


## The cool things to know about TRPM8!

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### ABSTRACT

Transient receptor potential melastatin 8 (TRPM8) channels play a central role in the detection of environmental cold temperatures in the somatosensory system. TRPM8 is found in a subset of unmyelinated (C-type) afferents located in the dorsal root (DRG) and trigeminal ganglion (TG). Cold hypersensitivity is a common symptom of neuropathic pain conditions caused by cancer therapy, spinal cord injury, viral infection, multiple sclerosis, diabetes, or withdrawal symptoms associated with chronic morphine treatment. Therefore, TRPM8 has received great attention as a therapeutic target. However, as the activity of TRPM8 is unique in sensing cool temperature as well as warming, it is critical to understand the signaling transduction pathways that control modality-specific activity of TRPM8 in healthy versus pathological settings. This review summarizes recent advances in our understanding of the mechanisms involved in the regulation of the TRPM8 activity.

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## Introduction

More than 50 years have passed since the first recording of the transient receptor potential (TRP) channel current in *Drosophila* in which the mutant channel generated a photoreceptor light response that decayed to baseline during prolonged light illumination [1]. Almost exactly 20 years later the mutant gene responsible for this phenomenon was identified and termed *trp* [2]. Since then, the complete identification and characterization of the large family of TRP channels have been completed, including the TRPC1 in the mid 1990 [3,4], followed by the vanilloid receptor TRPV1 [5], the camphor receptor TRPV3 [6] and, much later, the menthol receptor TRPM8 [7,8]. The TRPM8 channel was first discovered in mouse sensory dorsal root ganglia (DRG) neurons [9], although numerous TRPM8 isoforms have now been described in various mammalian tissues such as prostate, cardiovascular system, bronchopulmonary tissue, bladder and urogenital tract and many others [10]. This channel is expressed in peripheral sensory neurons of small size (unmyelinated C fibers) to transduce environmental cold stimuli [7,11,12]. Notably, its ability to sense cold is finely tuned in various species for better ambient temperature adaptation [13] and by taking part in the energy metabolism [14,15]. In a recent study it has

even been proposed that, along with TRPA1 and TRPV1 channels, TRPM8 underlies all thermosensation in mice [16]. Indeed, genetic manipulation of TRPM8 in mice leads to a loss of environmental cold sensation [11,17,18] and even the ability to detect warm [19]. In humans reduced TRPM8 expression leads to attenuated cold and pain sensation [20]. Conversely, hyperactivity of the channel plays a central role in disease pain states such as cold allodynia after inflammation or nerve injury [21,22]. A growing number of evidence link this channel to various tumors, such as prostate, pancreas, colon, breast, lung, and skin [23]. In addition, TRPM8 has been linked to migraine [24] but also to dry eye disease [25], irritable bowel syndrome, oropharyngeal dysphagia, chronic cough, or hypertension [26–29] and the list continues to grow.

It is, therefore, not surprising that attention to TRPM8 activity modulation has been getting lately by numerous research groups and pharmaceutical companies although, oftentimes, their results are conflicting. This review is not aimed at compiling all knowledge gathered to date regarding modulation of the TRPM8 activity but rather at updating and summarizing the new research results obtained in the last few years.

## TRPM8 channel

The TRPM8 channel is a voltage-gated, nonselective cation channel that contains six transmembrane segments (S1-S6) and large intracellular amino and carboxyl termini [30,31]. The S2 and S3 segments possess the binding sites for the cooling agents: menthol and icillin, respectively. The S4 segment and the region between S4 and S5 carry out the function of voltage sensing while the region between S5 and S6 forms the channel pore [30,31]. Like many TRP channels, TRPM8 has polymodal, distinct gating mechanisms, being activated by innocuous to noxious cool temperatures (10–28°C), membrane depolarization, cooling agents such as menthol and icilin and changes in osmolality and pH [7,8,11,12,32–37]. Most sensory modalities undergo calcium-dependent desensitization in which the activity of the channel decreases despite the continuous presence of the stimulus [7,38,39]. Indeed, removing the extracellular calcium markedly reduces desensitization [7]. Recently, Diver *et al.* provided insight into the structural basis of the desensitization process [40]. The authors showed the critical contribution of five negatively charged residues located in the S2 and S3 transmembrane helices and within the S2-S3 linker. They proposed a new model in which the channel had only two distinct conformational states, closed and desensitized, and upon activation does not undergo dramatic conformational changes within the S3-S4 transmembrane segments but rather only in the outer region of the pore.

## TRPM8 modulation by PIP<sub>2</sub>

Phosphoinositides are well-known modulators of a large number of ion channels at the plasma membrane. In the early 2000, B. Hille's group reported that recovery from muscarinic-induced inhibition of the M current was phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) dependent. His lab and others later established that PIP<sub>2</sub> was necessary to maintain the function of potassium-selective channels, including voltage-gated potassium (Kv) channels, inwardly-rectifying potassium (Kir) channels, and calcium-activated potassium (KCa) [41].

While TRP channels exhibit poor voltage sensitivity they have a tetrameric architecture similar to that of Kv channels. Accordingly, PIP<sub>2</sub> has been shown to

positively modulate the function of 20 out of 28 channels in the TRP superfamily, including TRPM8 [42]. Daniels *et al.* also reported that PIP<sub>2</sub> depletion could shift the channel voltage dependence toward more positive potentials, reducing the cold- and menthol-activated currents [38]. Later, another study showed that warm ambient temperatures (40°C) shift the channel temperature threshold through a calcium-independent mechanism involving PIP<sub>2</sub> [43]. The locus of PIP<sub>2</sub> binding has been under controversy until recently when it was shown, in the cryoEM structure of TRPM8, that PIP<sub>2</sub> binds in a pocket cradled by the pre-S1 elbow and S1 helix of one subunit and the S5 helix of the adjacent subunit [40,44].

Activation of Gq-coupled receptors stimulates the calcium-sensitive phospholipase C $\beta$  (PLC $\beta$ ) that cleaves PIP<sub>2</sub> into soluble inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and membrane-bound diacylglycerol. Therefore, the opening of TRPM8 channels leads to PLC $\beta$  activation and PIP<sub>2</sub> hydrolysis which activates TRPM8 and acts as a positive modulator of cold and menthol stimuli whereas PIP<sub>2</sub> depletion inhibits channel activity [7,38,39,45,46]. While PLC $\beta$  received attention in the modulation of TRPM8, the genetic manipulation of PLC $\delta$ 4 leads to an increase in cold- and menthol-induced current in DRG neurons [47], suggesting that other PLC isoforms that contain a calcium-binding motif could be involved in this process.

Another mechanism involved in PIP<sub>2</sub>-dependent regulation of TRPM8 activity has emerged lately. Phosphoinositide-interacting regulator of TRP (PIRT), a two transmembrane domain protein specifically expressed in the DRG, trigeminal ganglia, and enteric neurons of the peripheral nervous system, was first identified as a positive modulator of TRPV1 via PIP<sub>2</sub> interaction [48]. PIRT was then found to increase voltage dependence and sensitivity to menthol of the TRPM8 channel by having a synergistic effect with PIP<sub>2</sub> [49]. This regulatory mechanism was suggested to result from an increase in the local concentration of PIP<sub>2</sub> in complex with TRPM8 and PIRT [50]. However, the effect of PIRT appeared to be species specific since Hilton *et al.* found that while mouse PIRT enhanced mouse TRPM8 activity, human PIRT attenuated TRPM8 conductance [51]. This distinct regulatory mechanism provides real physiological significance in terms of species-specific fine-tuning of thermosensation.

## TRPM8 modulation by G proteins

Pain and heat are the two main cardinal symptoms of inflammation. Previous work revealed that inflammatory mediators, including BK and histamine, inhibited TRPM8 channel function, a process that could be implicated in core body temperature regulation.

While BK and histamine are known to act on Gαq-coupled receptors, several downstream signaling pathways have been proposed to promote channel inhibition.

Since PIP2 binding to TRPM8 maintains channel function, it was originally suggested that activation of the bradykinin B2 receptor could reduce TRPM8 activity via PLCβ activation and PIP<sub>2</sub> depletion [52]. Nevertheless, the mechanism by which G protein-coupled receptors (GPCRs) modulate TRPM8 has remained controversial over the years. Two studies made the surprising observation that GPCRs coupled to G<sub>s</sub> protein and activation of PKA were able to inhibit TRPM8 channels [53,54]. Moreover, clonidine activation of the α<sub>2</sub> adrenergic receptor, which signals through G<sub>i</sub> proteins and thus inhibit PKA activity, was also reported to inhibit TRPM8 [55] although another study found no effect of activating this pathway on TRPM8 inhibition in DRG neurons [56]. A later publication from Peter McNaughton's lab challenged the idea of an involvement of PIP2 degradation in mediating channel inhibition. They proposed that, upon bradykinin receptor activation, Gαq could directly bind to the channel and inhibit its activity evoked by cold or menthol [57,58]. This finding was observed using recombinant B2 receptor and TRPM8 channel in HEK cells as well as on native TRPM8 current recorded from isolated DRG neurons. To add to the complexity of TRPM8 regulation by G protein-coupled receptors, another study showed that stimulation of TRPM8 alone was able to mediate a metabotropic signaling via Gαq and calcium release from internal stores [59]. Liu et al. recently proposed an integrated mechanism in which Gαq enhances channel sensitivity to PIP2 levels [60] whereas another study, using DRG neurons and genetically engineered mice supported the sole role for Gαq in inhibiting TRPM8 following bradykinin receptor activation [61]. Therefore, it is still unclear to what extent TRPM8 function is controlled by Gαq and if the interaction of the channel with the G protein is able to compete with Gαq-coupled receptor activation.

## Regulation by phosphorylation

Several signaling cascades are involved in the regulation of TRPM8 activity through phosphorylation. Activation of PLCβ leads to not only the reduction of PIP2 levels but also to the activation of protein kinase C (PKC) that phosphorylates a large number of TRP channels including TRPM8. Various PKC isoforms (ε, α, and β) are present in the small unmyelinated C fibers, including the TRPM8-expressing subpopulation [62,63]. Previous studies showed that activation of PKC produced menthol-induced desensitization of TRPM8 as well as dephosphorylation and downregulation of channel expression in expression systems and rat neonatal DRG neurons [64–66]. However, none of the nine putative PKC phosphorylation sites of TRPM8 were involved in channel modulation and the authors concluded that PKC had an indirect effect on menthol-induced desensitization [65]. Activation of PKC did not increase the phosphorylation state of the channel but rather activated the calcium and calmodulin-dependent serine/threonine protein phosphatase calcineurin, indicating a dephosphorylation-induced desensitization process. Furthermore, PKC inhibitors failed to block desensitization [39] and menthol-induced TRPM8 current [45]. Recently, our group described a crosstalk relationship between TRPM8 and PKC in the context of opioid-induced cold sensitivity. Indeed, postoperative shivering and cold hypersensitivity are major side effects of acute and chronic opioid treatments, respectively. Our findings showed that activation of the μ-opioid receptor by morphine induced a PKCβ-dependent inhibition of TRPM8 desensitization (disinhibition) in response to cold or menthol [67]. The loss of TRPM8 desensitization was prevented by the use of a selective PKCβ blocker. We then identified a new PKC phosphorylation site in the carboxy terminus region located in the proximity of the S6-TRP box linker. The TRP box acts as a central determinant of channel gating evoked by voltage, menthol and cold and has several PIP2 binding sites that control channel gating [30,31,36,68]. Site-directed mutagenesis of this consensus site was able to abrogate the morphine-induced inhibition of TRPM8 desensitization.

Another kinase previously described for regulating TRPM8 is the protein kinase A (PKA). Previous studies showed that PKA activation inhibits TRPM8 channel in expression systems as well as DRG neurons

[53,54]. In contrast, inhibition of PKA, as a result of  $\alpha 2$  adrenergic receptors activation, was found to reduce TRPM8 activity in menthol-sensitive DRG neurons [55]. Using HEK cells heterologously expressing TRPM8, Pabbidi *et al.* found that Phorbol 12,13-Dibutyrate (PDBu), a PKC activator, and forskolin, a PKA activator, both downregulated TRPM8-mediated currents [66]. Of note, TRPM8 was found to in turn regulate PKA activity. In a recent study, PC12 cells exposed to oxygen-glucose deprivation were less susceptible to become apoptotic when TRPM8 was blocked. This antiapoptotic effect observed upon TRPM8 inhibition was mediated by cAMP production and PKA activation [69].

Much attention has been given to the contribution of TRPM8 in cancer, and particularly prostatic cancer [23]. Recent findings have shown that TRPM8 protein is a receptor for testosterone which directly binds to and promotes channel opening [70,71]. At picomolar concentrations, the application of testosterone induces full activation of the TRPM8 and interestingly mediates a cooling sensation in human skin [70]. In addition, testosterone can regulate the expression of the *TRPM8* gene through the androgen response element [70–72]. Still, controversy surrounds the mechanisms by which testosterone regulates TRPM8-mediated cold perception. A recent study reported the inhibitory effect of testosterone on TRPM8. At nanomolar concentrations, testosterone suppressed TRPM8-mediated currents in native dorsal root ganglion (DRG) neurons and HEK cells co-expressing recombinant TRPM8 and the androgen receptor, but not TRPM8 alone. The study identified a non-canonical signaling pathway downstream of the androgen receptor (AR) activation that involved Src, ERK, and Akt [73]. In contrast to earlier results, testosterone had no effect on TRPM8 in DRG neurons from AR null mice.

The tyrosine kinase Src plays a major role in cancer, neuronal growth, and synaptic plasticity [74]. Src is activated by testosterone and numerous inflammatory mediators or growth factors acting on tyrosine kinase receptors. As such, activation of the Nerve Growth Factor (NGF) receptor, TrkA, was first reported to induce an increase in the cold sensitivity of cultured rat DRG neurons [75]. In contrast to this study, Manolache *et al.* found only a modest effect of NGF on channel phosphorylation. However, they showed that TRPM8 was constitutively tyrosine phosphorylated by Src in

heterologous expression systems or DRGs. Pharmacological or genetic inhibition of Src reduced both TRPM8 tyrosine phosphorylation and cold-induced channel activation [76].

Lastly, TRP channel-associated factors, TCAF1 and TCAF2, have been shown to regulate TRPM8 channels in the prostate, with opposing effects [77]. In single-channel recordings, TCAF1 expression was able to increase TRPM8 channel trafficking to the membrane as well as channel gating. In contrast, TCAF2 completely silenced it. Importantly, this was shown to be achieved through PI3K phosphorylation of the channel as the use of wortmannin reversed this effect [77].

## Conclusions

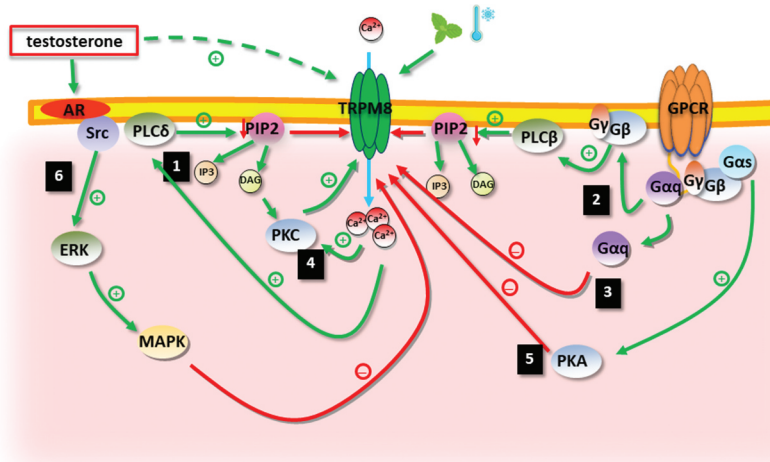
Although the role of TRPM8 channel in sensing environmental mild cold temperatures is well established, the knowledge regarding its regulation in health and diseases is still growing and controversial. Here we have reviewed recent findings on the regulation of the TRPM8 channel by phosphoinositide, G proteins and protein kinases, in heterologous expression systems, isolated DRG neurons, and animal studies (Figure 1).

The described molecular mechanisms will certainly have to be validated in larger animals and humans, particularly because of the species-specific sequence variation of TRPM8 that has been developed for thermal adaptation through evolution [13].

Clearly, new advances in knowledge regarding the physiological and pathological roles of TRPM8 along with the characterization of the natural ligands of the channel will guide researchers toward a better understanding of its regulatory mechanisms.

In fact, a recent study has proposed a role for TRPM8 activation in limiting the severity of human enterovirus, Coxsackievirus B virus infection [78]. Another one discovered a short TRPM8 isoform, termed 4TM-TRPM8, in mitochondria-associated endoplasmic reticulum membrane of keratinocytes and prostate epithelial cells [79]. Therefore, a better comprehension of TRPM8 function in thermosensation, pathogen infection, or cancer, and a deeper characterization of its expression in organs and cell types, will help to identify the relevant signaling pathways that can modulate TRPM8.





**Figure 1.** Identified signaling pathways involved in TRPM8 channel regulation. 1 – Activation of PLC $\delta$ 4 by calcium influx leads to PIP2 depletion and inhibition of TRPM8; 2 – Gq-coupled receptor-mediated stimulation of PLC $\beta$  leads to PIP2 depletion and inhibition of TRPM8; 3 – In response to the stimulation of the bradykinin receptor (B2R), G $\alpha$ q directly binds to and inhibit TRPM8; 4 – DAG and calcium influx mediate the activation of PKC, which contributes to a Ca $^{2+}$  mediated desensitization of TRPM8; 5 – Activation of Gs-coupled receptors leads to a PKA-mediated inhibition of TRPM8; 6 – Testosterone activates TRPM8 directly or indirectly through the AR signaling pathway that involves Src, ERK and MAPK. Abbreviations: AR-androgen receptor, DAG – diacylglycerol, ERK – extracellular signal-regulated kinase, GPCR – G protein-coupled receptor, IP3 – inositol trisphosphate, MAPK – mitogen-activated protein kinase, PIP2 – phosphatidylinositol bisphosphate, PKA – protein kinase A, PKC – protein kinase C, PLC-phospholipase C.

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