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Recent advances on the structure and the function relationships of the TRPV4 ion channel

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ABSTRACT
The members of the superfamily of Transient Receptor Potential (TRP) ion channels are physiologically important molecules that have been studied for many years and are still being intensively researched. Among the vanilloid TRP subfamily, the TRPV4 ion channel is an interesting protein due to its involvement in several essential physiological processes and in the development of various diseases. As in other proteins, changes in its function that lead to the development of pathological states, have been closely associated with modification of its regulation by different molecules, but also by the appearance of mutations which affect the structure and gating of the channel. In the last few years, some structures for the TRPV4 channel have been solved. Due to the importance of this protein in physiology, here we discuss the recent progress in determining the structure of the TRPV4 channel, which has been achieved in three species of animals (*Xenopus tropicalis*, *Mus musculus*, and *Homo sapiens*), highlighting conserved features as well as key differences among them and emphasizing the binding sites for some ligands that play crucial roles in its regulation.

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Introduction

The members of the superfamily of Transient Receptor Potential (TRP) channels have been described in many organisms, from yeast to vertebrates [1], where they are expressed in different cell lineages and perform multiple functions in both excitable and non-excitable tissues [2,3]. Some TRP channels are expressed in the membranes of cell organelles, where they play functions in their biology and signal transduction in response to stimuli such as changes in pH, oxidants, and osmomechanical forces, regulating processes such as endosomal and lysosomal function and trafficking, mitochondrial function, regulation of endoplasmic reticulum stress, among others [4]. TRP channels participate in signal transduction by controlling membrane potential and regulating intracellular Ca^{2+} concentrations [5].

Most TRPs are nonselective cation channels that respond to different stimuli [6–8], and contribute to various physiological functions including phototransduction, Ca^{2+} homeostasis, cell cycle modulation, changes in temperature and pH and/or noxious stimuli that may result in pain, among other roles [9–13].

The TRP superfamily of channels has been classified into seven main subfamilies based on the homology of their sequences: TRPC ("Canonical"), TRPV ("Vanilloid"), TRPM ("Melastatin"), TRPA ("Ankyrin"), TRPML ("Mucolipin"), TRPP ("Polycystin") and TRPN ("NOMPC-like" or no-mechanoreceptor potential C-like, only found in fish and invertebrates) [2,6,14]. Less well-described TRP subfamilies, which are found in invertebrates include TRPS ("Soromelastatin"), TRPVL ("TRPV-like"), TRPY/TRPF ("Yeast"), and even a subclade of TRPM [15–21].

In the case of the vanilloid subfamily, six members have been described: TRPV1-TRPV6. The first four have a lower selectivity for cations with a permeability ratio of PCa/PNa ~1–10; furthermore, they are responsive to physical and chemical stimuli and play a significant role in thermosensation, chemosensation, and nociception [7]. On the other hand, TRPV5 and TRPV6 channels are highly selective to Ca^{2+} (PCa^{2+}/PNa^{+} >100) and participate in regulating Ca^{2+} homeostasis in the kidneys and intestines [22].

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According to biochemical and structure–function studies [17,23–32], the members of the vanilloid subfamily are arranged as tetramers, where each subunit has six transmembranal regions, a pore-forming loop between the fifth and sixth transmembrane regions, and intracellular amino- and carboxyl-terminal regions with differences in their sequences and architecture, resulting in a distinctive biophysical feature [33–37].

In the early 2000's, researchers started examining TRP channel structures; still, the complexity of the hydrophobic environment where the ion channels are located, along with the flexibility of certain protein regions [38], restricted their experimental methods. Eventually, X-ray crystallography succeeded in solving crucial domains of these proteins [39–44] and then cryo-electron microscopy (Cryo-EM) techniques allowed scientists to determine the entire structure of several members of the TRPV subfamily of ion channels [35,36,45–53].

Here, we focus on pinpointing the structural details of one member of the TRPV subfamily of ion channels, which has been identified as an important regulator in normal physiology and disease: the TRPV4 channel. Particularly, we focus on studies using Cryo-EM and comparing these studies of TRPV4-channel structures solved in three different species (*Xenopus tropicalis*, *Mus musculus*, and *Homo sapiens*) to highlight the importance of different regions of the channel in its function.

General properties of TRPV4 channel

Biophysical properties

TRPV4 is a nonselective cation channel with a permeability sequence as follows: $Ca^{2+} > Mg^{2+} > K^+ \approx Cs^+ \approx Rb^+ > Na^+ > Li^+$ [54]. Specifically, permeability with respect to Na^+ is 6–10 and 2–3 for Mg^{2+} [55–57].

Since its early characterization more than 20 years ago as an osmosensitive Ca^{2+}-permeable channel [55,56,58,59], it has been demonstrated that TRPV4 is crucial in maintaining cell function by regulating Ca^{2+} concentrations in different cell types and organisms [60,61]. TRPV4's currents can be activated by several stimuli, as mentioned below and, in the presence of Ca^{2+}, the channel displays outward rectification in response to different voltages while when Ca^{2+} is absent, the current-voltage relationship, when only Na^+ is present, becomes more linear and even slightly inwardly rectifying. The conductance calculated from single-channel electrophysiological recordings for outward currents is 90–100 pS and 50–60 pS for inward currents [56,62].

Pharmacological properties

At present, we know that the TRPV4 channel can be activated by physicochemical stimuli like temperatures near 27°C [63], changes in osmolarity [55], mechanical stress [64], and protons (pH 4.5–7.5) [65]. This channel is also activated through its interaction with endogenously produced molecules, such as arachidonic acid (AA) and its derivatives, endocannabinoids [66], phosphatidylinositol 4,5-bisphosphate (PIP_2) [67], lysophosphatidylcholine (LPC) and its derivative, lysophosphatidic acid (LPA) [68,69], among others Figure 1(a).

It has also been demonstrated that some ligands of TRPV4 are natural products found in plant extracts like apigenin from celery and chamomile flowers [70]; eugenol from cloves, basil, cinnamon and nutmeg [71]; and bisandrographolide A (BAA) from *Andrographis paniculata*, commonly known as creat Figure 1(a) [72].

Additionally, synthetic molecules have been produced and reported to specifically regulate the activity of TRPV4 Figure 1(a) [73,74]. These include the following, which were used in experiments to solve the structure of TRPV4:

Agonists

- 4-alpha-phorbol 12, 13-didecanoate (**4αPDD**) [75]
- (*N*-((1S)-1-([4-((2S)-2-([(2,4-Dichlorophenyl) sulfonyl]amino)-3hydroxypropanoyl)-1-piperazinyl]carbonyl)-3-methylbutyl)-1-benzothiophene-2-carboxamide (**GSK1016790A**) [76]

Antagonists

- 2-Methyl-1-[3-(4-morpholinyl)propyl]-5-phenyl-N-[3-(trifluoromethyl)phenyl]-1 H-pyrrole-3-carboxamide (**HC-067047**) [77]

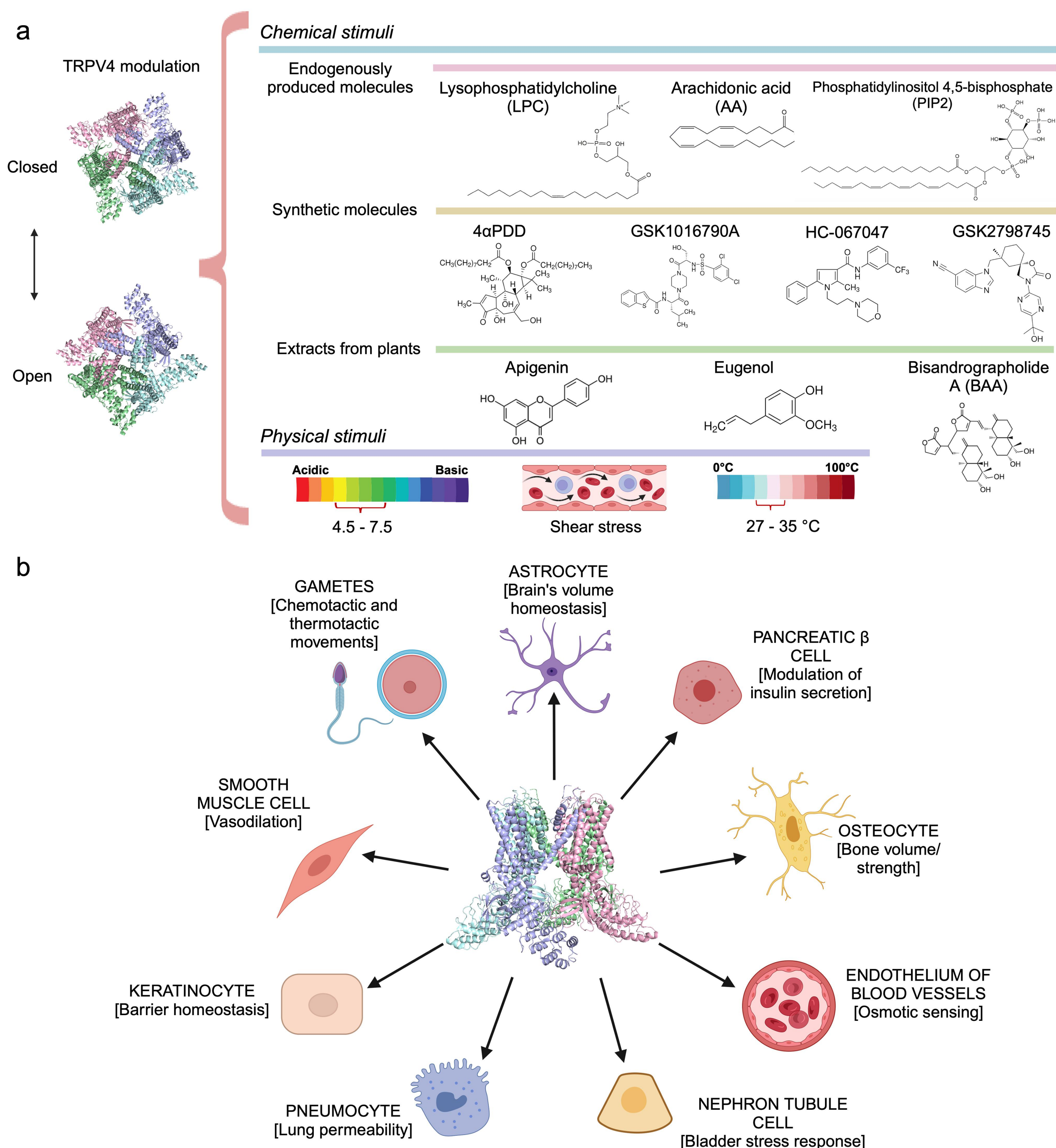

Figure 1. TRPV4 modulation and expression. a, the TRPV4 channel can respond to several stimuli, such as changes in temperature (27–35℃) or pH (4.5–7.5), shear stress, and endogenously produced molecules like LPC (lysophosphatidylcholine), AA (arachidonic acid), and PIP_2 (phosphatidylinositol 4,5-bisphosphate). Some synthetic ligands of TRPV4 function as agonists (i.e. 4-αPDD (4-alpha-phorbol 12, 13-didecanoate) and GSK1016790A ((*N*-((1S)-1-([4-((2S)-2-([(2,4-Dichlorophenyl)sulfonyl]amino)-3hydroxypropanoyl)-1-piperazinyl]carbonyl)-3-methylbutyl)-1-benzothiophene-2-carboxamide)) or antagonists (i.e. GSK2798745 (3-[[(5S,7S)-3-[5-(2-hydroxypropan-2-yl)pyrazin-2-yl]-7-methyl-2-oxo-1-oxa-3-azaspiro[4.5]decan-7-yl]methyl]benzimidazole-5-carbonitrile) and HC-067047 (2-methyl-1-[3-(4-morpholinyl)propyl]-5-phenyl-N-[3-(trifluoromethyl)phenyl]-1 H-pyrrole-3-carboxamide)) of the channel. Extracts from plants include BAA (bisandrographolide A), apigenin, and eugenol. b, examples of some types of cells that express TRPV4, along with their corresponding functions are depicted. PDB: 8T1B (3.0-Å resolution) [52]. Created with PyMOL and BioRender.com.

- 3-[[(5S,7S)-3-[5-(2-hydroxypropan-2-yl)pyrazin-2-yl]-7-methyl-2-oxo-1-oxa-3-azaspiro [4.5]decan-7-yl]methyl]benzimidazole-5-carbonitrile (**GSK2798745**) [78]

Functional roles and expression of TRPV4

When TRPV4 was discovered, it was initially named OSM-9 in the invertebrate *C. elegans* [58] and then VR-OAC [55], OTRPC4 [56], or VRL-2 [59] in vertebrates. Since then, our knowledge of its roles in physiology and advances in the details of its structure have come a long way. Activation of the TRPV4 channel translates into Ca^{2+} signals and, the responses vary depending on the cell type and tissue where it is expressed. TRPV4´s expression is widely distributed throughout the human body. It has been shown to be present in the cardiac, respiratory, urinary, muscle-skeletal, digestive, immune, endothelial, central, and peripheral nervous systems Figure 1(b).

For instance, TRPV4 is expressed in several cells of the respiratory system and functions by maintaining homeostasis of osmotic pressure. In the lungs, TRPV4 transduces several stimuli into Ca^{2+} signals and regulates the relaxation of the main pulmonary artery and the vasoconstriction of pulmonary circulation. Importantly, this channel plays an important function in preserving the integrity of the alveolar epithelial barrier (and skin barrier), where its activity can impact the severity of chronic asthma and, due to its sensitivity to mechanical forces, it can influence pulmonary injury induced by ventilators used to treat respiratory failure [79–81]. Activation of TRPV4 by mechanical forces also regulates the function of retinal cells such as ganglion cell soma-dendrite, microglia and Müller cells, suggesting that it plays roles in diseases like glaucoma and in the skeletal system where it is expressed in cells such as osteoblasts and chondrocytes, where it also participates in mechanotransduction [82].

As for the kidneys, TRPV4 is expressed in the distal convoluted tubule and in regions where transcellular osmotic gradients can develop, regulating osmotic balance by modifying water secretion in the kidney. In nephrons expressed in regions of the kidneys where there is no water permeability, TRPV4 contributes to the detection of osmotic stimuli and regulation of blood pressure in the presence of increased salt intake [55]. Interestingly, TRPV4's activation has been suggested to influence the severity and progression of polycystic kidney disease (PKD) [83].

Generalities of TRPV4's interactions and structure

Currently, we know that TRPV4 is assembled as a homotetramer [45,52,53,84]; although, it has been described that it can form heteromeric channels with the TRPP2 in an alternating 2:2 stoichiometry [39]; TRPC1 [85–88]; TRPC1 and TRPP2 [89], and most recently with TRPV3 [90].

In the case of the human TRPV4 homotetramer, each subunit consists of 871 residues and contains two layers: the bottom, also known as the cytoplasmic layer, which encompasses the amino- and carboxyl-terminal regions of the protein [67,91]; while the top layer, or transmembrane region, consists of six helices, where the first four α-helices (S1-S4) form a voltage sensor-like domain (VSLD), similar to other tetrameric voltage-gated ion channels (VGIC), while the S5 and S6 α-helices form the pore domain [45,52,53]. The *N*- and C-termini contain very characteristic domains such as a phosphoinositide binding domain (PDB), the ankyrin repeat domain (ARD), the proline-rich domain (PRD), the coupling domain (CD), the TRP box, the calmodulin-binding domain (CAM) and the PDZ-like domain Figure 2(a). Specific features of these regions are further detailed below.

The bottom or cytoplasmatic layer of TRPV4

N-terminal region

The N-terminal region of TRP channels has been shown to participate in the binding of molecules that regulate the activity of these proteins [23,29,96–100]. We will now detail some of these interactions in the N-terminus with different molecules and their effects on the structural conformation of the TRPV4 channel.

The N-terminus of TRPV4 contains a phosphoinositide binding site with the (++W++) characteristic sequence, where the positive charges correspond to lysine (K) or arginine (R) residues. This region allows the binding of PIP_2, sensitizing

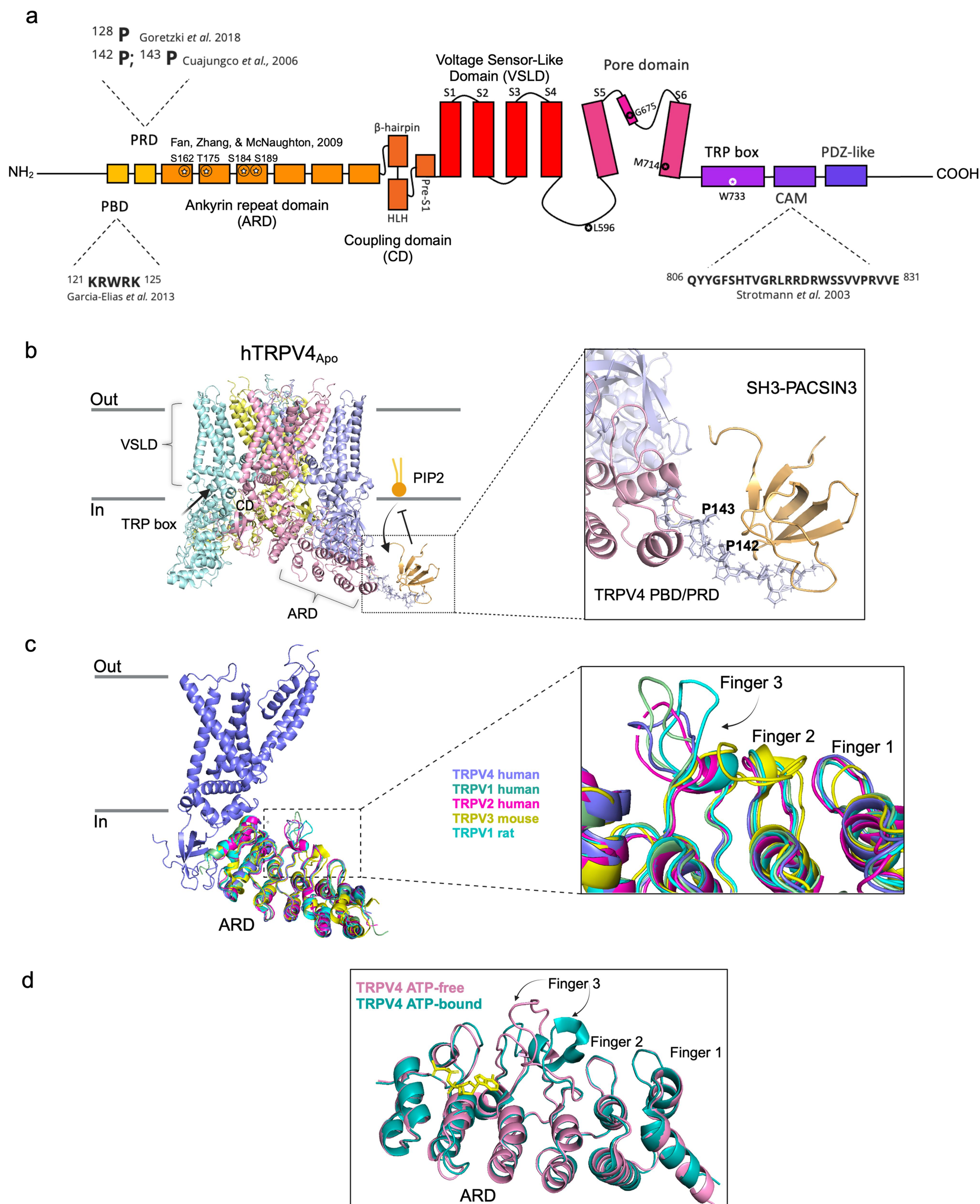

Figure 2. Domain organization of the TRPV4 channel. a, representation of a subunit of the hTRPV4 channel. From left to right: N-terminal region, which consists of the PIP_2 binding domain (PBD); proline-rich domain (PRD); Ankyrin repeat domain (ARD); and coupling domain (CD); the transmembrane region where the voltage sensor-like domain (VSLD, S1–S4 α-helices) and the pore domain (S5-S6 α-helices) are located; and the C-terminal region, which includes the TRP box and the calmodulin interaction site (CAM) and the PDZ-like domain. Capital and bold letters are amino acid residues that are part of the domains described or interaction sites with other proteins (numbers represent the positions of these amino acids). b, human TRPV4 in the apo state is

the channel to thermal or osmotic stimuli Figure 2(b) [67]. Moreover, the N-terminal region of each TRPV4 subunit also contains a proline-rich domain (PRD) to which the PACSIN3 protein (protein kinase C and casein kinase substrate in neurons 3) binds to the channel through proline residues 142 and 143 [101]; PACSIN3 acts as a negative modulator of the responses of TRPV4 to thermal and hypoosmotic stimuli [102] by reorienting and stabilizing the N-terminal region of TRPV4 away from PIP_2, which positively regulates TRPV4 Figure 2(b) [92].

The N-termini of TRPV channels all contain six ankyrin repeats domains (ARD) composed of two antiparallel helical structures joined by a turn for each repeat (anti-parallel helix-turn-helix motif), and with each joining to the next one through a loop or finger [103]. In general, the ARD is conserved in the vanilloid TRP subfamily including the human TRPV1 [93]; rat TRPV1 [94]; human TRPV2 [95]; mouse TRPV3 [104]; or human TRPV4 channels [105] Figure 2(c).

However, comparison of the structures resolved of the ARD of the TRPV4 channel by Inada et al. [43] with the structures of other vanilloid members show that the length and flexibility of the third finger of human TRPV4 differs when it is in the presence or absence of ATP. Moreover, this arrangement of finger 3 plays an important role in regulating channel activity; when ATP is bound to TRPV4, protein stability is favored. Studies have shown that several mutations reported along the ARD, including those in the third finger where the binding of ATP is impaired, cause dysregulation of the basal gating process or, in other words, an increase in the open probability or a “gain of function” phenotype Figure 2(d).

The ARD is involved in transport, anchoring, localization, and protein–protein interactions [106,107]. Currently, it is known that alterations in the sequence of TRPV4, especially in the ARD, can cause different diseases such as Brachyolmia, Charcot–Marie–Tooth type 2C disease, among other muscular atrophies and syndromes. Most of these have been associated with mutations in several amino acid residues within the ARD structure, which can result in “gain of function” effects on the channel [61,108–111].

In this sense, previously reported interactions between the ARD of TRPV4 and a GTPase named RhoA [112–115], have recently regained attention in the field. RhoA is a small GTPase that plays a crucial role in transmitting signals from outside the cell to its cytoskeleton. The structure of RhoA consists of a β sheet of six strands (β1–β6) surrounded by six short helices (α1–α6) joined by loops and a variable C-terminal region; additionally, regions corresponding to switches I and II regions or the ligand-binding pocket, adopt different conformations depending on whether RhoA is GDP or GTP bound Figure 3(a) [52,53,113,116].

Essentially, RhoA acts as a molecular switch by transitioning between an inactive GDP-bound state and an active GTP-bound state. In its active state, RhoA is bound to GTP and positively regulates downstream cytoskeletal-modulating proteins and is involved in a variety of processes such as focal adhesions, actin assembly, transcriptional activation, exocytosis, and regulation of smooth muscle contraction [113,116].

Most recently, the details of the binding of RhoA to TRPV4 were shown in two different studies using the Cryo-EM technique while resolving the structure of the channel. Both research groups overexpressed human TRPV4 in HEK293 cells but reported obtaining another additional protein density, which after analysis and comparison with other previously solved structures [116], was identified as RhoA Figure 3(b). The stoichiometry of RhoA molecules with respect to the

shown and its interaction with PIP_2 and PACSIN3 is represented; a zoom-in into the PRD shows that P142 and P143 are involved in these interactions with PACSIN3; other proline residues among the PRD are shown in purple sticks. c, comparison of the ARD between several members of the vanilloid subfamily, where the alignment shows mostly conserved structure among species. Only the human TRPV4 complete subunit is represented in a side view parallel to the membrane. d, zoomed-in view of the ARD of the human TRPV4 is shown, where finger 3 acquires a different conformation when it is unbound to ATP (yellow sticks). PDB 8T1B, 6L93, 2PNN, 2F37, 4N5Q, and 6F55 (resolutions were 3.0 Å, 4.47 Å, 2.70 Å, 1.70 Å, 1.9 Å and nuclear magnetic resonance structure, respectively) [43,52,92–95]. Created with PyMOL and BioRender.com.

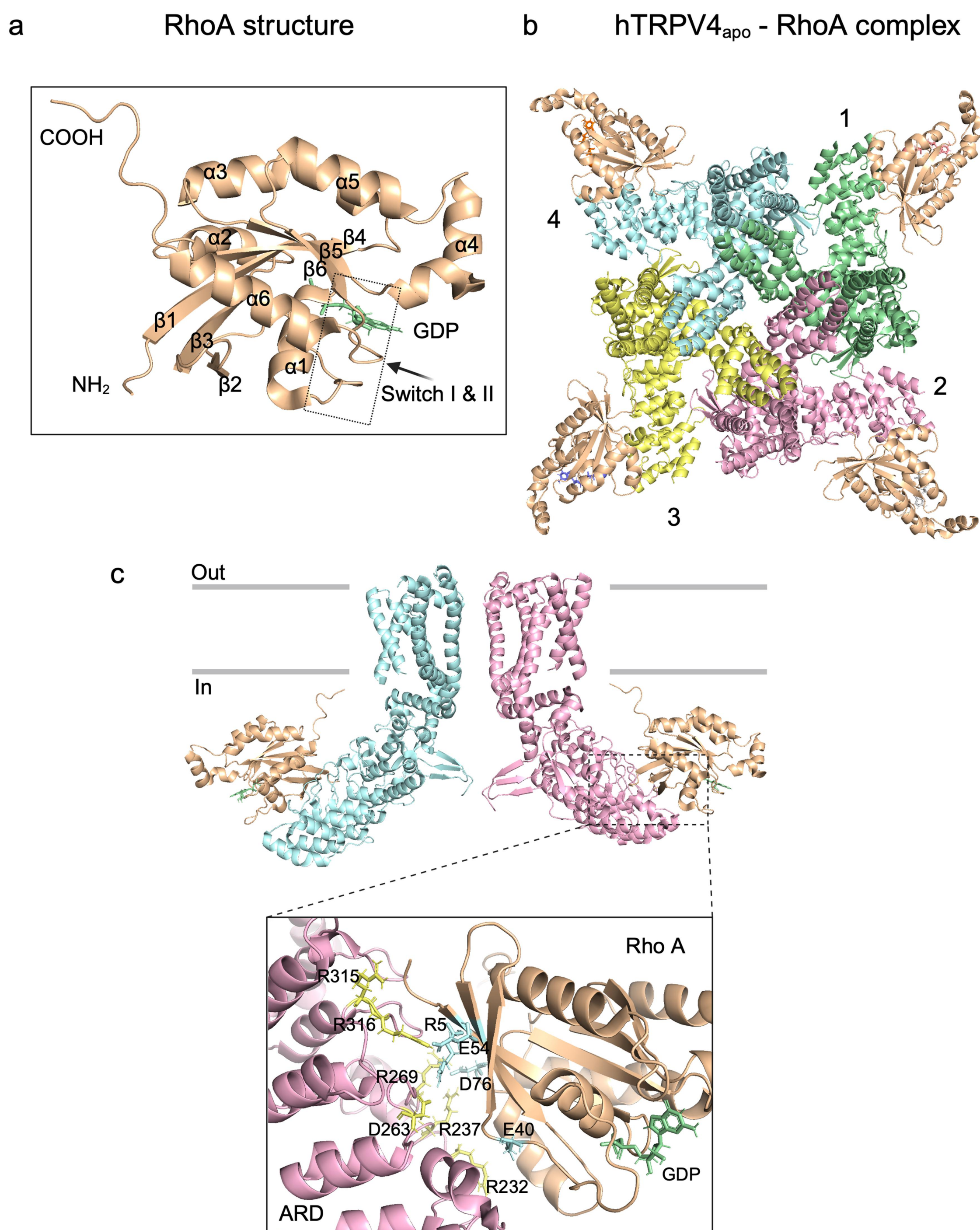

Figure 3. hTRPV4 interactions with RhoA GTPase. a, cartoon representation of the RhoA structure. The RhoA protein is bound to GDP (green sticks). b, the homotetramer of the human TRPV4 in complex with RhoA is shown; each subunit of TRPV4 is identified with a different color, RhoA (wheat) is shown interacting with the TRPV4 bottom layer; the stoichiometry is 1:1. c, lateral view of two subunits of TRPV4 in complex with RhoA and a close-up view of the interaction zone between the ARD of hTRPV4 (yellow sticks) and the β sheets of RhoA (blue sticks). PDB: 8FC9 (resolution 3.75 Å) [52,53]. Created with PyMOL and BioRender.com.

TRPV4 tetramer could be observed in a range from none to four molecules per channel, furthermore, this arrangement does not seem to result in major changes in the general structure of each subunit of TRPV4 [52,53].

However, in the hTRPV4-RhoA complex, no differences were observed in the presence of GDP or GTP [52,53]. On the other hand, the interface between hTRPV4-RhoA is generated through electrostatic interactions between the β1, β3, switch

I and II of RhoA, and AR2-AR5 of TRPV4. The sites in TRPV4 where strong interactions with RhoA are found are residues: R232, R237, D263, R269, R315 and R316 of TRPV4, while in RhoA residues R5, E40, E54, and D76 are involved Figure 3(c) [52].

It has been proposed that RhoA anchors to the membrane through its prenylated C-terminal region, forming an interface through three β-strands and one α-helix with the loops that join the fingers of the ARD of TRPV4, limiting its movement until activation of the channel can occur in the presence of a given stimulus [52,53]. Hence, it was concluded that the interaction between hTRPV4-RhoA results in mutual inhibition of the activity of these proteins, which was previously reported by structure–function studies of hTRPV4 [114,117–122].

Furthermore, it has been proposed that several mutations reported along the residues of the ARD of hTRPV4 [114,117–122], cause a loss of interaction with RhoA, leading to a bidirectional dysregulation since binding of RhoA to TRPV4 suppresses channel activity and, the binding of TRPV4 to RhoA inhibits activation of this GTPase [113]. These results agree with previous studies which suggested that mutations in the ARD of hTRPV4 can result in a gain-of-function, leading to the opening of the channel even when it is in a ligand-free state [61,108–110]. However, it is still unclear whether one single residue mutation is sufficient to cause the loss of interaction with RhoA, which leads to channelopathies due to the malfunction of TRPV4. Together, these results on the structure of TRPV4 provide valuable knowledge about its interaction with RhoA and its possible consequences in cell physiology.

It is important to also mention that RhoA plays pivotal functions in regulating cytoskeletal functions, modulating the transitions between the inactive GDP-bound state and an active GTP-bound state [123], which are important because the active state of RhoA leads to cell contraction and process extension by promoting actin polymerization and actomyosin contraction [114,124]. An interplay of regulation between RhoA and TRPV4 has been demonstrated by McCray et al. [114] to result in the modulation of the activities of both proteins and, in turn, this leads to cytoskeletal changes. The disruption in the interactions of TRPV4 and RhoA and to cellular outgrowth mediated by TRPV4, have been linked to mutations that lead to some neuropathies but not to mutations that produce skeletal dysplasias, which is conducive to a neuron-specific disease.

The N-terminus of TRPV4 contains an intrinsically disordered region (IDR) of about 150 amino acids, which remains unresolved. Interestingly, in a recent study, Goretzi et al. [125], have proposed that this region contains several constituents that can transiently couple and uncouple to regulate the activity of TRPV4. Such interactions were proposed to modulate the binding of lipids (i.e. PIP_2), of proteins that regulate TRPV4's activity and also participate in post-translational modifications in the IDR and ARD. By deleting stretches of several amino acids in the IDR and measuring the activity of the mutant TRPV4 channels, these authors proposed that there is an autoinhibitory region that acts on the PIP_2-binding site and negatively regulates TRPV4's activity. It was also proposed that the binding of lipids to the IDR and the PIP_2-binding region, wields a pull force on the ARD and helps transduce mechanical forces to the rest of the TRPV4 structure [125].

We will next discuss the features and relevance of another region in the bottom layer of the TRPV4 channel that also regulates its activity in homeostasis and pathological processes.

C-terminal region

All TRP channels contain a C-terminal region, which in turn encompasses important domains such as the TRP box, a calmodulin-binding domain (CAM), and a PDZ-like domain Figure 2(a). The TRP box (or TRP domain), is a sequence of six highly conserved amino acids found among the vanilloid subfamily, as well as in the TRPC, and TRPM subfamilies, that allow the functional coupling of the tetramers. In the case of the TRPA subfamily, although the sequence of amino acids differs in comparison to the other subfamilies mentioned, it has been demonstrated that the topology of the structure also acquires an alpha helix conformation [126]. In TRPV4 channels, this sequence interacts with the S4–S5 linker region of the same subunit

through a hydrogen bridge that regulates gating processes, which are also influenced by the polar–nonpolar interface that is established between the protein and the membrane Figure 4(a) [128,129].

There are also interactions between the TRP box and the S4–S5 linker where the side-chain indole is the hydrogen donor of the W733 residue, one that is highly conserved throughout TRPV4 channels of different species Figure 4(b), and residue L596 of the S4–S5 linker, whose carbonyl oxygen is the bridge acceptor. The hydrogen bond formed between L596-W733 maintains the TRPV4 channel in a closed state by orienting the S4-S5 linker along with the TRP box, which

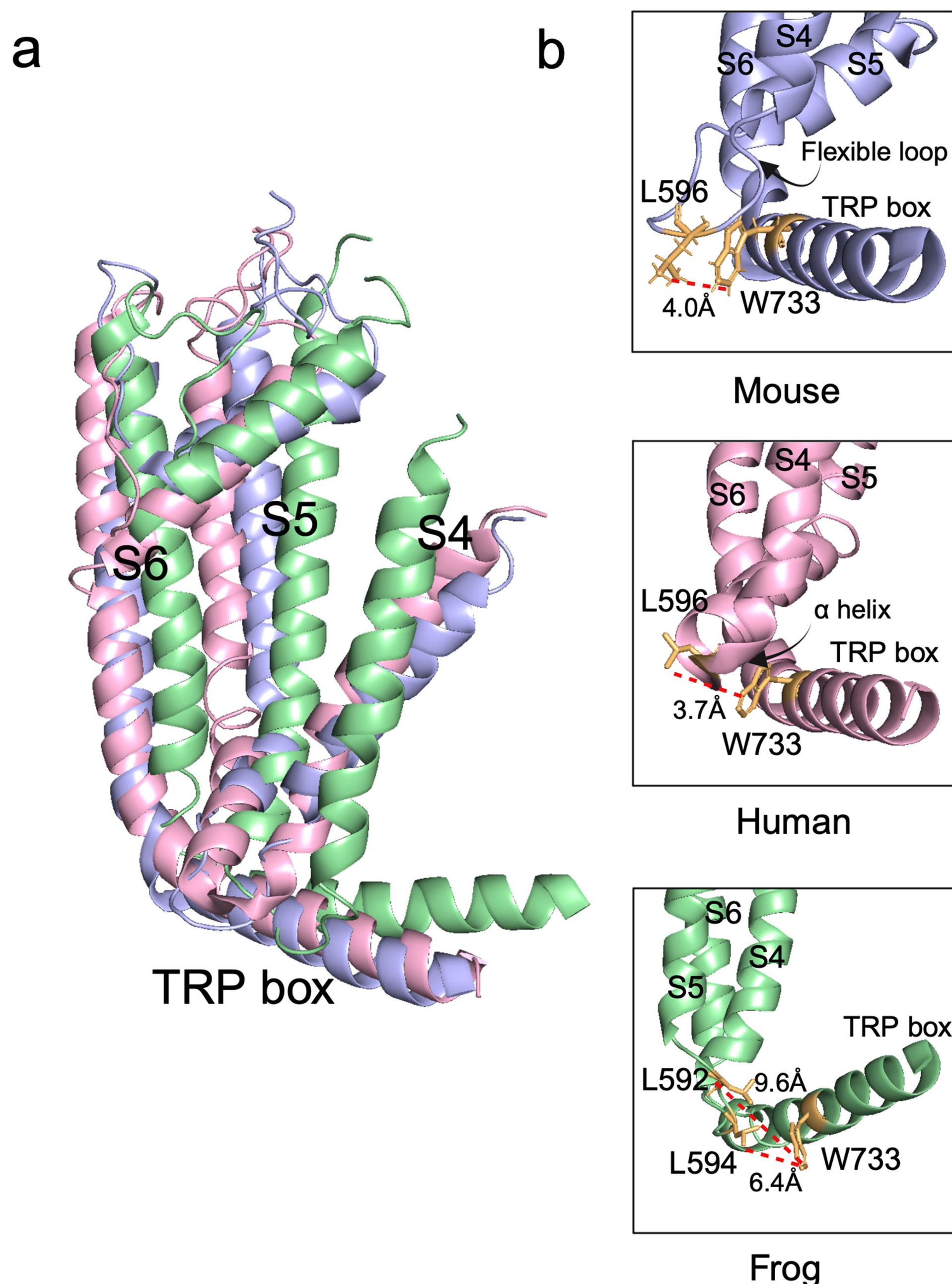

Figure 4. The S4–S5 linker/TRP box interface. a, alignment of the TRPV4 interface of different species as described in the text is shown in a lateral view, only the S4–S6 and the TRP box are represented. b, zoomed-in view of the interaction zone between the S4–S5 linker and the TRP box, where the amino acid residues L596 (human and mouse) or L592/L594 (frog) form a hydrogen bond with the conserved residue W733. The frog TRPV4 linker has the most flexible interface. The amino acid residues are shown in orange sticks and the hydrogen bridges are shown in dotted lines. PDB: 8J1D, 8T1B, and 6BBJ (resolutions of 3.59 Å, 3.00 Å and 3.80 Å, respectively) [52,53,127]. Created with PyMOL and BioRender.com.

impairs the movement of the S6 and the opening of the channel. Stimuli such as depolarization, membrane stretching, or heat, disrupt this hydrogen bond leading to gate opening [128]. Furthermore, it has been described that mutations in these two amino acids (i.e. L596P and W733R) are associated with the generation of skeletal dysplasias, associated with TRPV4 mutations with gain-of-function phenotypes due to increased open probability as a result of weak interaction between these amino acids [128,130,131].

When the human and mouse TRPV4 channels are compared, residue W733 in mouse TRPV4 is localized in a flexible loop, while in the human TRPV4, it is located at the end of the α-helix [52,127]. A closer look into the frog TRPV4 channel structures reveals that it exhibits a flexible loop that could shorten the distance of the interaction between either L592 or L594 [45]. These variations in the position along the S4–S5 linker possibly influence the stability of the hydrogen bridge and therefore the switching between closed or open states; however, despite these differences between species, all three structures maintain the same function of the S4–S5 linker/TRP box interface, giving stability to the closed state when no stimuli are present Figure 4(b). Specific features of the S4-S5 linker region in TRPV4 channels from different species are further detailed in the transmembrane layer section below.

In the C-terminal region, between residues 806 and 831, a CAM-binding site has been described and it has been shown that in the presence of $[Ca^{2+}]_i$, the spontaneous activity and the response of TRPV4 to hypotonic solutions has been suggested to be potentiated through a CAM-dependent mechanism [62]. This phenomenon is then followed by inhibition or inactivation, which is a stable non-conducting conformation of the open channel [62,132,133]. In the human TRPV4 channel, the CAM domain is also the binding site for inositol 1,4,5-trisphosphate (IP_3), which sensitizes the channel´s response, not only to mechanical and hypotonic stimuli, but also to epoxyeicosatrienoic acids (EETs) [134]. In addition, a self-inhibition domain has been proposed, where residues E797-P799 located immediately upstream of the CAM domain, participate in this type of down-regulation of the activity of the channel by establishing a salt bridge with an unidentified positively charged region that stabilizes the closed state of the channel [135]. Consistently, mutation of residue E797 leads the channel to a constitutively open state associated with the generation of skeletal dysplasia [133,135].

Finally, the PDZ-like domain (also known as PSD95/Dlg/ZO-1-like) near the end of the C-terminal region consists of a short sequence of four amino acids (DAPL) and participates in membrane trafficking of the channel, oligomerization of the tetramer and in the interaction with different proteins that regulate downstream signaling processes such as Yes-associated protein/transcriptional co-activator with PDZ-binding motif (YAP/TAZ), which is a transcription factor involved in inflammatory process, which seems to be activated by the influx of Ca^{2+} through TRPV4 channel of immune cells [130,134,136–138].

Moreover, the rigidity of the extracellular matrix participates in pathophysiological processes such as fibrosis in which epithelial–mesenchymal transition (EMT) is involved. TRPV4 has been linked to EMT as a mediator of its response to matrix stiffness and transforming growth factor β1 (TGF-β1). Sharma et al. [139] have suggested that inhibition of the activity of TRPV4 blocks matrix stiffness and EMT in response to TGF-β1, further supporting the role of the PDZ-binding motif of this channel and its interaction with matrix components on the physiology of cells.

The top or transmembrane layer of TRPV4

Voltage sensor-like domain

The S1-S4 helices form the VSLD, which flanks the S5 and S6 helices (pore domain) of the adjacent subunit in a domain-swapped arrangement; however, it has been demonstrated that the arrangement of the VSLD domain is different between TRPV4 channels from several species [45,52,53,127].

The structural arrangement of these regions in frog TRPV4 differs significantly from mouse and human [127], displaying closer proximity of the S3 and S4 from the VSLD to the pore domain Figure 4(b). The S3 establishes a key contact with

S6 in its entire length generating a zipper [45]. Additionally, in the frog TRPV4 channel, the S4–S5 linker structure is an ordered loop with greater flexibility [45], as compared to the α-helix present in the structures of mouse [127] and human TRPV4 channels Figure 4(b) [52,53]. Modifications in the S4–S5 linker, specifically shortening in length or changes in flexibility result in impairment of domain swapping, which affects the architecture and the gating of the channel [32,140,141]. For example, it has been shown that in the TRPV6 channel, the S4–S5 linker is a critical component in domain swapping, supporting the importance of this region among members of the TRPV subfamily of ion channels [32].

Pore domain

In TRPV4, the pore domain is located in the center of the tetramer and is formed by the S5-S6 helices and by a reentrant loop and helix motif. In the upper part of the TRPV4 pore structures there is a first constriction corresponding to the selectivity filter region (TIGMGD/E) (Figure 5). In the apo state, the frog and mouse TRPV4 channels have a cross-pore distance greater than 10 Å between the nearest amino acids in the narrowest region (residue G675, 10.6 Å; residue D682, 14.4 Å respectively) Figures (5a,b) [45,127]; both diameters are unable to prevent the passage of ions such Na^{2+}, K^{+} or Ca^{2+}, considering their Pauling radii [142]. Moreover, ion coordination in the mouse channel Figure 5(b) by this region is unlikely when its architecture is compared to other ion channels such as KcsA, considering that it requires greater closeness between the side chains of the residues [143].

In the human TRPV4 channel Figure 5(c), the distance between the carbonyls of G679 in the pore is reduced to 6.6 Å [52]; however, it is still wide compared to other channels of the same subfamily such as rat TRPV1 (4.6 Å, residue G643) [36]; rabbit TRPV2 (5.2 Å, residue G604) [144] or rat TRPV6 (4.6 Å, residue D541) [42], suggesting the entry of ions to the upper and middle pore cavity could occur in the closed state.

On the other hand, at the bottom of the pore domain, there is a region corresponding to the intracellular gate that controls the permeation pathway, present in other members of the vanilloid subfamily: M643 in rabbit TRPV2 [144]; M677 in mouse TRPV3 [47]; M578 in rabbit TRPV5 [48]; M577 in rat TRPV6 [42]) or I679 in rat TRPV1 [36] side chains are responsible for preventing the passage of cations in the closed state of the channel.

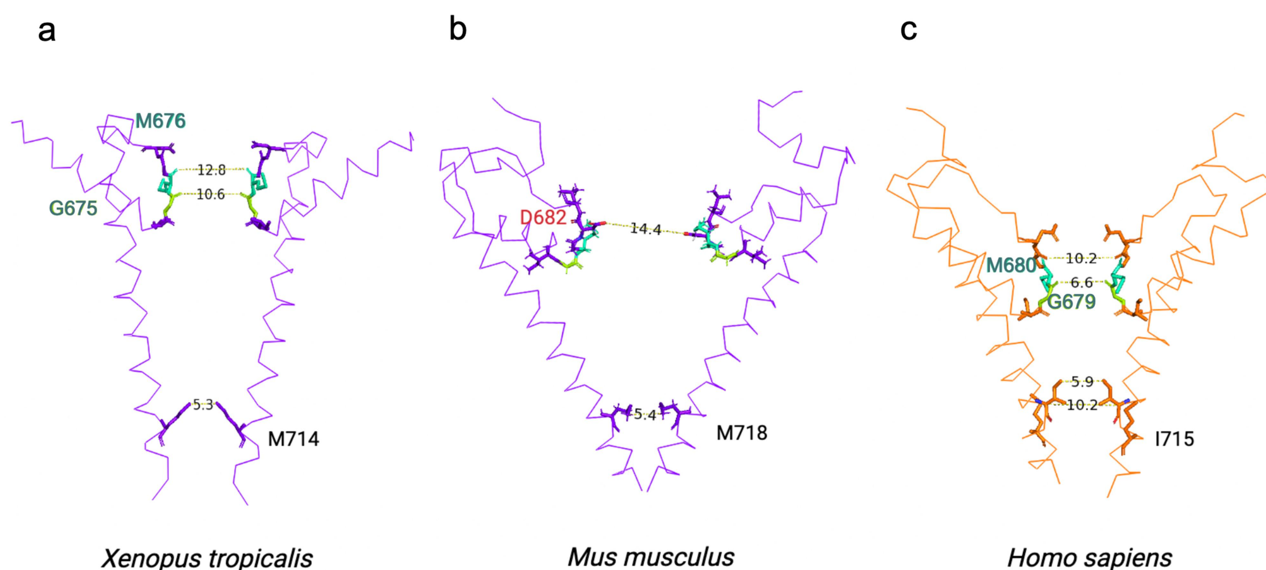

Figure 5. Pore domain of TRPV4. the pore regions of the TRPV4 channel from a, frog, b, mouse, and c, human are shown in the apo state; all of them have a selectivity filter in the upper region which differs in their cross-pore distances. The lower region of the pore domain contains the intracellular gate, where the distance between the side chains of the nearest amino acids is less than 6 Å, preventing the passage of ions. Mammalian ion TRPV4 channels show similarity in structure in contrast to the frog TRPV4, which displays a "tighter" conformation. The amino acids in the selectivity filter and the intracellular gate are shown as sticks. PDB: 6BBJ, 8J1D, and 8T1B (resolutions of 3.80Å, 3.59 Å and 3.00 Å, respectively) [45,52,127]. Created with PyMOL and BioRender.com.

In the frog and mouse TRPV4 channel, residues M714 and M718 are positioned homologously, and constrict the pore to a diameter of 5.3 Å and 5.4 Å Figures 5(a,c) [45,127]; however, in the human channel, the identity and position of the amino acid that restricts ionic conduction is I715, which has a diagonal distance of 5.9 Å between side chains or 10.2 Å between Cα Figure 5(c) [52]. The side chains of all three structures are directed to the central cavity and form an ion- and water-impermeable barrier (Figure 5) [52].

Interactions between the cytoplasmic and transmembrane layers

The TRPV4 channel tertiary structure has an arrangement where the *N*- and C-terminal regions interact through the coupling domain (CD) Figure 6(a). At the N-terminal region, the CD is located between the ARD and the S1 Figure 6(b), involving two beta strands (β1 and β2) which establish hydrogen bonds with a third beta strand (β3, black arrows in Figure 6(b) of the C-terminal region, and form an antiparallel beta sheet [52,53,127].

The interaction of the cytoplasmic domains promotes the nearing of distant regions in the primary structure that are important in the assembly and gating process of the channel. These regions include the TRP box, that runs along the lower part of the VSLD and comes into contact with key sites like the S4–S5 linker, the vanilloid pocket, and the helix-loop -helix motif (HLH) Figure 6(b). Any alteration in these sites of the structure influences the gating processes of the channel [52,53,127].

Modulation of TRPV4 channel by ligands

As previously mentioned, the TRPV4 channel has an exceptionally polymodal ability to respond to many physicochemical stimuli; additionally, its extensive distribution in different organs and its participation in physiology as well in pathologies caused by its malfunction or dysregulation have attracted the attention of different research groups, identifying TRPV4 as an important therapeutic target from which we yet need to learn more details of its function.

Several biophysical and biochemical studies have been performed and improved our understanding of the gating mechanisms and the role of modulators (endogenous or synthetic) of TRPV4 on its activity [61,73,145]. However, it is still necessary to complement this knowledge with structural studies that provide us with information about the changes, binding sites of modulators, and mutations that occur in TRPV4 [146]. Solved human and mouse TRPV4 structures have revealed details of agonist- and antagonist-bound open and closed states, giving us new insights into channels' gating processes. We will further discuss the binding site of synthetic modulators of TRPV4 and the structural mechanisms that determine both open and closed states.

The vanilloid pocket in the TRPV4 channel

According to the recently solved human and mouse TRPV4 structures, it seems more clear that certain compounds bind to the channels in a similar region, the vanilloid pocket, which lies within a cytosol-facing cavity between S1–S4 and the TRP box Figure 7(a) [52,53,127]. The apo structure of hTRPV4 shows that this cavity is composed mainly of aromatic and polar residues, where the polar side chains are distributed around the transmembrane helix top, while the aromatic rings are positioned in a central fashion Figure 7(b) [52]. This particular arrangement of the amino acid residues allows different ligands (agonists and antagonists) to enter the cavity and stably position themselves inside it.

The first synthetic TRPV4 selective agonist described was 4α-PDD, a non-PKC-activating phorbol ester [EC_{50} 200 nM in human, 370 nM in mouse] that has been widely used in many functional experiments [75,145,147–150].

Pioneering site-directed mutagenesis experiments had determined a tentative binding site for 4α-PDD within a pocket between S3 and S4, proposing residues Y556, L584, W586, Y591, and R594 as essential structural elements in the mechanisms of 4α-PDD-dependent TRPV4 gating [149,151]. In the currently solved open-state conformation structure of the human TRPV4 channel in complex with 4α-PDD, the binding site for this molecule has been identified within the cavity between the S1-S4 and the TRP helix bundle [52,53]. There, 4α-PDD is surrounded by residues S470, N474, S477, F524, Y553, Y591, S747, and F748, maintaining hydrogen bonds with N474 and Y591 Figure 7(c) [52].

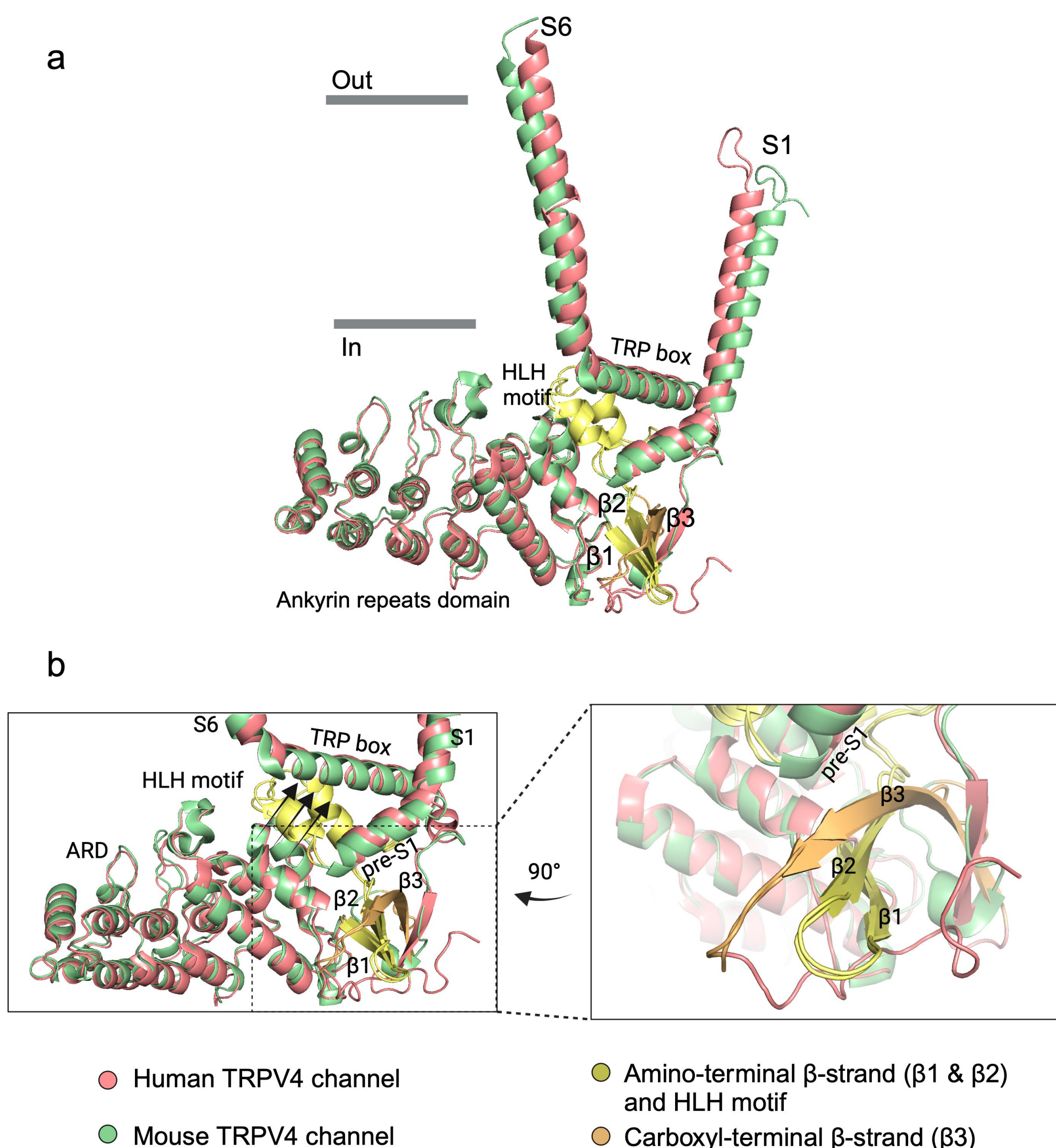

Figure 6. Contact domain of TRPV4. a, the cytoplasmic domains and helices S1 and S6 of the mouse and human TRPV4 channel are represented. b, zoomed-in views of the coupling domain (CD), where the β1 and β2 strands of the N-terminal regions interact with the β3 strand of the C-terminal region, which brings these two regions closer to each other. The arrangement of the tertiary structure allows certain domains such as the TRP box to come into contact with key areas of the protein for its regulation. The black arrows represent the movement of the TRP box toward the plasma membrane facilitated by the HLH motif. PDB: 8J1D and 8T1B (resolutions of 3.59 Å and 3.00 Å, respectively) [52,127]. Created with PyMOL and BioRender.com.

Furthermore, docking calculations and molecular dynamics simulation determined high flexibility within the 4α-PDD molecule with different conformations within the binding pocket [53]. However, these different modeled conformations showed that the aliphatic chain of 4α-PDD is stably localized outside the pocket toward the hydrophobic S1-S4 region, emphasizing the importance of the acyl-chain length as an essential element for the correct positioning of the terpenoid within the binding site Figure 7(c) [147].

It is also important to highlight that this binding site at the base of the S1-S4 bundle is an allosteric modulation site, which has been identified in all TRP members of the vanilloid subfamily [152], explaining why mutations that antagonize 4α-PDD effects also affect activation of the channel by other agonists such as 5,6-ETT and BAA, which could be sharing common binding sites [149,151].

Additionally, another human TRPV4 structure in the open-state conformation was solved, in

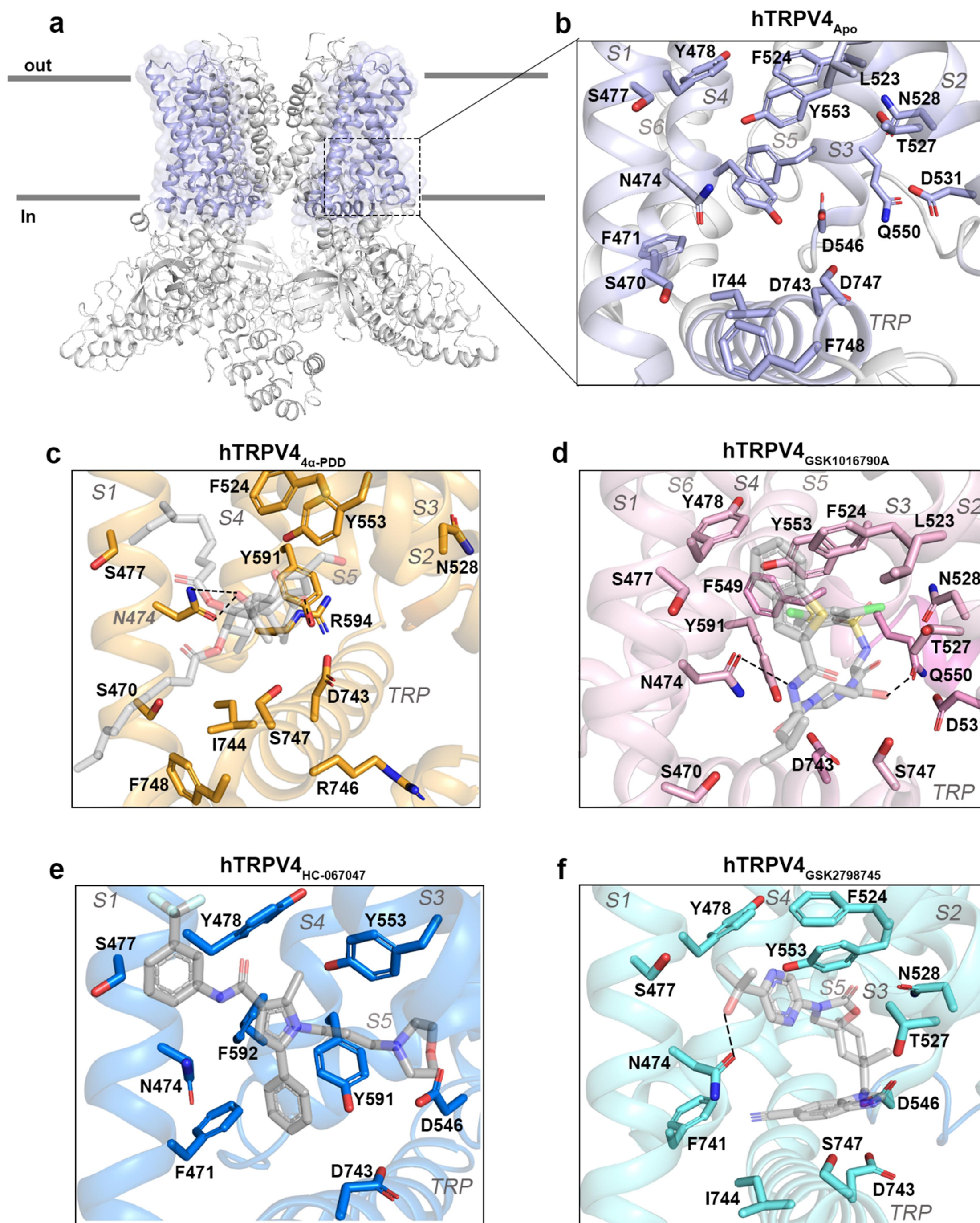

Figure 7. Human TRPV4 ligand binding site. a, schematic representation of the apo structure of TRPV4 channel (white ribbon) with the identified ligand binding site highlighted in light purple. b, zoomed-in view of the ligand binding pocket shown in a, (purple ribbon). c, zoomed-in view of the structure of TRPV4 in the open-state in complex with agonists 4α-PDD (orange ribbon) and d, GSK1016790A (pink ribbon). Closed-state structures in complex with antagonists are shown in e, HC-067047 (blue ribbon) and f, GSK2798745 (cyan ribbon). The side chains of the polar and aromatic residues essential for binding agonists and antagonists (shown in gray) are represented as sticks. Hydrogen bonds are represented as dashed lines. Both agonists and antagonists are stably positioned within the ligand binding pocket and share some residues with which they interact, such as S470, N474, F524, N528, Y553, Y591, D743, and S747. However, they are also closely surrounded by particular residues: 4α-PDD (F478); GSK1016790A (Q550, D531, F549, L523); HC-067047 (Y478, F592) and GSK2798745 (Y478, F524). PDB: 8T1B, 8FCA, 8FC7, and 8FC8 (resolutions of 3.00 Å, 3.41 Å, 3.30 Å and 3.47 Å, respectively) [52,53]. Created with PyMOL and BioRender.com.

complex with the synthetic compound GSK1016790A [EC_{50} = 2 nM in human, 18 nM in mouse] [76,153]. This structure shows that this agonist shares the same ligand-binding site as 4α-PDD Figure 7(d) [53]. GSK1016790A is a highly potent and selective agonist for the TRPV4 channel, used extensively in pharmacology studies of TRPV4 activation [154–161].

Also, molecular dynamics simulations showed that, unlike 4α-PDD, the conformational arrangement of GSK1016790A remains stable within the binding site and that among the closest residues surrounding it, both GSK1016790A and 4α-PDD share residues S470, S477, N474, F524, N528, Y591, T553, D743, and D747 for their binding to the channel. In contrast, residues L523, T527, D531, F549, Q550 and T478 seems to interact specifically with GSK1016790A only Figure 7(d) [53].

On the other hand, according to recent cryo-EM structures, these agonists are proposed to share the same ligand-binding site with synthetic antagonists. Nadezhdin et al. [52] have solved the human TRPV4 structure in closed-state conformation in complex with the molecule HC-067047 [IC_{50} 48 nM in human, 133 nM in rat, 17 nM in mouse] [77], which is a potent and selective antagonist for TRPV4 that has been used to study the pathophysiology of TRPV4 channel in both *in vitro* and *in vivo* models [61,162].

The structure shows that the HC-067047 molecule is positioned within the vanilloid pocket, surrounded by some residues shared with the agonists 4α-PDD and GSK1016790A (i.e. N474, S477, Y553, Y591, and D743), and, particularly with the residues F471, D546 and F592 Figure 7(e). Moreover, these residues identified for the binding of HC-067047 in the Cryo-EM structure, were also confirmed by functional assays in which substitutions for alanines were introduced in these sites, entailing a decrease in the inhibitory effect of the compound [52].

The compound GSK2798745 [IC_{50} 1.8 nM in human] [78,163] is the first TRPV4 antagonist to advance to clinical trials [163]. Kwon et al. [53] have solved the human TRPV4 structure in the closed-state conformation in presence of this synthetic molecule. They found that GSK2798745 is also surrounded by the residues shared with the agonists 4α-PDD and GSK1016790A (N474, S477, N528, Y553, Y591, D743 and S747), and like HC-067047, it interacts closely with F471 and D546, and with Y478 and F524 particularly Figure 7(f).

The solved mouse TRPV4 channel structure [127] has also provided detailed information about the mTRPV4 channel in a ligand-free state (apo), in the open-state in complex with agonist GSK1016790A, in complex with both GSK1016790A and ruthenium red (RR, a nonselective pore-blocking molecule) and, in complex with both GSK1016790A and Agonist-1 (or quinazoline-4(*3 H*)-one derivative 36) [164].

The apo structure of the mTRPV4 channel resembles a similar arrangement as the one described for the hTRPV4 channel Figure 8(a). In both structures, the intracellular gate shows the amino acid residue M718 (mouse) or I715 (human) of each subunit at a distance of less than 6 Å, which impairs ions influx Figure 8(a), right panel). On the other hand, the open-state structure of mTRPV4 in complex with GSK1016790A reveals that this agonist binds in the same pocket between the VSLD and the TRP box region as in the human TRPV4. The key residues for the binding of GSK1016790A in the mTRPV4 channel are S470, F524, and F549, while in the hTRPV4 the residues N474, Y478, Y591, Q550, T527, and D743 are involved Figure 8(b) [53,127].

Although in this review we only discuss the mTRPV4 apo and open structure in complex with GSK1016790A in order to compare it with the hTRPV4 structures, we consider that is also important to briefly highlight that Agonist-1 shares the binding site within the vanilloid pocket as well [127].

As for the structure solved with both GSK1016790A and RR, the structure solved by Zhen et al. [127] is the second TRPV channel structure resolved in the presence of RR, since the last one was in complex with the human TRPV6 channel [165]. The mTRPV4 structure with RR shows a classic pore-blocking mechanism in which RR binds to the extracellular region of the selectivity filter near residues D682 and M680 [127,166–174].

Structural mechanisms in the hTRPV4 gating

The structural changes of the human TRPV4 associated with channel activation by the binding of an agonist, whether 4α-PDD or GSK1016790A; or channel inhibition by the binding of an antagonist,

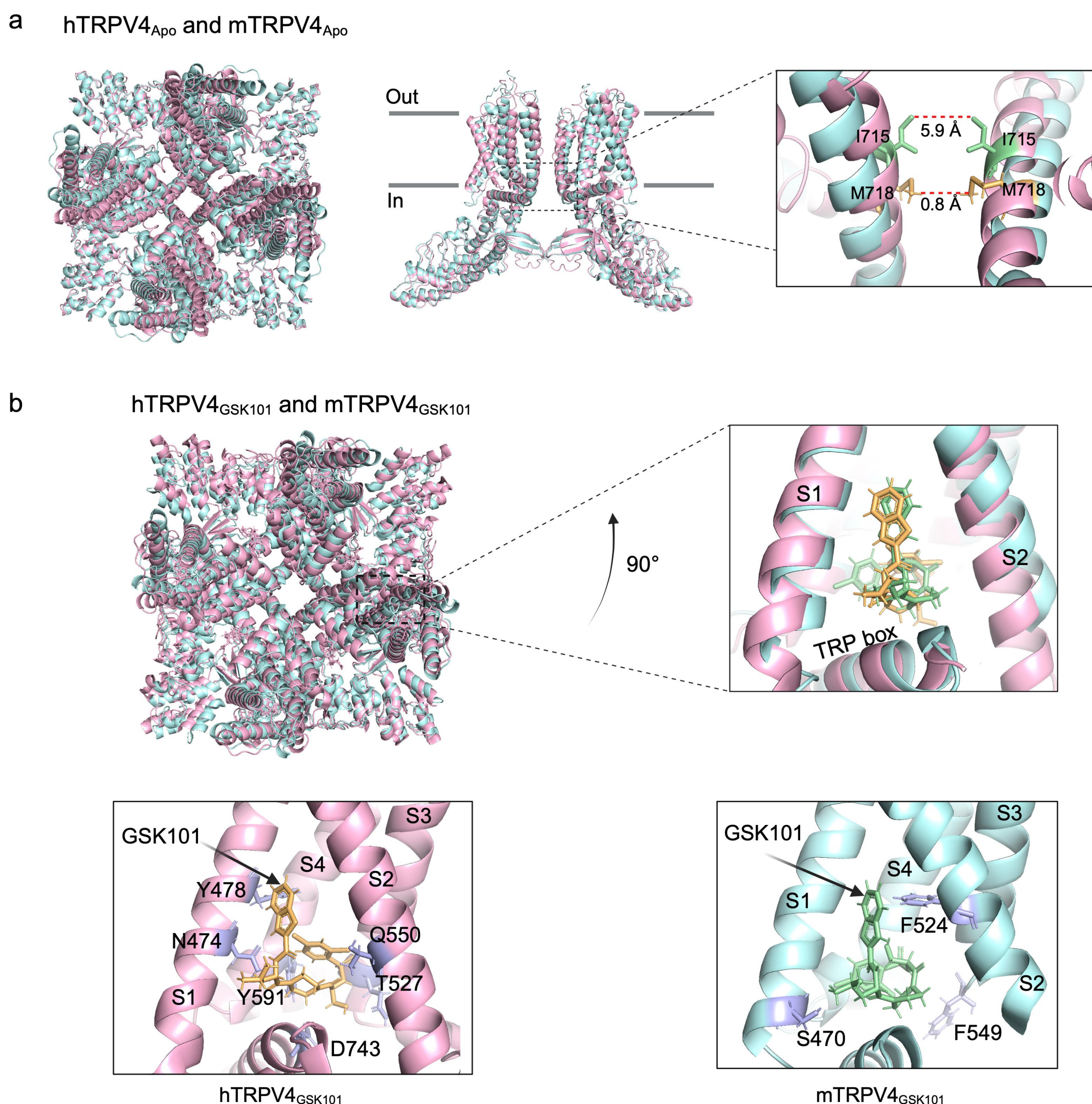

Figure 8. Comparison between hTRPV4 and mTRPV4 channels. a, schematic representation of the apo structure of human (pink ribbons) and mouse (cyan ribbons) TRPV4 channels along with a close-up of the pore domain with the identified intracellular gate residues at I715 (human, green sticks) or M718 (mouse, orange sticks) and its cross-pore distances. b, TRPV4 channel structure in the open state in complex with the agonist GSK1016790A. A zoom-in of the ligand binding pocket between the S1–S4 and the TRP box (human, pink ribbons; mouse, cyan ribbons) is shown. The key amino acid residues and the agonist structure are shown in purple sticks. PDB: 8J1D, 8FC9, 8J1F, and 8FC8 (resolutions are 3.59 Å, 3.75 Å, 3.62 Å and 3.47 Å, respectively) [53,127]. Created with PyMOL and BioRender.com.

whether HC-067047 or GSK2798745, involve transitions in different key domains, beginning in the amino acid residues within the binding site in the VSLD and ending until they reach the pore domain. It has been also proposed that residues D531, D546, Q550, and R594 within the S1–S4 region interact through polar contacts in the closed state Figure 9(a). When the interactions between residues within the S1–S4 are broken by the binding of an agonist, new hydrogen bonds are formed with both the agonist and residues of the TRP helix. In this fashion, residue Q550 interacts with the agonist, T594 forms a salt bridge with residue D743, and D531 forms a hydrogen bond with R746, which in turn, allows residue R746 to maintain an interaction with Y439, located in the CD that links the *N*- and C-terminal regions Figures (9a,b) [52,53].

Regarding the architecture of the intracellular gate in the open state, the solved structures [52,53] suggest that this region exhibits an increased distance between the gating residues (I715) of each subunit, allowing the movement of a hydrated ion through the channel Figure 9(c). As for the selectivity filter in the structures activated by 4α-PDD and

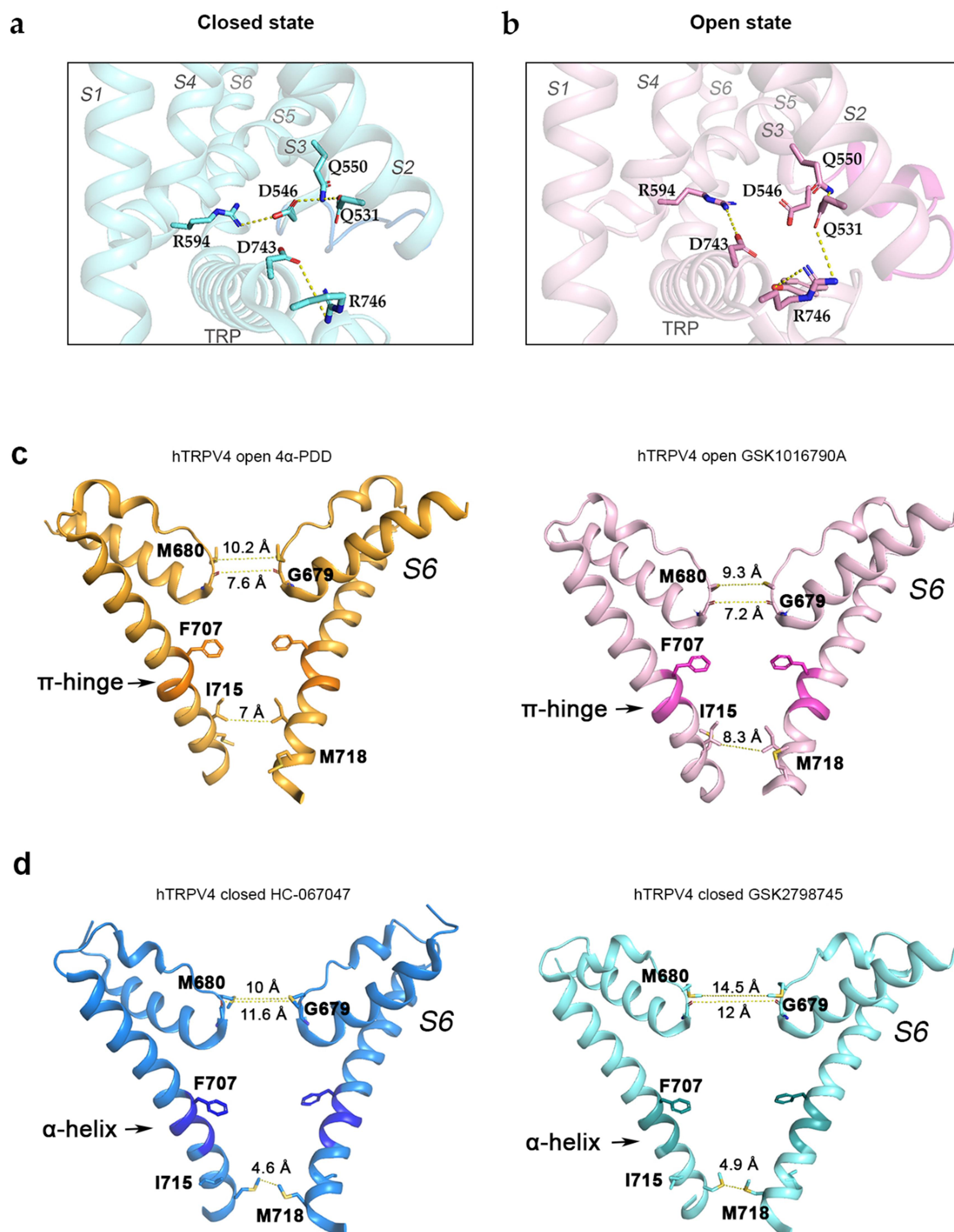

Figure 9. Structural changes in the closed and open states of hTRPV4. Close-up view of the ligand binding pocket showing the key residues that form the coupling interface between the S1–S4, CD, and TRP domains in the a, closed and b, open states. Dashed lines indicate hydrogen-bonds and salt bridges. Representation of the structural changes in the selectivity filter and the intracellular gate of the pore region caused by the binding of the agonists c, 4α-PDD (yellow structure) and GSK1016790A (pink structure), or antagonists d, HC-067047 (blue structure) and GSK2798745 (cyan structure). Dashed lines indicate the distances between gating (I715 in the open state and M718 in the closed state) and selectivity filter (G679, M680) residues in opposite subunits. Upon activation by the agonist, a transition from α to π secondary structure occurs in the S6 helix, inducing a helical bend (π-hinge). The binding of the antagonist promotes a transition from π to α secondary structure, inducing the formation of an α-helix. The position of residue F707 is highlighted since it putatively stabilizes the π-helices structure through H-C···π interactions. PDB: 8FCA, 8FC8, 8T1F, and 8FC7 (resolutions are 3.41 Å, 3.47 Å, 3.49 Å and 3.30 Å, respectively) [52]. Created with PyMOL and BioRender.com.

GSK1016790A [52,53], the distance between residues in the selectivity filter (G679 and M680) seems to be decreased Figure 9(c), as compared to TRPV4 structures in the closed state Figure 9(d), possibly allowing the coordination of cations [52,53].

Structure changes promoted by the binding of the antagonists HC-067047 and/or GSK2798745 [52,53] include the rotation in the middle of the S6, which allows for the transition from a π to α structure and a conformational change at the C-terminal end of the S6 that positions residues M718 of each subunit to form the intracellular gate and create a hydrophobic seal in the pore that blocks the passage of cations. Furthermore, the closed state bound to antagonists acquires a wide selectivity filter (G679 and M680) that inhibits the direct coordination of cations Figure 9(d) [52,53]. These studies also showed that the radius of the pore is wider when HC-067047 is bound to the channel (11.6 Å cross-pore distance) Figure 9(d),

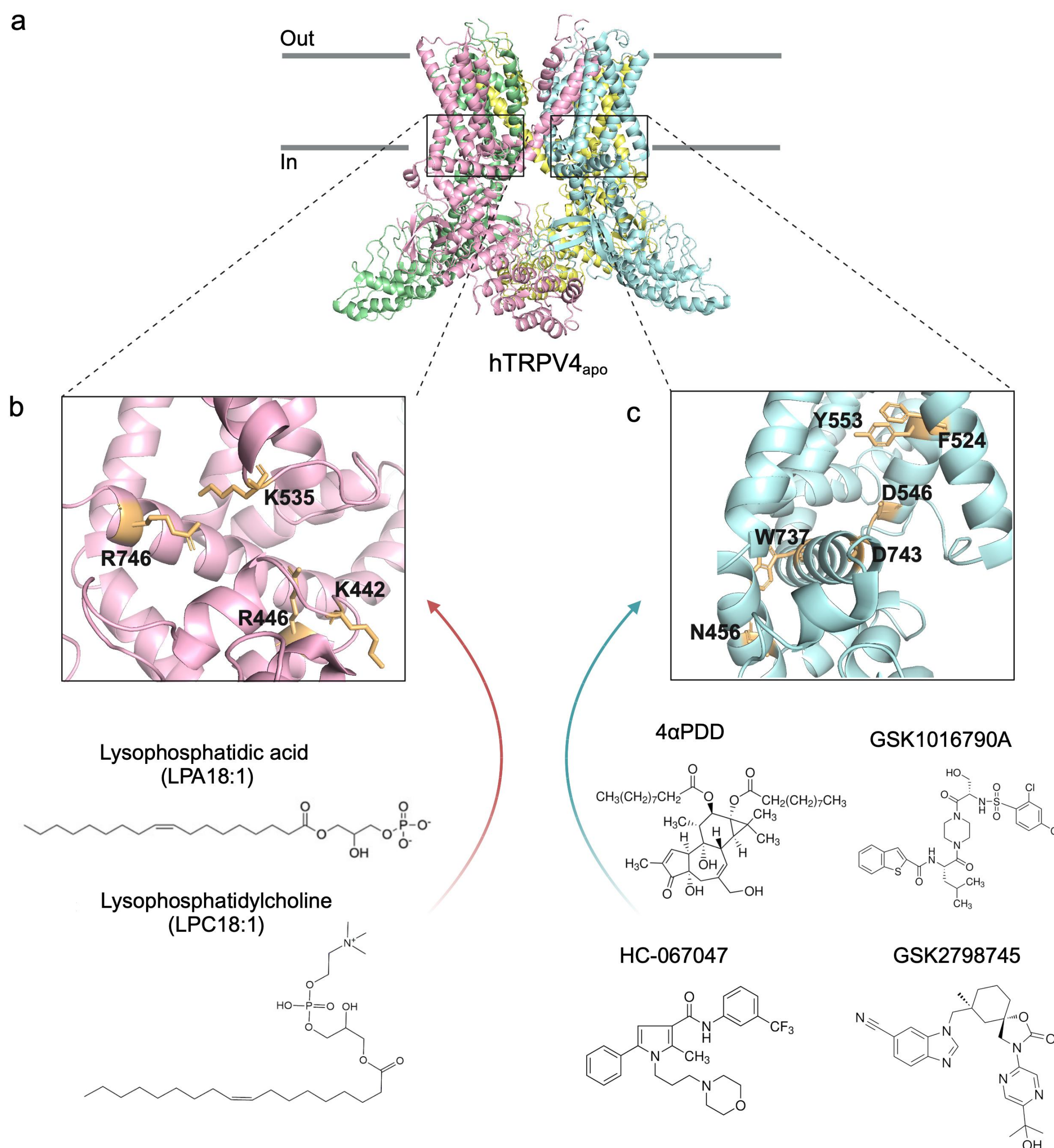

Figure 10. The “vanilloid pocket” in TRPV4. a, schematic representation of the apo structure of human TRPV4 channel in a parallel view with the membrane. Each subunit of the homotetramer is shown by a different color. A zoom-in of the ligand binding pocket between the S1–S4 and the TRP box where key amino acid residues for the binding of b, endogenous (pink ribbons, orange sticks) or c, synthetic (cyan ribbons, orange sticks) ligands are represented. The chemical structures of the modulators of the TRPV4 channel discussed in this review are shown as well. PDB: 8T1B (resolution 3.00 Å) [52]. Created with PyMOL and BioRender.com.

left panel), compared to the apo state (6.6 Å cross-pore distance) Figure 5(c) [52].

When GSK2798745 is bound to hTRPV4, the selectivity filter acquires a wide structure that impairs the coordination of cations (a distance of 14.5 Å between residues M680 from each subunit is observed), while the intracellular gate exhibits a distance which is too narrow for the flux of ions to occur (the distance between residues M718 among subunits is 4.9 Å) (Figure 9(d), right panel) [53].

In summary, based on the above-mentioned findings, it can be concluded that both agonists and antagonists can interact with the same ligand-binding sites of the TRPV4 channel, even sharing some of the residues in these sites. However, the molecular details of types of interactions or bonds vary with each ligand, possibly explaining the differences in the effects they exert and the potency of their modulation on the TRPV4 channel.

It is worth mentioning that very few endogenous ligands have been described for TRPV4. Our work group has recently shown that there is an interaction between LPA 18:1, an endogenous lipid associated with the generation of pain and many other functions [175], with the TRPV4 channel. Interaction of LPA18:1 with TRPV4 involves residues R746 of the TRP box, K442, R446 of the N-terminal region and K535 of the S2–S3 linker (Figure 10). When compared to activation of TRPV4 by GSK1016790A, single-channel current amplitude values in the presence of LPA18:1 are similar to those with GSK1016790A [69]. TRPV4 is also activated by the precursor of LPA18:1, LPC18:1 [68], by a mechanism that involves interaction with a positively charged residue (R746) localized in the TRP box of the channel. However, activation of TRPV4 by LPA18:1 and LPC18:1 occurs with different open probabilities (higher Po for LPC than for LPA) but with different single-channel conductances (lower for LPC), suggesting that this happens through a mechanism in which these agonists promote different open states. A similar phenomenon was observed by our group for LPA18:1 and TRPV1, where LPA18:1 activates the channel, partially through interactions with residue K710 in the TRP box of the channel [176], but also leads to a different conformational open state with higher conductance, as compared to the one produced by capsaicin [177].

Although no structure has been obtained for the endogenous ligands of the TRPV4 channel, Figure 10 highlights the binding sites we have found to be important for the binding of LPA18:1 and LPC18:1 and emphasizes the importance of the vanilloid pocket in the modulation of the gating process of the TRPV4 channel.

Conclusions

The TRPV4 channel is a tetrameric protein localized in the plasma membranes of different cells, where it regulates ion fluxes, particularly Ca^{2+}, in response to several stimuli. TRPV4 plays a crucial role not only in normal physiology but also in diseases or syndromes where its regulation is compromised. Although the different structures currently solved exhibit differences between them, they all agree that each subunit consists of an N-terminal region, where the PBD, PRR, and ARD are located, while the C-terminal region contains the TRP box, CAM binding site, and PDZ-like domain.

The transmembrane region contains helices S1–S6 and the VSLD, but among different species of TRPV4, especially the pore region and the S4–S5 linker, exhibit the most significant changes. Solved structures seem to share common binding sites for the synthetic modulators 4αPDD, GSK1016790A, GSK2798745, and HC-067047 in the vanilloid pocket, which also seems to play a crucial role in the modulation of the channel by endogenously produced molecules as well.

Recent advances in imaging techniques, such as Cryo-EM, have provided us with greater knowledge about the structure, function, and gating processes of TRPV4. It is crucial to emphasize the significance of not only obtaining the details of the structure of the human TRPV4 channel but also of its homologs in other species of animals (*Xenopus tropicalis*, *Mus musculus*), which serve as models for comparison and better understanding of how this biologically relevant protein functions. It is also important to consider that the resolutions of the TRPV4 structures are mostly around 3–3.8 Å, which are values like some of the other TRPV channels, although for a few of the latter, better quality structures have been obtained (~1.9–3.5 Å). Nonetheless, information

on specific molecular interactions of ligands with TRPV4 can be attained from the available structures for this channel.

We still have much to learn about how TRPV4 is regulated and surely many endogenous regulators of the activity of this channel have yet to be discovered.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contributions

R.S.H. and T.R. conceived the manuscript. R.S.H., M.B.A., and A.M.H.V. performed the literature search and produced the figures. R.S.H., M.B.A., A.M.H.V. and T.R. wrote the paper. T.R. supervised the work, reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Data availability statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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