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Structure-Driven Pharmacology of Transient Receptor Potential Channel Vanilloid 1 (TRPV1)

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List of non-standard abbreviations

APHC1: Analgesic Peptide *Heteractis Crispa*

ARD: Ankyrin repeat domain

CAP: Capsaicin

CPZ: Capsazepine

Cryo-EM: Single-particle electron cryo-microscopy

CTX: Charybdotoxin

DkTx: Double Knot toxin

Kv: Voltage gated potassium channel

LPA: Lysophosphatidic acid

MβCD: Methyl-β-Cyclodextrin

NaV: Voltage-gated sodium channel

RTX: Resiniferatoxin

VaTx: Vanillotoxin

TRPV1: Transient receptor potential vanilloid 1

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ABSTRACT

The Transient Receptor Potential Vanilloid 1 (TRPV1) ion channel is a polymodal receptor that mediates the flux of cations across the membrane in response to several stimuli including heat, voltage, and ligands; the best known agonist of TRPV1 channels is capsaicin, the pungent component of "hot" chilli peppers. Additionally, peptides found in the venom of poisonous animals, along with the lipids PIP2, lysophosphatidic acid, and cholesterol, bind to TRPV1 with high affinity to modulate channel gating. Here, we discuss the functional evidence regarding ligand-dependent activation of TRPV1 channels in light of structural data recently obtained by cryo-electron microscopy. This review focuses on the mechanistic insights into ligand binding and allosteric gating of TRPV1 channels and the relevance of accurate polymodal receptor biophysical characterization for drug design in novel pain therapies.

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Introduction

Pain sensation is triggered when the terminals of a specific subset of peripheral neurons called nociceptors are activated by noxious stimuli, such as irritant substances or heat. Specifically, the cationic non-selective transient receptor potential vanilloid1 (TRPV1) ion channel detects these stimuli and induces the opening of the channel pore and a subsequent increase in membrane permeability (Caterina et al., 1997). The TRPV1 channel is a polymodal receptor originally shown to be activated by capsaicin, heat, protons, (Tominaga et al., 1998), lipids (Hernandez-Garcia and Rosenbaum, 2014; Morales-Lazaro and Rosenbaum, 2015), and peptide toxins from some venomous animals (Bohlen et al., 2010; Hakim et al., 2015; Yang et al., 2015b). The TRPV1 channel has a tetrameric structure, with each subunit possessing six transmembrane domains, several ankyrin repeat domain (ARD), a conserved amino acidic sequence called the `TRP box' at the channel N-terminus, and a large intracellular carboxyl terminus (Liao et al., 2013). As with many other members of the TRP ion channel superfamily, TRPV1 has been the focus of intense research due to the intriguing nature of its polymodal activation mechanism (Latorre et al., 2007; Nilius et al., 2005; Tominaga, 2007), but until recently, experimental data lacked a structural framework to interpret functional findings.

A major breakthrough in the TRP channels field came from the laboratory of David Julius when the structure of the TRPV1 channel in different conformations was resolved using single-particle electron cryo-microscopy (cryo-EM) (Cao et al., 2013b; Liao et al., 2013). This picture showed that the TRPV1 channel shares many structural features with other ion channel of known structures, such as the Kv1.2 channel and the Kv1.2-2.1 paddle chimera (Long et al., 2005a; Long et al., 2007). The TRPV1 channel structure supported previous studies proposing that the TRPV1 channel is assembled as a tetramer (Kedei et al., 2001; Kuzhikandathil et al., 2001) and confirmed

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functional studies showing that the channel pore and corresponding intracellular activation gate(s), formed by the S5-S6 transmembrane segments, exist along the tetramer axis of symmetry (Jara-Oseguera et al., 2008; Oseguera et al., 2007; Salazar et al., 2009). The TRPV1 structure shows that a peripheral domain in each subunit, encompassing S1-S4 segments, is connected to the pore by a short linker, a feature also conserved in voltage-dependent potassium channels (Long et al., 2005a). The short stretch of 25 amino acids at the intracellular C-terminus, conserved in several TRP channels and known as the `TRP domain´, adopts an α-helical structure running parallel to the membrane (Liao et al., 2013). This TRP domain is tightly packed with the S4-S5 linker, a region that has been proposed to couple the voltage sensor domain with pore opening in voltage-gated ion channels (Long et al., 2007). The long ankyrin repeat domain (ARD) characteristic of many TRP channels interacts both with the ARD-S1 linker and the C-terminus of the neighboring subunit (Liao et al., 2013). The structure of the TRPV1 channel was obtained under several conditions: in the absence of ligand or the APO form; in the presence of capsaicin; and, in the presence of resiniferatoxin (RTX) and the double knotted toxin (DkTx) where both compounds acted as irreversible TRPV1 channel openers (Siemens et al., 2006). Among the several sensing modalities displayed by the TRPV1 channel, a number of unique features of the ligand-channel interaction make for an interesting study. One issue to consider is how the conformational changes triggered by small molecule binding are translated into the mechanical energy necessary to open the channel pore. Secondly, since the TRPV1 channel is a major component of pain pathways, suppressing this activity may lead to novel strategies that would alleviate pain stemming from several diseases (Julius, 2013).

This review focuses on the structural relationships between three major classes of ligands that are able to modulate the activity of TRPV1 channels: compounds that bind to the vanilloid binding site, peptides derived from poisonous animal venom extract, and lipids.

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Ligand binding at the vanilloid-binding pocket

Capsaicin, the pungent active compound of "hot" chili peppers, increases the cation permeability of sensory neuron membranes (Bevan and Szolcsanyi, 1990; Oh et al., 1996). However, its molecular target was not identified until the cloning of a functional cDNA encoding a protein of 838 amino acids -the TRPV1 channel- from dorsal root ganglia neurons (Caterina et al., 1997). This study showed that TRPV1 channels were activated by capsaicin, noxious heat, and protons, thereby revealing the channel's role as an integrator of painful stimuli (Caterina et al., 1997). Furthermore, a generation of transgenic mice lacking the TRPV1 channels exhibited impaired nociceptive responses induced by vanilloids (Caterina et al., 2000) and during inflammatory conditions (Davis et al., 2000). Once the target for capsaicin was identified, attention focused on determining the binding site within the TRPV1 channels and the operating mechanism for channel opening. Contrary to what is observed in rodents, dorsal root ganglia neurons from chicks are capsaicin insensitive even though they display heat-induced cationic currents (Wood et al., 1988). Taking advantage of the species-specific differences, Jordt and Julius (2002) showed that transferring transmembrane segments 2-4 from rat to bird TRPV1 channel restored capsaicin sensitivity (Jordt and Julius, 2002). Rabbits, like chicks, are also virtually insensitive to capsaicin; the EC_{50} of capsaicin-evoked currents of rat TRPV1 channel is 1000-fold lower than that of its rabbit counterpart. However, the rabbit TRPV1 channel becomes capsaicin-sensitive if leucine at position 547 or isoleucine at position 550 in segments S3 and S4 are substituted by the residues present at these positions in rats, a methionine and threonine, respectively (Gavva et al., 2004). These results suggest that residues contained in S3 and S4 constituted the capsaicin-binding site.

The structure of the rTRPV1 channel in the presence of capsaicin (PDB: ID 3JR5) did not reveal the precise orientation of the ligand at the vanilloid binding site, and thus specific

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interactions with residues forming the pocket cannot be inferred (Cao et al., 2013b). Figure 1a shows a capsaicin molecule (red) inside of the vanilloid binding pocket at the S3-S4 interface of the TRPV1 channel after molecular docking using Autodock Vina software (Trott and Olson, 2010). A recent study (Yang et al., 2015a) addressed this issue by performing a molecular docking of capsaicin at the capsaicin bound (HOLO) structure of the TRPV1 channel (Cao et al., 2013b) and found that capsaicin adopted a 'tail up-head down' orientation [Fig. 1b & c; see also (Poblete et al., 2015)]. Removal of six carbon atoms from the aliphatic tail of capsaicin produced a 6000-fold increase in the EC_{50}, but thermodynamic mutant cycle analysis (Hidalgo and MacKinnon, 1995; Ranganathan et al., 1996) revealed that the capsaicin tail is not energetically coupled ($\Delta\Delta G<1.5kT$) to any residue of the binding pocket (Yang et al., 2015a). The oxygen of the amide group connecting the capsaicin tail with the vanilloid ring is coupled with T550 in S4 by almost $2.2kT$. The vanilloid ring, despite showing extensive contacts with residues at the pocket during the molecular docking, was found to be energetically coupled only to E570 at the S4-S5 linker [Fig- 1b & c; (Yang et al., 2015a)]; this segment has been proposed to couple voltage sensor activation with channel opening in Kv channels (Long et al., 2005b) and is likely to play a similar role in coupling ligand binding to the opening of the lower activation gate in TRPV1. *In silico* characterization of capsaicin binding to TRPV1 channel by free energy methods also suggests that capsaicin adopts the `tail up-head down´ orientation (Poblete et al., 2015; Yang et al., 2015a). The atomic detail that MD provides also shows that capsaicin interacts with Y511 and T550 both directly and through water-mediated hydrogen bonds [Fig. 1c ; (Darre and Domene, 2015)].

Capsazepine (CPZ), a chlorinated compound with a K_d of approximately 220 nM obtained by capsaicin chemical modification [Fig. 1d (Walpole et al., 1994)], was originally described to specifically and competitively antagonize the capsaicin effects in rat dorsal root ganglia neurons (DRG) (Bevan et al., 1992). Species-specific effects of CPZ have been described in the past; the

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compound antagonizes capsaicin-mediated responses in TRPV1 from human, rat, and guinea pig, but displays a reduced effectiveness in the inhibition of heat- and pH-dependent activation of rat TRPV1 channels (rTRPV1) (McIntyre et al., 2001; Savidge et al., 2002). The rTRPV1 CPZ sensitivity phenotype is recovered after replacement of three rTRPV1 vanilloid-binding pocket residues with the corresponding human residues (I514M and V518L in S3 and M547L in S4) (Phillips et al., 2004). The reasons for such a differential inhibition have not been determined and more functional studies are required.

The structure of the TRPV1 channel in the presence of CPZ was recently obtained by Cryo-EM (Gao et al., 2016). Fig. 1e (right) shows CPZ into the vanilloid binding site in a head-up tail-down configuration, with the chlorine atom pointing towards the extracellular side of the membrane. Halogenation has proven to be a successful strategy for transforming a high affinity agonist into a potent competitive antagonist in RTX-related compounds (Kang et al., 2007); introduction of halogen atoms at the head of either capsaicin or resiniferatoxin transform the compounds into competitive antagonists (Appendino et al., 2005; Wahl et al., 2001) and the potency of these substituted competitive antagonists correlates with halogen size (I>Br>Cl>F) (Lim et al 2011). Even in the presence of the structural details that Cryo-EM provides, the mechanism of CPZ mediated inhibition and its dependence on the size of the halogen in other related TRPV1 inhibitors remain unknown, and functional data, combining site directed mutagenesis and chemical modification of the ligand are required.

The structure of the TRPV1 channel obtained by cryo-EM showed that the binding site of resiniferatoxin (RTX) (Fig 1f and g), a potent capsaicin analogue, is located close to Y511 and S512 in S3 and M547 and T550 in S4 (Cao et al., 2013b). The receptor activation response elicited by several TRPV1 agonists such as capsaicin, piperine, arvanil, olvanil, RTX, and SDZ-249665 was strongly correlated with compound hydrophobicity and pungency (Ursu et al., 2010). However, the

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underlying binding mechanism of these compounds was only recently solved when the diffusional pathway of capsaicin from the extracellular solution to the vanilloid binding site was characterized with molecular dynamics (MD) simulations of the TRPV1 channel structure inserted into a lipid bilayer (Hanson et al. 2015). Results showed that capsaicin flips from the external to the internal leaflets of the lipid bilayer and gains access to its binding site by lateral diffusion (Hanson et al., 2015). Diffusion across the plasma membrane may represent a substantial energy barrier that some hydrophobic ligands must surmount when attempting to reach a buried binding site. Thus, the correlation between potency and hydrophobicity reported by Ursu *el al.* (2010) may reflect the diffusional coefficient differences for each ligand when travelling from the outer to the inner membrane leaflet. These studies suggest that this energetic cost should be included in drug design *in silico* studies that attempt to tackle the shortcomings of current approaches.

Single channel recordings of TRPV1 channels suggest that both partial and full binding of capsaicin are able to open the channel pore (Hui et al., 2003). Furthermore, as shown with concatemers containing variable numbers of defective capsaicin binding sites, maximal activation of TRPV1 by capsaicin is independent of the number of available binding sites (Hazan et al., 2015). In line with the classic Monod-Wyman-Changeux model (MWC) that has previously been proposed for other ion channels, a fully functional ligand-sensitive TRPV1 channel with a single capsaicin binding site can be satisfactorily explained by allosteric gating (Altomare et al., 2001; Auerbach, 2012; Brauchi et al., 2004; Horrigan and Aldrich, 2002; Latorre et al., 2007; Matta and Ahern, 2007; Raddatz et al., 2014; Rothberg and Magleby, 1999).

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The mechanism for ligand binding-mediated channel opening is far from understood, but a comparison of closed and open TRPV1 channels structures obtained by cryo-EM shows a slight rearrangement of Y511 (Cao et al., 2013b; Liao et al., 2013). Comparison of the TRPV1 structures in different conformations suggests that conformational changes at the capsaicin binding pocket are directly transmitted to the lower activation gate through hydrogen bonding between D576 in the S4-S5 linker and M682 in S6 (Liao et al., 2013). Later analysis of both structures revealed a modest 10% decrease in the solvent accessible area (Elokely et al., 2016) and a small increase in the pore dimensions (around 2-3Å) in the lower gate (Cao et al., 2013a). These findings suggest that subtle conformational changes in the ligand binding pocket, such as a single side chain orientation, may be able to spread out toward the channel activation gates of the channel.

Molecular dynamics simulations of the hTRPV1 channel using the TRPV1 channel structure obtained by Cao et al. (Cao et al., 2013b) as a template, gave some hints on how ligand binding influences the operation of the channel activation gates. Feng et al (2015) showed that the APO structure, in the presence of RTX, increases the radius of the TRPV1 upper gate, while the lower gate remained closed. When simulations were performed in the presence of AMG9819, a potent TRPV1 channel antagonist, both gates remained in their nonconductive conformation suggesting that pore opening occurs in an non synchronous fashion after ligand binding (Feng et al., 2015). Despite comparison between the APO and RTX/DkTx structures showed differences in the interactions between side-chain of E600 and nitrogen atoms of Y653 and D654, all residues located in the S5-S6 loop, it is unknown how conformational changes at the vanilloid binding pocket are structurally linked to upper TRPV1 channel gate operation.

Thus, channel sensing module and channel gate activation induced by temperature, ligands, voltage, pH, and lipids, along with allosteric crosstalk, require further study. These issues are directly relevant to the design and development of novel TRPV1 modulators and pain inhibition

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therapies. One of the most physiologically relevant side effects of CPZ and other TRPV1 antagonists is homeostatic deregulation leading to severe hyperthermia (Gavva et al., 2008; Honore et al., 2009), and this has been a major impediment in late-stage clinical trials for TRPV1 inhibitors (De Petrocellis and Moriello, 2013). Dissection of TRPV1 channel structure/function dynamics may lead to the development of a novel class of selective TRPV1 inhibitors that are able to target the specific pain related TRPV1 sensing modules and avoid the aforementioned metabolic side-effects.

Peptide toxins as TRPV1 channels modulators

During evolution, venomous animals have developed a large repertoire of peptide toxins that bind to predators' ion channels and produce paralysis, pain and/or death. A subset of these peptides, the family of so-called inhibitor cystine knot (ICK) toxins, consist of peptides of 26-48 amino acids in length containing three disulfide bridges in their folded structure (Craik et al., 2001). The effect of two ICK toxins, charybdotoxin (CTX) and hanatoxin, in ion channel function has been particularly well characterized from the biophysical point of view. Moreover, these peptide toxins helped to reveal structural details of the Kv channel conduction pathway well before the crystal structure of this channel became available (Hidalgo and MacKinnon, 1995; MacKinnon and Miller, 1988). CTX binds to the outer face of the pore domain of potassium channels preventing ion permeation (Banerjee et al., 2013; Goldstein et al., 1994; MacKinnon and Miller, 1988; Miller et al., 1985; Naranjo and Miller, 1996) and hanatoxin prevents ion conduction through a different mechanism that involves the direct binding to the Kv channel voltage sensing apparatus (Lee et al., 2003; Phillips et al., 2005). These precedents suggest that a better understanding of the effect of peptide

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toxins on TRPV1 channel function will also shed light on functional features that are not revealed by the detailed, but static structural study pictures.

Vanillotoxins (VaTx1-3) are members of the family of ICK peptides isolated from the venom of the spider *Psalmopoeus cambridgei*. These compounds activate TRPV1 channels with an EC_{50} in the range of 0.45-9.9 µM and inhibit voltage dependent potassium channel Kv2.1 activity with an EC_{50} of approximately 7.4µM (Siemens et al., 2006). Hanatoxin, a close relative of VaTx, binds to the S3b-S4 helix in Kv channels stabilizing the voltage sensor in its resting state and thus preventing channel opening (Alabi et al., 2007; Milescu et al., 2007; Phillips et al., 2005). Although a comparison of Kv and TRPV1 channel crystal structures suggest conserved channel architecture, the binding of VaTx to TRPV1 seems to occur through a different mechanism. Alanine scanning of the entire region encompassed by S1 and S4 failed to produce a VaTx-insensitive rTRPV1 channel (Bohlen et al., 2010). Investigating the species-specific differences in TRPV1 channel behavior has turned out to be a successful strategy for the identification of molecular determinants of ligand binding in TRPV1 channels (Gavva et al., 2004; Jordt and Julius, 2002; Phillips et al., 2004). In the particular case of VaTx, TRPV1 channels from *X. Laevis* (xTRPV1) are insensitive to the toxin (Bolen et al 2010). Bohlen *et al.* (2010) took advantage of this by generating several chimeric channels between xTRPV1 and rTRPV1. This experimental strategy helped determine that a single residue mutation at the outer end of S6 - alanine 657 to proline - accounts for VaTx1 sensitivity of the TRPV1 channel in mammals (Bohlen et al., 2010).

The Double Knot toxin (DkTx), which was extracted from the venom of the Chinese bird spider *Ornithoctonus Huwena* is a 70 amino acid, bivalent peptide that targets TRPV1 and acts as an irreversible channel opener (Bohlen et al., 2010). The two separate knotted domains (K1 and K2) display high sequence homology (67%), but after enzymatic cleavage, were shown to possess a 10-fold difference in their EC_{50} (8.9µM for K1 and 0.97µM for K2) (Bae et al., 2012; Bohlen et al.,

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2010). The EC_{50} for DkTx is increased 100-fold at TRPV1 channels with the same mutation that accounts for VaTx sensitivity (A657P) compared to wildtype (WT). Other single point mutations such as I599A at the top of S5, F649A in the pore loop, and F659A in S6 disrupt channel activation by DkTx (Bohlen et al., 2010). The advent of membrane protein structural characterization by cryo-EM also revealed mechanistic insights of DkTx binding to TRPV1 channels. Comparison of APO (closed) TRPV1 channel structures (Liao et al., 2013) to channels bound to DkTx and resiniferatoxin (Cao et al., 2013b) revealed that DkTx binding increases the distance from 4.6 to 7.6 Å between the carbonyl atoms of Gly643, which is the narrowest point of the channel selectivity filter. Additional structural changes to the channel pore that promote ion conduction involve an increase in the distance between methionines at position 644 (from 5.9 to 13Å) and a shortening of the distance between aspartates 644 (from 15.6 to 13Å). Although these findings revealed TRPV1 channel modifications promoted by DkTx binding, the moderate resolution of the TRPV1 cryo-EM (~4Å) structure did not allow for the identification of the interaction surface of the TRPV1/DkTx complex. Recent work by Bae *et al.* (Bae et al., 2016) tackled this issue with a combination of techniques including nuclear magnetic resonance (NMR), MD simulations, *in-silico* refinement of novel cryo-EM structures of the TRPV1/DkTx complex, tryptophan (Trp) fluorescence, and electrophysiological assays, and this detailed structural characterization revealed that the channel/toxin interaction occurs in a relatively small surface of 556 and 655 A^2 for K1 and K2, respectively. The residues in each lobe responsible for DkTx binding were W11 and F27 in K1 and W53 and F67 in K2 (Fig. 2), and the model also suggested direct interaction of the peptide with F649, A657, T650, and Y631 at the channel surface (Bae et al., 2016). In addition, alanine replacement of a cluster of residues in S4, S6, and the pore helix (I599, F649 and F659) strongly influenced the effect of DkTx on the TRPV1 channel (Bohlen et al., 2010). Trp fluorescence experiments showed that K1 partitions more efficiently into the membrane, and since K2

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displayed a larger affinity for the channel than K1 (Bohlen et al., 2010), it has been proposed that the initial step of DkTx binding is the interaction of K1 with the membrane in order to increase the local concentration of DkTx and a later step involves the interaction of both lobes with the channel (Bae et al., 2016).

The replacement of residues I599 and F659 decreased DkTx effects on TRPV1 channel, but neither of these residues are in direct contact with the peptide, suggesting the existence of a more intricate network of interactions during DkTx-mediated TRPV1 channel gating (Bae et al., 2016). Figure 2a is a snapshot from a 10 ns-molecular dynamic simulation of the TRPV1 channel (PDB ID3J5Q) in the presence of DkTx from side (Fig. 2a) and upper (Fig 2b) views, and closer views of K1 lobe and K2 lobe interaction with the TRPV1 channel surface are shown in Fig. 2b and Fig. 2c, respectively. TRPV1 channel residues involved in DkTx binding are highlighted in red and the key residues at DkTx are displayed in atom representation using the VMD software (Humphrey et al., 1996).

The coexistence of toxin-lipid and toxin-protein interaction surfaces increase the lifetime of the channel/DkTx complex dramatically, and therefore, DkTx induces virtual irreversible opening of the WT form of the channel (Bae et al., 2016; Bohlen et al., 2010). However, the modulation exerted by lipids in the binding of DkTx to the TRPv1 channels seems to not be limited to this type of toxin. For example, the NaV1.7 channel inhibitor Hainatoxin I, a 30 amino acid peptide derived from *Haemadipsa hainana* spider venom, does not bind POPG membranes, but the presence of the inhibitor in the lipid phase increases significantly after introducing single and double point mutations at G7W and N24S. This increase in distribution of the lipid phase is accompanied by a 4-15 fold decrease in the EC_{50}, which indicates that lipid/peptide interactions play a role in stabilizing the Hainatoxin I/ NaV1.7 channel complex (Klint et al., 2015) as was shown to occur for DkTx (Bae et al., 2016).

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The work by Andreev *et al.* showed that a 56-amino acid peptide termed APHC1 (Analgesic Peptide *Heteractis Crispa*) obtained from the sea anemone *Heteractis crispa* inhibits capsaicin-meditated rTRPV1 channel activation with an EC_{50} of 54 nM and a maximal inhibition of 32% relative to the control response. Despite its moderate apparent affinity and low potency, systemic administration of APHC1 induced analgesic activity without producing hyperthermia in rodents during pain related behavioral tests (Andreev et al., 2008). These findings were extended to other related peptides found in *Hetereactis crispa*, including APHC2 andAPHC3, both of which display analgesic activity in rodents (Kozlov et al., 2009). Interestingly, even though APHC1 and APHC3 differ in only 4 out of 56 amino acids, only APHC3 is able to inhibit low pH-mediated activation of TRPV1 channels (Andreev et al., 2013). In agreement with these results, APHC3 was effective in displaying analgesic activity after intraperitoneal injection of acetic acid, a classic test for evaluating pain responses after tissue acidification (Andreev et al., 2013).

Changes in body temperature have been reported for several inhibitors of TRPV1 channels. AMG9810 inhibits capsaicin-, pH-, and temperature-mediated activation of TRPV1 channels and induces a dose-dependent increase in rat body temperature at a systemic level (Gavva et al., 2007). Overcoming a major problem of almost all TRPV1 inhibitors tested to date, APHC1 and APHC3 induce only a small, non-statistically significant 0.3-0.5ºC increase in body temperature. These finding not only make APHC1 and APHC3 excellent probes for biophysical studies that attempt to understand the structural basis of TRPV1 gating, but these compounds also display remarkable potential as drug design templates for pain inhibition.

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Modulation of TRPV1 channel gating by Lipids

Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) only represents a small fraction of lipids at the plasma membrane inner leaflet in eukaryotic cells, but this lipid is a key component of several signaling pathways (McLaughlin and Murray, 2005). PI(4,5)P_2 also directly modulates the function of several ion channels and transporters (Robertson, 2007; Suh and Hille, 2005), including members of the TRP channels family (Qin, 2007; Rohacs, 2014). The mode of action of PI(4,5)P_2 in TRPV1 channels has been the subject of debate [cf., (Cao et al., 2013a; Senning et al., 2014)], however, there is apparent consensus that PI(4,5)P_2 behaves as a TRPV1 channel agonist (Brauchi et al., 2007; Lukacs et al., 2007; Poblete et al., 2015; Senning et al., 2014; Ufret-Vincenty et al., 2015; Ufret-Vincenty et al., 2011). Brauchi *et al.* (2007) showed that alanine replacement of two positively charged residues (R701 and K710) located at the C-terminal region of the TRPV1 channel shifted the dose-response curve of PI(4,5)P_2 to the right. The mutation of these residues caused a 1000-fold increase in the EC_{50}, but had no effect on voltage or temperature gating of TRPV1, which suggests that two separate allosterically coupled sensing modules exist (Brauchi et al., 2007). This study was extended in work by Poblete *et al.* (2015) utilizing MD simulations of rTRPV1 crystal structures at 3.4 Å resolution (Cao et al., 2013b), site directed mutagenesis, and patch clamp electrophysiology; this study found that the neutralization of two positively charged residues at the S4-S5 linker (R575, R579) and K694 at the TRP domain decreased the apparent affinity of the TRPV1 channel for PI(4,5)P_2. MD simulations also showed that capsaicin and PI(4,5)P_2 induce opening of the lower activation gate at I679 in a process that involves Q561, K571 and K694 and causes an increase in the curvature of S6-TRP domain by ~20° when moving from the closed to open state. [Fig. 3 (Poblete et al., 2015)]. The Cryo-EM structure of TRPV1 channel in its APO form, shows an electron density at the interface between the S4 and the S5/S6 of adjacent subunits that corresponds to a phosphatidylinositol molecule (Gao et al., 2016). Despite there is a rough

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coincidence in the location of the phospholipid binding site inferred from Cryo-EM structure (Gao et al., 2016) and those proposed for PI(4,5)P_2 binding after electrophysiological experiments and MD simulations by Poblete et al. (2015), the position of the lipid in both studies is different. However, it is possible that the two observations can be reconcile if we consider that PI(4,5)P2 has two extra phosphate groups in PI(4,5)P_2 that can interact with the positively charged residues reported by Poblete et al. (2015) (see Figure 3).

PI(4,5)P_2 induces the opening of the Kir2.2 potassium channel and regulates the cell´s resting potential (Hilgemann et al., 2001). The crystal structure of Kir2.2 was obtained in the presence of a short chain derivative of PI(4,5)P_2 that promotes channel opening (Hansen et al., 2011). Comparisons with the binding site for PI(4,5)P_2 proposed by Poblete *et al.* (2015) showed similarity to the Kir2.2 crystal structure (Hansen et al., 2011) in terms of pocket electrostatics and distance from the channel pore (Poblete et al., 2015). These features make it tempting to speculate that the mechanism for PIP2-dependent gating is conserved in TRPV1 and distantly related ion channels, but additional experimental evidence is required to confirm this.

As stated above, based on structural and functional studies, it has been proposed that the S4-S5 linker plays a pivotal role in coupling the channel sensing modules with the pore domain in voltage dependent K-channels (Chowdhury et al., 2014; Long et al., 2005b; Lu et al., 2002). For TRPV1, structural data shows that the S4-S5 linker appears to be close to the `TRP domain´ located at the proximal C-terminal region. In agreement with functional studies, it has been suggested that these domains may constitute the machinery that couples the channel sensing modules to the activation gate (Boukalova et al., 2010; Brauchi et al., 2007; Cao et al., 2013b; Susankova et al., 2007; Taberner et al., 2014; Yang et al., 2015a). The binding site of the PI(4,5)P_2 is therefore nontrivial as it seems that this location may be related to the channel activation gate, although the modulation of channel gating has not been exhaustively dissected as a function of PI(4,5)P_2

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chemistry. However, this may eventually lead to the discovery of novel compounds from the *de novo* design of TRPV1 channels modulators.

Lysophosphatidic acid (LPA) plays a role in many cellular processes including cell migration, apoptosis, cell differentiation, and angiogenesis (Oude Elferink et al., 2015). It has been proposed that LPA is the trigger for neuropathic pain through a signaling cascade involving the LPA receptor and the Rho-Rho kinases pathway (Inoue et al., 2004). As shown in rats, LPA potentiates TRPV1 activity in dorsal root ganglia neurons during bone cancer via an indirect mechanism involving protein kinase C-epsilon (Pan et al., 2010) and this potentiation of TRPV1 channel activity occurs after the blockage of the signaling pathways associated with LPA. Given that the effect of LPA was markedly reduced using extracellular LPA applications, an intracellular LPA binding site was hypothesized to mediate these actions (Nieto-Posadas et al., 2012). Deletion of the channel region comprising residues 777-821 rendered the channel LPA-insensitive, suggesting that the LPA binding site is located at the C-terminus of the channel. Further charge neutralization of R701 and K710 showed that these residues, which had previously been proposed to stabilize the PIP2 binding pocket (Brauchi et al., 2007), are key components in LPA-dependent potentiation of TRPV1 channel activity (Morales-Lazaro and Rosenbaum, 2015; Nieto-Posadas et al., 2012).

Cholesterol, an abundant component of biological membranes, is involved in membrane mechanical stability, fluidity, and subdomain organization (Garcia-Saez and Schwille, 2010). Cholesterol directly modulates the function of several ion channels, and at least three cholesterol-binding motifs have been described to date (Levitan et al., 2014). Depletion of cholesterol from membranes of dorsal root ganglia (DRG) neurons via methyl-β-cyclodextrin (MβCD) application decreases both capsaicin-induced responses and TRPV1 immunoreactivity. These effects where specific for TRPV1 and not $P2X_3$ receptors, suggesting that cholesterol is involved in the stability of lipid rafts where TRPV1 channels are located (Liu et al., 2006). Consistent with this hypothesis,

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pharmacological disruption of lipid rafts in DRG neurons by the enzyme sphingomyelinase decreases the calcium influx in response to capsaicin (Szoke et al., 2010). To avoid trafficking effects that had previously been reported by Liu *et al.* (2006), Picazo-Juarez *et al.* (2011) assessed the effects of cholesterol levels on TRPV1 channel function in excised membrane patches. Data from excised membrane patches suggested that cholesterol specifically binds to rTRPV1 channels; an increase of cholesterol levels decreased capsaicin-evoked currents, while the increase of cholesterol's stereoisomer epicholesterol did not. The sensitivity of rTRPV1 channels to cholesterol was affected by mutations at isoleucine 585 in S5, a region wherein the sequence contains a cholesterol-binding motif between residues 579 to 586 (Picazo-Juarez et al., 2011). These finding suggest that cholesterol levels are critical for TRPV1 channel function in several ways and a decrease in its levels may produce structural disruption of membrane elements that are key for ion channel function/assembly. On the other hand, increases in cholesterol levels inhibit channel activity possibly by trapping the channel in its closed state (Picazo-Juarez et al., 2011).

Concluding Remarks

The TRPV1 channel is a polymodal receptor whose activation is driven by ligands, heat, voltage and lipids (Baez-Nieto et al., 2011; Nilius and Voets, 2004). This channel has become an attractive target for developing novel pain inhibitors due to its role in nociceptive and inflammatory responses. The structure of the TRPV1 channel has been resolved by cryo-EM (Cao et al., 2013b; Liao et al., 2013), and this work has provided most of the structural details for protein-ligand docking algorithms that rapidly evaluate the binding of thousands of compounds from virtual libraries (Sousa et al., 2013). However, the coexistence of several activation modules in the same protein is a double-edge sword; while the dissection of the channel activation modes and their allosteric crosstalk results are fascinating from a biophysical point of view, the polymodal nature is

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a pharmacologist's nightmare that complicates the transit from *in vitro* assays to clinical trials. Several molecules acting as potent TRPV1 channel inhibitors displayed analgesic activity during *in vivo* assays, but were also found to produce hyperthermia or impaired heat sensing, rendering them unsafe for human use (Lee et al., 2015). Thus, future work will require function-oriented pharmacology which includes the suppression of activation of the module that is sensitive to decreases in external pH, a widespread phenomenon during inflammatory processes (White et al., 2011). To achieve this goal, a detailed biophysical understanding of ion channel function will be necessary. Future efforts should also include a search for the structural determinants of each sensing module and aim to understand how these domains are functionally connected.

Authorship contribution

Wrote or contributed to the writing of the manuscript: Latorre, Diaz-Franulic, Gonzalez-Nilo

Performed Molecular Dynamics simulations and data analysis: Sepulveda and Cáceres-Molina

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Footnotes

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Figure Legends

Figure 1. The Vanilloid Binding Pocket of the TRPV1 channel. a) Side view of the TRPV1 channel bound to a capsaicin molecule (red) embedded in a POPC lipid bilayer in a 140Å periodic box containing 100 mM of KCl. b) Chemical structure of capsaicin (CAP). Right: Lowest energy conformation (ΔG=-7.2 kcal x mol^{-1}) from fifty independent docking simulations of capsaicin into the vanilloid binding pocket of TRPV1 (PDB:3J5Q) where residues involved in capsaicin binding (Y511, T550 and E570) are pointed by arrows. d) Left: Chemical structure of capsazepine (CPZ). Capsazepine into the vanilloid binding pocket of TRPV1 (PDB:5IS0) where residues involved in capsazepine binding I514 and V518 in S3 and M547are pointed by arrows. e) Left: Chemical structure of Resiniferatoxin (RTX). f) Resiniferatoxin into the vanilloid binding pocket of TRPV1 (PDB: 5IRX). Transmembrane segments of the TRPV1 channel are color coded as: S3 in light blue, S4 in green, S4-S5 linker in purple and S6 in blue.

Figure 2. Binding of the Double Knotted Toxin (DkTx) to the TRPV1 channel. a) Side view of the TRPV1 channel bound to DkTx (K1 lobe and linker in green and K2 lobe in light blue) embedded in a POPC lipid bilayer in a 140Å periodic box containing 100mM of KCl. b)Upper view of the system showed in a). c) Closer view of the K1 lobe and the K1-K2 linker (green) where the key residues in toxin binding are showed in licorice representation and labeled in light blue. Residues at the channel showed to be involved in DkTx mediated channel opening are colored in red. d) Closer view of the K2 lobe (light blue) where the key residues in toxin binding are showed in the licorice

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representation (light blue) Residues at the channel showed to be involved in DkTx mediated channel opening are colored in red.

Figure 3.Atomistic representation of PI(4,5)P_2 binding site in TRPV1 channel a) Side view of TRPV1+PI$(4,5)_2$ complex where PI$(4,5)_2$ bound at the interface of two adjacent subunits is colored in yellow. b) Closer view of the PI$(4,5)_2$ binding pocket in TRPV1 channel. Key residues involved in TRPV1+PI$(4,5)_2$ interaction are R575, R579 and K694 according to after Poblete et al. (2015).

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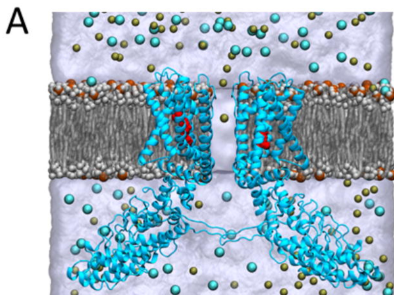
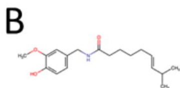
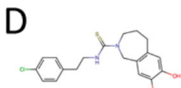
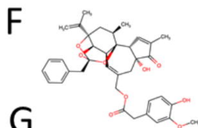
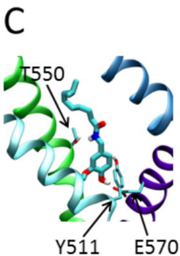
Figure 1

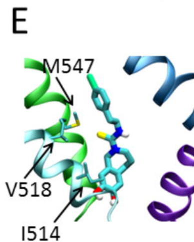












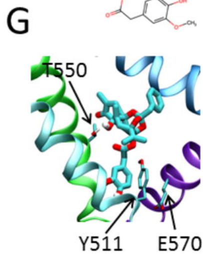
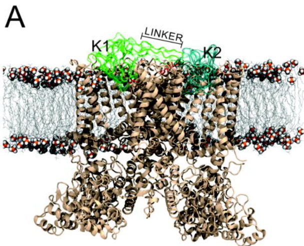
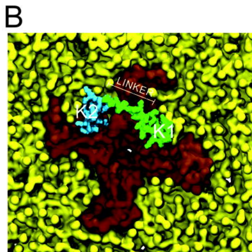
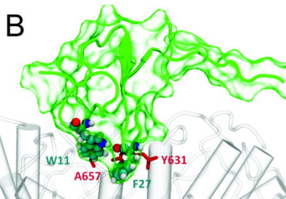


Figure 2







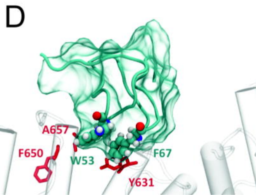
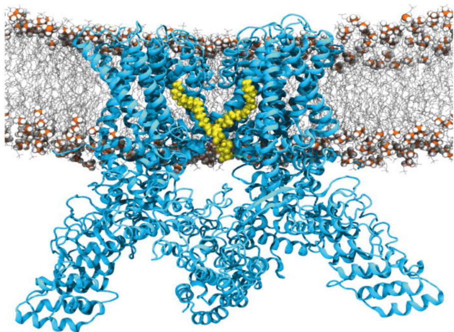


Figure 3

A



B

R579

PI(4,5)P_2

R575

K694