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Molecular Physiology of TRPV Channels: Controversies and Future Challenges

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Abstract

The ability to detect stimuli from the environment plays a pivotal role in our survival. The molecules that allow the detection of such signals include ion channels, which are proteins expressed in different cells and organs. Among these ion channels, the transient receptor potential (TRP) family responds to the presence of diverse chemicals, temperature, and osmotic changes, among others. This family of ion channels includes the TRPV or vanilloid subfamily whose members serve several physiological functions. Although these proteins have been studied intensively for the last two decades, owing to their structural and functional complexities, a number of controversies regarding their function still remain. Here, we discuss some salient features of their regulation in light of these controversies and outline some of the efforts pushing the field forward.

INTRODUCTION

Without the ability to sense changes in temperature and chemical signals, organisms would not be able to respond by adopting behaviors to contend with or avoid these stimuli. Hence, an important challenge in physiology has been to elucidate the molecular identity of the receptors that permit their detection.

Among these molecules are transient receptor potential (TRP) ion channels, which are found in the membranes of many types of cells and tissues from diverse organisms, where they perform essential roles in physiology. TRP channels are subdivided into seven subfamilies: TRPC (canonical), TRPA (ankyrin), TRPV (vanilloid), TRPM (melastatin), TRPML (mucolipin), TRPP (polycystin), and TRPN (no mechanoreceptor potential C-like or NOMPC-like) (1); the last one is found only in invertebrates and fish (2, 3). Many of these proteins have been clearly shown to be activated by several stimuli, and their activity is regulated by multiple mechanisms.

TRP channels that respond to changes in temperature are known as thermoTRPs, and they detect not only innocuous and noxious heat and cold but also osmotic, mechanical, and/or chemical stimuli. Like other ion channels, the activity of TRPs is tuned by several molecules. Other than their agonists, these include lipids, kinases, calmodulin (CaM), and ATP that indirectly affect their function or directly bind to the channels to influence their activity and expression.

The last decade has proven to be particularly fruitful in this field of study, with exciting new results in structural biology on TRP channels and the 2021 Nobel Prize in Physiology and Medicine awarded to David Julius and Ardem Patapoutian for their discoveries improving our understanding of how thermal and mechanical stimuli are transduced. The study of TRP channels saw its beginnings at the end of the 1960s. Experiments performed by Cosens & Manning (4) used *Drosophila melanogaster* that had acquired a spontaneous mutation, resulting in transient, as opposed to sustained, electroretinogram responses to long light flashes, leading to blindness in the insects (5). Several other studies detailed the behavior of the mutant, and the term transient receptor potential or trp (5–7) was coined, which would become the foundation upon which the whole family described to date would be named. The *trp* gene was cloned in 1989 and was shown to encode for an eight-helix transmembrane protein (8, 9), localized in the rhabdomere, supporting its role in phototransduction (5, 6, 8, 10).

It was later also suggested that the *trp* gene might encode for an inositide-activated Ca^{2+} (calcium) channel involved in refilling intracellular Ca^{2+} stores. Another protein analogous to the *trp* gene product was identified, TRP-like (TRPL), leading to the suggestion that the light response of *Drosophila* is mediated by channels composed from the *TRP* and *TRPL* gene products (11). The first direct evidence of the *Drosophila trp* gene encoding an ion channel came from a study by the Montell lab (12).

Several studies, including the first sequencing and cloning of mammalian TRP channels (13, 14), that preceded the cloning of the first mammalian TRPV channel proved the existence of neurons that responded to pungent compounds, protons, and temperature. Among these, some showed that capsaicin could produce a hot, prickling, and/or painful sensation when placed on the tongue of humans (15, 16). They also indicated that protons could activate capsaicin-responsive neurons (17) and that sensory neurons could respond to stimulation with capsaicin, piperine, and zingerone (pungent compounds from hot chili peppers, black pepper, and ginger, respectively) (18, 19).

Moreover, the cellular basis of the response to painful stimuli had been scarcely investigated. It was known that dorsal root ganglia (DRG), which contain a mixed population of myelinated and unmyelinated primary sensory neurons that innervate the skin surface and other parts of the body, could carry information on temperature, noxious stimuli, and touch. At the end of 1996, Cesare &

McNaughton (20) demonstrated that cultured neurons from the DRG responded to heat stimuli of an intensity that would produce a sensation of pain and that the response could be sensitized by bradykinin, a pain-inducing peptide produced during inflammation or injury to tissues.

Then, at the end of 1997, the mammalian TRPV1 (then termed VR1 or vanilloid receptor 1) was cloned by David Julius's lab and shown to be a capsaicin- and heat-sensitive nonselective cation ion channel (21), triggering an exponential development in the study of TRP channels. Subsequently, several proteins were cloned, shown to respond to chemical, thermal, and mechanical stimuli, and later included in the newly born family of TRP channels.

In this review, we focus on the TRPV subfamily of ion channels to which TRPV1 belongs. We discuss the general biophysical properties and functions and emphasize some of the current controversies in the field.

TRPV CHANNELS: FUNCTION AND GENERAL STRUCTURAL PROPERTIES

TRPV channels (TRPV1–TRPV6) are multimodal proteins that are gated by various mechanisms. They function as mechanical sensors and hygrosensors in insects, chemical and mechanical sensors in nematodes, and as chemical, thermal, and osmotic sensors in vertebrates (3, 21–30).

TRPV1 is primarily expressed by small-diameter neurons of sensory ganglia, such as DRG and trigeminal ganglia, and responds to noxious heat (>43°C), small chemical agonists and animal peptide toxins, protons (produced during tissue injury, ischemia and inflammation), fatty acids, and other stimuli (21, 31–33), generally leading to pain perception. TRPV1 also participates in the physiology of non-neuronal tissues, playing roles in the function of respiratory airways (34), for example, in cough; in smooth muscle and endothelial cells, contributing to control of the vasculature induced by chronic hypoxia (35); and in keratinocytes, where it may participate in low-pH-promoted skin proliferation (36).

TRPV2 is expressed in sensory neurons, where it senses changes in temperature (>52°C). It acts as a cannabinoid and lipid receptor but also detects mechanical and osmotic stimuli. It is also localized in the brain, where it may be involved in autonomic regulatory tasks such as appetite and cardiovascular function, and at the cerebellum and retina. In tissues other than the brain, it responds to hypotonicity, stretch, and shear stress (37, 38), playing important roles in heart function. For example, it has been found that mice lacking the expression of TRPV2 exhibit severe cardiac dysfunction. It has also been suggested that this is due to the absence of the channel in the intercalated disks that sense mechanical forces produced by nearby cardiomyocytes and ensure that the myocardium functions in synchrony (39).

The TRPV3 channel participates in the detection of warm temperature and pain and is mainly expressed in skin, but it can be found in hair follicles, tongue, nose, palate, colon, sensory ganglia, brain, and testes (40, 41). It participates, among other functions, in itch, hair development, and skin barrier function. Initial activation of this channel requires temperatures above 50°C, but lower temperatures (~30°C) can also activate it because it displays use-dependent and hysteretic temperature-dependent activation (42).

TRPV4 is expressed in many tissues and responds to temperatures near 27°C, mechanical stress, hypoosmotic conditions, and some compounds (25). In vascular endothelial cells it contributes to vascular tone and blood pressure regulation, and TRPV4 has mechanotransduction roles in several tissues. In retinal cells, it contributes to glaucoma, regulates relaxation of the main pulmonary artery and vasoconstriction of pulmonary circulation by controlling the integrity of the lung walls, and affects conditions such as asthma. TRPV4 may also be involved in pulmonary injury due to the mechanical force of ventilators used to treat respiratory failure. Finally, it also

contributes to kidney function, regulating the osmotic balance by modifying water secretion in this organ (26); to the skeletal system (43), where it is required for mechanotransduction; to preserving the skin barrier (44); to regulating hair follicle growth (45); and to itch responses in some pathologies (46).

Studies have not yet demonstrated that TRPV5 and TRPV6 channels are modulated by temperature and are different from other TRPV subfamily members. Localized at the apical membrane of Ca^{2+}-transporting epithelia, these two channels respond to 1,25-dihydroxyvitamin D_3 and are important for regulating blood Ca^{2+} levels in higher organisms. TRPV5 is mainly expressed in the distal convoluted tubules and connecting tubules of the kidney, and TRPV6 is found in the exocrine tissues, intestine, and kidney. TRPV5 participates in tuning Ca^{2+} reabsorption in the kidney and determines the level of urinary Ca^{2+} excretion, which affects trabecular and cortical bone thickness (47). TRPV6 contributes to intestinal Ca^{2+} absorption, transcellular Ca^{2+} transport in regions of the tubular system of the kidneys, and Ca^{2+}-dependent sperm maturation (48).

Structurally, TRPV channels contain four subunits, each with intracellular N and C termini, six transmembrane helices (S1–S6), and a pore region (S5-P-S6). The pore contains a selectivity filter that, in turn, is constituted by a re-entrant pore (P)-loop and small-pore helix between S5 and S6. Like voltage-gated potassium (Kv) channels, they possess an S1–S4 region, which forms a voltage-sensing-like domain (VSLD). The pore regions interact with the VSLD of the neighbor monomer in a domain-swapped manner. In some TRPV channels, the S5–S6 region undergoes conformational changes that open the pore in response to temperature and ligands by affecting the activation gate located in S6 (49, 50).

The selectivity filters of TRPV1–4 show the most sequence conservation compared to TRPV5–6. In TRPV5–6, four acidic residues are located in the outer vestibule of the pore and selectivity filter, presumably allowing for the high Ca^{2+} selectivity observed in these channels (51, 52). In contrast, TRPV1–4 lack an acidic residue that can bind Ca^{2+} in the selectivity filter, allowing for higher permeability of monovalent cations (53–56).

Sequence analysis has shown the presence of a pore turret formed by an unstructured loop region with 15–25 residues between S5 and the pore helix. This turret can be found in TRPV1, TRPV2, and TRPV4 channels (56–58). The structure for this pore turret has been partially solved for the squirrel TRPV1 channel (58) and completely solved for the rat TRPV2 channel (57). All TRPV channels contain six N-terminal ankyrin repeats in each subunit, giving rise to the ankyrin repeat domain (ARD), where each repeat is usually composed of approximately 30 residues. ARDs participate in subunit–subunit interactions and bind molecules such as ATP, CaM, and allicin that modulate the function of the channels (59–64). Adjacent monomers interact at the ARD through a β-sheet region consisting of one β-strand from the C terminus and two β-strands from the N-linker, a region between the ARD and S1, that contains a helix-turn-helix motif and a short helix immediately before S1 (pre-S1) (50, 52, 55).

Finally, the C terminus contains an alpha helix that runs parallel to the membrane, and that lodges into the S1–S4 bundle, the TRP domain, which can interact with the membrane proximal or linker domain in the N terminus of a neighboring subunit. The S4–S5 linker can interact with the S1–S4 bundle, TRP domain, and pore domains to regulate channel gating.

The recent solving of high-resolution structures has facilitated recognition of the presence of some molecules previously suggested to regulate the function of TRP channels. This also helps us understand how some protein regions in some members of the TRP family are involved in gating of the channels in response to different stimuli.

REGULATION BY PHOSPHOLIPIDS

PIP2

Like other TRP ion channels (65–73), the activity of TRPVs is regulated by lipid molecules such as phosphoinositides, glycerophospholipids, and sterols, such as cholesterol, which has been discussed elsewhere (74, 75). Although there is much to learn about the impact of regulation of TRP channels by lipids in organismal physiology, and contrasting results have been obtained by different research groups, some inferences have been made. Interactions with phospholipids may result in different excitability states in the cells that express TRP channels by sensitizing (72, 76) or desensitizing (77–80) them. For example, some lipids have been shown to either raise or decrease the temperature threshold of some TRP channels, resulting in either positive or negative regulation in the presence of activating stimuli (33, 81–83), which may influence the sensation of pain. Other studies have suggested that modulating the activity of TRP channels by lipid molecules influences brain capillary function (84), cell division and cancer-associated pain (85, 86), asthmatic conditions (87), and itch (46, 88).

A widely discussed topic is the regulation of some TRPVs by phosphatidylinositol 4,5-bisphosphate (PIP2), a phospholipid with asymmetric distribution in eukaryotic membranes (89). PIP2 controls several cellular processes and the function of many ion channels.

Differing effects of PIP2 on TRPV1 function have led to controversy about how this phospholipid modulates the channel. It was first proposed that PIP2 functions as a negative regulator of thermal and chemical activation through interactions with its distal C terminus (79), but other studies showed that depletion of PIP2 produced desensitization of the channel (90, 91) and that PIP2 rather functions as a positive regulator, binding to the TRP domain of TRPV1 (92, 93). Using artificial bilayers containing TRPV1 and PIP2, researchers later showed that rapid activation of the channel with heat (33–39°C) could be achieved in the presence of PIP2 and that it is required for heat-induced channel activity (94) in this experimental model, as well as in intact cells (95). However, incorporating TRPV1 into liposomes containing PIP2 showed that the channel activates at higher temperatures and with higher concentrations of capsaicin, indicating PIP2's role as a negative modulator (82). A binding pocket that could coordinate PIP2, through interactions with four residues (Q561, R575, R579, and K688) located in regions conformed by the S4–S5 linker and S6, was pinpointed before structures were available (93). Mutations in these sites alter binding of PIP2 and also modulate the recovery of the channel from desensitization in such a way suggesting that PIP2 is required for channel opening (96). The high-resolution structures now available for TRPV1 illustrate that there is a residing phosphatidylinositol (PI) molecule, but not PIP2, in the vanilloid-binding site (58, 97). The authors proposed that it putatively functions as a competitor of vanilloid agonists that needs to be dislodged from the site for activation by ligands and/or heat (97).

Patch-clamp experiments using excised inside-out membrane patches expressing TRPV1 showed that application of PI to the cytoplasmic leaflet results in inhibited channel activity in the presence of PIP2. They also indicate that substitution of residues interacting with PI (but not capsaicin) results in decreased inhibition by PI and augmented sensitivity to capsaicin, suggesting that PI does actually act as a competitive vanilloid antagonist (98). However, a possible interaction of PI with a site different from the vanilloid-binding pocket was also proposed, as PI enhances the activity of TRPV1 when coapplied with high capsaicin concentrations and in the absence of PIP2 (98). Together, the data suggest that there can be activation or inhibition of TRPV1 due to the presence of two phosphoinositide-binding sites and that binding of PIP2, rather than PI, at the activating site is more favorable (98).

Part of the contrasting results on the modulation of TRPV1 by PIP2 may stem from the asymmetry of the plasma membrane, because reports showing opposite effects of PIP2 on TRPV1 function depend on the leaflet of the cell membrane where the phospholipid is located (98). For example, potentiation of TRPV1 by capsaicin is observed when PIP2 is applied to the intracellular leaflet of the membrane, while inhibition is present if the molecule resides in both leaflets (99–103).

In TRPV2, a homologous PIP2-binding region has not been identified or modeled because of the lack of high resolution in the structure and the ordered density in the C-terminal region. However, several positively charged residues present in the pre-S1, S1, S2, and C-terminal domain loop could allow binding of PIP2 and other negatively charged lipids (54). TRPV2, like TRPV1, undergoes Ca^{2+}-dependent desensitization after activation by 2-aminoethoxydiphenyl borate or temperature. Whole-cell patch-clamp experiments have shown that TRPV2 currents were desensitized by lower PIP2 concentrations caused by phospholipase C (PLC) Ca^{2+}-dependent activation (104), indicating that PIP2 facilitates channel activation. However, when experiments with TRPV2 were performed in liposomes, supplementation with PIP2 almost obliterated channel activity (57). However, similar to TRPV1, these results for TRPV2 may be related to the side of the membrane where PIP2 levels are modified; in liposomes, it is not straightforward to control the side that contains PIP2.

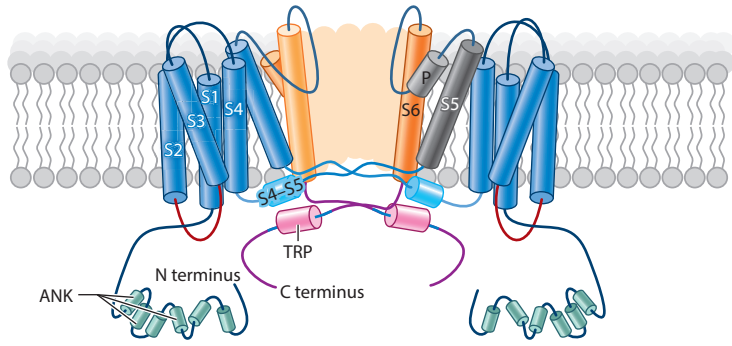
Depletion of PIP2 was found to promote activation of TRPV3. In addition, resting levels of this phospholipid in the membrane are sufficient to inhibit the channel in its resting phase, and residues R696 and K705 in the TRP domain of the channel participate in the inhibition of TRPV3 by PIP2 (77). Solved TRPV3 channel structures in the closed state reveal the presence of a two-tail lipid density in the vanilloid site that could correspond to PI, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, or phosphatidylglycerol.

There is also contrasting information on the effects of PIP2 on the activity of TRPV4. Some studies have shown that TRPV4 is sensitized by PIP2 through interactions with the channel's N-terminal region, which may undergo conformational rearrangements upon PIP2 binding that lead to its sensitization in response to heat and hypotonicity (60, 105). In contrast, different studies using mouse brain capillary endothelial cells have shown that TRPV4's activity is inhibited by PIP2, as depletion of the phospholipid sensitizes the channel (84). Moreover, PIP2 was shown to interact with the ARDs of TRPV4, and gain-of-function mutations in which PIP2 binding and sensitivity are abolished (80) have been associated with several human diseases (106, 107). Recent cryo-electron microscopy (cryo-EM) and X-ray structures of TRPV4 channels describe the presence of unidentified lipid-like structures in the selectivity filter of the channel (56). Together, these data suggest that PIP2 might bind to different sites and produce distinct effects on the channel's activity.

In the case of TRPV5, cryo-EM experiments were performed in the presence of a lipid soluble form of PIP2, dioctanoyl PIP2 (diC8 PIP2), and the solved structure shows it binding to a site between the N-linker (R302 and R305), S4-S5 linker (K484), and S6 (R584). Interactions of diC8 PIP2 produce rearrangements, which include changes in the orientation of residues in the selectivity filter as well as widening of this region and of the lower gate to allow ion flow and global conformational changes in the S6, TRP helices, and S4–S5 linker (52) (**Figure 1**).

The structures also show the presence of several lipid-like densities, which may correspond to phosphatidylcholine in the lower segment of the S1–S4 domain and to cholesterol in a pocket similar to the vanilloid-binding site (108), as also reported for TRPV6 (109). For all these ion channels, the exact identity of these lipids still remains unknown.

Structures of the homologous TRPV6 channel had suggested that PIP2 could interact with S4 and S5 or with S1, S5, and the pore loop (109). However, molecular dynamics modeling



Channel	PIP2-binding regions
TRPV1	Proximal and/or distal C terminus, S4–S5 linker, S6, and TRP domain
TRPV2	Proximal C terminus
TRPV3	TRP domain
TRPV4	N terminus
TRPV5–6	N terminus, S4–S5 linker, and TRP domain

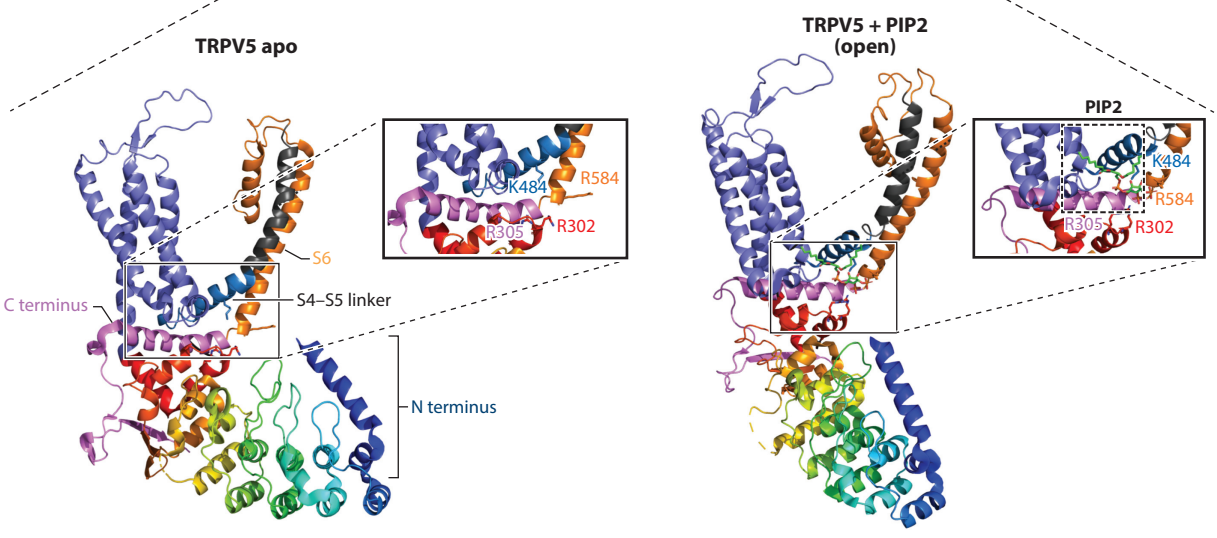


Figure 1

General features of transient receptor potential vanilloid (TRPV) channels and their interactions with phosphatidylinositol 4,5-bisphosphate (PIP2). TRPV channels are tetramers formed by subunits with six transmembrane segments (S1–S6), with intracellular N and C termini and pore helix (P) and a filter loop between S5 and S6. The N terminus contains six ankyrin repeats, and the C terminus comprises the TRP helix, a region conserved among these channels. The table shows regions where PIP2 has been described to interact with TRPV channels. Interactions with different regions of a channel may be responsible for different outcomes in its activity. The structure of TRPV5 is the only one where PIP2 has been identified. Comparison of the apo or closed state (*left*; PDB 6DMR) and PIP2-bound or open states (*right*; PDB 6DMU) (52) showed that, when PIP2 is not bound to the channel, the N and C termini and the S4–S5 linker interact to maintain the channel in its basal state. However, binding of PIP2 to these regions induces a conformational change that breaks those interactions leading to the opening of the channel.

predicted that PIP2 binding also occurs with residues R302, R305, K484, and R584, and functional experiments showed that mutation of these residues resulted in lower affinity to the phospholipid, so the exact binding site has not been clearly defined (52). Interactions between the N/C region, formed by the N-terminal pre-S1-helix and the C terminus, and the L/C region, composed of the S4–S5 linker to C-terminal TRP-helix, promote autoinhibition of TRPV6. Further functional examination of the residues with which PIP2 binds identified three cationic amino acids (K484, R589, and R632) in the S5 and C terminus. Researchers proposed that PIP2 binding resulted in the disruption of these inhibitory interactions between the N/C and L/C regions and activation of the channel (110).

In summary, the effects of PIP2 on some of these channels remain controversial, and more studies will be required to fully solve the PIP2-binding sites in different channels. It will also be necessary to understand how PIP2 binding leads to the stabilization of open or closed states and how this result varies in different subtypes of TRPV channels. Finally, we will need to define how the cell-specific context influences the effects of this phospholipid on TRPV channels.

Glycerophospholipids

Fatty acids have been shown to modulate the activity of TRP channels. Among these molecules are lysophosphatidic acid (LPA) and its precursor, lysophosphatidylcholine (LPC). It has been shown that LPA directly binds to TRPV1 and activates it in DRG neurons and in a heterologous cell-expression system (33), which was also later confirmed in synthetic liposomes containing TRPV1 (82). These experiments also showed that this endogenously produced glycerophospholipid, whose levels are increased under pathological situations that include ischemia and certain types of cancer, produced acute pain through the activation of TRPV1 (33).

More recently, studies demonstrated that LPC interacts with TRPV4, activating this channel and leading to a cascade of events that result in the generation of itch associated with primary biliary cholangitis (46). As for the other TRPV channels, only TRPV2 is activated by LPC through G protein–coupled receptors (85), although it has not yet been clarified completely if it can, like with TRPV1, directly bind to the channel (111, 112).

CONDUCTANCE CHANGES AS A FUNCTION OF CHANNEL ACTIVATION

The conductance and selectivity of ion channels determine how many ions and which ones flow through their pores and constitute important signature features of their function. TRPV1 channels may lose their ionic selectivity as a function of prolonged exposure to activators and allow permeation of large ions such as $NMDG^+$ (N-methyl-D-glucamine), as measured by reversal potential changes in bi-ionic solutions, where Na^+ is present on one side of the channel and $NMDG^+$ on the opposite side. This was initially explained by proposing a conformational change produced by the long-term exposure to capsaicin, which was termed pore dilation (113). Dilation of the pores of TRPV2–TRPV4 has been suggested (114–116), although only TRPV1 has been studied in detail (113). Experiments in P2X purinergic ion channels suggest that they also undergo a slow conformational change upon prolonged activation in which the ion selectivity changes, resembling pore dilation (117).

However, a reexamination of this phenomenon in P2X receptors provides a better understanding of pore dilation in ion channels, including TRP channels. This work showed that changes in the equilibrium potential (V_{rev}) occur in the presence of $NMDG^+$ in the external solution during sustained activation in bi-ionic solutions, but they are the result of intracellular Na^+ depletion

and extracellular $NMDG^+$ accumulation (118), phenomena that are exacerbated in whole-cell recording conditions.

P2X receptors and TRPV1 channels may allow for slow but significant permeation of large molecules such as $NMDG^+$ and dyes, even if pore dilation does not occur. Hence, the observed passage of dyes (113) and local anesthetics (119) through TRPV1 could occur even if pore dilation was absent and could be explained rather by slow permeation to large cations. Thus, the existence of pore dynamic selectivity changes in TRPV channels remains to be demonstrated.

The pore of TRPV1 indeed seems to be a flexible structure. An example of this is that LPA produces not only activation of the channel with a high open probability, as evidenced from single channel recordings, but also a novel phenomenon consisting of an increase in the single-channel current, as compared to capsaicin (120). This behavior has not been reported in other ion channels in the presence of ligands that produce maximal open probability. These experiments show that the increase in current amplitude is not due to changes in membrane properties, nor is it due to an increase in membrane surface charge (due to the insertion of phosphate groups of LPA). Rather, the interaction with residue K710 in the TRP box of TRPV1 was responsible for the presence of a different larger conductance open state with LPA, as compared to the one obtained with capsaicin (120). Perhaps also relevant to pore dilation is that excised-patch experiments with TRPV1, which allow for tight control of ionic conditions, as compared to whole-cell experiments, did not show changes in selectivity. When the patch was excised, prolonged activation with capsaicin or LPA in solutions that contained $NMDG^+_{out}/Na^+$ did not produce changes in the selectivity of the channel to these ions (120), as opposed to what was reported in whole-cell experiments with TRPV1 (113). Hence, the phenomenon of pore dilation in TRPV channels should be reexamined.

TRPVs AND MECHANOSENSATION

The role of TRP channels in mechanosensation is also actively debated. Since 2000, research has suggested that TRPV4 is involved in mechanosensation when cells swell in response to osmotic changes (i.e., in the kidney) (25, 26, 121) or to high mechanical force such as that generated by lung ventilators (122). Evidence for the role of this channel in mechanosensation has come from animals lacking the expression of TRPV4 (29, 123–126). For example, chondrocytes in cartilage detect and respond to fluctuations in the physical microenvironment and mechanical loading in the joints. This allows them to maintain the integrity and physical properties of the extracellular matrix where they are embedded, and disruption of these homeostatic processes can result in osteoarthritis and cartilage degradation. Mice lacking TRPV4 expression are prone to developing osteoarthritis (29), indicating that deficiencies in mechanotransduction are the result of TRPV4 absence. Mechanical activation of TRPV4 may occur indirectly in response to production of certain molecules such as phospholipase A2 (PLA2), which participates by producing arachidonic acid, and P450 epoxygenase, which metabolizes AA and 5′,6′-EET (5′,6′-epoxyeicosatrienoic acid) and activates TRPV4 (127).

TRPV4 is abundantly present in the epithelium of rodent and human bladders, and it has been shown that infusion of a TRPV4 agonist results in bladder overactivity, which is not present in TRPV4 knockout mice (128). In cultured rodent urothelial cells, activation of TRPV4 results in Ca^{2+} influx and increased ATP release, suggesting TRPV4's pivotal role in mechanosensory transduction in the bladder (128).

Two decades later, the mechanisms that confer this channel with its putative mechanical force-sensing capabilities are still under debate. TRPV4's expression patterns in mechanosensitive cells and organs substantiate the channel's role in mechanotransduction. There are several examples of the profound effects that mutations in TRPV4 have in human physiology and, in fact, the

role of TRPV4 in mechanical sensing is stressed by its function in cartilage and bone. In this sense, TRPV4 mutants have been associated with conditions such as arthropathy, peripheral neuropathy, and skeletal dysplasia (129, 130). Bones remodel in response to mechanical loading during physical activity (131). Regeneration of osteoblasts that form the bone is also important for skeletal homeostasis, and TRPV4 has been linked to these processes, where it participates in mechanotransduction, which is needed for oscillatory fluid shear that depends on Ca^{2+} signaling. Moreover, it has been shown that TRPV4 and the mechanosensory Piezo1 and Piezo2 (132) channels influence stretch-dependent Ca^{2+} responses in chondrocytes, but TRPV4 participates more in physiologically relevant strain and the Piezo channels contribute during harmful levels of strain (133).

To determine whether these channels are directly activated by mechanical forces in chondrocytes and whether the cells answer to different types of forces, mice chondrocytes were exposed to mechanical forces either at the point of contact between the cell and its surrounding matrix or to cell membrane stretching. In these experiments, Piezo1 and TRPV4 activated in response to forces transmitted between cells and the extracellular matrix, but only Piezo1 responded to cell membrane stretch, suggesting that chondrocytes distinguish among various types of mechanical forces (29). Nonetheless, the mechanisms by which TRPV4 is activated by mechanical stimuli at the cell-substrate interface, as well as the factors regulating this activation, are not yet understood. A recent study addressing how substrate mechanics and cytoskeletal components impact mechanical activation of TRPV4 found that it is modulated differently to Piezo1, raising the possibility that these two channels participate in fine-tuning mechanosensitivity. The authors found that increased substrate roughness produced minor changes in the activity of TRPV4 (134).

It has also been suggested that TRPV1 is necessary for osmosensory functions. Experiments on neurons in the organum vasculosum lamina terminalis, the osmosensor in the brain, have shown that TRPV1 knockout mice display attenuated water intake in response to systemic hypertonicity (135). Other experiments have shown that TRPV1 splice variants, where amino acids in the N-terminal region are absent, are expressed in arginine-vasopressin-releasing neurons in the supraoptic nucleus, which control body fluid homeostasis. These TRPV1 variants may be essential to osmosensitivity of arginine-vasopressin-releasing neurons (136). TRPV2 may also respond to osmotic swelling in vascular smooth muscle cells (137), but its mechanosensitivity seems to be secondary to modulation of PIP2 levels by mechanical stimuli (138).

A recent study (139) addressed the involvement of several TRP channels in mechanotransduction, including TRPV4 and TRPV2, to clarify their elusive role as primary transducers of mechanical forces. The study used a combination of expression systems, including liposomes and *Caenorhabditis elegans* neurons. The results show that the tested TRP channels were largely unresponsive to mechanical forces, such as tension from membrane stretching, and that their so-called mechanical responses were due to actions of molecules released during stretch (139). Further studies are required to determine whether TRPV channels are directly gated by mechanical forces or modulated through interaction with molecules produced by mechanical processes such as osmotic or shear stress.

TEMPERATURE ACTIVATION OF TRPV CHANNELS

Several members of the TRPV subfamily are classified as thermoTRPs (TRPV1–4), that is, channels that are directly activated by changes in temperature. TRPV1–4 are generally considered to be activated by increases in temperature (21, 22, 25, 30). TRPV5 and TRPV6 are regarded as not activated by temperature, as these two channels show a high basal activity at room temperature. However, there are no published data demonstrating that they are, in fact, insensitive to temperature.

For example, it is not known whether in the absence of PIP2, when the channels are closed, TRPV5 and TRPV6 can be activated by temperature.

Heat activation in general remains a poorly understood phenomenon, and TRPV1 is the best studied channel in this regard. The search for a molecular mechanism underlying heat (or cold) activation has centered on finding protein regions that affect temperature activation, while no general physical principles of heat activation have been dilucidated beyond the understanding that it likely involves a change in heat capacity of one or several heat sensors, which is associated with a large change in enthalpy and entropy (140, 141). Of all thermoTRPs, TRPV4 activates at the lowest temperatures (~30°C), TRPV1 shows activation at around 40°C, and TRPV2 and TRPV3 activate above 50°C. TRPV1 and TRPV4 show an increased probability of opening as a direct result of increased temperature. TRPV1 undergoes a rapid, nonreversible heat desensitization (142), whereas the presence of desensitization in TRPV4 is documented but has not been fully explored (143). In contrast, TRPV2 and TRPV3 undergo a sensitization process, in which an initial heat stimulus does not produce full channel activation, and several subsequent stimuli are needed for the channel to reach its maximal activation (144). The molecular origin of heat desensitization of TRPV1 may be the result of an interaction between the N and C termini of the channel (145), and the molecular basis that underlies heat sensitization in some, but not all, thermoTRP channels remains poorly understood. However, although TRPV3 and TRPV4 respond to temperature, as discussed below, there is debate on whether they are important for heat detection in mice (146). Nonetheless, there is evidence that their contribution to temperature detection in these animals might be specific to their genetic background (147, 148).

Several results have been obtained by applying mutagenesis and chimera construction. A significant number of regions of the channel protein can affect activation by heat. Early work showed that amino acid residues located in the extracellular part of S6 and the pores of TRPV1 and TRPV3 are required for increased temperature to open the channels and suggested that interactions mediated by these channel regions stabilize one or more of the multiple open states during heat activation (149, 150). Subsequently, it was shown that the pore turret, a long extracellular loop, can modulate the temperature range of activation of TRPV1 (151) but is not necessary for activation per se (152). However, a recent study showed that deletion of the pore turret does not affect temperature sensitivity of the squirrel TRPV1 (58). More recently, it was shown that the entire pore domain of TRPV1 channels can be transplanted into the heat-insensitive Shaker Kv channel, and these chimeric channels can be opened in response to increases in temperature at negative voltages, almost in the same way as TRPV1 (153). Nothing is known about the possible role of the pore domain of TRPV2 and TRPV4 in heat activation.

Another channel region clearly shown to play a role in heat activation of TRPV channels is the linker domain or membrane proximal domain. This region spans residues from the ARD to the first transmembrane helix. It contains multiple and complex interactions with the TRP domain of the same subunit and the ankyrin repeats of an adjacent subunit through the β-sheet formed by β-strands of the amino and carboxy terminus. It has been shown that this domain is sufficient to determine the enthalpy and the temperature threshold for activation between TRPV1, TRPV2, and TRPV4 channels, behaving as a transplantable protein domain (154).

An early result in which the carboxy termini of heat-activated (TRPV1) and cold-activated (TRPM8) channels were swapped suggested that at least part of the temperature activation mechanism might be determined by this region of the proteins (155). The fact that several regions of the channel protein seem to affect temperature activation has led to the suggestion that a single, discrete temperature sensor might not exist, unlike voltage-sensor domains in voltage-gated channels or ligand-binding regions in ligand-activated channels. This view is compatible with the current understanding that TRPV and TRP channel gating generally involves multiple allosteric

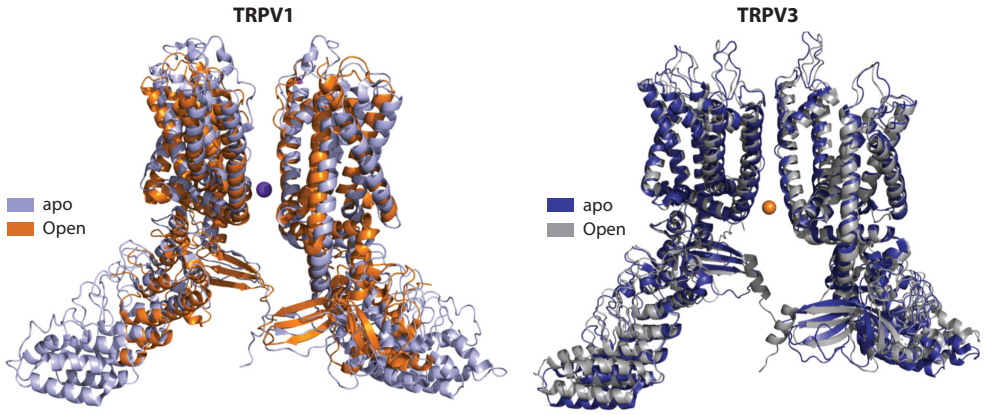


Figure 2

Ligand- and/or heat-induced conformational changes in transient receptor potential vanilloid (TRPV) channels. Structures for TRPV1 in apo (4°C; PDB 7LP9) and open (42°C plus capsaicin; PDB 7LPE) (49) states and of TRPV3 in apo (4°C; PDB 7MIJ) and open (42°C; PDB 7MIO) states (161). Superposition of TRPV1 closed and open structures (*left*) schematizes global conformational changes, which include reorganization of the cytoplasmic domains. For TRPV3 (*right*), changes in the S2–S3 linker and cytoplasmic domains are also shown, but these changes seem to be more discrete than those observed for TRPV1.

interactions between an equally large number of discrete closed and open states (156, 157). To understand the mechanisms of temperature activation, it is critically important to have a consensus definition of what constitutes a temperature sensor. Although no such agreed upon definition exists, a heat sensor would be a region of the protein that can absorb heat and convert it to a change in conformation. Currently it is not possible to measure heat absorption in specific regions of a protein, but structural experiments might provide information regarding conformational changes.

The availability of an increasing number of structures of TRPV channels can help in rationalizing the seemingly disparate results outlined above. Of particular importance are recent studies that have solved structures after exposing the channel protein preparation to varying temperatures.

When comparing structures of TRPV1 obtained by cryo-EM at 4°C and 48°C in the presence of the activator capsaicin, Kwon et al. (49) observed structural classes compatible with the interpretation that the channel transitions to an open state through intermediate, closed conformations. Importantly, the authors described heat-induced conformational changes in multiple regions of the channel in the presence of capsaicin (**Figure 2**), with some corresponding to previously described segments or individual amino acids that influence heat activation. Importantly, this study showed that several amino acid residues undergo changes in their exposure to solvent, a phenomenon that had been postulated to contribute to the change in heat capacity associated with channel activation. Another conformational change resolved by these structures corresponds to the ARD. Previous work had shown that this domain regulates temperature activation in another thermoTRP channel, TRPA1 (158). Also, it had been previously shown that the isolated ARD of TRPV1 undergoes a heat-dependent conformational change similar to protein denaturation, suggesting that it can function as a thermal sensor (159). The cryo-EM TRPV1 structure at 4°C plus capsaicin shows resolved ARDs (PDB 7LPA). However, the 48°C (PDB 7LPC) and 48°C plus capsaicin (PDB 7LPE) structures indicate that the first ankyrin repeats in the N terminus become unresolved when the channel opens, suggesting a large change in conformation accompanied with increased mobility. Moreover, the authors suggest that the conformational changes in the ankyrin repeats initiate the heat-induced opening of the channel (49).

As mentioned before, full activation of TRPV2 and TRPV3 by heat occurs only after a sensitizing period. The enthalpy of activation of these channels is almost twice as big as that of TRPV1, indicating higher heat sensitivity, but sensitized channels are now able to respond to lower temperatures with less apparent enthalpy (144, 160). A recent cryo-EM study solved TRPV3 structures over a range of temperatures, finding different results to those of TRPV1, which was solved in the presence of both heat and capsaicin (49), suggesting a different structural mechanism (50, 161) (**Figure 2**). These experiments show that specific N- and C-terminal domain interactions are altered between the closed and heat-activated structures, without major changes to the ARD. Importantly, this study with TRPV3 shows that in the closed state, the vanilloid-binding site contains a lipid, which abandons the site in the heat-activated, open conformation (161). Curiously, this mechanism had been previously proposed for activation of TRPV1 by increased temperature (97) but is not substantiated by the recent cryo-EM structures at high temperatures (49).

A reasonable hypothesis derived from these structural experiments in TRPV3 is that the mechanism of sensitization in channels such as TRPV2 and TRPV3 is the slow heat-dependent removal of the inhibitory lipid. This hypothesis suggests that the inhibitory lipid in TRPV1 would dissociate more readily than the corresponding inhibitory lipids in TRPV3 or TRPV2.

Interestingly, heat-dependent sensitization has been found to be well modeled by irreversible gating schemes, in which the channel irreversibly transitions between initial closed states before reaching a reversible channel opening equilibrium (144). In an analogous fashion, heat-induced desensitization of TRPV1 has been shown to be explained by a similar nonreversible scheme (**Figure 3**), this time with the desensitizing transition being irreversible. Research shows that the heat-dependent desensitization process cannot be reversed even after prolonged cooling (142). It is possible that these heat-induced irreversible gating transitions in TRPV1–3 might reflect irreversible conformational changes similar to protein denaturation, which might be unraveled by future structural studies.

THE FUNCTIONAL STATE OF STRUCTURES

The recent abundance of relatively high-resolution structures of almost all families of TRP channels has placed us in a unique position in which we expect that long-standing questions will be answered with a simple picture. However, although some structural models derived from electron densities have a resolution <2 Å, most are >3 Å and do not always allow an unequivocal assignment of amino acid side chains. These models still need to be validated by other experimental results, and the inferred structures may not represent physiologically relevant channel conformations. Nonetheless, the growing number of structures allows comparisons across experimental conditions and different types of channels, and these efforts allow inferences of general structural features and mechanisms across TRP channels.

A recent multiple sequence analysis of more than 2,800 protein sequences of TRP channels allowed some general conclusions to be reached on these channels as well as TRPV channels. The pattern of conserved amino acid residue interactions suggested that TRP channels possess a voltage-sensor VSLD that is less solvated and more rigid than the voltage-sensing domain of Kv-type channels (162). This implied that the VSLD in TRP channels does not contribute to voltage sensing. However, there are always exceptions and, in this case, it has been shown that TRPP2 exhibits a large gating charge, consistent with a significant conformational change of the S4 in the VSLD (163). The multiple sequence analysis also identified that the pore helix in TRP channels is decoupled from the S6 helix, as opposed to Kv channel pore domains. This may show that the selectivity filter in TRPV1–4 channels is highly flexible, again in contrast to the mostly fixed selectivity filter in potassium and possibly TRPV5–6 channels, which agrees with functional and structural studies of select TRPV channels.

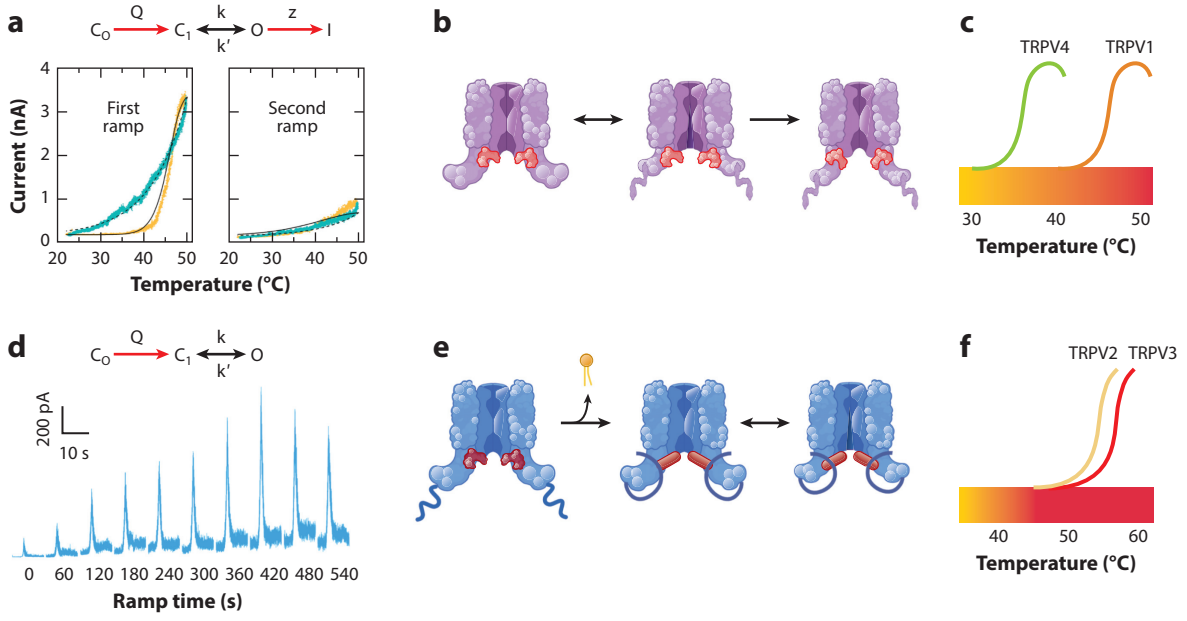


Figure 3

Irreversible processes in transient receptor potential vanilloid (TRPV) channel gating by temperature. (*a*, *left*) TRPV1 channel gating in response to activation by a temperature ramp is highly asymmetric or hysteretic, implying the presence of an irreversible early transition. (*a*, *right*) The response to a second identical temperature ramp is highly diminished, indicating the presence of temperature-dependent desensitization or inactivation. The kinetic scheme employed to account for this current kinetics is shown on top of the panel and includes two irreversible transitions: an early transition and a late transition, leading to the desensitized state with the rate constant z. (*b*) A diagram of the activation and desensitization of TRPV1 in the presence of temperature and ligand based on Reference 49. Desensitization is thought to involve a different interaction between the N and C termini (*red*). (*c*) Ranges of temperature activation of TRPV4 and TRPV1 channels. The curves show that the open probability increases with heat and then decreases, indicating desensitization. (*d*) TRPV2 currents in response to temperature ramps from 22 to 54°C applied at the indicated times. Gating is facilitated with each ramp until the channels fully respond. The simple gating scheme that has been proposed to account for this facilitation is shown on the top of the panel. It includes an initial, irreversible transition between closed states that is highly temperature dependent (rate constant Q) and leads to an equilibrium channel opening transition with equilibrium constant k/k′. (*e*) Diagram of the temperature activation and desensitization of TRPV3 based on Reference 50. Upon opening by heat, a region of the C terminus changes its conformation from a loop that wraps around the linker domain to an alpha helix (*red*), while the disordered N terminus interacts with the C terminus (*dark blue*). (*f*) Ranges of temperature activation of TRPV2 and TRPV3 channels. Panels *a*, *c*, *d*, and *f* adapted from images created with BioRender.com.

Functional studies using substituted cysteine accessibility mutagenesis in TRPV1 showed that gating occurs by a conformational change at the S6 helix, which acts as a gate for the flux of cations (164). These experiments demonstrated that the same conformational change is gated by capsaicin binding or elevated temperature and suggested that the S6 segments form an inner gate similar to that in Kv channels (164).

Many early TRPV1 structures (53, 165) obtained with and without ligand activators showed that the inner gate was indeed expanded in what seemed to be an open conformation when the channel was ligand-bound. However, the selectivity filter also presented an enlarged radius compared to the closed structure. This has been observed in TRPV1 (53, 58, 165) and TRPV2–4 channel structures and led to the idea that TRP channel opening is mediated by a selectivity filter gate in addition to the inner, S6 bundle-crossing gate (54, 56, 165, 166). This gating scheme with two gates controlling ion flux has not been substantiated by functional evidence. Substituted

cysteine accessibility mutagenesis experiments have shown that the selectivity filter can allow state-independent movement of ions as small as silver, implying that it does not act as a gate, at least in thermoTRP channels (167). It is possible that the different conformations of the selectivity filter capture intrinsic conformational dynamics of this module and molecular dynamics simulations could help define these dynamics. Unfortunately, molecular dynamics simulations to explore permeation and dynamics have been carried out systematically only on systems that exclusively include the pore domain of TRPV1 (168) and TRPV6 (169) and impose artificial constraints that preclude observation of long-term conformational changes. However, a recent study with TRPV3 has provided full-length channel molecular dynamics simulations of the permeation process in the open-state structure determined at high temperature (161).

A recent multiple sequence analysis employing alignment of both sequence and structures using hundreds of cryo-EM and crystallographic structures found that a comparison of the dimensions of the inner S6 gate across putatively open or closed structures showed no correlation between the gate being open and the presence or absence of an activator (170). Furthermore, the observed maximum radius of the TRPV channel's S6 gate is ~4.4 Å, barely large enough to allow entry of a hydrated cation. Moreover, it is much smaller than the required >5 Å for TRPV1 to allow entrance of quaternary ammonium ion blockers in the open state (171, 172).

This systematic study (170) suggested that many if not all of the TRPs, including TRPV channel structures determined so far in the presence of activators, represent a desensitized state and that the open state of any TRP channel is yet to be determined. Molecular dynamics simulations employing techniques such as computational electrophysiology could help settle whether these open structures sustain high-conductance ion permeation (173).

CONCLUSIONS

Over the past two decades, from the cloning of TRPV channels to the recent resolution of their structural details, several studies have demonstrated the roles of TRPV channels in physiology and pathophysiology and their responses to a large diversity of physical and chemical stimuli. They have also unveiled the interactions of TRPV channels with diverse molecules. TRPV channels are activated and modulated by several stimuli, which permits them to be finely regulated in different cellular and organ contexts, yielding a rich variety of responses. However, the complexity of their responses adds to the difficulty of clearly defining some of their functional traits. It is also important to consider that some of the reported discrepancies regarding factors such as their modulation by some molecules respond to different experimental conditions that had not been initially considered or even may be due to different isoforms of the channels used.

In this review, we have outlined some, but not all, of the leading controversies in the field in terms of the function and regulation of TRPV channels by providing a link, based on the available information, between their structural features and their functional responses to different stimuli. We still have much to learn about these ion channels and how they interact with other proteins. For example, it is known that the activity of TRPV channels, except TRPV2 (104), is modulated by CaM. Among TRPV channels, TRPV1, TRPV3, TRPV5, and TRPV6 are negatively regulated by CaM (174–179), whereas TRPV4 is positively regulated by this protein (180). Structures for TRPV channels bound to CaM have been solved only for TRPV5 (52, 108) and TRPV6 (181), which are inactivated by Ca^{2+} through the binding of CaM to their C-terminal regions (178, 179). Recent controversy in the study of TRPV channels stems from their newly obtained structures in the presence of CaM. Examination of one TRPV5 structure shows one CaM molecule bound to the C terminus at the entrance of the pore (52). Another study uncovered more than one CaM density; one CaM was described as binding to the C terminus and interacting with the intracellular

gate, and the other two CaMs were bound to neighboring subunits (108). As for the closely related TRPV6 channel, the solved structure exhibits a 1:1 stoichiometry, where one TRPV6 tetramer binds one CaM molecule (181). Moreover, one of two CaM lobes plugs the channel by inserting a side chain into the pore of TRPV6 (181). Functional studies using concatemers with mutant CaM-binding sites will provide valuable information to further clarify how TRPV channels interact with this protein.

Changes in the activity, mutations, or posttranslational modifications of TRPV channels result in diseases that affect humans. In this sense, it is important to emphasize that refining our understanding of how TRP channels function will allow for the development of better strategies to control pain, itch, and other pathologies.

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