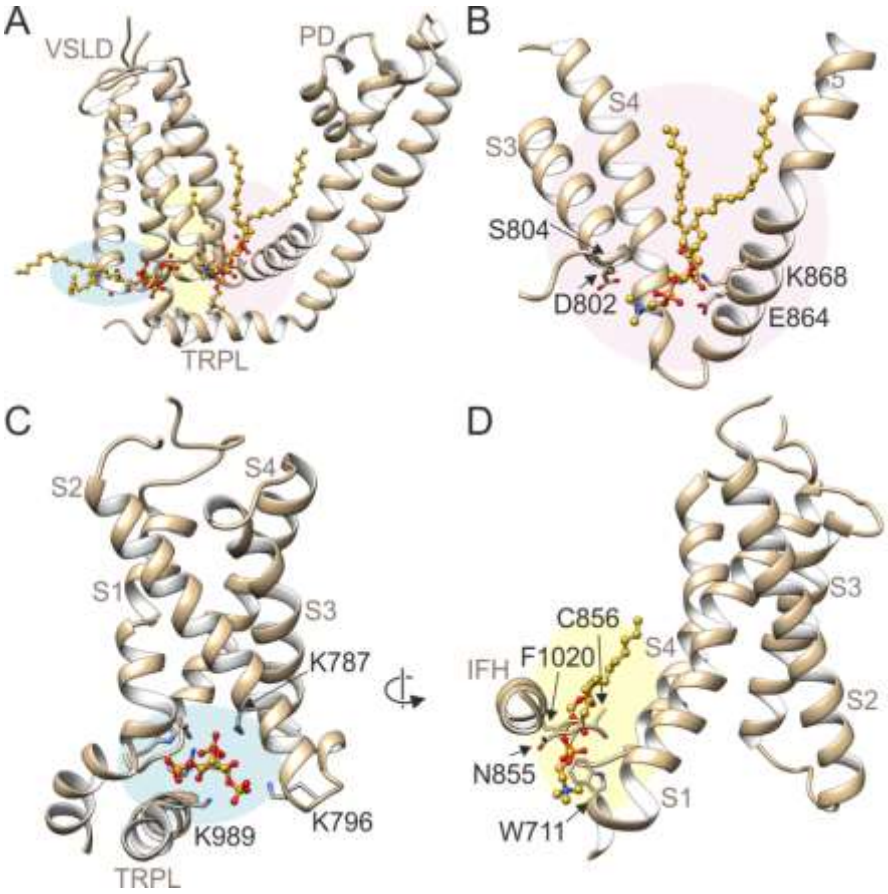
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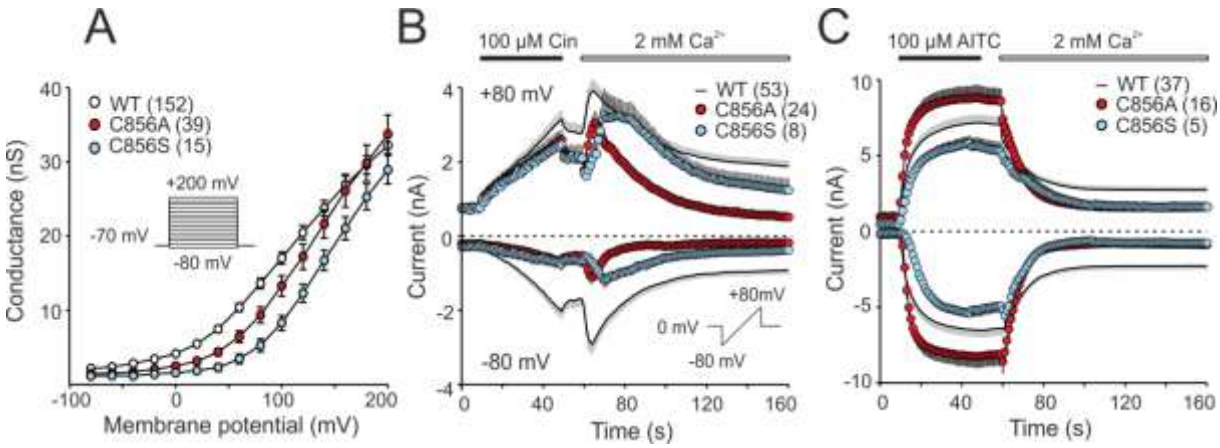
Figure 5


A
B
C
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C856A (39)
C856S (15)
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-70 mV
-80 mV
Conductance (nS)
Membrane potential (mV)
100 µM Cin
2 mM Ca2+
+80 mV
WT (53)
C856A (24)
C856S (8)
Current (nA)
+80mV
0 mV
-80 mV
Time (s)
100 µM AITC
2 mM Ca2+
WT (37)
C856A (16)
C856S (5)