Polymodal Ligand Sensitivity of TRPA1 and Its Modes of Interactions

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Understanding the mechanism of peripheral pain sensation has progressed remarkably in recent years, in part thanks to the discovery of transient receptor potential (TRP) ion channels in sensory neurons. Among these ion channels, the TRPA1 subtype is attracting attention because of its ability to sense a wide spectrum of damage signals from external and internal environments, which provides for a novel paradigm for the modulation of ion channels. In this Perspective, we focus on the TRPA1 ligands and cofactors, which are grouped in terms of their reactivity and binding fashions. The mechanism(s) of binding and possible pharmacological implications on TRPA1 will also be discussed because the polymodality of TRPA1 in signal sensing suggests that this channel may be a promising target for pain modulation.

The Journal of General Physiology

Introduction

Currently, the literature on TRPA1 is increasing exponentially, reminiscent of that on TRPV1 in the late 1990s. The scientific interest in TRPV1 reflects the idea that TRPV1 is a novel and promising therapeutic target for pain relief. While waiting for clinically effective TRPV1 modulators, our knowledge has expanded about the nature of peripheral pain sensation, including our understanding of heat- and acid-induced pain, neurogenic inflammation, the roles of cytoplasmic modulators, pharmacophores of TRPV1 ligands, and information related to ion channels as pain sensors. TRPA1 appears to be another multiplayer that may have a comparable impact on the elucidation of pain mechanisms and the design of new tools for pain modulation. Using the experiences and lessons from TRPV1 and other pharmacological advances, researchers are rapidly and efficiently constructing information on TRPA1 ligands, and synthetic antagonists have been released as investigational reagents (McNamara et al., 2007; Petrus et al., 2007). Here, we look at important ligands of TRPA1 and their unique modes of ligand–receptor interaction and propose future directions of TRPA1 research.

Reactive Electrophilic Species (RES)

Despite their structural heterogeneity, many TRPA1 ligands share a common (and unusual) feature: electrophilicity. The known TRPA1 ligands—cinnamaldehyde, supercinnamaldehyde, mustard oil, diallyl disulfide, acrolein, *N*-methylmaleimide, and iodoacetamide—contain a highly reactive electrophilic carbon moiety, and these compounds are all RES. Recently, two independent groups found that these compounds bind covalently to TRPA1, resulting in channel activation (Hinman et al., 2006; Macpherson et al., 2007a). The reactive carbons of those ligands form (The α,β-unsaturated carbonyl groups of the ligands react with the -SH groups of cysteines on the channel, thereby forming covalent adducts. In the case of iodoacetamide, alkylation adducts are formed.) Michael adducts by binding with specific N-terminal cysteine residues on TRPA1. Hinman et al. (2006) also found that a lysine residue on the N-terminal covalently reacts with mustard oil's electrophilic carbon and contributes to TRPA1 activation. Three different cysteine residues have been proposed to be involved in the covalent binding (Hinman et al., 2006; Macpherson et al., 2007a), but only one cysteine (C622 in mouse TRPA1, which corresponds to C619 in human TRPA1; Fig. 1) was identified in both studies. Future studies, including structural approaches, are required to explain whether this discrepancy is owing to species differences or other factors.

Allicin activation of TRPV1 has also been reported to be mediated via adduct formation with a cysteine residue within the cytoplasmic TRPV1 N terminus (Salazar et al., 2008). Indeed, many studies of TRPA1 ligands predict covalent interactions rather than traditional lock and key receptor–ligand interactions. In addition to the compounds listed above, a variety of RES known to elicit pain or contribute to inflammation have been reported as TRPA1 activators: formalin, acetaldehyde, 4-oxononenal, 4-hydroxynonenal, 15-deoxy-delta 12,14-prostaglandin J2 (15d-PGJ2), prostaglandin A2 (PGA2), 8-iso PGA2, etc. The thiol reactivity of these compounds might confer their ability to activate TRPA1 (Bang et al., 2007; Macpherson et al., 2007b; McNamara et al., 2007; Trevisani et al., 2007; Andersson et al., 2008; Taylor-Clark et al., 2008a,b).

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Abbreviations used in this paper: DAG, diacylglycerol; GPCR, G protein–coupled receptor; IP3, inositol triphosphate; PGA2, prostaglandin A2; PIP2, phosphatidylinositol-4,5-bisphosphate; PLC, phospholipase C; RES, reactive electrophilic species; RNS, reactive nitrogen species; ROS, reactive oxygen species; THC, δ-9-tetrahydrocannabinol; TRP, transient receptor potential.

The Rockefeller University Press $30.00
J. Gen. Physiol. Vol. 133 No. 3 257–262
www.jgp.org/cgi/doi/10.1085/jgp.200810138

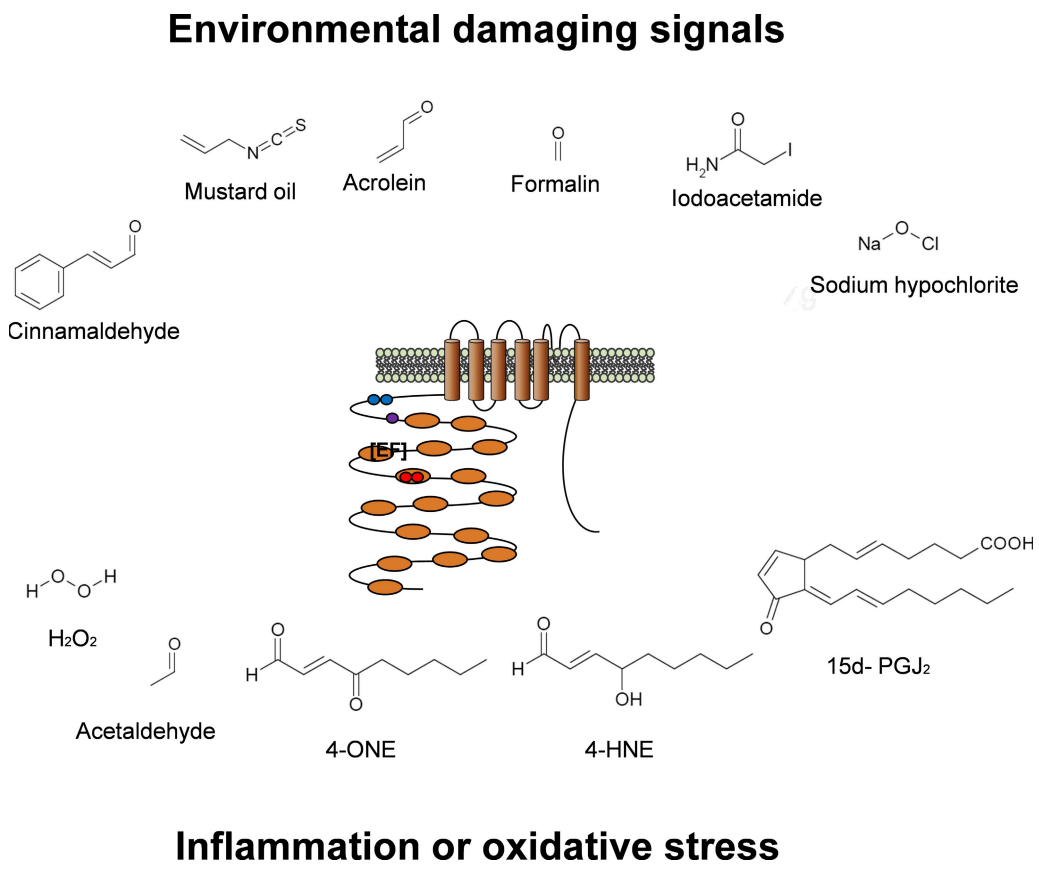

Figure 1. Electrophilic TRPA1 ligands and the cysteine residues in TRPA1, which covalently bind to the ligands, leading to channel activation. Exogenous chemicals are listed at the top, and physiological ligands are listed at the bottom. The critical cysteine residues are indicated by circles (red, from mouse TRPA1; blue, from human TRPA1; purple, from both). EF, the location of the Ca^{2+}-binding EF-hand domain.

However, other nucleophilic amino acids like lysine or histidine can also be an important target for the compounds, as in the case of mustard oil (Lin et al., 2005; Hinman et al., 2006).

Adduct formation of electrophilic xenobiotics or drug metabolites with proteins or DNA is a generally accepted mechanism for carcinogenesis and chemical toxicity (Liebler, 2008). In this respect, TRPA1 might represent a novel target for toxic substances, and it is intriguing that TRPA1 was initially found to be expressed in cancer cells, raising the question of the importance of endogenous RES ligands in cancer cell homeostasis (Jaquemar et al., 1999). TRPA1 also may function as a molecule in which these substances initiate neurological damage, or it can play a beneficial role as a sensory alarm for harmful environmental signals. It can even serve as a trigger for a tissue-protective mechanism against damaging signals, such as enzymes in detoxification processes. Other sensory TRP channels also contain large numbers of cysteine residues, and those cysteines are likely vulnerable to access by and covalent attack from RES. However, it is surprising that, except for TRPA1, only TRPV1 has been reported to be able to transduce covalent binding to channel gating. Thus, a sensory role of TRPA1 as a major detector of a wide set of damaging ligands can be hypothesized.

The molecular mechanism linking covalent binding to channel gating has not been fully elucidated, but it has been shown that a single amino acid change (A946 or M949 in rat TRPA1) in the S6 region determines whether TRPA1 is activated or inhibited by the ligands (Chen et al., 2008). These results suggest that this region may couple the energy contained in the covalent binding to channel gating. In the same study, however, the mutant channels were unable to switch the action of mustard oil to TRPA1 inhibition, and the location of the critical domain for coupling may depend on ligands.

Reactive Oxygen Species (ROS)

Many endogenous RES, including the compounds mentioned above, are generated under oxidative stress. RES result from oxidative damage to normal cellular metabolites by ROS, with the chemical reactivity being transferred to RES. Three laboratories have focused on direct oxidative attack of TRPA1 without RES as intermediates. Two studies using an excised inside-out patch clamp method showed that H_2O_2 activates TRPA1, suggesting that TRPA1 is activated directly or at least through a

membrane-delimited pathway (Andersson et al., 2008; Sawada et al., 2008). The other group used mutated human TRPA1, in which inert amino acids were substituted for the three critical cysteines and lysine. The mutant TRPA1 showed impaired sensitivity to H_2O_2 and OCl^- (Bessac et al., 2008). Andersson et al. (2008) found that the OH· radical, which is produced from H_2O_2 by the Fenton reaction (Fe^{2+}-catalyzed conversion of H_2O_2 to OH· radical in the presence of iron) also has the potential to activate TRPA1. It is likely that these ROS promote disulphide bond formation between vicinal thiol residues because the ROS-induced TRPA1 activation can be reversed by the application of dithiothreitol, which reduces the disulphide bond but does not affect Michael adducts. The activities of several TRP ion channels (TRPM2, TRPM7, TRPC3, TRPC4, TRPC5, TRPV1, and TRPV4) can be regulated by oxidative stress, although the molecular mechanisms remain elusive (Miller, 2006; Susankova et al., 2006; Yoshida et al., 2006). Many of the above TRPs are also expressed in sensory neurons. Surprisingly, both Andersson et al. (2008) and Bessac et al. (2008) failed to observe any contamination with other TRPs' activity in the TRPA1 responses to ROS in cultured sensory neurons, which indicates that TRPA1 may dominate sensory neuronal ROS sensitivity in their experimental conditions.

Reactive nitrogen species (RNS), such as nitric oxide donors, can also activate TRPA1 (Sawada et al., 2008), but it is not known whether the same residues are targets for the RES/ROS attack as well as the RNS-induced nitrosylation. The above studies have also not covered the following key questions: Although the direct interaction of ROS or RNS with TRPA1 is very likely, does TRPA1 also have a separate but membrane-delimited redox partner to buffer oxidative attacks, which would allow finer tuning of its activity? Does TRPA1 use a specific endogenous antioxidant to maintain its oxidative state independent of the overall cellular oxidative level? Is the ROS/RNS-TRPA1 connection a critical contributor to the development of chronic pain (Chung, 2004)? Do additional inflammatory changes, including low pH, amplify the duration or efficacy of the effect of these reactive substances?

Natural Compounds and Drug Pharmacology

TRPA1 can also detect many non-electrophilic phytochemicals and some synthetic compounds, probably in the traditional receptor–ligand binding fashion. These types of ligands, such as δ-9-tetrahydrocannabinol (THC) and icilin, display rapid reversibility. Their responses are tolerant to substitution of critical cysteine residues for the covalent attack, suggesting that traditional ligands use separate TRPA1 binding sites (Hinman et al., 2006; Macpherson et al., 2007a). Unlike RES ligands, THC does not need cofactors such as inorganic polyphosphates to activate TRPA1 (Cavanaugh et al., 2008; see below). Thus, the mechanism that couples binding to gating in noncovalent ligand-evoked TRPA1 activation is likely to be independent of that in covalent ligand–evoked activation. The chemical structures of plant-derived TRPA1 agonists and antagonists are heterogeneous (for example, menthol, eugenol, camphor, THC, citral, etc.). Future expansion of knowledge about natural ligands will be needed to determine whether TRPA1 has multiple binding sites for noncovalent interaction. Further, as more detailed information becomes available, we can isolate common or major pharmacophores.

The ligand preference of TRPA1 for covalent binding has been discussed (Peterlin et al., 2007). TRPA1 likely has blunted size/shape specificity compared with other chemoreceptors, leading to sensitivity to a wide array of environmental damage signals. One simple example of this phenomenon is that the covalent TRPA1 agonists acrolein (trans-2-propenal), trans-2-pentenal, and cinnamaldehyde are all α,β-unsaturated aldehydes, but they have chains (or a ring) of different sizes. The main drawback to this ability appears to be that the dissociation from reactive ligands and the return to basal activity for the next signal generation are delayed. For instance, the responses to mustard oil (allyl isothiocyanate) or *N*-methyl maleimide last far longer than their perfusion periods (Hinman et al., 2006; Macpherson et al., 2007a). Although not particularly rigorous, some steric stringency was also observed in a recent study (Taylor-Clark et al., 2008b). PGB2, which seems to contain a structurally less exposed electrophilic carbon, failed to exhibit significant reactivity with TRPA1, whereas PGA2 and 15d-PGJ2, which have a relatively open electrophilic carbon, robustly activate TRPA1. AP18 is a synthetic antagonist that is structurally related to cinnamaldehyde, but its carbons seem less reactive. Its TRPA1 antagonism is rapidly reversible, indicating that it probably exerts the effect noncovalently (Petrus et al., 2007).

Because TRPA1 mediates multiple types of pain sensation, drug development targeting this ion channel seems active. Due to its low structural specificity for electrophilic ligands, covalent adduct formation might not be the major modulatory method for TRPA1 activity that will carry clinical impact. Furthermore, a covalent ligand, cinnamaldehyde, also activates TRPV3 and inhibits TRPM8 in the millimolar range (Macpherson et al., 2006). One can imagine another type of low specificity for one covalent ligand to other related TRPs, although it remains to be determined whether this phenomenon is caused by cinnamaldehyde's attack on particular cysteine residues of TRPV3 or TRPM8. Specificity issues are important; looking back on the aspirin-like cyclooxygenase inhibitors, few effective agents that covalently modify specific cyclooxygenase subtypes have been developed (Kalgutkar et al., 1998). Practical concerns exist regarding difficulties in filtering selective covalent TRPA1 ligands that are free from adverse effects on other tissues

(Chen et al., 2008). On the other hand, from a wider perspective, many existing clinical medicines covalently modify enzyme activities (Robertson, 2005). Integrated approaches—for example, combining new technologies such as the click chemistry method (covalent ligands bound to receptors can be rapidly monitored by tagging alkynes to the ligands and reacting with azide-containing visualizing reagents) may open a novel pool of covalent ligands and enable simultaneous examination of adverse effects caused by reactivity with off-targets (Evans et al., 2005; Macpherson et al., 2007a). URB597, which is predicted to activate TRPA1 in a noncovalent binding manner according to its pharmacological profile, is an originally covalent fatty acid amide hydrolase inhibitor (Niforatos et al., 2007). The nature of the covalent interaction between fatty acid amide hydrolase and URB597 was recently redefined using click chemistry, and moreover, this method helped design more reactive and selective derivatives of the original compound (Alexander and Cravatt, 2005).

Ca^{2+} and Cold

The sensitivity of TRPA1 to intracellular Ca^{2+} levels has been proposed (Jordt et al., 2004; Nagata et al., 2005). Two recent independent studies revealed that Ca^{2+} directly binds to the N-terminal EF-hand Ca^{2+}-binding domain of TRPA1, leading to channel activation (Doerner et al., 2007; Zurborg et al., 2007) (Fig. 1). This information also extends the horizon of our conceivable ideas regarding TRPA1 roles. That is, TRPA1 now seems to be one of the typical receptor-operated channels for signaling modes that use the G protein–coupled receptor (GPCR)–phospholipase C (PLC)–Ca^{2+} pathway in sensory neurons. This hypothesis was supported by Zurborg et al. (2007), with experiments using muscarinic receptor signaling systems and EF-hand domain mutants. Proinflammatory mediators such as bradykinin, ATP, and serotonin are released around sensory neurons and evoke acute excitatory responses from these neurons, resulting in inflammatory pain. These mediators also act on their own GPCRs and commonly use the PLC pathway. Indeed, TRPA1 was previously shown to be a downstream effector for bradykinin (Bandell et al., 2004; Bautista et al., 2006). In these studies, Ca^{2+} also was shown to be important for TRPA1 activation (Bautista et al., 2006); lipid metabolites of PLC, such as diacylglycerol (DAG), also contributed to TRPA1 activation (Bandell et al., 2004).

TRPA1 can be an electrical amplifier in sensory neuronal firing. In other words, Ca^{2+} that flows into the cytosol through other excitatory ion channels subsequently stimulates TRPA1, thereby accelerating depolarization. The interaction of native Ca^{2+}-activated nonselective cation channels and native TRPA1 gave support to this hypothesis (Cho et al., 2003). Excessively increased intracellular Ca^{2+}, however, is able to desensitize TRPA1 activity, which narrows the actual Ca^{2+} range where this hypothesis is working (Nagata et al., 2005; Akopian et al., 2007).

Both Zurborg et al. (2007) and Doerner et al. (2007), using TRPA1 mutants with substituted amino acids in the EF-hand domain, have shown that Ca^{2+} binds to the N-terminal EF-hand domain of TRPA1, thereby activating TRPA1. However, the amino acids that were critical for Ca^{2+} binding differed between the two studies. In particular, Doerner et al. (2007) found no difference in Ca^{2+}-evoked activation between the D468 mutant (corresponding to the D466 mutant of Zurborg et al., 2007) and the wild type, whereas the mutant of Zurborg et al. (2007) was not activated by Ca^{2+}. There was also a discrepancy in Ca^{2+} responses between the S470 mutant used by Doerner et al. (2007) and the corresponding S468 mutant used by Zurborg et al. (2007). Further study is needed to precisely isolate the key binding sequences or to define the Ca^{2+}-binding site.

Cold activation of TRPA1 has been disputed. The ability of TRPA1 to directly sense Ca^{2+} may endow this channel with its sensitivity to cold, according to data showing a cold-evoked elevation in intracellular Ca^{2+} levels in nontransfected HEK293 cells (Zurborg et al., 2007). However, results from recent studies challenge this idea (Sawada et al., 2008; Karashima et al., 2009). Cold-activated TRPA1 currents were readily detected using whole cell, cell-attached, and excised membrane patch clamp methods under completely Ca^{2+}-depleted conditions, indicating that cytosolic factors such as intracellular Ca^{2+} are not necessary for cold activation. Moreover, Karashima et al. (2009) showed that the voltage-dependent gating kinetics of TRPA1 were significantly affected by cold. In fact, there is little information available regarding molecular determinants of temperature sensing and their gating-coupling mechanisms, not only for TRPA1, but also for most thermosensitive TRP channels and further studies are required (for review see Bandell et al., 2007 and Latorre et al., 2007). Cross-chimeric studies and high-throughput mutagesis screening would be helpful. Recent domain-swapping approaches between heat and cold receptors (TRPV1 and TRPM8), conjugated with structural insights, have shown that these channels have temperature-sensing modules at the cytoplasmic C terminal (Brauchi et al., 2006, 2007). A different region (five amino acids located in the putative sixth transmembrane helix and adjoining the extracellular loop within the pore region), critical for TRPV3 heat sensibility, was isolated in a high-throughput random mutagenesis study (Grandl et al., 2008).

Cofactors

PLC action mediates various GPCR-induced signals. Activated PLC hydrolyzes membrane phosphatidylinositol-4, 5-bisphosphate (PIP2) into DAG and inositol triphosphate (IP3), which in turn evokes Ca^{2+} release from ER, possibly leading to TRPA1 activation. Evidence is accumulating

that the PLC substrate PIP2 also has a regulatory role in TRPA1 activity. Protease-activated receptor 2, a GPCR, is expressed in sensory neurons and is cleaved and activated by trypsin under inflammatory conditions. Dai et al. (2007) reported that activated protease-activated receptor 2 subsequently activates PLC, thereby sensitizing TRPA1. In this study, they argued that DAG or protein kinase C was not involved in TRPA1 sensitization; rather, a decrease in plasma membrane PIP2 resulting from consumption by PLC disinhibits TRPA1. This result was once confirmed by Kim et al. (2008), who showed that PIP2 directly inhibits TRPA1 using an excised inside-out patch configuration. However, inconsistent data have also been reported, showing that PIP2 depletion is involved in TRPA1 desensitization (Akopian et al., 2007). Separate mechanisms may underlie TRPA1 sensitization and desensitization processes despite some components being overlapped. Which role (sensitizing or desensitizing TRPA1) of PIP2 is dominant in physiological conditions is an open question. Furthermore, questions can be posed as to why Ca^{2+} most likely released via IP3-ER signaling failed to directly activate TRPA1, and why DAG, which has been reported to be a potential TRPA1 activator in the bradykinin–PLC pathway, also had no effect in the above studies. Different onsets or durations of the action of these molecules, or different contributions by particular downstreams in each GPCR pathway, might be possible explanations, but experimental evidence is still lacking.

The Kim group continues to produce interesting data about cytosolic modulators of TRPA1 activity. The cytoplasm has endogenous phosphate-containing molecules, including nucleotides, IP3, inorganic polyphosphates, etc. In their screening, inorganic polyphosphates such as pyrophosphate and polytriphosphate were shown to aid in the activation of TRPA1 (Kim and Cavanaugh, 2007). The supporting effect of the polyphosphates was most conspicuous during activation by electrophilic species binding covalently to N-terminal cysteine residues. In fact, the activation effect of RES is readily lost in the cell-free excised membrane configuration. This phenomenon leads to the hypothesis that the cytoplasm contains cofactors essential to coupling the covalent binding and the channel gating. So far, the polyphosphates are the sole candidates for this type of cofactor. Another study from the same laboratory showed that supporting cofactors are also required for TRPA1 activation by Ca^{2+} (Cavanaugh et al., 2008). They showed that a THC binds to the intracellular region of TRPA1, and that the THC-induced TRPA1 activation is not affected by the absence or presence of cofactors. Collectively, the polyphosphate cofactors are thought to participate only in the TRPA1 conformational changes that couple RES attacks to the gating. Structural studies of TRPA1 will shed light on the ligand-specific gating mechanisms.

Conclusion

Research has expanded our understanding not only of the various ligand interactions of TRPA1, but also of its physiological functions. Many details of the interactions between extraneous signals or physiological factors and TRPA1 still await close analysis. For example, the mechanism(s) underlying the antagonism or synergism between non-electrophilic ligands and covalent modifiers remains largely unknown. Which paradigm would be more appropriate to elucidate different thermal sensitivities (and mechanosensitivity) of TRPA1 from various species (mammalians, fruit flies, and worms) — modular analysis narrowing down sensing units versus examining whether Ca^{2+} or other physiological ligands mediate physical sensitivity — would also be an interesting question. With regard to the relationship between intrinsic channel properties and TRPA1 pharmacology, voltage connection has not been thoroughly studied. In the near future, many questions are likely to be answered that will greatly advance our knowledge about TRPA1-involved pain mechanisms and methods for its pharmacological modulation.

This work was supported by a grant (code M103KV010016-07K2201-01610) from Brain Research Center of the 21st Century Frontier Research Program, and by a grant (code R01-2007-000-20493-0) funded by the Ministry of Education, Science and Technology of the Republic of Korea.

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