TRPA1 and Cold Transduction: An Unresolved Issue?

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TRPA1 was originally characterized as a cold-activated ion channel; however, since the initial description there has been some disagreement about its role in cold sensation. Here, we review the literature on the function of TRPA1 as a cold sensor. Many studies have demonstrated that TRPA1 can be activated by cold when expressed in heterologous systems, but its role as a cold sensor in native peripheral sensory neurons remains uncertain. We argue that TRPA1 is activated indirectly by cold via a background Ca^{2+} influx seen in cells during cooling. This background cold response is present in most heterologous cell types, but not in sensory neurons. Thus, we propose that cold sensitivity of TRPA1 is indirect and is dependent on cellular context.

Introduction

The transient receptor potential (TRP) proteins form a large family of cation channels that are grouped into six main subfamilies: the TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), and TRPA (ankyrin) groups. Six of these channels, TRPV1, TRPV2, TRPV3, TRPV4, TRPM8, and TRPA1 are expressed in the peripheral nervous system and can be gated by temperature when expressed heterologously. These channels have been termed thermoTRPs and proposed to function as temperature transducers in mammals (Dhaka et al., 2006; Talavera et al., 2008). TRPA1 is the most recent addition to the thermoTRP list and was originally proposed to be a noxious cold sensor. However, there is disagreement on its activation by cold and its role in temperature sensation. Here, we discuss the principal points of this controversy and show how it might be resolved using current knowledge of TRPA1.

The Identification of TRPA1

TRPA1 was first isolated in 1999 in a screen for transformation-sensitive proteins in cultured fibroblasts (Jaquemar et al., 1999). It was named p120 and shown to be similar in sequence to other proteins belonging to the TRP channel family. Four years later in a bioinformatics screen, Story et al. (2003) identified TRPA1 as a novel thermoTRP. They searched for predicted cDNA sequences containing six transmembrane domains and N-terminal ankyrin repeats, both of which are common characteristics of TRP channels. Using reverse transcriptase–PCR from mouse trigeminal and dorsal root ganglia (DRG) RNA, they cloned TRPA1 and obtained a theoretical protein with 14 predicted N-terminal ankyrin domains, followed by a six-transmembrane domain. This channel was termed ANKTM1 and after an extensive analysis examining its expression and function, it was proposed to be a candidate receptor for noxious cold temperature.

One of the most striking discoveries from this initial analysis was the finding that TRPA1 is only expressed at significant levels in a small population of sensory neurons of the DRG and trigeminal ganglia. Of interest, these neurons appeared to be peptidergic nociceptors in that they also contained calcitonin gene-related peptide (CGRP) and TRPV1, but they did not express the other cold-sensitive TRP channel, TRPM8. Thus, TRPA1 could have a potential function in detecting nociceptive stimuli.

Evidence that TRPA1 is a cold sensor came from experiments examining heterologous expression of the channel. Using patch clamping and calcium microfluorimetry from Chinese hamster ovary (CHO) cells expressing TRPA1, Story et al. (2003) demonstrated that at temperatures <17°C, or in the presence of the cooling agent icilin, TRPA1 channel activity was significantly increased. This was an important finding because skin cooling <17°C evokes a sensation of pain rather than innocuous cold in man (Dhaka et al., 2006). Furthermore, in cultures of mouse DRG neurons, they showed that there is a small population of cold-sensitive neurons that do not contain TRPM8 but are $TRPV1^+$. Because this population corresponded well to the number of neurons expressing TRPA1 (3.6% of all DRG neurons) and had a similar activation temperature, it was reasoned that TRPA1 was the noxious cold sensor.

These findings tied in with the hypothesis that specific thermoTRP channels with defined temperature

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Abbreviations used in this paper: CGRP, calcitonin gene-related peptide; CHO, Chinese hamster ovary; DRG, dorsal root ganglia; TRP, transient receptor potential.

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thresholds determine temperature sensitivity in mammals. Thus, painful temperatures <17°C are detected by the ion channel TRPA1 expressed on nociceptors, neurons expressing TRPM8 mediate innocuous cool and menthol sensation, and neurons (and keratinocytes) expressing TRPV channels sense warm and hot temperatures.

Other Functions of TRPA1

Shortly after the characterization of TRPA1 as a noxious cold sensor, two studies demonstrated that TRPA1 is also a receptor for several pungent chemicals that evoke pain when applied to the skin (Bandell et al., 2004; Jordt et al., 2004). One of these compounds, mustard oil, has long been used as an algesic agent to evoke nociceptive behavior in animal models of pain. Another, called cinnamaldehyde, was also demonstrated to have pro-nociceptive activity in mice. Thus, these novel agonists could now be used as tools to identify $TRPA1^+$ neurons in cultured sensory neurons.

An additional finding from these studies was that TRPA1 also functions as a receptor-operated channel. Growth factors or proinflammatory peptides such as bradykinin acting via G protein–coupled receptors could activate TRPA1. This was shown to occur downstream of phospholipase C pathways and gave the first indication that intracellular Ca^{2+} concentration is an important factor in the gating of TRPA1 (Jordt et al., 2004).

Is TRPA1 a Cold Sensor?

The identification of relatively selective TRPA1 agonists allowed for the analysis of TRPA1 channel activity in cultured DRG neurons and triggered several investigations into the cold sensitivity of $TRPA1^+$ neurons. However, the picture that has emerged from these studies is complicated, and there is no clear consensus as to whether TRPA1 is cold sensitive or not.

In an early study, Bandell et al. (2004) used cinnamaldehyde in calcium microfluorimetry experiments to distinguish $TRPA1^+$ neurons in mouse DRG. They reported that cinnamaldehyde activates 3.3% of the neurons, and that most of the cinnamaldehyde-sensitive neurons were sensitive to cold. However, data that followed from several groups using TRPA1 agonists, in particular mustard oil, cast doubt upon the function of TRPA1 as a cold sensor in cultured sensory neurons.

Jordt et al. (2004) analyzed populations of trigeminal neurons according to their response to cold and mustard oil. They found no correlation between mustard oil and cold sensitivity, and reported that most of the mustard oil–sensitive neurons (96%) were cold insensitive. Additionally, they were unable to evoke cold-activated currents in TRPA1-expressing HEK293 cells or oocytes, a finding supported by Nagata et al. (2005).

Similarly, in cultured DRG neurons, several studies showed no correlation between TRPA1 pharmacology and cold sensitivity (Reid, 2005; Munns et al., 2007). In these experiments, mustard oil or cinnamaldehyde were used to identify the TRPA1-expressing neurons. Mustard oil activates a larger population of neurons than cinnamaldehyde, and it is not clear if this is because it is a stronger activator of TRPA1 or because it also activates another unknown receptor (Bandell et al., 2004). Munns et al. (2007) analyzed the response of mouse DRG neurons to both compounds, and, interestingly, the percentage of cold-sensitive neurons within the two populations was in both cases only around 20% (Munns et al., 2007).

In contrast, Sawada et al. (2007) reported that most mustard oil–sensitive neurons in mouse DRG were cold sensitive (119 out of 135), and that heterologously expressed TRPA1 responded to cold. They suggested that TRPA1 could be activated by cold only when the temperature decreased slowly; thus, the rate of temperature change used in the experiments could influence the results (Reid, 2005; Sawada et al., 2007). However, Munns et al. (2007) investigated this hypothesis and performed experiments with both fast and slow cold ramps. No difference was found in the proprieties of the cold-sensitive population in terms of distribution, kinetics, or sensitivity to menthol and to mustard oil (Munns et al., 2007).

Further complications on the function of TRPA1 emerge from expression analyses. Reported values for the proportion of cells in the DRG expressing TRPA1 mRNA range from 3.6 to 56.7% (Story et al., 2003; Jordt et al., 2004; Kobayashi et al., 2005; Nagata et al., 2005; Caspani et al., 2007). In addition, there is some disagreement about which subclass of nociceptive neuron expresses TRPA1. Nociceptors can be divided into two major types: peptidergic nociceptors that express CGRP and substance P, and non-peptidergic nociceptors that bind the isolectin IB4. Two groups have reported that TRPA1 is predominantly coexpressed with CGRP (Story et al., 2003; Bautista et al., 2005); however, we have found that the majority of $TRPA1^+$ neurons (>90%) are co-labeled with IB4 and can thus be classified as non-peptidergic nociceptors (Caspani et al., 2007).

Hjerling-Leffler et al. (2007) combined calcium imaging with vital IB4 staining to study the developmental expression of TRP channels in subpopulations of cultured sensory neurons. They demonstrated that in adult mice, TRPA1 is expressed in both peptidergic and a large population of $IB4^+$ nociceptors, and that cold sensitivity corresponds with TRPA1 expression only in peptidergic nociceptors. Furthermore, they observed the appearance of a population of cold-sensitive sensory neurons at embryonic day 12.5, well before the onset of TRPM8 and TRPA1 expression (Hjerling-Leffler et al., 2007).

Intriguingly, in cultured vagal sensory neurons that innervate the viscera, it has recently been demonstrated that there is a very good overlap between TRPA1 pharmacology and cold responsiveness (Fajardo et al., 2008). Cold sensitivity was closely correlated with activation by TRPA1 agonists, and furthermore, cold responses could

be blocked by TRPA1 antagonists and were reduced in TRPA1 knockout mice. In the same study, and using similar protocols, DRG neurons were also examined. Here, the authors found little correlation between cold sensitivity and TRPA1 expression, suggesting that somatic and visceral sensory neurons use entirely different mechanisms for cold transduction.

Many factors have been proposed to explain the controversy of cold sensitivity of TRPA1; for example, differences in the preparation of cells, the addition of growth factors to culture medium, and different types of stimulation protocol used to cool cells. It is also possible that cold sensitivity of TRPA1 can be more reliably measured at the peripheral endings of sensory neurons in the skin and that TRPA1 is more readily activated by cold in vivo.

TRPA1 Function In Vivo

Several groups have investigated TRPA1 function in vivo; however, here too, a clear consensus on its role in cold detection has not emerged. An initial indication that TRPA1 might have a role in cold sensation came from a study examining TRPA1 expression in animal models of inflammatory and neuropathic pain (Obata et al., 2005). A common symptom of chronic pain in humans is an increased sensitivity to cold temperatures, or cold allodynia (Kehlet et al., 2006). This feature (which is also observed in animal models) leads to pain and discomfort from temperatures that are normally perceived as being innocuously cool. It was therefore assumed that changes in the expression of TRPA1 might contribute to this phenomenon. Obata et al. (2005) reported an increase in the number of neurons expressing TRPA1 after injury and showed that by blocking this with TRPA1 antisense oligonucleotides, cold allodynia was reduced. However, we have measured TRPA1 expression in similar experimental settings and reported a decrease in the expression level of TRPA1 (Caspani et al., 2007).

A human study using the TRPA1 agonist cinnamaldehyde was also not able to substantiate the role of TRPA1 as a cold sensor (Namer et al., 2005). In a series of psychophysical studies, a 40% cinnamaldehyde solution was applied to the forearm of healthy human volunteers, and quantitative sensory testing was performed. Cinnamaldehyde evoked significant spontaneous pain, but no sensation of cold, and actually lowered cold threshold temperatures. In contrast, application of the TRPM8 agonist menthol produced cold hyperalgesia in the subjects.

The generation and analysis of TRPA1 knockout mice was anticipated to resolve whether TRPA1 functions in cold sensation. However, TRPA1-null mice from two independent laboratories produced conflicting results. Kwan et al. (2006) observed decreases in the cold sensitivity of TRPA1-null mice in behavioral experiments. They measured paw withdrawal responses from a cold plate set at 0°C and responses to brief evaporative cooling evoked by acetone. However, differences in thermal sensitivity were only significant in female mice. In contrast, Bautista et al. (2006) reported that the lack of functional TRPA1 channel had no effect on cold sensation in mice. They analyzed cold sensitivity in vitro using cultured trigeminal neurons and in vivo by assessing behavioral responses to acetone and a cold plate. There were no differences in the cold sensitivity of wild-type and TRPA1-null mice in any of these assays (Bautista et al., 2006).

More recently, several studies examining TRPM8-null mice have clearly demonstrated the importance of this ion channel to cold sensation (Daniels and McKemy, 2007). Cold responses and the number of cold-sensitive sensory neurons are strongly reduced in TRPM8-null mice but are not completely abolished, supporting the idea that an additional cold transduction pathway is present. Interestingly, these residual cold-sensitive neurons in TRPM8-deficient mice are not mustard oil sensitive, indicating that TRPA1 does not underlie the cold response (Bautista et al., 2007).

Differences in Cold Activation of Native versus Heterologous Expression of TRPA1

As discussed above, most evidence from cultured sensory neurons indicates that TRPA1 is not a noxious cold sensor in its native somatic sensory neuron environment. However, several groups have observed cold responses in cells expressing TRPA1 heterologously. Using identical cold-stimulation protocols, we and others have been able to record cold activation of TRPA1 in HEK293 cells, but not in DRG neurons. This indicates that differences in cold stimulation do not account for the lack of cold sensitivity of TRPA1 in neurons (Reid, 2005; Zurborg et al., 2007). Rather, there must be important differences between heterologous expression systems and neurons.

In a comprehensive review of cold sensation, Gordon Reid highlights the fact that a cold receptor is a neuron and not a molecule (Reid, 2005). A single molecule such as TRPA1 in an expression system will only give limited insight into the behavior of an intact cold receptor, and receptor activity results from the interaction between a transducer and its cellular context (Reid, 2005). We have investigated the concept of cellular context and cold activation of TRPA1 by examining how intracellular signaling pathways, in particular calcium signaling, modify gating.

Several studies have shown that Ca^{2+} is an important modulator of TRPA1 function. It is required for a full response to agonists such as mustard oil and is involved in desensitization of the channel (Jordt et al., 2004; Nagata et al., 2005; Garcia-Anoveros and Nagata, 2007). More recently, we and another group have shown independently that intracellular Ca^{2+} directly activates TRPA1 and that this activation is mediated by a putative EF-hand domain present in the N terminus of the channel (Doerner et al., 2007; Zurborg et al., 2007). Because of

the ubiquitous nature of Ca^{2+} signaling it is clear that direct gating of TRPA1 by Ca^{2+} will allow the channel to participate downstream of many different types of activation pathway.

From examining cold and Ca^{2+} responses of TRPA1-expressing HEK293 cells, we have put forward a model that explains, at least in part, the discrepancy between different studies. In our experiments, we used calcium microfluorimetry and observed responses to cold stimuli in both control mock–transfected cells and TRPA1-expressing cells. The responses in TRPA1-expressing cells were much stronger than in control cells; however, the activation temperature threshold in both groups was remarkably similar (17°C). This observation suggests that the trigger for the response to cold does not change upon expression of TRPA1, only the amplitude of the response. We therefore propose that cold activates an as yet unknown mechanism in control HEK293 cells, which produces an increase of intracellular Ca^{2+}. When cells express TRPA1, the increase in Ca^{2+} activates TRPA1 and consequently the response to cold is much stronger. Indeed, blocking Ca^{2+} activation of TRPA1 results in a drop of the cold response to the level of the background response in control cells.

To investigate this idea further, we undertook patch clamp experiments where we buffered intracellular Ca^{2+} concentration with EGTA in the patch pipette and measured currents over a range of voltages. Many TRP channels have been demonstrated to be weakly voltage dependent and upon activation with an agonist shift their voltage activation curve to more negative values (Talavera et al., 2008). We therefore reasoned that analyzing voltage dependence of TRPA1 would greatly increase the sensitivity of our measurements. We performed two types of experiments to examine activation of TRPA1 by cold. First, we buffered intracellular Ca^{2+} to nominally zero concentrations and measured voltage activation at room temperature and 16°C. Under these conditions, we never observed any shift in the voltage dependence of TRPA1 at lower temperatures. Second, to exclude the possibility that intracellular Ca^{2+} is required for activation of TRPA1 by cold, we measured activation of TRPA1 at a calculated intracellular Ca^{2+} concentration of 10 μM. Here, we found that decreasing the temperature actually reduced the activation of TRPA1 by Ca^{2+}, as would be expected from a channel that is not gated by cold (Zurborg et al., 2007).

Our experiments were performed in HEK293 cells; however, we and others have also observed background cold responses in most common cell-expression systems, including COS cells, HeLa cells, CHO cells, and *Xenopus* oocytes (Klionsky et al., 2007; Hamada et al., 2008). Klionsky et al. (2007) tested the cold sensitivity of TRPA1 in CHO cells by measuring the uptake of $^{45}Ca^{2+}$ during cooling. They observed a small increase in $^{45}Ca^{2+}$ uptake in control cells that was strongly amplified in the presence of TRPA1, indicating that a similar mechanism is present in CHO cells. Indeed, the only cell type where we have not observed background cold responses is cultured DRG neurons. Here, activation of ionic currents by cold is tightly regulated, and only defined populations of neurons respond to cooling. We found little overlap between cold-responsive neurons and TRPA1 expression, implying that even indirectly, TRPA1 has little involvement in the detection of cold stimuli by somatic sensory neurons (Caspani et al., 2007). Intriguingly, visceral vagal sensory neurons do display a strong correlation between cold sensitivity and TRPA1 expression, and it is possible that in these neurons, indirect activation of TRPA1 might play a role in cold sensitivity (Fajardo et al., 2008).

Not all data support a model of indirect activation of TRPA1 by cold. Sawada et al. (2007) performed inside-out single-channel recordings under calcium-chelating conditions and observed activation of TRPA1 by cold stimuli. They reported that cold has two direct effects on TRPA1: it decreases its conductance but increases the open probability of the channel (Sawada et al., 2007). This is in contrast to our data on voltage activation of TRPA1 and previous studies testing the cold sensitivity of TRPA1 in single-channel recordings (Nagata, 2007; Zurborg et al., 2007).

Conclusions

Since the initial description of TRPA1 as a novel thermoTRP channel (Story et al., 2003), there has been disagreement about its function as a noxious cold sensor. Although TRPA1 can be activated by cold when expressed heterologously, most groups report a lack of overlap between TRPA1 expression and cold sensitivity of cultured somatic sensory neurons. We suggest that TRPA1 is only indirectly activated by cold and that this occurs via an increase in intracellular Ca^{2+} concentration in heterologous cells. Because cooling does not evoke unspecific rises in Ca^{2+} concentration in DRG neurons, TRPA1 is probably not involved in cold detection in these cells.

Several issues remain concerning the role of TRPA1 in cold transduction in vivo. Separate mouse knockout studies have reported conflicting results on cold sensation in TRPA1-null mice (Bautista et al., 2006; Kwan et al., 2006). It is possible that these are complicated by the presence of the other cold-sensitive TRP channel, TRPM8, a problem that could be resolved by examining TRPM8 and TRPA1 double knockout mice for cold sensitivity. In the absence of any function for TRPA1 in cold sensation, the crucial question is, What is the identity of the other cold sensor?

Note added in proof. A recent study has reported activation of TRPA1 by cold in a Ca^{2+}-independent manner via a shift in the voltage activation curve of the channel (Karashima, Y., K. Talavera, W. Everaerts, A. Janssens, K.Y. Kwan, R. Vennekens, B. Nilius, and T. Voets. 2009. *Proc. Natl. Acad. Sci. USA.* 106:1273–1278).

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