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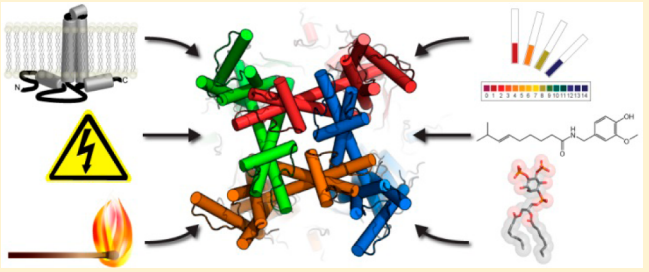
Understanding Thermosensitive Transient Receptor Potential Channels as Versatile Polymodal Cellular Sensors

Jacob K. Hilton,[†] Parthasarathi Rath,[†] Cole V. M. Helsell,[†] Oliver Beckstein,[‡] and Wade D. Van Horn*[,†]

[†]Center for Personalized Diagnostics, Magnetic Resonance Research Center, and Department of Chemistry and Biochemistry, Arizona State University, 551 East University Drive, PSG-106, Tempe, Arizona 85287, United States

[‡]Center for Biological Physics and Department of Physics, Arizona State University, 550 East Tyler Mall, Tempe, Arizona 85287, United States

ABSTRACT: Transient receptor potential (TRP) ion channels are eukaryotic polymodal sensors that function as molecular cellular signal integrators. TRP family members sense and are modulated by a wide array of inputs, including temperature, pressure, pH, voltage, chemicals, lipids, and other proteins. These inputs induce signal transduction events mediated by nonselective cation passage through TRP channels. In this review, we focus on the thermosensitive TRP channels and highlight the emerging view that these channels play a variety of significant roles in physiology and pathophysiology in addition to sensory biology. We attempt to use this viewpoint as a framework to understand the complexity and controversy of TRP channel modulation and ultimately suggest that the complex functional behavior arises inherently because this class of protein is exquisitely sensitive to many diverse and distinct signal inputs. To illustrate this idea, we primarily focus on TRP channel thermosensing. We also offer a structural, biochemical, biophysical, and computational perspective that may help to bring more coherence and consensus in understanding the function of this important class of proteins.



TRP channels are a family of diverse ion channels with broad physiological roles found primarily in higher organisms. These protein channels share structural homology with voltage-gated potassium and related ion channels (Figure 1). Since cloning and sequencing of the first transient receptor potential (TRP) ion channel in 1989, this protein superfamily has emerged as a diverse group of cellular sensors.[1] With the identification of temperature, gustatory, and mechanosensing TRP channels in the late 1990s and early 2000s, TRP channels have been widely associated with sensory physiology. Many investigations have shown that in addition to significant roles in sensory biology, TRP channels are involved in a wide variety of physiological and pathophysiological roles. Unlike many protein families in which characteristic functional attributes identify family members, TRP channels are sufficiently evolutionarily diverse such that they are defined solely by their sequence, perhaps underscoring the ability of TRP channels to function in a variety of biological contexts.[2] In addition to high levels of sequence and functional diversity among family members, individual TRP channels have been evolutionarily fine-tuned and multipurposed in a species-dependent manner. This molecular speciation complicates comparative studies and seems to convolute attempts to elucidate molecular mechanisms. Many TRP channels are modulated by a number of distinct stimuli, which compounds this problem.

The inherent polymodal modulation of themoTRP channels is illustrated by TRPV1, which is the most thoroughly studied family member (Figure 2). In humans, TRPV1 is the primary heat sensor and is activated by elevated temperatures.[3,4] It is also activated by pH, changes in membrane potential (voltage), divalent cations (Mg^{2+} and Ca^{2+}), phosphoinositide lipids, a small membrane protein named PIRT, and perhaps mechanical force.[3,5−10] TRPV1 is also directly and indirectly modulated by an array of natural and synthetic compounds, including capsaicin from spicy chili peppers, THC (Δ9-tetrahydrocannabinol) from cannabis, and a variety of toxins originating from spiders, fungi, and bacteria.[3,11−15] While a given TRP channel may be sensitive to a wide variety of stimulatory inputs, TRPV1 and other TRP channels have evolved to fill specific species-dependent roles. For example, human and rodent TRPV1 channels are sensitive to capsaicin, while amphibian and avian TRPV1 are not.[16] Similarly, above physiological temperatures, human TRPV1 activates and thus fulfills its role as a molecular thermometer.[4] Vampire bats, on the other hand, utilize a truncated TRPV1 isoform in specialized organs to detect infrared radiation, allowing for thermal imaging of prey.[17]

This work briefly highlights the polymodal modulatory nature of thermosensitive TRP channels and the importance of these channels in roles outside of sensory biology and uses this as a platform to examine controversial modes of modulation by proteins, lipids, and thermal stimuli. We suggest that the inherent diversity of TRP channels and their means of channel modulation give rise to apparently opposing experimental

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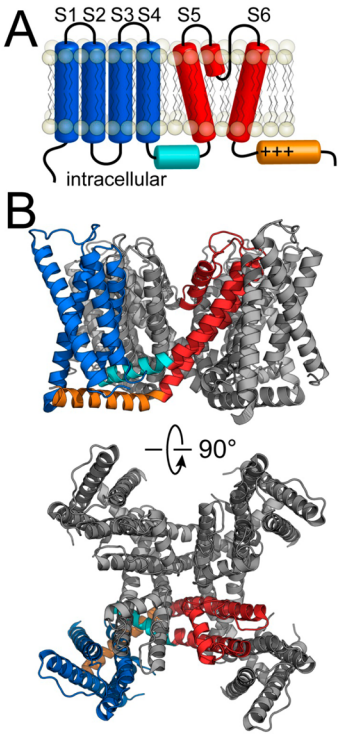

Figure 1. Architecture of TRP channels. (A) The membrane domain structure of TRP channels is homologous to that of voltage-gated potassium channels. The sensor domain is comprised of helices S1−S4 (blue). This domain is linked to pore domain transmembrane helices S5 and S6 (red) by an S4−S5 α-helix linker (cyan). The C-terminal juxtamembrane region is comprised of an α-helical segment (orange) that includes a short segment of generally conserved basic residues that have been implicated in PIP_2 regulation of TRP channels. (B) Structural view of the membrane regions of the functionally relevant tetrameric TRPV1 channel from Protein Data Bank entry 3J5P with the same coloring as panel A. The bottom panel is an extracellular view of the membrane regions of TRPV1.

results. Finally, we offer some yet to be investigated avenues that may help identify molecular mechanisms of thermosensation that have so far eluded previous investigations.

TRP CHANNELS BEYOND SENSORY PHYSIOLOGY

Of the 27 TRP channels identified in mammals, 10 have been identified as thermosensors, or thermoTRP channels.[18] While many are familiar with TRPV1 and TRPM8, which function as heat and cold sensors, respectively, a number of other TRP channels have been implicated as thermosensors, including TRPV2-V4, TRPA1, TRPM2, TRPM4, TRPM5, and TRPC5.[18,19] These ion channels are well-known as vanguards of the somatosensory system; however, research is increasingly revealing a wide variety of physiological roles beyond temperature and ligand sensing. Here, we highlight a few recent results that illustrate some of these diverse physiological roles.

TRPM8 is the primary cold sensor in higher organisms, and several studies have shown that TRPM8 regulates body temperature.[20−23] Mice and rats experience a transient drop in core body temperature following administration of TRPM8 antagonists.[20,22] This is apparently due to thermoregulatory systems of the brain misinterpreting the body's core temperature feedback system; consequently, thermogenesis factors such as vasoconstriction, oxygen consumption, brown adipose tissue thermogenesis, and cold-avoidance behavior are suppressed, resulting in a lower core temperature. Perhaps unsurprisingly, activation of TRPM8 channels by agonists, such as menthol and icilin, has an opposite physiological hyperthermic effect.[21,23] The thermoregulatory effect of TRPM8 was shown to be connected to the cold hyperalgesia symptom of opiate withdrawal via a direct interaction with the opioid G-protein-coupled receptor (GPCR), OPRM1.[24] Upon activation of OPRM1 by morphine, TRPM8 and OPRM1 are co-internalized, resulting in a reduction of cold- and menthol-activated currents in vitro. In wild-type mice, morphine treatment causes cold analgesia, whereas TRPM8 knockout results in a weakening of this effect.[24] The thermoregulatory role of TRPM8 also has downstream physiological effects. In a recent study, TRPM8 activation in brown adipose tissue was shown to upregulate expression of a UCP1, the mitochondrial uncoupling protein that bypasses ATP synthesis and harnesses the transmembrane proton gradient to produce heat.[25] Further, mice fed a high fat diet supplemented with a TRPM8 agonist gained significantly less weight than nonsupplemented mice, suggesting that TRPM8 plays a role in obesity and body weight regulation.[25]

TRPM8 has also been shown to be involved in insulin regulation. TRPM8 knockout mice exhibit increased rates of insulin clearance, which is apparently the result of an increased level of liver expression of an insulin-degrading enzyme.[26] The precise mechanism underlying this effect is unclear, given that TRPM8 is not expressed in either the pancreas or liver; however, TRPM8 afferent neurons do innervate the hepatic portal vein.[26]

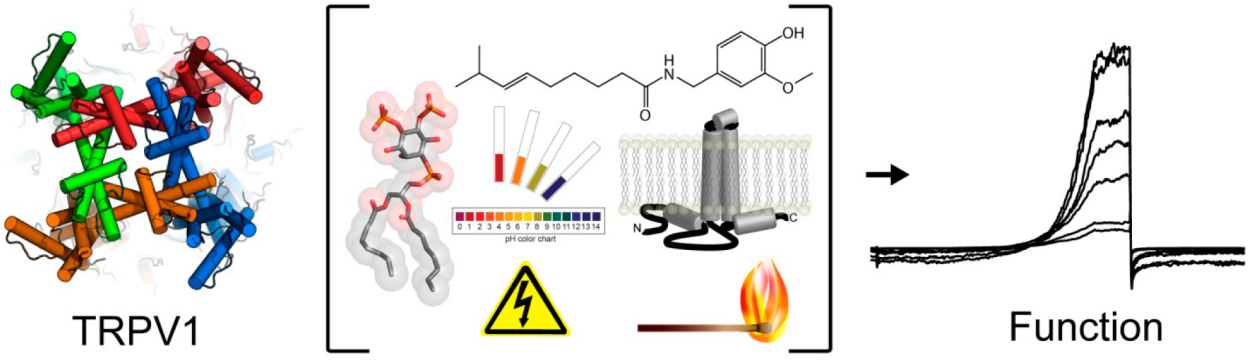

Figure 2. Polymodal modulation of thermosensitive TRP channels. TRPV1 is regulated, modulated, and gated by a variety of diverse stimuli. These include chemical compounds, such as capsaicin; lipids, such as phosphoinositides; and membrane proteins, such as PIRT. In addition, TRPV1 function is modulated by heat, voltage, and pH. The polymodal nature of TRPV1 modulation exemplifies a general feature of thermosensitive TRP channels.

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Taken together, while TRPM8 is best known for its role in cold thermosensation, it is implicated in a wide variety of physiologically important roles.

TRPV1 is a heat-activated channel that is also sensitive to a number of noxious stimuli.[27] It was the first human thermosensing channel identified, and is one of the most studied TRP channels. Its role in thermosensing is well-established, but recent reports suggest more complex physiological roles. For example, TRPV1 knockout mice have longer lifespans and maintain a youthful metabolic profile, the downstream effects of inactivation of a calcium signaling cascade that results in improved glucose homeostasis.[28] In addition, TRPV1 expressed in osteoclasts is involved in regulating bone resorption via crosstalk with GPCR cannabinoid receptors. Mice models of osteoporosis with either genetic or pharmacological inactivation of TRPV1 have restored osteoclast quiescence and improved bone density and morphology.[29]

TRPM2 is activated by H_2O_2 and other agents that produce reactive oxygen species (ROS), in addition to intracellular adenosine diphosphate ribose (ADPr). It is involved in a diverse array of physiological roles, and new roles continue to be identified. Recently, TRPM2 was shown to play an important role in neuritogenesis during embryonic brain development.[30] In this process, axonal growth and retraction must be properly balanced as the brain is shaped. Neurons that lack TRPM2 activity have neurites that are significantly longer than those of native cells.[30] This study also linked TRPM2 to the lysophosphatidic acid (LPA) signaling pathway, which is known to induce neurite retraction. LPA activates poly-ADPr polymerase 1, which results in an increased level of production of ADPr, an endogenous activator of TRPM2.[30]

TPRM2 is also expressed in mucosal mast cells, where it is involved in the degranulation process in response to antigen stimulation. Oda and co-workers demonstrated that TRPM2-mediated calcium influx is important in this process, and TRPM2 inhibition improved symptoms in a mouse model of food allergy.[31]

Finally, Numata et al. demonstrated that ADPr- and cyclic ADPr-induced TRPM2 currents were identical to hypertonicity-activated currents in HeLa cells.[32] Further experiments revealed that a splice variant of TRPM2 is likely the hypertonicity-induced cation channel in HeLa cells. TRPM2 appears to be activated downstream of CD38, an ectoenzyme responsible for exporting ADPr and cADPR out of the cell. siRNA knockdown of both TRPM2 and CD38 significantly reduced the increase in the regulatory volume in response to hypertonic challenge.[32] While the nature of the crosstalk between CD38 and TRPM2 remains unclear, the finding suggests an interesting role of TRPM2 in cell volume regulation, an important factor in cell proliferation and apoptosis.

These highlights are just a few of the extensive roles thermosensitive TRP channels play in diverse aspects of physiology, but this list is far from comprehensive.[33] Nonetheless, thermoTRP channels clearly play significant roles beyond sensory physiology.

VARIATION IN THERMOSENSITIVE TRP CHANNEL MODULATION

Surprising differences in modulation have been documented between thermosensitive TRP orthologues, even in closely related species (Figure 3). TRPA1 is a prime example of species-dependent differences, which has probably contributed to the controversy surrounding this channel's function.[34] TRPA1 is a chemical and thermosensitive nociceptor in animals. Sequence variation between species likely accounts for the observed differences identified in TRPA1 function. As an example, human and mouse TRPA1 have 80% identical sequences, though a lower level of conservation is found between more distantly related species. TRPA1 serves as a heat sensor for reptiles, while it is reportedly a cold sensor in rodents and other mammals.[35,36] A screen of potential new TRPA1 antagonists revealed that trichloro(sulfanyl)ethyl benzamides inhibit human TRPA1; however, some of the compounds elicited far smaller inhibitory effects on rat TRPA1, while others even activated it, demonstrating differential pharmacological profiles between the channels of the two species.[37] Similarly, caffeine activates mouse TRPA1 but inhibits human TRPA1; the mouse channel activation by caffeine can be reversed to inhibition by a point mutation.[38−40] There is substantial evidence of evolutionary differentiation of mammalian TRPA1 from other animals: pit vipers express TRPA1 as an infrared sensor, and TRPA1 appears to be a heat sensor in *Drosophila melanogaster*.[41−43] Indeed, the function of TRPA1 as a nociceptor is well-conserved throughout many species, but there are many examples of species-dependent differences: for a more thorough analysis of this literature, refer to the review by Chen and Kim.[44]

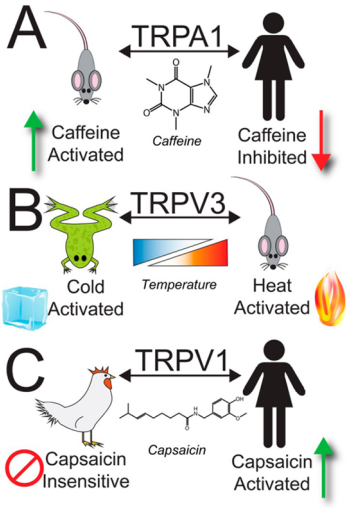

Figure 3. Examples of variation in TRP channel function between species. (A) Caffeine activates mouse TRPA1 but attenuates the activity of human TRPA1. (B) Evolutionary divergence has produced cold-sensitive TRPV3 in western clawed frogs, whereas TRPV3 has been implicated as a heat sensor in mice and other mammals. (C) Avian orthologues of TRPV1 are insensitive to capsaicin, a key agonist of the mammalian channel.

In addition to functional differences for TRP channels between species, it is clear that specific isoforms of TRP channels expand the functional properties for a given channel in a given species. This idea is highlighted in *Drosophila* TRPA1 studies that carefully characterized four channel isoforms.[42,45] Two *Drosophila* TRPA1 isoforms are heat-sensitive, while the other two isoforms are not temperature-sensitive. Interestingly, the *Drosophila* TRPA1 isoforms exhibit differential expression profiles, apparently physiologically tailored to either heat- or pain-sensing neurons.

While TRPA1 is a particularly rich example of apparent speciation and complex regulation, it is not unique in this regard. The IR-sensitive TRPA1 found in pit vipers is not the only TRP channel that has evolved to fill this functional niche: in vampire

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bats, a TRPV1 isoform serves a similar function.[46] Western clawed frogs developed a cold-sensitive TRPV3, whereas mammalian TRPV3 is likely a heat sensor.[47] There is conflicting evidence about the contributions of TRPV3 and TRPV4 to heat sensitivity that may potentially be explained by strain differences in mice.[48]

Species-dependent pharmacology has been shown for many compounds for a number of thermosensitive TRP channels. A putative TRPM8 orthologue in clawed frogs is activated by menthol, but with fine-tuned thermosensitivity across a temperature range much lower than that of the mouse and rat channels.[49] Similarly, chickens and other avians express TRPV1 orthologues that are insensitive to capsaicin, a key agonist of the mammalian channel.[50] The candidate anti-inflammatory compound JYL-1421 blocks capsaicin responses in rat TRPV1, but not in the human or monkey channels.[51] Likewise, phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV) is a strong agonist of rat TRPV1 but has no discernible effect on human TRPV1.[52] Zebrafish TRPV1 orthologues lack residues that are likely essential for endocannabinoid binding, suggesting that zebrafish TRPV1 may be insensitive to endocannabinoids known to interact with the mammalian channels.[53] Rat and mouse TRPV2 are activated by 2-aminoethoxydiphenyl borate (2-APB), a known modulator of many TRPs and store-operated calcium channels, but human TRPV2 is not responsive at all.[54]

Even for well-established endogenous regulators, a given thermosensitive TRP channel's modulatory profile can be complicated. In the case of PIP_2 (phosphatidylinositol 4,5-bisphosphate), an endogenous inner-leaflet membrane lipid known to interact with many ion channels and most TRPs, several channels have produced conflicting accounts of activation and inactivation. There is emerging and broad agreement that PIP_2 activates TRPV1 in a physiologically relevant context.[55] However, experiments with PIP_2 in artificial liposomes produce an inhibitory effect.[56] This may be explained by the fact that these liposomes do not exhibit the leaflet segregation of PIP_2 found in natural membranes, so the localization of PIP_2 in these experiments may not accurately reflect the endogenous biological context. However, the sensitivity of functional data to such minute differences demonstrates the intrinsic diversity of TRP channel modulation at a fundamental level. A TRP-like *Drosophila melanogaster* channel (dTRPL) has been reported to be both inhibited and strongly activated by PIP_2.[57,58] Similarly, TRPC4 may exhibit isoform-specific interaction with PIP_2.[59] For a thorough analysis of these issues, see the review of phosphoinositide regulation of TRP channels by Rohacs.[55]

In 2008, Dong and co-workers characterized a small two-span membrane TRP channel modulatory protein named PIRT (phosphoinositide interacting regulator of TRP), analogous to other β-subunits like the KCNE family for voltage-gated potassium channels.[9] In these studies, PIRT activated TRPV1 channels, and PIRT knockout mice showed reduced sensitivity to heat and capsaicin. PIRT has since been implicated in pruritus, or itch sensation, with PIRT knockout mice exhibiting minimal scratching in response to injection of histamine and non-histamine pruritogens.[60] A more recent report revealed that PIRT also potentiates TRPM8 currents in a manner similar to that of TRPV1. PIRT knockout mice exhibited impaired response to cold temperatures, while electrophysiology recordings showed increased current density in response to cold or menthol stimuli when PIRT was co-expressed with TRPM8.[61] These results reveal another layer of complexity in thermosensitive TRP channel modulation, and given the diverse physiological roles of TRP channels, this functional heterogeneity is potentially achieved by modulatory proteins, including PIRT.

Interestingly, like TRP channels, PIRT contains a putative PIP_2-binding site. Initially, it was proposed to be necessary for PIP_2-mediated activation of TRPV1.[9] However, later work by Carmen et al. called that conclusion into question, as inside-out excised patches showed that PIRT had no effect on PIP_2-dependent TRPV1 activation.[8] In any case, from in vivo studies, PIRT seems to modulate TRPV1 activity, but questions about the interplay, interactions, and intricacies among the channel, PIRT, and PIP_2 remain to be answered. For example, PIRT was shown to bind directly to TRPV1;[9] however, the binding site and stoichiometry of this interaction have yet to be elucidated, and the role of the phosphoinositide-binding region of PIRT is unclear.

Differential modulation has been demonstrated in multiple thermosensitive TRP channels, suggesting that it is not an incidental feature of a few family members. Perhaps these distinct modulatory modes have allowed them to function as polymodal sensors well beyond the realm of sensory biology. This issue presents a potential problem for functional studies of TRP channels, because it can be difficult to discern spurious results from genuine differences in systems. Further study and careful analysis must be devoted to differential modulation of TRP channels to habilitate animal models and further drug development.

TRP CHANNELS AS MOLECULAR THERMOMETERS

One of the most interesting features of some TRP channel family members is their functional role in thermosensing. TRP channel thermosensitivity is an intrinsic property of the proteins, which are directly activated or gated by temperature. This is evidenced by purified channels reconstituted into artificial membranes. Rohacs and co-workers expressed and purified full-length rat TRPM8 from *Escherichia coli*, which was incorporated into a 3:1 POPC/POPE planar bilayer and subjected to single-channel electrophysiology measurements. Their results show that TRPM8 purified and reconstituted in a nonbiological bilayer produces cold-evoked currents with steep temperature dependence similar to results observed in cell membranes.[62] Similarly, Julius and co-workers have expressed and purified a truncated functional form of rat TRPV1 from Sf9 insect cells. The resulting protein was incorporated into soybean polar lipid extract-based liposomes for electrophysiology measurements probing thermal sensitivity.[4] The outcomes mirror those seen from recombinant TRPM8 studies and indicate that elevated temperature produces direct activation of TRPV1. These results clearly show that thermosensitivity is an inherent property of these channels. Despite the inherent thermosensitivity of TRPM8 and TRPV1 (and presumably the other thermosensitive TRP channels), to date there is no coherent understanding of the temperature-dependent mechanism at the molecular level. Regardless, a number of important ideas, frameworks, and mechanisms have been proposed, including experimentally testable hypotheses.[27,63−65]

Part of the challenge in identifying a mechanism for TRP channel thermosensation arises because the nature of the protein region (or regions) used to sense temperature has not been isolated. A number of studies have reported on regions that affect TRP thermosensitivity. In aggregate, the outcomes of these studies do not isolate a specific region or domain and are generally contradictory.[64] The resulting regions are spread throughout the channels in both sequence and structure space

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(Figure 4). For example, a number of studies have identified the N-terminal extramembrane ankyrin repeat domains (ARDs) of

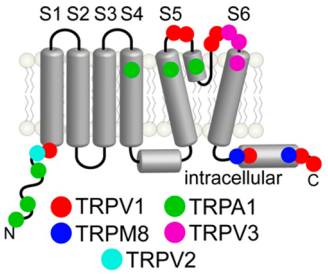

Figure 4. Reported regions of TRP channels important for thermosensing. For a number of TRP channels, various regions have been implicated as being crucial to thermosensing. Thermosensing regions are plotted on the monomer topology diagram of a generic TRP channel. Colors represent different thermosensitive TRP channels as follows: TRPV1 (red),[112−119] TRPM8 (blue),[91,114] TRPA1 (green),[35,40,42,120,121] TRPV3 (purple),[115,122] and TRPV2 (cyan).[112] Each colored circle represents a published account of a region involved in thermosensing.

TRPV1 and TRPA1 as being involved in thermosensation. However, TRPM8 has no ARDs, and TRPA1 has significantly more ARDs than TRPV1. This result suggests that the ARDs are not key to thermosensing or indicates that the TRPM8 thermosensing mechanism is distinct from that of TRPV1 and TRPA1, which admittedly could be the case as the channels are relatively divergent (TRPV1 and TRPM8 sequences are ∼11% identical). Indeed, thermoTRP channels in general have a low degree of sequence homology, even between members of the same family (Figure 5). The lack of consensus between functional studies intended to isolate regions and mechanisms of thermosensing TRP channels has interesting implications in its own right. It suggests that unlike other ion channel properties, such as voltage- or ligand-dependent channel activation and ion selectivity (which are driven by localized structural regions), TRP channel thermosensation may be delocalized over structural space dependent on a new, yet to be identified mechanism. An alternative explanation for the challenges in isolating a TRP channel thermosensor is that using mutations, chimeras, and/or truncations may not be the most viable method for probing thermosensation mechanisms because these changes generally have unknown and unintended thermodynamic consequences, which can result in energetic perturbations that change the functional output without inherently perturbing thermosensation.[63]

Arising from the challenges in isolating a temperature-sensitive domain or subdomain, the hypothesis has emerged that perhaps TRP thermosensing regions are structurally dispersed over multiple domains. Regardless, important strides in understanding thermosensation are forthcoming. It is clear from quantitative electrophysiology-based thermodynamic studies that TRP channels have relatively large magnitude changes in enthalpy ($\Delta H \geq \pm 100$ kcal/mol) upon channel activation.[66,67] The magnitudes of ΔH are more than an order of magnitude larger than for many other reported proteins undergoing conformational change and are similar to the reported values for classical protein unfolding studies.[68] Interestingly, for the cold sensing protein TRPM8, the ΔH of activation is exothermic

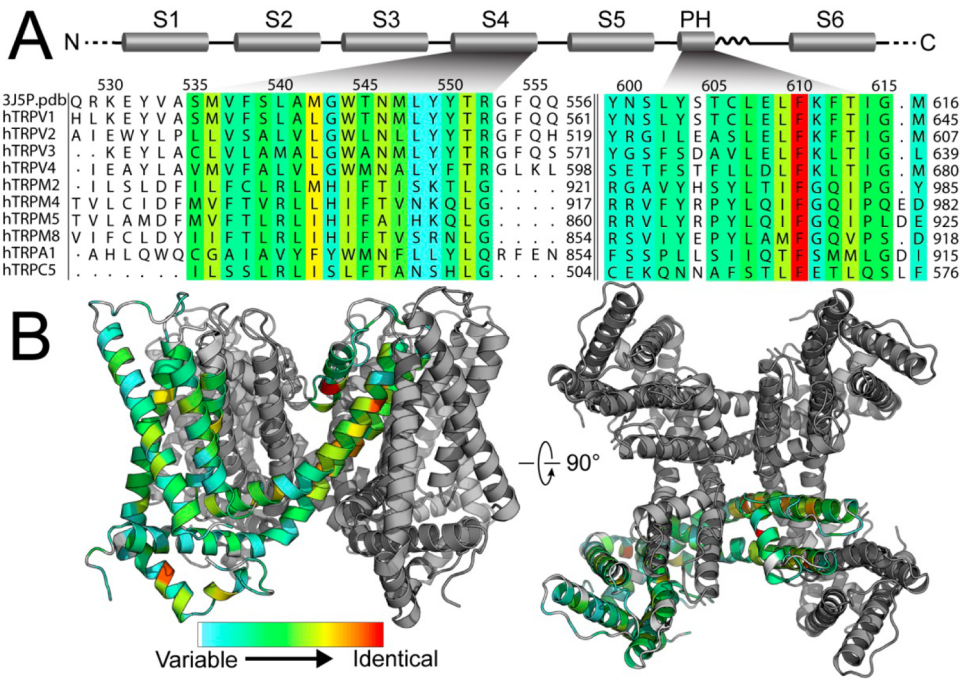

Figure 5. Low-level sequence conservation in human thermosensitive TRP channels. (A) Multiple-sequence alignment of the S4 and pore (PH) helices in known human TRP thermosensors represented using ALINE.[123] These two helices were chosen because, unlike strongly voltage-gated channels, the S4 helix is relatively poorly conserved in TRP channels, whereas the pore helix (PH) appears to contain the only residue that is absolutely conserved in the transmembrane region of human thermoTRPs. There is little to no homology in the loop regions and limited homology in the helices. This alignment was generated using ClustalX[124] to iteratively add multiple sequences to a pairwise structural alignment of human TRPM8 to the apo structure of rat TRPV1 (Protein Data Bank entry 3J5P) generated using MUSTANG.[125] Sequence similarity is indicated with a color spectrum from cyan to red using ALSCRIPT Calcons.[126] White, cyan, and red indicate subthreshold conservation, weak conservation, and absolutely conserved residues, respectively. (B) The sequence conservation is mapped to membrane regions of the rTRPV1 structure using the same colors given above.

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Table 1. Structures of TRP Channels and TRP Channel Domains

channel	source	domain studied[a]	expression system	method	resolution (Å)	Protein Data Bank entry
TRPV1[71,72,127−129]	rat	ARD (101−364) and ATP	*E. coli* BL21(DE3)	X-ray	2.7	2PNN
		C-terminus (767−801) and CaM	*E. coli* BL21(DE3)	X-ray	1.95	3SUI
		full-length*	*Saccharomyces cerevisiae* BJ5457	cryo-EM	19	N/A
		near-full-length (110−603//627−764)	baculovirus transduction in HEK293S GnTI$^-$	cryo-EM	3.4	3J5P
		with DkTx and RTx†			3.8	3J5Q
		with capsaicin†			4.2	3J5R
TRPV2[130−132]	rat	full-length	*S. cerevisiae* BJ5457	cryo-EM	13.6	5688[b]
	human	ARD (68−319)	*E. coli* DL41	X-ray	1.7	2F37
	rat	ARD (75−321)	*E. coli* BL21(DE3)	X-ray	1.65	2ETB
TRPV3[133]	mouse	ARD (118−367)	Rosetta (DE3)	X-ray	1.95	4N5Q
TRPV4[134,135]	human	ARD (149−397) and ATP	*E. coli* BL21(DE3)	X-ray	2.85	4DX1
	rat	full-length‡	baculovirus-infected Sf9 cells	cryo-EM	35	N/A
TRPV6[136]	mouse	ARD (44−265)	*E. coli* BL21(DE3)	X-ray	1.7	2RFA
TRPA1[137]	mouse	full-length§	*S. cerevisiae* BJ5457	negative stain EM	16	5334[b]
TRPM2[138]	human	full-length‡	HEK 293	negative stain EM	28	N/A
TRPM7[139,140]	rat	coiled coil (1230−1282)	*E. coli* BL21(DE3)pLysS	X-ray	2.0	3E7K
	mouse	α-kinase (1549−1828)	baculovirus-infected Sf9 cells	X-ray	2.8	1IAJ
TRPC3[141]	mouse	full-length	HEK 293	cryo-EM	15	N/A
TRPP2[142,143]	human	coiled coil (833−872)	Rosetta2 (DE3)	X-ray	1.9	3HRN, 3HRO
	human	EF hand (720−796)	BL21(DE3) codon Plus RIL	NMR	1.9[c]	2KQ6
	human	EF hand (680−796)	BL21(DE3)	NMR	4.2[c]	2KLD, 2KLE
TRPP3[144]	human	coiled coil (699−743)	Rosetta (DE3)	X-ray	2.8	4GIF

[a]Numbers indicate residues used for structural studies. ARD and CaM stand for the ankyrin repeat domain and calmodulin-binding domain, respectively. Full-length or near-full-length channels were reconstituted in various artificial hydrophobic environments such as *n*-decyl β-D-maltoside (asterisk), Amphipol A8−35 (dagger), *n*-dodecyl β-D-maltoside (double dagger), and Fos-choline 12 (section). [b]Reference numbers for the EMDataBank. [c]Predicted equivalent resolution.[145]

($\Delta H < 0$), suggestive of an enthalpy-driven activation as given by $\Delta G = \Delta H - T\Delta S$. On the other hand, for the heat-sensing TRPV1, ΔH is endothermic, which suggests that the free energy of activation is entropy-driven. The free energy of activation for thermosensitive TRP channels is similar to that of other ion channels (~10 kcal/mol), which demands that the large ΔH of thermosensitive TRP channels be compensated by significant changes in entropy (ΔS).[69] To this end, Clapham and Miller have proposed a thermodynamic model of TRP thermosensitivity mirroring early protein folding studies in which the temperature-dependent conformational change of these channels is driven by a change in heat capacity (ΔC_p).[63] Historical protein studies have found that temperature-dependent enthalpies and entropies are common and arise from a change in heat capacity. At a given temperature (T), the ΔH can be related to a reference enthalpy (ΔH°) at the reference temperature (T°) as a function of ΔC_p, where

$$\Delta H = \Delta H^\circ + \Delta C_p(T - T^\circ)$$

Similarly, a temperature-dependent change in entropy, ΔS, includes a ΔC_p term

$$\Delta S = \Delta S^\circ + \Delta C_p \ln\left(\frac{T}{T^\circ}\right)$$

These temperature-dependent thermodynamic entities give rise to nonlinear protein stability curves, which dictate how ΔG varies as a function of temperature, with the form

$$\Delta G = \Delta H^\circ - T\Delta S^\circ + \Delta C_p\left[T - T^\circ - T\ln\left(\frac{T}{T^\circ}\right)\right]$$

Thus, changes in heat capacity can impact the temperature dependence of conformational changes, including potentially that of thermosensing TRP channels.[70]

One prominent feature of the ΔC_p-dependent thermosensing hypothesis is that both positive and negative enthalpies of TRPM8 and TRPV1 can be explained thermodynamically because transitions that have changes in heat capacity between states have, by definition, protein stability curves that are nonlinear where transitions can be either enthalpy- or entropy-driven, analogous to protein heat and cold denaturation. The most important outcome of this hypothesis is that it can be tested experimentally. It is clear from early protein folding studies what underlies changes in heat capacity. These underpinnings include changes in electrostatics, secondary structure, etc. However, it has been recognized for decades that changes in heat capacity usually correlate extremely well with changes in solvation. For example, a hydrophobic residue undergoing a transition to a hydrophilic environment results in a large and positive ΔC_p. Moreover, changes in solvent accessibility can be observed in experimental and computational studies that may provide a method for linking the existing thermodynamics to structural investigations.

ON THE PATH TO A STRUCTURAL BIOLOGICAL MECHANISM OF TRP THERMOSENSING

Understanding thermodynamic measurements in terms of structure is at best inherently challenging. However, the emerging progress in TRP channel structural biology suggests optimism. The laboratories of Cheng and Julius recently reported three high-resolution structures of a truncated but

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functional rat TRPV1 channel in apo and agonist-bound states.[71,72] Their structures are the first to include membrane regions of a TRP channel at high resolution, and their studies highlight important features of agonist-dependent channel gating. While seminal to understanding TRP channel function, the structures do not elucidate anything in particular regarding TRPV1 thermosensation. In addition to the recent TRPV1 structures, there are a handful of other low-resolution structures and high-resolution structural domains from some TRP family members as detailed in Table 1.

Beyond thermosensitive TRP channels, there are a number of other proteins and nucleic acids that have physiologically relevant temperature-dependent responses, which may provide insight into TRP channel thermosensitivity.[73−75] One relevant example is DesK, a bacterial histidine-kinase thermosensor. DesK is a bifunctional membrane enzyme that is regulated between kinase and phosphatase activities by changes in temperature. Recent studies indicate that the temperature-dependent switch in DesK is a membrane proximal helical segment that undergoes a transition between coil and helix conformations.[76] Interestingly, this conformational change alters the solvent accessibility of a patch of hydrophilic residues to and from the membrane, suggesting a mechanism that would produce significant changes in heat capacity (ΔC_p) as has been proposed for TRP channel thermosensing.[63]

Recently, Chanda and co-workers used rational protein design on the Shaker voltage-gated potassium channel ($K_v1.2$), engineering significantly increased thermosensitivity at magnitudes on par with those of thermosensitive TRP channels.[77] The Shaker channel was used because it is well-studied and has negligible inherent temperature sensitivity. At the heart of the design principle was the idea that the ΔC_p could be changed and thus thermosensitivity altered by modifying the polarity of residues that undergo a change in solvent accessibility as a function of gating. Chanda and co-workers focused on mutating the membrane interfacial residues of helices S1−S3 and charge-bearing S4 helix residues of the voltage-sensing domain and were able to confer temperature sensitivity to the Shaker channel (Figure 6). The outcome of this work supports the hypothesis that TRP channel thermosensitivity is strongly dependent on ΔC_p. Similarly, the outcomes suggest that alterations of a single structural domain, like the voltage-sensing domain of the Shaker channel, are sufficient to confer thermosensitivity.

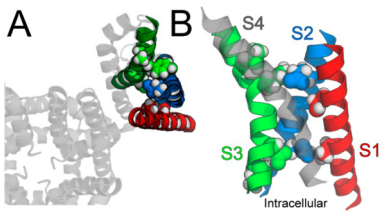

Figure 6. Residues and regions of the Shaker channel modified to confer thermosensing functionality. (A) Extracellular view of a portion of the pore domain (bottom left) with the voltage-sensing domain (top right) from the Shaker channel. Residues that were rationally mutated in helices S1−S3 are highlighted; these mutations generally increase the temperature sensitivity of a non-thermosensing channel. These residues are posited to change solvent accessibility with domain conformational changes and are focused around the interface of the S4 helix. (B) Close-up view of the residues mutated to increase the thermosensitivity of Shaker. The reduction in the number of the S4 helix charged residues significantly increased the thermosensitivity. The S4 helix is colored gray and is transparent.

IDENTIFYING A TRP-SPECIFIC THERMOSENSOR

The only conserved structural domains in all thermosensing TRP channels are the pore and sensor domains (helices S5 and S6 and helices S1−S4, respectively). As a result, it should be possible to employ a "divide and conquer" approach to overexpress these (and other) structural domains of TRP channels on the path to identifying a thermosensor structural domain. Given the apparent thermodynamic similarities between thermosensing proteins and protein unfolding, one can tap into the immense and well-developed field of protein folding to gain mechanistic and thermodynamic insight into thermosensing, including answering the basic question of whether thermosensing is accomplished by a specific domain or is diversified over many structural domains and ultimately what the nature and magnitude of the temperature-dependent conformational changes are.

Calorimetry data would be the gold standard for identifying and validating a thermosensing region. In particular, differential scanning calorimetry (DSC) provides a direct measurement of the molar heat capacity as a function of temperature that can be used to detect the change in enthalpy of the temperature-dependent transition. DSC data can also provide the midpoint of the transition temperature and with further analysis the change in entropy and free energy. These values would be directly comparable to existing values obtained from functional studies and therefore very useful for potentially confirming the isolation of a thermosensing domain. There are downsides to this approach. The main limitation is that with a modern sensitive DSC instrument, one would still likely need milligram levels of pure and folded protein to ensure accurate and reliable calorimetry data, the same quantities needed for NMR and X-ray structural studies.

In the absence of calorimetry data, reversible temperature-dependent conformational changes can be measured by a handful of spectroscopic techniques (circular dichroism, fluorescence, NMR, etc.) and subjected to van't Hoff analysis to obtain estimates of the changes in enthalpy, entropy, and heat capacity. For example, far-ultraviolet circular dichroism (CD) offers a relatively sensitive, label-free, and straightforward method for subjecting potential thermosensing proteins and domains to thermodynamic analysis.[78] For far-UV CD to properly detect a temperature transition, there must be a change in secondary structure, which seems likely if the structural transition is similar to that of DesK.

In addition to DSC and CD measurements, solution NMR is well-suited to thermosensing studies for a number of reasons. Both voltage-sensing and pore domains from other channels have been investigated by solution NMR.[79−81] The benefits for solution NMR are that the experiments are capable of probing atomic-resolution protein dynamics over biological time scales, including the millisecond time scale regime during which protein conformational changes generally occur. This information could be used in comparative studies of thermosensitive TRP channels to validate the outcomes with existing electrophysiology data. Lastly, modern NMR hardware makes it straightforward to probe structure, dynamics, and thermodynamics in a temperature-dependent manner from 0 to 80 °C, making this technique particularly promising for TRP thermosensing studies. One classic study made use of relaxation dispersion experiments to probe conformational dynamics and energetics between states of a membrane-bound enzyme.[82] Such methods would be very

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relevant to a TRP channel thermosensing domain. While it is unlikely with current methods that solution NMR could be used to determine the structure of a full-length TRP channel, it is feasible to determine the structures of the sensor domain or a tetrameric pore domain at distinct temperatures.

In addition to NMR structural studies, advances in other structural techniques suggest that either cryo-electron microscopy (EM) or X-ray crystallography could potentially be able to determine the structure of a full-length thermosensing channel in distinct states. It is clear from the work of Cheng and Julius that high-resolution cryo-EM can be used to determine TRP channel structure. With new advances in EM software, which includes the ability to perform single-particle reconstruction on conformationally heterogeneous samples and the use of time-resolved cryo-EM instrumentation, which can freeze samples on a time scale on the order of milliseconds, it may be possible to trap and determine the structures of TRP channels in distinct conformations as a function of temperature.[71,83]

One last promising structural technique that could have important implications for understanding the mechanism of thermosensitive TRP channels is the use of time-resolved serial femtosecond X-ray crystallography (SFX). This emerging technique relies on intense femtosecond pulses from an X-ray laser that interact with a stream of small protein crystals causing X-ray diffraction prior to sample destruction. A recent example coupled time-resolved SFX with a yellow light laser to specifically activate photosystem (PS) II, resulting in the ability to probe structurally the conformational changes in PSII associated with water splitting, which is central to photosynthesis.[84] Modifications on this theme, where instead of a light to activate PSII, one could modulate the temperature, perhaps by altering the distance between the sample injector and X-ray laser, could give way to probing structurally the conformational changes central to TRP channel thermosensitivity. Given the advances in understanding of TRP channels and membrane protein structural biology, it seems likely that it is only a matter of time before the mechanism of TRP thermosensing emerges.

X-ray crystallography and cryo-EM may provide structures at medium to high resolution, and NMR can reveal a structural, dynamic, and thermodynamic context. Computational approaches can yield insights that are complementary to the insights of these and other experimental approaches previously discussed. Computer simulations take static protein structures as input but are able to reveal the details of conformational changes, protein−membrane interactions, or protein−solvent interactions at the atomic level and can also provide dynamic information.[85] Computer simulation output is often sufficient to construct mechanistic molecular models. Computational methods model the interactions between atoms with physical or heuristic interatomic interaction potentials and utilize computational algorithms to sample the conformational dynamics of a given channel.

The gold standard of these studies are molecular dynamics (MD) simulations that provide a means of computationally exploring the dynamics of structural models of, for instance, a TRP channel embedded in a lipid bilayer.[86] On the basis of atomic-resolution experimental structures, MD simulations show the interactions of the channel with the solvent (ions and water molecules) and the membrane lipids and allow for qualitative and quantitative analysis of thermodynamic and kinetic properties of the channel.[87−90] To date, few TRP channel MD studies have been conducted because of the lack of reliable atomic-resolution input structures, although a number of studies combined experiments with short (<20 ns) simulations of TRPV1 models based on Kv, HCN, or the KcsA channel structures.[91−94] The first atomic-resolution structures were published in late 2013, and as a result, the first MD simulations based on these experimental input structures are only now beginning to emerge.[71,72] Recently, the TRPV1 selectivity filter of the isolated pore structure was simulated to probe its interaction with sodium, potassium, and calcium ions.[95] This study concluded that the selectivity filter of TRPV1 is highly flexible and hypothesized that the cryo-EM-observed selectivity filter is not optimized for ion conductance.[72] Another recent study combined experimental mutagenesis, electrophysiology, and MD simulations to identify a binding site for $PI(4,5)P_2$.[96] Both studies utilized equilibrium MD simulations of a few hundred nanoseconds that explicitly contained all atoms of the protein, lipid, and solvent molecules. Similar simulations will continue to yield further insights, especially when they are conducted over longer time scales to increase the level of sampling of solvent and protein degrees of freedom. Unbiased equilibrium MD is particularly useful because no specific assumptions are made about the protein of interest and the system evolves naturally, driven by thermal fluctuations. However, because of the great computational demands, equilibrium MD is currently limited to tens of microseconds on standard supercomputers and hundreds of microseconds on special purpose machines.[97] Importantly, enhanced sampling methods can be employed to obtain equilibrium properties over longer time scales, including conformational changes underlying channel gating.[98−101] MD-based methods also exist to compute thermodynamic information that may be useful to correlate experimental observables such as single-channel conductance or thermodynamics with structural conformational changes.[102,103] Because TRP channels are temperature-sensitive, temperature-based ensemble methods, such as temperature replica exchange (typically coupled with Hamiltonian exchange), are of particular interest.[104,105] Nonequilibrium MD simulations that include the membrane potential have been used to study ion permeation of other channels in a voltage-dependent manner and have yielded observables such as completer current−voltage curves and ion selectivity, as well as new insights into voltage sensor dynamics.[106−109] Thus, computational methods have demonstrated their usefulness in bridging the gap between structure and function, in particular for ion channels, and therefore present another meaningful technique for dissecting the function of thermosensitive TRP channels.

One of the primary questions that needs to be addressed by these techniques is whether the thermosensitivity of TRP channels is governed by discrete modular domains or distributed throughout the channel in smaller, structurally unrelated regions. As explained earlier, random mutagenesis studies have so far not yielded definite conclusions for distinguishing these two hypotheses. Testing the dispersed thermosensing hypothesis will require high-resolution structural information from full-length or near-full-length native channels to identify regions of the channel that are involved in temperature-dependent conformational change. The cryo-EM or X-ray crystallography techniques discussed above could provide high-resolution insight into these changes. Residues or regions that are identified as potential contributors to channel thermosensitivity could then be probed and tested functionally in a hypothesis-driven approach. Chowdhury et al. demonstrated an excellent example of such an approach by utilizing the wealth of structural information about the Shaker channel to rationally guide experiments.[77] In this

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context, one of the major challenges limiting fundamental TRP studies is the ability to produce sufficient quantities of pure, reconstituted, and biologically relevant TRP channels and related domains. As this hurdle is overcome, more biophysical, structural, and computational investigations will follow, yielding new insight into this important class of proteins.

As mentioned above, thermosensitive TRP channels are inherently susceptible to multiple distinct stimuli. In addition, a number of studies have identified TRP channel orthologs with species-dependent functionality. The TRP channel activation and modulation landscape is further expanded by reports of isoforms and heteromultimerization with additional diversified functional output.[110] It thus stands to reason that TRP channels are exquisitely balanced for sensitivity to a variety of distinct inputs, which likely is at the heart of reported challenges isolating and identifying mechanisms associated with PIP_2, PIRT, and thermosensing-based gating. This idea is supported by recent molecular phylogenetic studies that indicate TRP channels originally evolved in non-neuronal cells and later in animal evolution acquired neuronal functionality associated with sensory biology.[111] The intrinsic plasticity of TRP channels notwithstanding, important inroads are being made, pointing toward a more complete understanding of how these proteins are integrated into many diverse physiologically important signal transduction pathways.

In addition to providing a brief synopsis of the state of thermosensing TRP channel studies, we offer a perspective of a few structural and biophysical methods that should complement the existing functional electrophysiology data that have been foundational for the current understanding of these fascinating channels. These types of studies are needed to test specific hypotheses that have recently emerged, and we are hopeful they will lead to a better understanding of the molecular intricacies of thermosensation, with a long-term view of elucidating how these channels sense temperature and the means by which they are able to integrate numerous diverse stimuli.

AUTHOR INFORMATION

Corresponding Author

*E-mail: wade.van.horn@asu.edu. Telephone: (480) 965-8322. Fax: (480) 965-2747.

Notes

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ABBREVIATIONS

TRP, transient receptor potential; PIRT, phosphoinositide-interacting regulator of TRP; ARD, ankyrin repeat domain; CaM, calmodulin-binding domain.

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