REVIEW

Gain-of-Function Mutations in the Transient Receptor Potential Channels TRPV1 and TRPA1: How Painful?

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Summary

Gain-of-function (GOF) mutations in ion channels are rare events, which lead to increased agonist sensitivity or altered gating properties, and may render the channel constitutively active. Uncovering and following characterization of such mutants contribute substantially to the understanding of the molecular basis of ion channel functioning. Here we give an overview of some GOF mutants in polymodal ion channels specifically involved in transduction of painful stimuli – TRPV1 and TRPA1, which are scrutinized by scientists due to their important role in development of some pathological pain states. Remarkably, a substitution of single amino acid in the S4-S5 region of TRPA1 (N855S) has been recently associated with familial episodic pain syndrome. This mutation increases chemical sensitivity of TRPA1, but leaves the voltage sensitivity unchanged. On the other hand, mutations in the analogous region of TRPV1 (R557K and G563S) severely affect all aspects of channel activation and lead to spontaneous activity. Comparison of the effects induced by mutations in homologous positions in different TRP receptors (or more generally in other distantly related ion channels) may elucidate the gating mechanisms conserved during evolution.

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Introduction

The family of Transient Receptor Potential (TRP) ion channels mediates numerous sensory transduction processes, such as thermosensation, mechanosensation, sensation of irritative chemicals and different kinds of taste (for more information, see the TRP channel database: http://www.iuphar-db.org/DATABASE/FamilyMenuForward?familyId=78). As the voltage dependent potassium (K_v) channels, the TRP channels consist of four subunits, each with N- and C-terminal cytoplasmic domains, six transmembrane regions (S1-S6) and the central pore lined by S5 and S6 together with the loop between them (Fig. 1A) (Moiseenkova-Bell and Wensel 2011, Kalia and Swartz 2013). Among TRP channels, TRPV1 and TRPA1 are known to be specialized to transduce painful stimuli in mammals (Moran *et al.* 2011, Nilius and Owsianik 2011, Nilius *et al.* 2012). High temperature, low pH and some natural pungent compounds such as capsaicin act on TRPV1 to elicit nociception. TRPA1 is targeted by many environmental irritants including allyl isothiocyanate, responsible for the pungent taste of horseradish, and cinnamaldehyde from cinnamon. The activity of both channels is also modulated by voltage, but their sensitivity to depolarization is much lower than in K_v channels. Different parts of TRP channels are responsible for the sensitivity to different agonists (Nilius and Owsianik 2011, Winter *et al.* 2013). Due to the resulting allosteric gating, it is necessary to have in mind the complex character of TRP channels when evaluating the

mutational analysis data. Gain-of-function (GOF) mutation may enhance the responses of the channel to one type of stimuli, but sensitivity to other agonists may be unchanged or even diminished. Amino acid substitutions which affect gating can lead to altered

phenotype regarding to all aspects of channel activation (Myers *et al.* 2008b, Minor 2009, Pertusa *et al.* 2012). Such GOF mutations are invaluable in elucidating the mechanisms of signal transduction from different domains of the protein complex to channel gating.
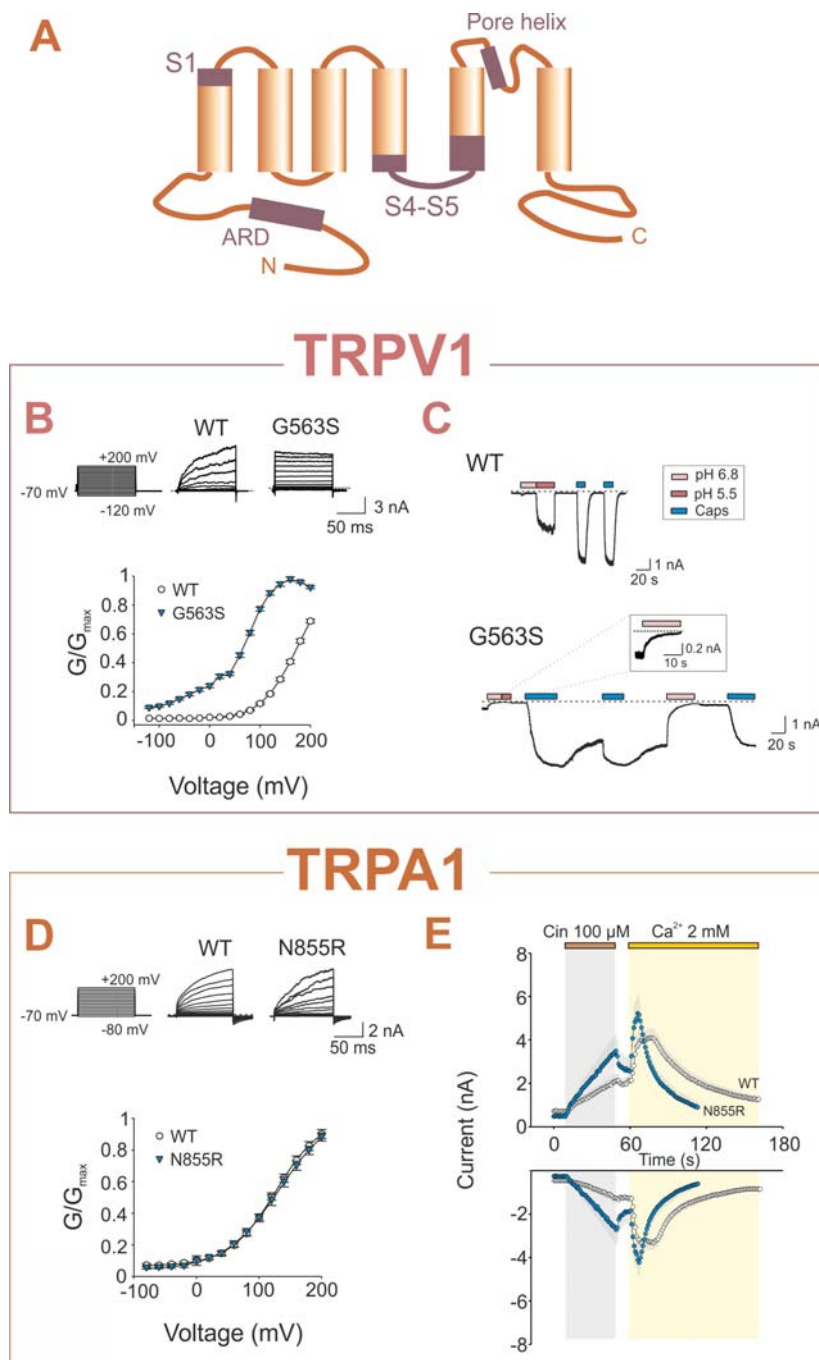

Fig. 1. **(A)** The topology of TRP channel subunit, that is formed by six transmembrane spanning domains (in orange), N- and C-cytoplasmatic ends. N-terminus contains ankyrin repeat domain (ARD), that is important in channel regulation. The regions containing gain-of-function mutation of TRP channels are depicted in darker color – extracellular part of the first transmembrane domain S1, loop between S4 and S5 helix (S4-S5) and the pore helix situated in the linker between S5 and S6. **(B)** Upper traces, representative whole cell patch clamp current recordings in response to voltage steps (from −120 mV to +200 mV) in HEK293T cells transfected with wild-type TRPV1 and G563S mutant. Lower trace, normalized conductance-voltage relationship obtained from steady state currents at the end of the pulse. The voltage depencence of G563S mutant is shifted towards less depolarizing voltages compared to wild-type TRPV1. **(C)** The sample recording of whole cell current responses of wild-type TRPV1 (upper trace) and G563S mutant (lower trace) to consecutive applications of low pH and 1 µM capsaicin. Holding potential −70 mV. Note the inhibitory effect of protons (pH 6.8) on TRPV1-G563S channel function, shown in inset. **(D)** Upper traces, representative current traces of wild-type TRPA1 and its N855R mutant in response to indicated voltage step protocol (voltage steps from −80 mV to +200 mV; increment +20 mV; holding potential −70 mV). Lower trace, normalized conductance-voltage relationship for wild-type TRPA1 (white symbols) and N855R mutant (blue symbols) fitted with Boltzmann equation. The data were obtained using the same voltage step protocol as shown in upper traces. **(E)** Time course of representative whole cell currents through human TRPA1 measured at +80 mV (upper trace) and −80 mV (lower trace). The horizontal bars above the records indicate the duration of 100 µM cinnamaldehyde and Ca^{2+} application. The chemical sensitivity of N855R mutant is increased and the inactivation kinetics is faster than in wild-type TRPA1. The voltage dependence of N855R mutant is unchanged. The data represent the means ± standard error.

GOF mutants in TRPV1 – proton sensitized or inhibited?

Several GOF mutants of the TRPV1 ion channel were revealed by unbiased genetic screening of a

randomly generated population of TRPV1 mutants, which were tested for the ability to cause growth defects when expressed in yeast strains (Myers *et al.* 2008a). Two of the uncovered mutations, K160E and K155E, are located in the ankyrin repeat domain (ARD). These two

lysine residues were previously shown to modulate TRPV1 sensitivity and desensitization properties by binding ATP and calmodulin (Lishko *et al.* 2007). The study of Myers and colleagues (2008a) also revealed the important role of the outer pore region in TRPV1 gating. When mutated, the phenylalanine F640 and adjacent threonine T641 residues located in the putative flexion between pore helix and selectivity filter render the TRPV1 channel constitutively active with increased basal activity and enhanced chemical sensitivity. The authors hypothesised that F640L and T641S mutations mimic the proton-potentiated state, as they were insensitive to proton-mediated potentiation.

In our project aimed at mutational analysis of the transmembrane region of the TRPV1 ion channel, we depicted several overactive mutants with strikingly similar phenotypic properties comprising increased basal activity and voltage sensitivity, altered kinetics of capsaicin-induced responses and incomplete deactivation after capsaicin washout. In contrast to wild-type TRPV1, low pH neither activates nor potentiates these GOF mutants, but instead stabilize their resting conformation (Pertusa *et al.* 2012) (Fig. 1B,C). These findings were surprising to us, considering that the mutations are located in distant regions of the protein subunit, such as (i) extracellular portion of S1 (R455K mutation), (ii) lower part of S4 and S4-S5 linker (R557K and G563S) and (iii) pore helix (T633A) (Boukalova *et al.* 2013).

Uncovering the GOF mutations in the S4/S4-S5 region is consistent with its putative role in voltage sensing and signal transduction from peripheral domains (S1-S4) to the pore-forming domain subsequently leading to the opening or closing of the channel (Boukalova *et al.* 2010). On the other hand, finding of constitutively active mutant with single amino acid substitution in the peripheral region of TRPV1 protein complex was not expected. So far, R455K is the only overactive mutation found in the S1-upper S4 region of TRPV1 protein complex, which forms binding capsule for chemical agonists (Jordt and Julius 2002, Gavva *et al.* 2004), but which direct role in ion channel gating was not presumed. According to previously published homology model of TRPV1 tetramer (Brauchi *et al.* 2007), it seems that arginine R455 and threonine T633 in the pore helix of adjacent subunit could be in close proximity. We hypothesized that, in analogy to K_v channels, S1-pore interface between neighbouring subunits of TRPV1 might serve to stabilize conformations associated with channel

gating (Lee *et al.* 2009). This presumption could explain the similarity of R455K and T633A mutant phenotypes and the crucial role of arginine in S1 in TRPV1 gating.

The most prominent feature of the GOF mutants described recently by us is the lack of low pH-induced activation. Conversely, relatively mild acidification of the extracellular solution inhibits the spontaneous activity and also the inward current remaining after capsaicin washout in R455K, R557K, G563S and T633A (Fig. 1C). Unlike in the outer pore region mutants F640L and T641S (Myers *et al.* 2008a), the phenotype of these mutants cannot be simply explained as mimicking the proton-potentiated state of TRPV1, as they failed to be activated by severe acidification of the extracellular solution (pH 5.5). In addition, in R455K, T633A and the S4-S5 overactive mutants we observed significantly slowed kinetics of the first capsaicin-induced response, which does not correspond to low pH-stimulated wild-type TRPV1 characterized by much faster onset rate of responses elicited by capsaicin.

In R455K, R557K, G563S and T633A, the washout phase of current responses after removal of capsaicin was markedly slowed down and the current did not deactivate to the basal level. The subsequent application of capsaicin led to rapid augmentation of the current amplitude, which in some cells was followed by again much slower onset phase (Fig. 1C). It appears that once exposed to capsaicin, the closed state of mutant ion channels is acutely destabilized. On the other hand, mild acidification of the extracellular space (pH 6.8) resulted in rapid and irreversible deactivation of the basal activity and also the current remaining after capsaicin washout. Overall, it seems that the overactive mutants can adopt at least two modes of activation and it is possible to switch between them using either capsaicin (to open the channel and leave it opened) or low pH (to close the channel and leave it closed). The question is whether this feature is unique for the GOF mutants, or whether it reflects innate phenotypic aspects of wild-type TRPV1 unmasked by the mutations. The later option is favoured by the fact that the same phenotype is found in several TRPV1 mutants with single amino acid substitution in different parts of the protein.

Gain-of-function: which function of the many?

High throughput unbiased functional screens and a combination of molecular evolution-selection

approaches helped to reveal a number of interesting mutants in TRPV1, TRPV3 and TRPM8 channels (Bandell *et al.* 2006, Grandl *et al.* 2008, 2010, Myers *et al.* 2008a). Pools of many thousands of mutants have been explored using random mutagenesis and calcium imaging in transfected HEK293 cells which led to the identification of some key regions of these channels that are likely to be important for channel activation or pore permeation (Pertusa *et al.* 2012). However, for the 250 residue transmembrane part of the TRPV1 channel subunit, for example, the theoretical number of possible sequences is 20^{250} which is a number exceeding our imagination and all our experimental capacities. Moreover, for the thermosensitive TRP channels, the prototypically polymodal ion channels, it is important to test as many aspects of function as possible for each mutant because the activation modes can be readily uncoupled. No doubt, the chance of discovering a mutation that changes the function of an ion channel has to be greatly increased by combining an appropriate analogy, reasoning and informed intuition.

In our recent project focused on the sixth transmembrane domain of the human transient receptor potential ankyrin receptor subtype 1 (TRPA1) channel (Story *et al.* 2003, Jordt *et al.* 2004), we aimed to identify residues that are likely to be core elements of the gating mechanism (Benedikt *et al.* 2009). This channel can be activated by a vast number of pungent and irritant chemicals that either covalently interact with (isothiocyanates, cinnamaldehyde, acrolein, allicin, oxidants and lipid peroxidation products) or bind to TRPA1 (cannabinoids, icilin, eugenol, thymol, nicotine), and can also be activated by deep cooling ($<17\,°C$) or depolarizing ($>+100$ mV) voltages (Bandell *et al.* 2004, Corey *et al.* 2004, Bautista *et al.* 2005, Macpherson *et al.* 2005, Nagata *et al.* 2005, Sawada *et al.* 2007, Caceres *et al.* 2009, Hu *et al.* 2009, Karashima *et al.* 2009, Andrade *et al.* 2012, Nilius *et al.* 2012). Using mutagenesis, electrophysiology and sequence homology with certain potassium and sodium channels, we identified several residues within the S6 inner pore-forming region that contribute to allyl isothiocyanate (AITC) and voltage-dependent gating. We found that alanine substitution in the conserved mid-S6 proline (P949A) strongly affected the activation/deactivation and ion permeation. The P949A was functionally restored by substitution with a glycine but not by the introduction of a proline at positions –1, –2 or +1, which indicated that, just like in K_v channels, a flexible residue in the middle of S6 is

structurally required for the normal functioning of the TRPA1 channel. Notably, we found a residue N954, at which alanine substitution generated a constitutively open (i.e. GOF) phenotype, suggesting a role in stabilizing the closed conformation. In a prospective unbiased functional screen, this mutant would probably remain unrecognized or misinterpreted as a loss-of-function, because AITC, at first, only slightly potentiates the currents through the N954A channels, but this is followed by a complete inactivation at negative membrane potentials (Benedikt *et al.* 2009). Our results also pointed to important functional roles for the two distal glycines G958 and G962, comprising the distal G*XXX*G-motif. The G958A "GOF" mutation strongly decreased the inactivation rate of AITC-induced whole-cell currents, whereas the G962A mutation led to a dramatically delayed onset of the secondary phase of AITC-induced activation, indicating that the distal G962 stabilizes the open conformation. G958, on the other hand, provides additional tuning leading to decreased channel activity. It seems likely that these two glycines play a role similar to that of the distal bi-glycine motif G$(X)_3$G of the conserved IS6 domain of the $Ca_V1.2$ and $Ca_V2.3$ channels (Raybaud *et al.* 2006).

In fact, for the polymodal TRPA1 ion channel, it is not easy even only to define what is the "gain-of-function" because the channel has various activation modes and undergoes desensitization which depends on many factors, including its own activity (Story *et al.* 2003, Jordt *et al.* 2004, Nilius and Owsianik 2011, Nilius *et al.* 2012). For example, by performing the systemic neutralization of 27 positively charged residues within the C-terminal tail of human TRPA1, we identified eight residues that are important to the allosteric regulation of the channel by chemical and voltage stimuli (K969, R975, K989, K1009, K1046, K1071, K1092, and R1099). In addition, we revealed three charge-neutralizing "GOF" mutants (R975A, K988A, and K989A) which exhibited higher sensitivity to depolarizing voltages, indicating that these residues may be directly involved in the voltage-dependent regulation (Samad *et al.* 2011). However, the currents induced by AITC at negative membrane potentials were small or indistinguishable from wild-type in these mutants.

GOF mutants in the distal C-terminal acidic motif of TRPA1

An ubiquitous and physiologically important modulator of TRPA1 are calcium ions (Ca^{2+}), which enter

through the open channel, or are released from internal stores, and dynamically control channel opening probability, unitary conductance, ion selectivity (Nagata *et al.* 2005, Cavanaugh *et al.* 2008, Patil *et al.* 2010, Nilius and Owsianik 2011), but also surface expression levels (Schmidt *et al.* 2009). Intracellular Ca^{2+} ions activate the channel at micromolar concentrations (EC_{50}=0.9-6 µM) (Doerner *et al.* 2007, Zurborg *et al.* 2007) and strongly potentiate chemically and voltage-induced responses (Wang *et al.* 2008). This potentiation is followed by an almost complete and irreversible inactivation, and both processes are accelerated at higher intracellular concentrations of Ca^{2+}. In our recent study, we identified the residues within the distal C-terminal domain of TRPA1 that when mutated affected the Ca^{2+}- and voltage-dependent gating of the channel (Sura *et al.* 2012). This study revealed several acidic residues in the TRPA1 cytosolic C-terminus that play important roles in Ca^{2+}-dependent modulation and may represent a candidate region for the site of Ca^{2+} binding. According to our molecular dynamics simulations, the $I^{1074}ISETEDDDS^{1083}$ motif has been shown to form a Ca^{2+} binding loop, in which the two residues D1080 and D1082 are predicted to be crucial for binding Ca^{2+}, whereas the side chains of I1074 and E1077 are in contact with the calcium ion using their main chain carbonyl oxygen atoms. We identified residues E1073, E1077, D1080, D1081, and D1082, the specific properties of which are not involved in cinnamaldehyde activation but all appear to be most important for the modulation of the TRPA1 channel by Ca^{2+}. Notably, we found two mutants in this region, E1077A and E1077K, in which the degree of potentiation of cinnamaldehyde responses by 2 mM Ca^{2+} was markedly reduced obviously due to their initial close-to-saturation state at +80 mV. We reasoned that this sensitizing effect might reflect either a gain-of-function (constitutively active) phenotype or tonic activation due to an increased expression of the mutant channels on the cell surface. In this region, we identified two strong consensus phosphorylation motifs containing S1076 and T1078, both predicted to be targeted by casein kinase CK2. Therefore, we tested additional mutants in which either serine or threonine were replaced by either alanine or aspartate to mimic the nonphosphorylated and phosphorylated forms of the TRPA1 protein, respectively. We found that the nonphosphorylatable mutant S1076A and the phosphorylation-mimicking S1076D mutant channels were normal in all general

aspects of functionality. Mutation T1078A exhibited substantially reduced responses to voltage and cinnamaldehyde and was capable of strong potentiation by 2 mM Ca^{2+}, whereas mutation T1078D resulted in TRPA1 channels whose conductance to voltage relationships were also strongly augmented and shifted toward less depolarizing potentials. These findings indicate that the functional changes caused by other substitutions in this region are likely to be steric or local, rather than related to changes in the phosphorylation-dependent membrane insertion of the channels. We, however, did not exclude the possible involvement of these amino acid residues in the recently proposed mechanism by which localized Ca^{2+} influx upon channel activation controls TRPA1 functionality through its acute translocation to the membrane (Schmidt *et al.* 2009).

GOF mutant of TRPA1 – pain-related channelopathy

The TRPA1 channel has been recently linked to a heritable human pain disorder (Kremeyer *et al.* 2010). In the S4-S5 linker of TRPA1, the missense mutation N855S that leads to increased channel activity has been revealed as a cause of familial episodic pain syndrome manifested as paroxysmal pain induced by tiredness, fasting or cold (Kremeyer *et al.* 2010). In their study, the authors demonstrate that the N855S mutant exhibits a 4-fold increase in inward currents on activation by cinnamaldehyde, menthol, the endogenous aldehyde 4-hydroxynonenal, or cold at normal resting membrane potentials. Removing extracellular Ca^{2+} ions shifted the voltage-dependent activation by +20 mV in both the wild-type and the N855S mutant, but under the Ca^{2+}-free conditions, the cooperativity of voltage-dependent gating decreased only in N855S, but not in the wild-type TRPA1 channels. This finding was interpreted as indicating that this mutation might destabilize the closed conformation of the channel, which could account for a more general effect on channel gating (Smith-Maxwell *et al.* 1998) and is consistent with the proposed functional role for the S4-S5 linker in the gating of other thermosensitive TRP channels (Brauchi *et al.* 2007, Voets *et al.* 2007, Boukalova *et al.* 2010). It is generally accepted that the electrophilic compounds, such as allyl isothiocyanate activate TRPA1 through covalent binding at specific cysteine residues on the intracellularly located N-terminus (Hinman *et al.* 2006, Macpherson *et al.* 2007, Kang *et al.* 2010). It is, however, not known how

covalent cysteine modifications translate into TRPA1 activation. Simulations based on a low-resolution electron density map indicate that the chemical signal can be readily conveyed through cytoplasmic domains to the intracellular channel gate (Cvetkov *et al.* 2011). Although to discern the individual activation pathways in TRPA1 is very difficult even if we knew the actual structure of the channel (Cvetkov *et al.* 2011), it can be supposed that, analogous to K_v channels, the conformational changes within the S1-S4 "sensors" are converted by the inner S4-S5 linker helices directly into gate opening and closing through the motions of the S6 inner helices which dilate (open) and constrict (close) the pore entryway (Long *et al.* 2005a,b, 2007, Brauchi *et al.* 2007, Benedikt *et al.* 2009, Salazar *et al.* 2009). Interestingly, we found that the introduction of a positively charged residue at position N855 (N855R) increased the TRPA1-mediated current responses to cinnamaldehyde and speeded up the inactivation kinetics without affecting conductance to voltage relationships (Fig. 1D,E). This indicates that the mutation altered the chemical but not the voltage activation pathway of the TRPA1 channel.

Future perspectives

Chronic pain conditions, resulting from disease, injury or inherited, cause unimaginable suffering and constitute a huge burden for the individual and society. Both, TRPV1 and TRPA1 are implicated in acute and chronic pain states and are intensively studied as potential therapeutic targets (Brederson *et al.* 2013). Mutations in genes encoding ion channels associated with nociceptive pathways, e.g. voltage-gated sodium, potassium and calcium channels, TRP channels, ASIC channels or purinergic receptors, have been described to cause a variety of pathological states (Cregg *et al.* 2010, Lampert *et al.* 2010, Raouf *et al.* 2010, Nilius and Voets 2013, Waxman 2013). It is likely that with ongoing research further mutations will emerge and therefore future structural and functional studies will be necessary to enhance our understanding of the unique features of these channels and to elucidate how their pain-related functioning can be manipulated to therapeutic advantage.

Conflict of Interest
There is no conflict of interest.

Acknowledgements

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