Ion channels involved in cold detection in mammals: TRP and non-TRP mechanisms

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Abstract Substantial progress in understanding thermal transduction in peripheral sensory nerve endings was achieved with the recent cloning of six thermally gated ion channels from the TRP (transient receptor potential) super-family. Two of these channels, TRP melastatin 8 (TRPM8) and TRP ankyrin 1 (TRPA1), are expressed in dorsal root ganglion (DRG) and trigeminal ganglion (TG) neurons, are activated by various degrees of cooling, and are candidates for mediating gentle cooling and noxious cold, respectively. However, accumulating evidence suggests that more than just these two channels are involved in cold sensing in mammals. A recent report described a critical role of the voltage-gated tetrodotoxin-resistant sodium channel Na_v1.8 in perceiving intense cold and noxious stimuli at cold temperatures. Other ion channels, such as two-pore domain background potassium channels (K2P), are known to be expressed in peripheral nerves, have pronounced temperature dependence, and may contribute to cold sensing and/or cold hypersensitivity in pain states. This article reviews the evidence supporting a role for each of these channels in cold transduction, focusing on their biophysical properties, expression pattern, and modulation by pro-inflammatory mediators.

Keywords Pain · Sensory · Thermal · Dorsal root ganglion · Inflammation

Introduction

Changes in ambient temperature are detected by specialized nerve endings in the skin that are able to convert these physical stimuli into electrical activity. A clearer understanding of the transduction mechanism has emerged only in the last decade, when several ion channels from the TRP (transient receptor potential) super-family directly gated by temperature have been cloned (Reid 2005; Bandell et al. 2007). Two of these thermoTRP channels [TRP melastatin 8 (TRPM8) and TRP ankyrin 1 (TRPA1)] are activated by cooling and are obvious candidates for a crucial role in cold transduction. However, recent findings are not entirely consistent with a simplified though attractive scheme in which TRPM8 is the sole detector for moderate cooling, while TRPA1 senses noxious cold. Our aim is to review the recent literature on TRPM8, TRPA1, and other ion channels that may be involved in shaping the response to cold temperature in peripheral sensory nerve endings. It should be noted however that careful interpretation is necessary regarding the properties of specific cold-sensing peripheral neurons, as TRPM8 and TRPA1 expression as well as the neuronal responses to cold and TRP channel agonists differ substantially between trigeminal ganglion (TG) and dorsal root ganglion (DRG) neurons.

TRPM8 as a sensor for gentle cooling

Cloning, expression pattern, and biophysical properties

The first hint for the existence of a nonselective cation channel expressed in sensory neurons and activated upon cooling was provided in 2001 (Reid and Flonta 2001b). Using the patch clamp technique, the existence of a cold-

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activated current in a subpopulation of small rat DRG neurons was demonstrated. This current was strongly and dose-dependently sensitized by (–)-menthol and adapted with a time constant of ~60s in the presence of prolonged cooling, in good agreement with properties of intact cold receptors in other species [Kenshalo and Duclaux 1977 (monkey); Schafer et al. 1986 (cat)]. Soon thereafter an ion channel from the mouse, TRPM8, was cloned, expressed in both DRG and TG, and gated by a moderate decrease in temperature, with a threshold of ~25°C (McKemy et al. 2002; Peier et al. 2002). The biophysical properties of TRPM8 were strikingly similar to those reported for the native cold- and menthol-sensitive current: a reversal potential close to 0 mV, indicating a nonselective cation channel, and a sensitization by menthol manifested in a shift of the threshold to warmer temperatures. Within the DRG, the mRNA for TRPM8 was found to be expressed predominantly in a subpopulation of small unmyelinated (C-fiber) neurons lacking nociceptive markers such as TRPV1, calcitonin gene-related peptide (CGRP), and substance P (Peier et al. 2002). This issue, related to the expression of TRPM8 in neurons that are functionally distinct from nociceptors, has been subject to intense debate and is still controversial. It may be speculated that this non-nociceptive subclass of TRPM8-expressing neurons could be responsible for TRPM8-mediated analgesia (see below). Nevertheless, several authors have shown using functional assays that, at least in rat and mouse DRG or TG, a significant fraction of cold- and menthol-sensitive (and thus very likely TRPM8-expressing) neurons are also activated by the TRPV1-specific agonist capsaicin [Reid et al. 2002 (rat DRG culture); McKemy et al. 2002 (rat DRG culture); Xing et al. 2006 (rat acutely dissociated DRG neurons); Viana et al. 2002 (mouse TG culture)] or by noxious heat [Okazawa et al. 2004 (rat DRG culture)]. More recent evidence was provided for the expression of TRPM8 in C and thinly myelinated A δ fibers that terminate in the superficial layers of the spinal dorsal horn (mostly lamina I), as well as for the substantial overlap between TRPM8 and TRPV1 (Takashima et al. 2007; Dhaka et al. 2008). The functional consequence of this overlap between TRPM8 and TRPV1 is not entirely understood, but it may underlie the phenomenon of paradoxical activation of low threshold cold receptors by noxious heat (Dodt and Zotterman 1952).

Modulation by inflammatory mediators and TRPM8-mediated analgesia

Altered cold sensitivity is a known feature of inflammatory conditions and neuropathies. Activation of G protein-coupled receptors (GPCRs) that stimulate phospholipase C (PLC) by a variety of inflammatory mediators such as bradykinin and nerve growth factor (NGF) leads to a

diminished cold sensitivity of TRPM8 (Premkumar et al. 2005; Abe et al. 2006). The effect is mediated by protein kinase C (PKC) and the downstream activation of a protein phosphatase (Premkumar et al. 2005). According to other authors, a crucial modulatory influence on TRPM8 is exerted by phosphatidylinositol 4,5-bisphosphate (PIP₂). This molecule has a positive effect on TRPM8 channel function, and PIP₂ depletion following the activation of PLC leads to pronounced channel desensitization (Liu and Qin 2005; Rohacs et al. 2005). Work in our own laboratory demonstrated that the pro-inflammatory mediators bradykinin and prostaglandin E₂ (PGE₂) desensitize native TRPM8 channels, and this effect can be prevented by inhibition of PKC and protein kinase A (PKA), respectively (Linte et al. 2007).

TRPM8 channels expressed both peripherally and in the central terminals of cold-sensitive neurons in the spinal cord appear to mediate analgesia; activation of TRPM8-expressing nerve fibers leads to glutamate release in the dorsal horn and stimulation of group II/III metabotropic glutamate receptors (mGluRs) located both pre- and post-synaptically, which then trigger signaling cascades that inhibit the activity in first and/or second order nociceptors (Proudfoot et al. 2006). Interestingly, it was recently shown that both peripheral and spinal activation of TRPM8 leads to decreased excitability of spinal locomotor networks and slower locomotor rhythms also via inhibitory group II/III mGluRs (Mandadi et al. 2009). As both expression level and activity of TRPM8 change following inflammation, this may have profound effects on locomotor activity in chronic pain states.

TRPM8 null mutant mice

Important progress in understanding the pathophysiological role(s) of TRPM8 was made by three recent studies reporting on TRPM8 null mutant mice. The most important finding was that genetic ablation of TRPM8 leads to pronounced deficits in innocuous cold and noxious cold sensing. TRPM8 knockout animals are impaired in temperature preference tests and fail to react to a reduction in temperature between 27 and 10°C (Bautista et al. 2007). However, at temperatures equal to or below 10°C, even TRPM8^{–/–} mice display aversive behavior, indicating that additional cold-sensing mechanisms are recruited in this temperature range. Similar results were reported by Dhaka et al. (2007), while Colburn et al. (2007) showed that TRPM8 null mutants spend equal amounts of time at 23 and 5°C. Moreover, in two reports there was no difference between genotypes in pain behavior evoked in the cold plate test at –1°C (Dhaka et al. 2007) and 10, 0, and –5°C (Bautista et al. 2007), while a third group described a strongly increased response latency in TRPM8 knockout

mice on a cold plate at 0°C (Colburn et al. 2007). In conclusion, while there appears to be a consensus that TRPM8 is required for moderate (or innocuous) cold sensing in a wide range of temperatures, the actual involvement of this channel in detecting noxious cold remains to be established.

TRPA1 as a sensor for noxious cold

Cloning, expression pattern, and biophysical properties

TRPA1 (initially named ANKTM1) was amplified by Story et al. (2003) from the mouse, using a bioinformatic analysis approach based on screening for cDNAs that have six transmembrane domains (6TM) as well as ankyrin domains, both features of the thermosensitive TRP channels. TRPA1 mRNA is quite abundant in rodent peripheral ganglia, being expressed in ~50% of DRG neurons, ~35% of TG neurons, and ~30% of nodose ganglion neurons, in all cases the staining being confined to small cells (Nagata et al. 2005). Co-expression studies indicated that TRPA1 is expressed in a subpopulation of peptidergic C-fiber nociceptor neurons that also express the vanilloid receptor subtype 1 TRPV1, but not TRPM8 (Story et al. 2003). However, a low level of co-expression for TRPM8 and TRPA1 (3.3% of all rat DRG neurons express both mRNAs) was found by a later study (Kobayashi et al. 2005). TRPA1 is a slightly outward rectifying, nonselective cation channel ($P_{Ca}/P_{Na} \sim 0.84$) (Story et al. 2003). Calcium ions have a strong influence on channel gating, accelerating activation but also leading to inactivation in a voltage-dependent manner (inactivation is reduced at positive potentials) (Nagata et al. 2005).

According to other authors, however, calcium release from stores is sufficient to activate TRPA1 (Jordt et al. 2004; Zurborg et al. 2007). Recombinant mouse TRPA1 was activated by strong cooling (~17°C) and icilin (a synthetic compound that also activates TRPM8), but not menthol (Story et al. 2003). Another group failed to record any cold-induced activity of recombinant rat TRPA1 (rTRPA1) but demonstrated that rTRPA1 was activated by the pungent compound allyl isothiocyanate (AITC, also known as mustard oil) and by Δ^9 -tetrahydrocannabinol, the active compound of marijuana (Jordt et al. 2004). A plethora of chemical agonists of TRPA1 were subsequently discovered, including pungent compounds such as cinnamaldehyde, methyl salicylate, allicin (from garlic), acrolein (2-propenal, from exhaust smoke), sesquiterpenes, caffeine, and inflammatory mediators (bradykinin, NO, A-, and J-series prostaglandins) (Bandell et al. 2004; Bautista et al. 2005, 2006; Escalera et al. 2008; Nagatomo and Kubo 2008; Takahashi et al. 2008; Taylor-Clark et al. 2008). Even menthol was shown to have a bimodal action on TRPA1, as it activates the

channel at low-micromolar concentrations and blocks it at higher ones (Karashima et al. 2007).

In agreement with a nociceptive function of TRPA1, the channel is activated by highly reactive chemical products of oxidative stress, including hydrogen peroxide and endogenous aldehydes (4-hydroxynonenal) (Macpherson et al. 2007b; Andersson et al. 2008). Most of these reactive compounds activating TRPA1 act by covalent binding to cysteine residues located in the intracellular N-terminal domain of the channel (Hinman et al. 2006; Macpherson et al. 2007a). A variety of other chemical agents exert their proalgesic action via TRPA1 activation, including formalin (McNamara et al. 2007), the antimycotic agent clotrimazole (Meseguer et al. 2008), the L-type calcium channel blockers 1,4-dihydropyridines (Fajardo et al. 2008a), the general volatile anesthetics desflurane and isoflurane, the i.v. anesthetics propofol and etomidate (Matta et al. 2008), and even the local anesthetic lidocaine (Piao et al. 2009).

More recent studies described additional modalities of chemical activation of TRPA1: intracellular calcium ions acting via an EF-hand domain located in the N-terminal domain directly gate TRPA1 (Doerner et al. 2007; Zurborg et al. 2007), while alkalization of the internal milieu activates TRPA1 both in whole-cell and inside-out excised patch mode, most likely through covalent modification of N-terminal cysteines (Fujita et al. 2008). Finally, osmotic stimuli (hypertonic but not hypotonic solutions) activate both recombinant rTRPA1 and rat DRG neurons, which are sensitive to the TRPA1 agonist AITC (Zhang et al. 2008). It can be concluded that TRPA1 appears to be a truly polymodal receptor in the pain pathway, as it is activated by a wide variety of chemical agents acting through different molecular mechanisms and also by mechanical and thermal stimuli.

Modulation by inflammatory mediators

TRPA1 is the target of signaling cascades involved in nociceptor sensitization. A variety of pro-inflammatory compounds released by the injured tissue itself or by recruited immune cells (macrophages, mast cells, neutrophils, etc.) amplify the response to pain-producing stimuli. From early studies on TRPA1, it became clear that this channel plays a key role in inflammatory pain, as it is activated by bradykinin via PLC stimulation and diacylglycerol (DAG) production (Bandell et al. 2004). In addition to direct activation of TRPA1, bradykinin also sensitizes the TRPA1 response to AITC in a PLC- and PKA-dependent manner (Wang et al. 2008). The relevance of these findings was confirmed by the fact that acute behavioral responses to bradykinin as well as bradykinin-induced thermal and mechanical hyperalgesia are massively attenuated in TRPA1 null mutant mice (Bautista et al. 2006;

Kwan et al. 2006). TRPA1 activity is potentiated following protease-activated receptor 2 (PAR2) activation via PLC and PIP_2 degradation, which may be involved in the proalgesic effect of inflammatory proteases tryptase and trypsin (Dai et al. 2007). Moreover, TRPA1, alongside TRPV1, is one of the targets of NGF, as long term exposure to this neurotrophin leads to upregulation of TRPA1 mRNA and to an increase in the amplitude of AITC-evoked currents in cultured TG neurons from the rat (Diogenes et al. 2007). In general, given the activation of TRPA1 by increased intracellular calcium concentration, any signaling pathway involving PLC, inositol triphosphate (IP_3), and calcium release from stores would be expected to recruit TRPA1 as an active player.

TRPA1 gating by intense cold: conclusions from native tissue, expression systems, and null mutant mice

In what follows we shall focus on the putative role of TRPA1 as a cold sensor. A controversy arose as to whether recombinant TRPA1 was cold-sensitive or not. After the initial paper reporting the cloning of TRPA1 and its activation by cooling below $\sim 17^\circ\text{C}$ (Story et al. 2003), two other groups failed to activate TRPA1 by applying noxious cold (Jordt et al. 2004; Nagata et al. 2005). The reason for this discrepancy is not clear, as both groups used strong cooling ($\sim 5^\circ\text{C}$ and $\sim 15^\circ\text{C}$, respectively), and recombinant TRPA1 was from both mouse (Nagata et al. 2005) and rat (Jordt et al. 2004). Recently however accumulating evidence seems to favor the gating of TRPA1 by cold; two recent papers described activity induced by cooling mediated by mTRPA1 in heterologous expression systems, both at the whole-cell level (Sawada et al. 2007; Karashima et al. 2009) and in excised inside-out patches (Sawada et al. 2007), with a temperature threshold close to that initially reported of $\sim 17^\circ\text{C}$. A similar debate was generated regarding the activation of TRPA1 by cooling in cultured DRG or TG neurons (Bandell et al. 2004; Jordt et al. 2004; Babes et al. 2004; Munns et al. 2007). Interestingly, a recent report demonstrated that cold sensing in nodose ganglion neurons is almost exclusively mediated by TRPA1 in both the rat and the mouse, unlike the rat DRG, where a role for TRPA1 as a sole cold detector in a subset of cold-sensitive neurons could not be assigned (Fajardo et al. 2008b). Whether TRPA1 is a cold sensor in vivo is still a matter of debate, even after the investigation of TRPA1 null mutant mice by three independent groups (Kwan et al. 2006; Bautista et al. 2006; Karashima et al. 2009). Thus, while two of the groups found altered sensitivity to noxious cold in TRPA1 $^{-/-}$ animals (Kwan et al. 2006; Karashima et al. 2009), the other group reported no effect of TRPA1 deletion on cold sensitivity in vitro (TG neurons in primary culture) or in vivo in a variety of

behavioral tests (acetone, cold plate, temperature preference) (Bautista et al. 2006). The reason for these discrepancies is not clear.

Other thermo-sensitive ion channels that (may) participate in cold sensing

The first attempt to investigate cold sensing at the cellular level was carried out on cultured sensory neurons from the rat and led to the conclusion that cooling closes a background potassium conductance, leading to depolarization and action potential firing (Reid and Flonta 2001a). Work from another laboratory proposed a similar model for cold transduction in which a decrease in temperature activates sensory nerve endings by closing a leak potassium channel, while activation of cold-insensitive neurons is prevented by the action of a different transient outward potassium channel acting as an excitability brake (Viana et al. 2002). Two-pore domain (K2P) potassium channels have been described (named TREK-1 and TRAAK) with the required features for a cold transducer: active at resting membrane potentials, strongly temperature-dependent (sensitized by warming and inhibited by cooling) and highly expressed in DRG (Maingret et al. 2000; Talley et al. 2001; Kang et al. 2005). Accumulating evidence indicates that K2P channels are subject to modulation by inflammatory mediators; TREK-1 is inhibited by low extracellular pH and lysophosphatidic acid via stimulation of PLC and by PGE2 via cAMP signaling, which may contribute to increased excitability and inflammatory sensitization of peripheral nerve endings (Alloui et al. 2006; Cohen et al. 2009). Interestingly, both TREK-1 and TRAAK are highly co-expressed at the protein level with thermoTRP channels, including TRPM8 (Yamamoto et al. 2009). However, behavioral investigation of TREK-1 and TRAAK null mutant mice revealed no change in cold sensitivity (Alloui et al. 2006; Noel et al. 2009). Interestingly, when both TREK-1 and TRAAK were absent, a strong increase in cold sensitivity was measured both in vitro and in vivo, in behavioral assays (Noel et al. 2009), suggesting that these background potassium channels oppose the excitatory action of cooling and contribute to the fine tuning of the thermal sensitivity of cold receptors. However, to what extent the effect of removing the K2P channels TREK-1 and TRAAK is specific to cold sensing or is merely due to a general increase in overall neuronal excitability is not entirely clear. It should be noted that this model does not agree with the original role assigned to leak channels, namely that they could mediate excitation of specialized sensory nerve endings by cold through closing and subsequent depolarization (Reid and Flonta 2001a; Viana et al. 2002).

Other potassium channels that appear to be involved in shaping cold sensitivity in the peripheral nervous system belong to the family of voltage-gated potassium channels K_v1. Differential expression of excitatory agents (TRPM8 channels) and excitability brakes (4-AP-sensitive, *Shaker*-

Fig. 1 Schematic of a cold-sensitive nerve ending that may express TRPM8 or TRPA1 (rarely both), the heat-activated background K2P channels TREK-1 and TRAAK, and the voltage-gated TTX-resistant sodium channel Na_v1.8. **a** At 32°C, TRPM8 and TRPA1 are closed, while TREK-1 and TRAAK contribute to the negative resting membrane potential. The nerve ending is silent, or it fires at low frequency. **b** At moderately cold temperatures, TRPM8 (but not TRPA1) channels are open and allow sodium and calcium ions to flow in and generate a receptor potential, which in turn activates Na_v1.8, triggering action potentials. Inhibition of TREK-1 and TRAAK amplify the receptor potential. **c** At noxious cold temperatures, both TRPM8 and TRPA1 channels are open. The cation influx through these channels depolarizes the membrane and triggers Na_v1.8-dependent action potentials. K2P channels are closed leading to an increased membrane resistance, lowering the threshold for Na_v1.8. **d** Modulation of cold-sensitive nerve endings by inflammatory mediators is illustrated using bradykinin (BK) and prostaglandin E2 (PGE2) as examples. Stimulation of BK receptors (B2) leads to activation of phospholipase C (PLC), depletion of PIP₂, and generation of diacylglycerol (DAG) and inositol tri-phosphate (IP₃). Calcium is released from stores (ER) and directly activates TRPA1. Protein kinase C (PKC) is activated and, together with PIP₂ depletion, drives desensitization of TRPM8. PLC sensitizes TRPA1 in a protein kinase A (PKA)-dependent manner. PGE2 signaling leads to activation of adenylate cyclase (or PLC, depending on the receptor type) and PKA-mediated phosphorylation, which in turn inhibits TRPM8 and TREK-1 and positively modulates Na_v1.8

like K_v1 potassium channels generating the I_{KD} current) defines the temperature threshold of activation in cold-sensitive TG neurons, such that low threshold innocuous cold receptors are characterized by high TRPM8 and low K_v1 expression, while for the high threshold cold receptors the situation is just the opposite (Madrid et al. 2009). As expected, pharmacological blockade of I_{KD} leads to enhanced nocifensive responses to cold exposure in wild type and TRPA1^{-/-} mice, indicating that the resulting hyperalgesia is not mediated by TRPA1.

Hyperpolarization-activated nonselective cation channels of the HCN family generate the so-called I_h current, are widely expressed in sensory neurons of the DRG and TG (in particular the HCN1 and HCN2 subunits), and appear to be involved in generating the ongoing electrical activity typical of peripheral cold receptors. This feature of cold receptors is not required for the transduction event per se but seems to participate in defining the sensitivity to small changes in ambient temperature. Moreover, the I_h current has a significant contribution to the ability of neurons to respond to stimuli with high frequency bursts of action potentials, a pattern that is also encountered in cold receptors and which may be involved in temperature coding by the nervous system. HCN1 null mutant mice display a reduction in their nocifensive response in the cold plate test, and pharmacological blockade of I_h by subcutaneous injection of ZD7288 had the same effect (Orío et al. 2009).

Experiments carried out in our laboratory, as well as studies of other investigators, have provided evidence for cold-sensing mechanisms independent of TRPM8 or

TRPA1, at least in the primary culture model (Babes et al. 2006; Munns et al. 2007). Using a combination of calcium microfluorimetry and patch clamp, we have identified a novel type of cold-sensitive neuron with a transient response to a cooling step that was not activated or sensitized by the TRPM8 and TRPA1 agonists menthol or cinnamaldehyde (Babes et al. 2006). The time course of this rapid adaptation to cold fits very well with the fast component of cold receptor adaptation measured in vivo (in the time range of seconds; Darian-Smith et al. 1973; Kenshalo and Duclaux 1977; Campero et al. 2001), which cannot be accounted for by TRPM8 or TRPA1, as both display much slower desensitization during cooling.

Na_v1.8 is a voltage-gated, TTX-resistant sodium channel selectively expressed in small-diameter sensory neurons with nociceptive function (Akopian et al. 1996). Interestingly, electrically evoked action potentials in intact nerve endings are abolished by 1 μ M TTX at 32°C, indicating that at this temperature the rather high voltage threshold of Na_v1.8 is not normally reached. Remarkably, upon cooling, TTX-blocked fibers became electrically excitable again. Cooling closes some background potassium channels and inhibits the activity of the Na/K-ATPase, leading to an increased membrane resistance and therefore amplifying the voltage change induced by any depolarizing current (Zimmermann et al. 2007). The consequence is that Na_v1.8 is being recruited at low temperatures by stimuli that would not have reached its threshold at normal skin temperatures around 32°C. In agreement with this hypothesis, Na_v1.8 null mutant mice displayed drastically reduced pain behavior in the cold plate test (Zimmermann et al. 2007). At low temperatures, TTX-sensitive sodium channels in nociceptive endings enter a state of prolonged inactivation, becoming unavailable for electrical signaling, which explains the impaired sensory and motor functions in the cold. However, the organism's ability to detect threatening situations is preserved by the recruitment of Na_v1.8, which takes over the function of generating electrical impulses at low temperatures in a specialized set of pain-sensing nerve endings (Zimmermann et al. 2007).

Conclusion

The picture of cold sensing emerging from this plethora of molecular, cellular, behavioral, and genetic studies is a rather complex one that cannot be reduced to a simplified scheme in which TRPM8 senses gentle cooling while TRPA1 is responsible for the pain induced by cold. These cold-activated TRP channels (with a possible contribution from other channels as yet unidentified) appear to be involved in the actual transduction event, in which a reduction in ambient temperature produces a depolarizing

current and a subsequent receptor potential (Fig. 1). At this point, however, other channels come into play, as depolarization activates voltage-dependent potassium channels (composed of K_v1 subunits) acting as an excitability brake. The differential expression of these channels in functionally distinct subpopulations of cold-sensitive neurons determines the balance between inward and outward currents, thus defining the extent of the depolarization and setting the temperature threshold for eliciting Na_v1.8-dependent action potentials, which are then propagated along peripheral axons and into the central nervous system, leading to the conscious perception of cold. The complexity of cold sensing is increased by the fact that thermosensitive ion channels are substantially modulated by inflammatory mediators, leading to changes in temperature threshold and abnormal cold sensing in pathological pain states.

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