CHAPTER FOUR

TRPA1 Channels: Chemical and Temperature Sensitivity

Willem J. Laursen[1,2], Sviatoslav N. Bagriantsev[1,*] and Elena O. Gracheva[1,2,*]

[1]Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT, USA
[2]Program in Cellular Neuroscience, Neurodegeneration and Repair, Yale University School of Medicine, New Haven, CT, USA
*Corresponding author: E-mail: elena.gracheva@yale.edu, sviatoslav.bagriantsev@yale.edu

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Abstract

Transient receptor potential ankyrin 1 (TRPA1) is a polymodal excitatory ion channel found in sensory neurons of different organisms, ranging from worms to humans. Since its discovery as an uncharacterized transmembrane protein in human fibroblasts, TRPA1 has become one of the most intensively studied ion channels. Its function has been linked to regulation of heat and cold perception, mechanosensitivity, hearing, inflammation, pain, circadian rhythms, chemoreception, and other processes. Some of these proposed functions remain controversial, while others have gathered considerable experimental support. A truly polymodal ion channel, TRPA1 is activated by various stimuli, including electrophilic chemicals, oxygen, temperature, and mechanical force, yet the molecular mechanism of TRPA1 gating remains obscure. In this review, we discuss recent advances in the understanding of TRPA1 physiology, pharmacology, and molecular function.

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1. INTRODUCTION

TRPA1 (transient receptor potential ankyrin 1, also known as ANKTM1) is a polymodal ion channel that belongs to a functionally and structurally diverse group of ion channels known as transient receptor potential (TRP) channels. It was originally discovered as an obscure molecule in cultured human fibroblasts (Jaquemar, Schenker, & Trueb, 1999), and later received considerable attention as a putative cold sensor in rodents (Story et al., 2003). TRPA1 is expressed in a specific subset of somatosensory neurons of trigeminal, dorsal root, and nodose ganglia, and exhibits pronounced promiscuity in terms of activation: the channel can interact with an increasingly long list of identified ligands, including the electrophilic compounds in pungent spices, industrial pollutants, and various external irritants (Julius, 2013; Nilius, Appendino, & Owsianik, 2012). TRPA1 is also activated by endogenous inflammatory mediators and plays a significant role in transducing nociceptive signals associated with tissue damage and inflammation (for reviews see Bautista, Pellegrino, & Tsunozaki (2013), Julius (2013)). Indeed, TRPA1 is expressed specifically in peptidergic C-fibers, which release an endogenous neurogenic inflammatory cocktail, containing CGRP (Calcitonin gene-related peptide), substance P, and neurokinins, resulting in the initiation of cellular responses and tissue inflammation.

A growing number of studies implicate TRPA1 in the development of migraine headache (Benemei, Fusi, Trevisan, & Geppetti, 2013). This notion is supported by the fact that umbellulone (the active compound of the so-called "headache tree") activates TRPA1 channels within the trigeminal system (Zhong et al., 2011), thereby initiating CGRP release, leading to significantly increased meningeal blood flow (Kunkler, Ballard, Oxford, & Hurley, 2011). Recently, additional support was given to the idea of TRPA1 as a mediator of pain in humans when a gain-of-function mutation was identified and found to be associated with a familial episodic pain syndrome (Kremeyer et al., 2010).

Itch (pruritus) is a very important protective physiological response to different irritants, including insect bites, poisonous plants, and pollutant allergens. Not long ago, TRPA1was shown to be involved in the itch pathway and proposed to act as an integrator of multiple histamine-independent signaling cascades (Bautista, Wilson, & Hoon, 2014; Oh et al., 2013; Wilson et al., 2011).

In some invertebrate and vertebrate species, including fruit flies, *Caenorhabditis elegans*, pit-bearing snakes, frogs, and lizards, the TRPA1 channel evolved as a temperature sensor (Chatzigeorgiou et al., 2010; Gracheva et al., 2010; Kang et al., 2012; Saito et al., 2012; Viswanath et al., 2003). It is interesting to note that, in these animals, sensitivity of TRPA1 to temperature often appears to come at the expense of chemical activation, with thermo-activated orthologues displaying decreased chemosensitivity (Cordero-Morales, Gracheva, & Julius, 2011; Gracheva et al., 2010; Saito et al., 2012) (see Table 4.1).

Nonetheless, its role as a polymodal sensor of such a variety of stimuli including temperature and noxious irritants—combined with its described role in episodic pain syndrome, inflammation and migraine—makes TRPA1 an attractive target for analgesic intervention. A thorough understanding of channel activation mechanisms will therefore be crucial for the development of effective and specific therapeutics.

2. ACTIVATION AND REGULATION OF TRPA1 BY CHEMICAL COMPOUNDS

TRPA1 is a nonselective cation channel that contains six transmembrane domains, a huge cytoplasmic N terminus (~720 amino acids), and a cytoplasmic C terminus. It is distinguishable from other members of the TRP channel family by the presence of numerous regulatory ankyrin repeats within the N terminus (Jaquemar et al., 1999; Story et al., 2003) (Figure 4.1). Ankyrin repeats are 33-amino-acid-long structural motifs involved in protein–protein interactions. This region of the TRPA1 channel is thought to play a key role in the regulation of gating and integration of multiple stimuli, including temperature, multiple classes of chemicals, and cytoplasmic calcium (Cordero-Morales et al., 2011; Nilius, Prenen, & Owsianik, 2011; Zurborg, Yurgionas, Jira, Caspani, & Heppenstall, 2007).

2.1 Chemical activation of TRPA1 by covalent modification

The most well-characterized mechanism of TRPA1 activation occurs through covalent modification of the thiol groups of conserved cysteine residues that are located in the "linker" region of the channel that connects the ankyrin repeats with the transmembrane domain (Hinman, Chuang, Bautista, & Julius, 2006; Macpherson, Dubin, et al., 2007) (Figure 4.1). There are several pungent culinary compounds that activate TRPA1 via electrophilic attack on these cysteines, including allyl isothiocyanate

Table 4.1 Temperature and chemical sensitivity data for TRPA1 orthologues from different animal species

Temperature and chemical sensitivity of TRPA1 orthologues

Species	Cold/heat	Threshold (°C)	Q_{10}	AITC (EC_{50})	References
Human (*Homo sapiens*)	Cold?	≤17	N.D.	Sensitive (62 μM)	Story et al. (2003), Cordero-Morales et al. (2011)
Rhesus macaque (*Macaca mulatta*)	Insensitive	–	–	Sensitive (75.5 μM)	Chen et al. (2013)
Rat (*Rattus norvegicus*)	Cold?	≤15	N.D.	Sensitive (11 μM)	Story et al. (2003), Jordt et al. (2004)
Mouse (*Mus musculus*)	Cold?	≤18	N.D.	Sensitive (0.39 μM)	Story et al. (2003), Sawada et al. (2007), Zhou et al. (2013)
Chicken (*Gallus gallus domesticus*)	Heat	39.4 ± 1.1	N.D.	Sensitive (N.D.)	Saito et al. (2014)
Rattlesnake (*Crotalus atrox*)	Heat	27.6 ± 0.9	13.7	Sensitive (>2000 μM)	Gracheva et al. (2010)
Rat Snake (*Elaphe obsoleta lindheimeri*)	Heat	37.2 ± 0.7	8.8	Sensitive (>500 μM)	Gracheva et al. (2010)
Python (*Python regius*)	Heat	32.7 ± 1.3	N.D.	Sensitive (>500 μM)	Gracheva et al. (2010)

Boa (*Corallus hortulanus*)	Heat	29.6 ± 0.7	N.D.	Sensitive (>500 μM)	Gracheva et al. (2010)
Anole (*Anolis carolinensis*)	Heat	33.9 ± 0.8	45.71	Sensitive (N.D.)	Saito et al. (2012)
Frog (*Xenopus tropicalis*)	Heat	39.7 ± 0.7	59.24	Sensitive (N.D.)	Saito et al. (2012)
Zebrafish (*Danio rerio*)	Insensitive	–	–	Sensitive (N.D.)	Prober et al. (2008)
Fruit fly (*Drosophila melanogaster*)				Sensitive (277.6 μM)	Cordero-Morales et al. (2011), Kang et al. (2010), Kang et al. (2012), Zhong et al. (2012)
TRPA1(A)	Heat	29.7 ± 0.3	9		
TRPA1(B)	Heat	27.8 ± 0.4	116		
TRPA1(C)	Insensitive	–	–		
TRPA1(D)	Heat	≥34	N.D.		
Mosquito (*Anopheles gambiae*)	Heat	34.2 ± 1.8	4	Sensitive (N.D.)	Wang et al. (2011), Kang et al. (2012)
TRPA1(A)		25.2 ± 0.9	200		
TRPA1(B)					
Nematode (*Caenorhabditis elegans*)	Cold?	≤18	N.D.	Insensitive	Chatzigeorgiou et al. (2010), Kindt et al. (2007)

N.D., Not Defined.

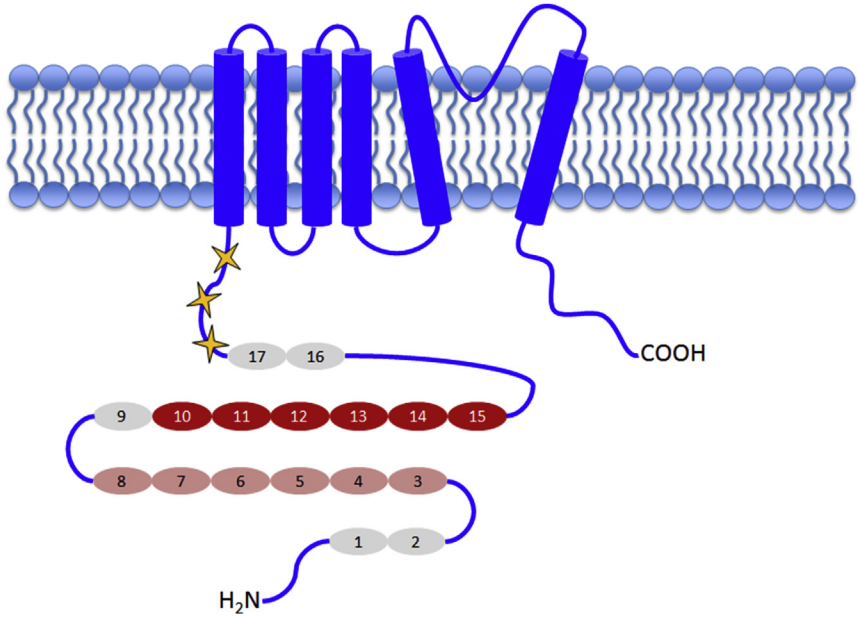

Figure 4.1 Topological organization of a snake TRPA1 channel. Ankyrin repeats are shown as numbered ovals. The primary and enhancer modules important for determining heat sensitivity correspond to repeats number 10–15 and 3–8, respectively. Stars in the linker region between the transmembrane domains and ankyrin repeat domain depict conserved cysteine residues involved in channel activation by electrophiles. (See the color plate.)

(AITC; found in horseradish, wasabi, and mustard) (Bandell et al., 2004; Jordt et al., 2004), allicin and diallyl disulfide (from raw garlic) (Bautista et al., 2005; Macpherson et al., 2005), and cinnamaldehyde (cinnamon) (Bandell et al., 2004), among others (Figure 4.2). These molecules are widely used in the laboratory to explore physiological roles of TRPA1 and to determine structure–function relationships.

Electrophilic compounds are capable of forming reversible adducts with thiol groups (Hinman et al., 2006; Macpherson, Dubin, et al., 2007). Structure–function analyses identified several key conserved cysteine residues (Cys619, Cys639, and Cys663 for human TRPA1) in the linker region between the cytoplasmic N terminus and the transmembrane domains of the channel that are necessary prerequisites for proper channel activation by electrophiles (Hinman et al., 2006) (Figure 4.1). However, different TRPA1 channel orthologues, such as those from human and rattlesnake, exhibit significantly different sensitivities to AITC, despite the presence of all three conserved cysteine residues (Gracheva et al., 2010). This

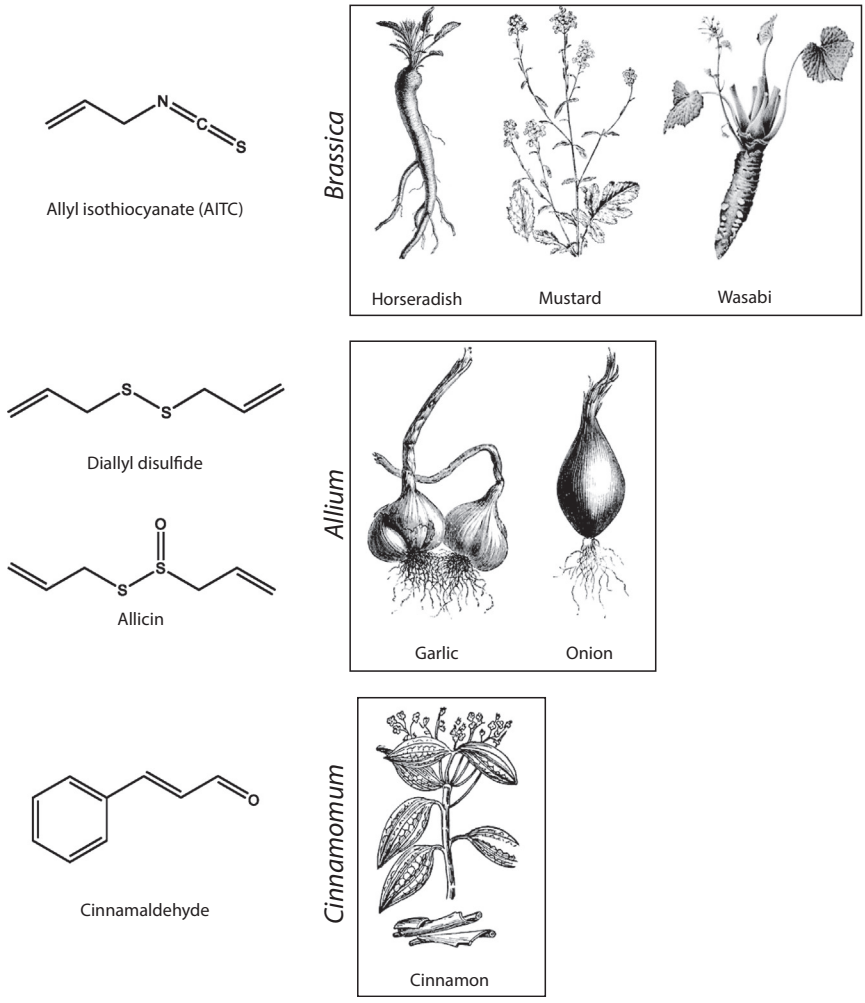

Figure 4.2 Examples of electrophilic activators of TRPA1 from three different plant genera.

observation suggests that other regulatory elements may be involved in determining sensitivity to electrophilic ligands.

In addition, a number of other plant-based compounds have been identified as TRPA1 agonists: gingerol (ginger), thymol (thyme), oleocanthal (olive oil), eugenol (cloves), methyl salicylate (wintergreen), Δ^9-tetrahydrocannabinol (psychoactive compound in marijuana), and carvacrol (oregano, marjoram)—just to name a few (Bandell et al., 2004; Jordt et al., 2004; Peyrot des Gachons et al., 2011; Xu, Delling, Jun, & Clapham, 2006).

Interestingly, some of these chemicals, like carvacrol and oleocanthal, do not act through the reactive cysteine residues, suggesting the presence of an alternative activation mechanism (Peyrot des Gachons et al., 2011). Nevertheless, all these compounds are associated with a spicy, chemesthetic sensation upon ingestion owing to activation of TRPA1. It is believed that the evolution of TRPA1 agonists in these plants may have arisen as self-defense strategy in the molecular arms race against attacks by pests and consumption by herbivores.

From a physiological point of view, TRPA1 plays an important protective role because it can detect the presence of less-than-appetizing (i.e., potentially harmful) environmental pollutants such as toluene diisocyanate, hypochlorite, and H_2O_2 (industrial pollutants); H_2S; as well as α, β-aldehydes from smoke (acrolein, crotonaldehyde, etc.) (Andersson, Gentry, Moss, & Bevan, 2008; Bautista et al., 2006; Bessac, Sivula, von Hehn, Escalera, & Cohnet, 2008; Taylor-Clark, Kiros, Carr, & McAlexander, 2009). Like AITC and other organosulfur activators, these electrophiles covalently interact with the conserved linker cysteines.

Moreover, TRPA1 appears to mediate the irritating effects of exposure to formalin, another widespread environmental pollutant (Macpherson, Xiao, et al., 2007; McNamara et al., 2007; Yonemitsu et al., 2013). TRPA1-knockout animals display significantly attenuated pain responses when injected with a dilute formalin solution into the hindpaw (Macpherson, Xiao, et al., 2007; McNamara et al., 2007). Inhalation of these environmental irritants activates TRPA1 and leads to depressed respiration rates, coughing, and bronchial contraction (Bessac & Jordt, 2010). Therefore, the ability to detect the presence of these hazardous chemicals, even at low concentrations, can be beneficial to limit injurious exposure.

The military has exploited this protective physiological function of TRPA1 as a noxious chemosensor for decades. 1-chloroacetophenone (Mace®), dibenz[b,f][1,4]-oxazepine, 2-chlorobenzylidene malononitrile, and chloropicrin are extremely effective tear gases and riot control agents. These electrophiles are also some of the most potent TRPA1 agonists identified (Bessac et al., 2009; Brone et al., 2008). With half-maximal activation (EC_{50}) values in the pico- to nanomolar range for heterologously expressed TRPA1, these alkylating agents lead to profuse lachrymation, pain, coughing, and respiratory distress. Genetic deletion of the channel, mutation of the reactive cysteine residues, or treatment with TRPA1 antagonists can significantly reduce pain behavior in vivo, suggesting a potential treatment strategy for exposed patients (Bessac et al., 2009; Brone et al., 2008).

In addition to external irritants, endogenous signals, such as mediators of inflammation, also make use of the reactive thiol moieties to activate TRPA1 nociceptors (Andersson et al., 2008; Bautista et al., 2013; Takahashi et al., 2008; Taylor-Clark, Ghatta, Bettner, & Undem, 2009; Taylor-Clark et al., 2008). Following injury, activation of the cyclooxygenase pathway leads to the production of inflammatory mediators known as prostaglandins. The prostaglandin 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), one of the molecules whose production leads to hyperalgesia associated with the carrageenan-induced laboratory model of inflammation, was shown to activate a specific subset of dissociated dorsal root ganglion cells (Takahashi et al., 2008). It was subsequently demonstrated that this molecule could activate TRPA1 via the conserved cysteines in the N terminus. Specifically, the α,β-unsaturated moieties of 15d-PGJ_2 are believed to alkylate TRPA1 via a Michael addition reaction (Andersson et al., 2008). When assessed by calcium imaging, mutations in specific N-terminal cysteine residues of human TRPA1 are able to significantly reduce activation by 15d-PGJ_2 (Takahashi et al., 2008).

Other compounds associated with the inflammatory milieu include NO, H_2O_2, and 4-hydroxynonenal, a product of lipid peroxidation due to release of reactive oxygen species (Basbaum & Woolf, 1999; Schneider, Porter, & Brash, 2008). All these molecules have been found to activate TRPA1, albeit via a slightly different mechanism than 15d-PGJ_2 (Andersson et al., 2008; Macpherson, Xiao, et al., 2007; Takahashi et al., 2008; Trevisani et al., 2007). Unlike15d-PGJ_2, the interactions of these molecules with the N-terminal cysteines are not inhibited by the presence of the reducing agent dithiothreitol. This is consistent with nitrosylation or oxidation of the sulfhydryl group, as opposed to alkylation by 15d-PGJ_2 (Figure 4.3).

2.2 Noncovalent activation of TRPA1

As is the case for the monoterpenic phenol carvacrol and several of the agonists mentioned earlier in the text, TRPA1 can be activated by mechanisms independent of covalent modifications. For instance, a decrease in intracellular pH can activate the channel. Studies have shown that weak acids such as acetic acid, formic acid, and others can cause activation (Wang et al., 2011). This represents another mechanism for activation of TRPA1 at areas of inflammation due to local acidosis near the injury site. Sensitivity of TRPA1 to acidic pH has also been demonstrated to mediate the stinging, pungent effects of carbonated drinks. The carbon dioxide (CO_2) in these

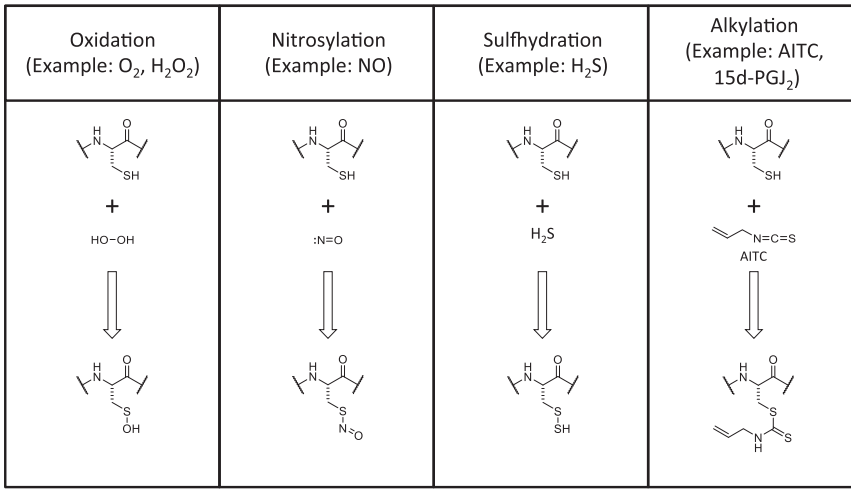

Figure 4.3 Mechanisms of cysteine modification by oxidation, nitrosylation, sulfhydration, and alkylation.

beverages is able to diffuse into the cell, thereby lowering intracellular pH and activating TRPA1 (Wang, Chang, & Liman, 2010).

Oxygen (O_2) is another gas for which TRPA1 is suggested to serve as a sensor (Takahashi et al., 2011). Because it is required for mitochondrial respiration, oxygen levels must be carefully monitored. Signaling by neurons in sensory and vagus nerves alerts the animal to hypoxic conditions. On the other hand, elevated levels of oxygen (hyperoxia) can have toxic effects and cells will respond to deviations from normoxia in either direction. Genetic mutation of TRPA1 in mice prevents signaling from sensory and vagal nerves in response to hypoxia or hyperoxia. During states of normoxia, TRPA1 activation is prevented by the actions of prolyl hydroxylases (PHDs). At near atmospheric oxygen levels, the action of these O_2-dependent enzymes is believed to result in the hydroxylation of a conserved proline residue within the N terminus (Pro394 of human TRPA1), thus inhibiting channel activation during normoxia. When O_2 levels drop, activity of PHDs decrease and the channel is freed from inhibition. In contrast, hyperoxia has been shown to override PHD-mediated inhibition to activate TRPA1 via direct oxidation of reactive cysteine residues by O_2 (Takahashi et al., 2011) (Figure 4.3).

Chronic metal toxicity has become a very hot topic in sensory physiology and pathology. Over the past decade, a number of studies suggested a role for TRPA1 in this condition, with results showing direct activation

and modulation of channel function by divalent cations, including zinc, copper, cadmium, and calcium (Andersson, Gentry, Moss, & Bevan, 2009; Gu & Lin, 2010; Hu, Bandell, Petrus, Zhu, & Patapoutian, 2009; Miura et al., 2013; Zurborg et al., 2007). Animals lacking TRPA1 display attenuated irritation and nociception after zinc chloride injection (Gu & Lin, 2010). Single amino acid mutagenesis studies identified N-terminal Cys614, C-terminal Cys1021, and His983 as important prerequisites for activation by zinc. Although it is not clear how the last of these residues is involved, it is interesting to note that cysteine/histidine residues are commonly found in zinc interaction sites of other proteins (Hu et al., 2009).

2.3 Receptor-operated activation of TRPA1

Like many other TRP channels, TRPA1 activation can result as a downstream consequence of signaling cascades generated by other receptors. Coexpression of TRPA1 with phospholipase-C (PLC)-coupled receptors like the bradykinin receptor (B_2R) (Bandell et al., 2004; Wang, Dai, et al., 2008) and the M1 muscarinic acetylcholine receptor (Jordt et al., 2004) show typical receptor-operated channel behavior. In heterologous expression systems, activation of either of these proteins alone results in minimal noticeable currents on the plasma membrane. However, when these receptors are coexpressed with TRPA1, the magnitude of the signal increases dramatically, an effect that can be reversed by treatment with PLC inhibitors. However, the exact mechanism(s) by which PLC signaling leads to TRPA1 activation remains controversial and activation of this pathway results in the formation of a number of products that have been proposed to regulate TRPA1 function (Dai et al., 2007; Nilius, Owsianik, & Voets, 2008; Rohacs, Thyagarajan, & Lukacs, 2008; Wang, Chang, Waters, McKemy, & Liman, 2008; Zurborg et al., 2007).

Phosphatidylinositol-4,5-bisphosphate (PIP_2) is a membrane phospholipid that acts as a substrate for cleavage by PLC. PIP_2 can also function as an anchor for a variety of proteins. Of particular relevance to this review, PIP_2 has been suggested to elicit both stimulatory and inhibitory effects on members of the TRP channel family (Qin, 2007). Although not as well understood or as well studied as the interaction of PIP_2 with the Transient receptor potential, subfamily V, member 1 channel, there is still some evidence to suggest that PIP_2 may display dual regulation over TRPA1. One study found that desensitization in response to agonist stimulation was delayed by addition of exogenous PIP_2. Furthermore, reduction of PIP_2 levels by application of neomycin increased the rate of desensitization (Nilius

et al., 2008). On the other hand, another group found that application of PIP_2 led to inhibition of TRPA1 potentiation by bradykinin (Dai et al., 2007). Similarly, a third group found that application of PIP_2 to inside-out patches of mouse TRPA1-expressing HeLa cells did not activate the channel and, in fact, when applied subsequently to AITC, actually inhibited TRPA1 currents. Removal of PIP_2 either through polylysine treatment or through the use of an antibody led to increased AITC-activated current in whole-cell configuration (Kim, Cavanaugh, & Simkin, 2008).

At the same time, it is well known that PLC signaling can lead to increases in cytosolic calcium. Ca^{2+} itself has been suggested to play major roles in both activation and desensitization of TRPA1 (Wang, Chang, Waters, McKemy, & Liman, 2008; Zuborg et al., 2007). Moreover, it has been proposed that cold-induced activation of TRPA1 reported for some heterologous systems may be indirect, resulting from Ca^{2+}-induced activation (Zuborg et al., 2007). TRPA1 contains a putative intracellular EF-hand motif suggested to mediate the effects of Ca^{2+}. This hypothesis has proven to be somewhat controversial, however, as different groups have reported conflicting results when this region is mutated (Wang, Chang, et al., 2008). Although the precise mechanisms remain to be elucidated, it does appear that Ca^{2+}-mediated potentiation/desensitization are mediated by cytosolic rather than extracellular Ca^{2+}, as mutations that limit TRPA1 Ca^{2+} permeability are shown to abrogate both these processes, while application of Ca^{2+} to the cytosolic face of excised patches was able to restore them (Wang, Chang, et al., 2008).

In the past few years, PLC-independent receptor-operated activation pathways for TRPA1 were also described. As mentioned earlier, TRPA1 has been linked to the pathophysiology of histamine-independent itch (Wilson et al., 2011). Several converging pathways are capable of activating TRPA1 to mediate this response. While some of these signals occur through PLC pathways, activation of Mas-related G protein-coupled receptor A3 (MrgprA3), the receptor for the infamously pruritogenic antimalarial drug chloroquine (CQ), leads to TRPA1 activation independent of PLC. Interestingly, signaling by the $G\beta\gamma$ subunit has been proposed to mediate the effects of CQ receptor activation and treatment with subunit chelators or small molecule inhibitors prevent TRPA1 activation.

By acting as a downstream target of different signaling cascades, TRPA1 is able to further diversify the list of ligands to which it effectively responds. This role also endows the channel with an important function as an amplifier and integrator of signals from a wide range of both internal and external sources.

To understand the role of TRPA1 in vivo, it will be of utmost importance to develop a thorough knowledge of activation mechanisms for the plethora of proposed ligands. This knowledge will be critical to control for potentially physiologically undesirable effects resulting from channel activation by unidentified cellular components in the nonuniform heterologous expression systems currently used to investigate TRPA1 function.

3. TEMPERATURE SENSITIVITY OF TRPA1

Temperature is a potent regulator of TRPA1, but unlike sensitivity to electrophilic compounds—conserved in all known TRPA1 orthologues except nematodes—temperature sensitivity of TRPA1 varies significantly between species.

3.1 TRPA1 in mammals

In the somatosensory system, TRPA1 is expressed in a subset of TRPV1-positive neurons (Kobayashi et al., 2005; Mishra & Hoon, 2010; Story et al., 2003) and initially the channel was suggested as a cold sensor in the sub-Transient receptor potential, subfamily M, member 8 range (Kwan et al., 2006; Story et al., 2003). Since then, this hypothesis has been refined (McKemy, 2013), leading to the prevalent notion that TRPA1 contributes to cold sensitivity in injury-evoked or pathological conditions (del Camino et al., 2010; Knowlton, Bifolck-Fisher, Bautista, & McKemy, 2010).

In an attempt to understand the potential contributions of TRPA1 as a cold sensor in vivo, knockout mice were independently developed by two groups (Bautista et al., 2006; Kwan et al., 2006). Despite the fact that the animals were created using similar genetic schemes, there is disagreement about the resulting phenotype. Notably, one group reported the absence—even in WT (wild type) mice—of menthol-insensitive, cold-activated trigeminal neurons that fit TRPA1 pharmacological profiles (Bautista et al., 2006). The same group also found no significant differences between TRPA1-knockout and WT animals in terms of either the latency to lift the paw in cold plate assays, flinching due to acetone-induced cooling of the paw (Bautista et al., 2006), or by temperature preference assay (Bautista et al., 2007). These results led them to conclude that TRPA1 does not behave as a cold sensor in vivo. In contrast, Kwan et al. (2006) reported dramatically decreased paw lifting in TRPA1-knockout mice exposed to a 0 °C cold plate as well as decreased behavioral responses to acetone application. A number of other conflicting reports have emerged either supporting or

contradicting the notion of TRPA1 as a noxious cold sensor (del Camino et al., 2010; Fajardo, Meseguer, Belmonte, & Viana, 2008; Karashima et al., 2009; Knowlton et al., 2010; Kwan & Corey, 2009; Kwan, Glazer, Corey, Rice, & Stucky, 2009). Of note, a recent study showed that specific ablation of TRPV1-positive neurons in adult mice leads to disappearance of TRPA1 neurons, but does not abolish animal sensitivity to painful cold, further arguing against a role of TRPA1 in nonpathological detection of noxious temperatures (Pogorzala, Mishra, & Hoon, 2013).

Interestingly, while there remains considerable debate as to the role of TRPA1 as a noxious cold sensor, the channel does not appear to be involved in thermoregulation. This is demonstrated by the lack of change in core body temperature induced by genetic knockout (Bautista et al., 2007) or treatment with channel-specific antagonist (Chen et al., 2011). An effect on thermoregulation is also not observed in cold-exposed TRPA1-knockout animals or antagonist-treated rats when core body temperatures are decreased below the reported TRPA1 activation threshold of 17 °C (Oliveira et al., 2014). These findings are in stark contrast to other thermo-TRPs such as TRPV1, inhibition of which results in hyperthermia (Gawa et al., 2007).

Perhaps the most controversial data exist with regard to temperature activation of mammalian TRPA1 orthologues in heterologous systems by cold (Caspani & Heppenstall, 2009). A number of studies have reported potent activation of mammalian TRPA1 at ~17 °C (del Camino et al., 2010; Karashima et al., 2009; Kremeyer et al., 2010; Sawada et al., 2007; Story et al., 2003), while others claimed the absence of a response (Cordero-Morales et al., 2011; Jordt et al., 2004; Knowlton et al., 2010; Nagata, Duggan, Kumar, & Garcia-Anoveros, 2005; Zurborg et al., 2007). The reason for this discrepancy may reside in the differences in experimental conditions, such as expression system, expression level, ionic composition of recording solution, etc. In addition, much of the controversy may be due to species-specific properties of TRPA1 orthologues. In other temperature-sensitive TRP channels, species differences are not as pronounced. For example, all tested mammalian TRPV1 and TRPM8 orthologues are, respectively, heat and cold sensitive, and a similar behavior, perhaps erroneously, was expected from TRPA1.

Along these lines, a recent report documented a side-by-side comparison of mouse, rat, human, and rhesus monkey TRPA1 in identical experimental conditions (Chen et al., 2013). The study showed that while all four molecules are potentiated by AITC, cold activates only mouse and rat

orthologues, while primate channels are insensitive to temperature changes in the 8–24 °C range.

3.2 TRPA1 in insects and worms

As a heat sensor, TRPA1 was originally identified in *Drosophila melanogaster* (Viswanath et al., 2003). It was demonstrated that insect TRPA1 is a polymodal ion channel that can be activated by temperature and electrophilic compounds (Kang et al., 2010). Later, the same group identified two isoforms of TRPA1 that play distinct physiological roles (Kang et al., 2012). The longer TRPA1(A) isoform is less temperature sensitive than TRPA1(B), with reported Q_{10} values of 9 and 116, respectively. The latter is localized to specific thermosensory neurons and is involved in temperature discrimination, whereas TRPA1(A) is expressed in chemosensory neurons and plays a key role in chemical sensitivity rather than heat perception. Mosquitoes have similar gene organization and use the same molecular strategies to produce two different isoforms of TRPA1 with distinct temperature activation profiles, suggesting a common evolutionary trend in insects for modulating TRPA1 function (Kang et al., 2012). Moreover, two additional isoforms (TRPA1(C) and TRPA1(D)) were cloned from *Drosophila* larvae (Zhong et al., 2012). These two channels are expressed in nociceptors and activated by AITC. However, TRPA1(C) is not temperature sensitive, whereas TRPA1(D) can be activated by heat at temperatures $\geq$34 °C.

In contrast, nematode TRPA1 is proposed to function as both a mechanosensor and cold sensor in a distinct population of sensory neurons (Chatzigeorgiou et al., 2010; Kindt et al., 2007). *Caenorhabditis elegans* TRPA1 is a polymodal ion channel, but compared to other orthologues, it is not activated by chemical compounds such as AITC (see Table 4.1). Moreover, recent studies revealed an additional role for TRPA1: worm TRPA1 is proposed to detect thermal changes in the environment and, as a result, promote longevity at cold temperatures (Xiao et al., 2013).

3.3 TRPA1 in fish, birds, reptiles, and amphibians

Zebrafish (*Danio rerio*) contain two genes that encode TRPA1a and TRPA1b. Both these molecules are expressed specifically in sensory neurons. Unlike the invertebrate channel, TRPA1a and TRPA1b are activated only by chemical compounds, not temperature (Prober et al., 2008). At the physiological level, TRPA1b (but not TRPA1a) is implicated in nocifensive responses to chemical irritants.

A recent report presented the first characterization of an avian TRPA1 channel (Saito et al., 2014). TRPA1 cloned from chickens (*Gallus gallus domesticus*) is activated by heat with an average threshold of ~39.4 °C. Furthermore, the channel is responsive to classic noxious electrophilic TRPA1 agonists as well as sensitive to the agricultural bird repellant methyl anthranilate. This finding is especially interesting because it represents the first characterization of a TRPA1 orthologue from a nonmammalian homeotherm. The fact that the channel responds selectively to heat as opposed to activation by cold, as is proposed for some mammalian TRPA1 channels, suggests that the directionality of thermal-sensing properties of the channel did not change coincident with the evolution of homeothermy. Along these lines, it should be noted that, based on amino acid sequence, chicken TRPA1 displays closer similarity to green anole lizard (*Anolis carolinensis*) TRPA1 (82%) than to human and mouse TRPA1 (64% and 65%, respectively).

In frogs, lizards, and snakes, TRPA1 serves as a detector of noxious heat and chemical irritants (Gracheva et al., 2010; Saito et al., 2012). In more specialized species such as pit-bearing snakes (boas, pythons, and rattlesnakes), TRPA1 plays a crucial role in the detection of infrared radiation emitted by warm-blooded animals (Gracheva et al., 2010). This enhanced thermal sensitivity comes at the cost of chemical sensitivity to electrophilic compounds with these heat-activated orthologues displaying significantly reduced sensitivity to electrophiles (Table 4.1). Taking into consideration that insect TRPA1s are also heat sensitive, this may suggest that heat sensitivity evolved and adjusted multiple times during evolution to support adaptability to environmental conditions as well as to unique feeding habits. The ability to separate modalities (temperature/chemical/mechanical activation) in order to prevent conflicting sensory input may be the key to the adaptive significance and functional diversification that TRPA1 has attained throughout the course of evolution.

3.4 TRPA1: Molecular mechanism of temperature sensitivity

Little is known about the molecular mechanism of temperature sensitivity in TRPA1. As discussed above, some TRPA1 orthologues are activated by heat, and others by cold, which at first sight may suggest profound differences in the temperature-sensing apparatus of these channels. However, as counterintuitive as it may seem, activation by heat and cold may represent similar conformational changes that come hand in hand when considered from the point of view of thermodynamics of heat- and cold-induced protein folding.

Basic thermodynamic considerations dictate that an ion channel with a nonzero change in heat capacity between open and closed conformations will be active at the extreme sides of the temperature spectrum. With regard to temperature-gated ion channels, it is expected that a cold-activated ion channel will also be activated by heat. However, practical limitations prevent us from assessing the full temperature range, so we often observe only one arm of the U-shaped activation profile, and, based on the results, call the channel either a cold or a heat sensor. While these considerations (for details, see (Clapham & Miller, 2011)) await experimental support (Chowdhury, Jarecki, & Chanda, 2014), we note here that this thermodynamic framework may provide an elegant explanation as to why different TRPA1 orthologues exhibit seemingly incompatible temperature activation properties. For example, rattlesnake TRPA1 is activated by heat (Gracheva et al., 2010), while the ~60% identical mouse orthologue is activated by cold (Chen et al., 2013; Story et al., 2003). It was suggested that the point of minimum in the U-shaped activation curve can be left or right shifted by slightly changing the amino acid composition without significantly affecting heat capacity of the channel's temperature-sensing element (Clapham & Miller, 2011). As a result, the practically testable temperature window will shift, leaving only the cold- or heat-activated "arm" of the U-curve accessible for experimental analysis. In practical terms, this means that alterations in the amino acid composition may lead to the apparent reversal of temperature properties, manifested as cold or heat activation in different TRPA1 orthologues.

Alternatively, temperature-dependent activation of TRPA1 may be explained using modular allosteric gating and coupling models (Jara-Oseguera & Islas, 2013; Qin, 2013; Salazar, Moldenhauer, & Baez-Nieto, 2011; Voets, 2012). Regulation of channel gating in these scenarios takes place via allosteric coupling to a heat sensor module. In this way, temperature-induced channel activation may occur without relying on changes in heat capacity. Interestingly, when temperature-dependent coupling parameters are included in their model, Oseguera and Islas (2013) point out that a channel exhibiting allosteric coupling may also display a U-shaped temperature activation profile, similar to predictions from the heat capacity model.

How temperature is translated into opening of the TRPA1 pore is a matter of intense research. It is of great interest to determine whether the temperature-sensing module of TRPA1 has defined structural boundaries, or whether different parts of the molecule collectively contribute to the formation of such a sensor. If the temperature-sensing module has defined

boundaries, does it include gating elements? As of this writing, these questions have not been definitively answered.

The fact that both cold- and heat-activated thermo-TRP channels retain their temperature responses when reconstituted in artificial bilayers suggests that temperature responses and directionality are not determined by membrane lipid composition (Cao, Cordero-Morales, Liu, Qin, & Julius, 2013; Zakharian, Cao, & Rohacs, 2010). Chimeras and mutagenesis experiments in TRP channels, including TRPA1, provide further support for this idea. Transposition of ankyrin repeats from the N terminus of fly or rattlesnake TRPA1 confers heat sensitivity onto the human orthologue without changing AITC responses (Cordero-Morales et al., 2011). Additionally, a random mutagenesis screen in mouse TRPA1 identified three single-point mutations in ankyrin repeat 6 that are reported to confer warmth-induced activation on the channel while maintaining sensitivity to chemical responses (Jabba et al., 2014). If we assume that heat and AITC open the channel at the same molecular gate, then these findings suggest that the ankyrin repeats of the heat-sensitive TRPA1s contain functional prerequisites for heat activation. The existence of a heat "module" within the same ankyrin repeats indicate that such evolutionarily distant species as flies and snakes undertook similar molecular strategies for developing heat sensitivity in TRPA1 (see Figure 4.1). These observations suggest that the N terminus of the temperature-insensitive human TRPA1 does not contain the heat module. However, substitution of the entire N and C termini in fly TRPA1 with human domains fails to abolish heat sensitivity (Wang, Schupp, Zurborg, & Heppenstall, 2013), indicating that regions beyond the ankyrin repeats also contribute to temperature activation. Indeed, in many insects, such as flies, mosquitoes, and lice, alternative splicing of the extreme N-terminal region was shown to be critical for defining the steepness of temperature responses (Kang et al., 2012). Collectively, these data strongly support the existence of heat-sensing modules in the N terminus of TRPA1, separate from the regions sensing electrophilic compounds, such as AITC.

Single or multiple amino acid substitutions in the pore region of TRPA1 can obliterate temperature responses without affecting chemical activation (Chen et al., 2013; Wang et al., 2013). These data clearly establish a critical role of the pore region in temperature gating. It remains unclear, however, if the gate only responds to temperature-induced conformational changes in the N terminus, or if temperature causes structural rearrangements in various parts of the channel, including the cytosolic and transmembrane domains, which then leads to opening of the channel gate.

To complicate the matter, the location of a functional temperature-activated gate in TRPA1 has not been clearly defined. In the absence of structural data, it is reasonable to assume that, by analogy with voltage-gated potassium channels, the region between transmembrane helices 5 and 6 of TRPA1 will form the ion-conducting pore. In many potassium channels, the pore domain contains two gates: the extracellular selectivity filter-based gate and the intracellular gate formed by the inner helices, topologically analogous to transmembrane segment 6 in TRPs (Mathie, Al-Moubarak, & Veale, 2010). Whether or not any of these regions, or both, are opened by changes in temperature, is unclear. This aspect is critical for understanding the molecular mechanism of TRPA1 gating by temperature and chemical compounds.

A thorough understanding of the biophysics of temperature-induced TRPA1 activation mechanism(s) may be critical for understanding and manipulating channel function in vivo. It will be fascinating to see if the same (or similar) mechanisms apply to other temperature-activated ion channels. We look forward to progress on this topic in the near future.

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