Chapter 3
Involvement of TRPV1-ANO1 Interactions in Pain-Enhancing Mechanisms

Y. Takayama and Makoto Tominaga

Abstract Primary sensory neurons detect potentially dangerous environmental situations via many "sensor" proteins located on the plasma membrane. Although receptor-type cation channels are thought to be the major sensors in sensory neurons, anion channels are also important players in the peripheral nervous system. Recently, we showed that transient receptor potential vanilloid 1 (TRPV1) interacts with anoctamin 1 (ANO1, also called TMEM16A) in primary sensory neurons and that this interaction enhanced TRPV1-mediated pain sensation. In that study, we induced ANO1 currents by application of capsaicin to small DRG neurons and showed that ANO1-dependent depolarization following TRPV1 activation could evoke more action potentials. Furthermore, capsaicin-evoked pain-related behaviors in mice were strongly inhibited by a selective ANO1 blocker. Together these findings indicate that selective ANO1 inhibition can reduce pain sensation. We also investigated non-specific inhibitory effects on ion channel activities to control ion dynamics via the TRPV1-ANO1 complex. We found that 4-isopropylcyclohexanol (4-iPr-CyH-OH) had an analgesic effect on burning pain sensations through its inhibition of TRPV1 and ANO1 together. Additionally, 4-iPr-CyH-OH did not have clear agonistic effects on TRPV1, TRPA1, and ANO1 activity individually. These results indicate that 4-iPr-CyH-OH could function globally to mediate TRP-ANO1 complex functions to reduce skin hypersensitivity and could form the basis for novel analgesic agents.

Keywords TRP channel · Anoctamin 1 · Isopropylcyclohexanol · Acute pain

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3.1 Introduction

Transient receptor potential (TRP) channels are involved in a diverse range of physiological functions, including pain sensation. TRP channels expressed in primary sensory neurons can be activated by several different physical and chemical stimuli, including temperature changes and irritants [34]. However, the output phenotypes produced by these stimuli are not solely dependent on TRP channel activities. As is well known, almost all TRP channels have high calcium permeability [9]. This calcium influx could affect other calcium-dependent proteins located within a micrometer range of the channel pore [22]. Anoctamin (ANO) is one of the calcium-dependent proteins [1, 26, 37]. We recently showed that the calcium-activated chloride channel ANO1 (also known as TMEM16A) can be strongly activated by calcium influx through TRPV1 activation and that TRPV1-ANO1 interaction is involved in pain enhancement [30]. This chapter reviews the recent findings concerning TRP interactions in sensory systems and potential strategies for pharmacological control of the ion dynamics.

3.2 TRPV1: ANO1 Interaction

Both TRPV1 and ANO1 are expressed in primary sensory neurons and are involved in acute pain sensation [30]. TRPV1 is activated by various natural ligands, including capsaicin, resiniferatoxin, bivalent tarantula toxin, acid, and noxious heat [13]. Rat TRPV1 is phosphorylated at Ser502 and Ser800 by protein kinase C epsilon (PKCε) activated in response to signaling by G protein-coupled receptors (GPCR), including the bradykinin receptor and P2Y receptor [32, 38]. This PKCε phosphorylation is mediated by A-kinase anchoring proteins [38]. Because phosphorylation reduces the threshold for TRPV1 activation, phosphorylated TRPV1 can be activated at temperatures lower than core body temperature [32]. This characteristic is thought to be involved in molecular mechanisms that cause inflammatory pain. Therefore, TRPV1 is a primary target for pain therapy. However, the chloride channel ANO1 is also thought to play a major role in generating pain signals in primary sensory neurons due to its heat sensitivity and immediate activation following GPCR activation [3, 18]. ANO1 directly interacts with the IP_3 receptor on the endoplasmic reticulum (ER) membrane [11]. Interestingly, TRPV1 and ANO1 are also co-expressed in small dorsal root ganglia (DRG) neurons [2]. We previously demonstrated an interaction between TRPV4 and ANO1 in choroid plexus epithelial cells [29]. Similar to TRPV1, TRPV4 has high calcium permeability (Na^+:Ca^{2+} = 1:10). Therefore, calcium entering the cell rapidly induces ANO1 activation followed by secretion of fluids such as cerebrospinal fluid, saliva, and tears [6, 29]. We thus investigated whether TRPV1-ANO1 interaction occurs in DRG neurons and the physiological relevance of this interaction.

Typically, we began by conducting an electrophysiological analysis using whole-cell patch-clamp recording in HEK293T cells expressing TRP channels and ANO1. The main composition of the bath and pipette solutions in these assays is N-methyl-D-glutamine chloride (NMDG-Cl), and the free calcium in the pipette solution was maintained at 100 nM using 5 mM O,O′-Bis (2-aminophenyl)ethyleneglycol-N,N,N′,N′-tetraacetic acid (BAPTA). To study TRPV1-ANO1 interactions, we activated TRPV1 by applying 300 nM capsaicin, which is approximately the half effective concentration, although in DRG neurons the concentration is 1 μM [15]. Under these conditions, large chloride currents that could induce cell shrinkage at −60 mV holding potential were observed in cells expressing TRPV1 and ANO1, but not cells expressing TRPV1 or ANO1 alone. Moreover, these currents were abolished in a calcium-free bath solution and a reversal potential shift occurred in NMDG-aspartate bath solution. These results clearly suggest that calcium influx through TRPV1 activation strongly induces ANO1 activation. Furthermore, immunoprecipitation results indicated that TRPV1 and ANO1 directly interact. Thus, TRPV1 directly and functionally interacts with ANO1 although ANO1 alone could be activated by global calcium increases depending on ER calcium stores and voltage-gated calcium channels on plasma membrane [12].

3.3 Pain-Enhancing Mechanisms in DRG Neurons

The physiological activity of ANO1 is dependent on concentration differences in extracellular and intracellular chloride. Interestingly, in many DRG neurons, the intracellular chloride concentration is reportedly higher than in other neurons, such as those in the central nervous system [20]. The equivalent potential in DRG neurons containing high chloride can reach −20 mV, and the resting potential is approximately −60 mV. Therefore, ANO1 activation should induce depolarization due to chloride efflux and neuronal excitations. To examine this possibility, we performed the same experiments as those for HEK293T cells using isolated small DRG neurons. In whole-cell patch-clamp recordings, capsaicin-induced currents decreased by half following application of the selective ANO1 inhibitor T16Ainh-A01 with a physiological ion concentration in the bath solution (NaCl base solution containing 2 mM $CaCl_2$). The capsaicin-induced current is composed of cations and chloride movements, even though capsaicin-mediated neuronal excitation in DRG neurons was thought to depend only on TRPV1 function. Moreover, action potentials evoked by capsaicin applications were almost completely inhibited by T16Ainh-A01. Together, these results indicate that a TRPV1 and ANO1 interaction should also occur in DRG neurons in the presence of high intracellular chloride concentrations.

However, the efficacy of this interaction remained unclear because some DRG neurons have low concentrations of intracellular chloride. In these neurons, ANO1 could induce hyperpolarization with TRPV1 activation. To clarify whether ANO1 activation following TRPV1 activation is involved in pain generation but not pain

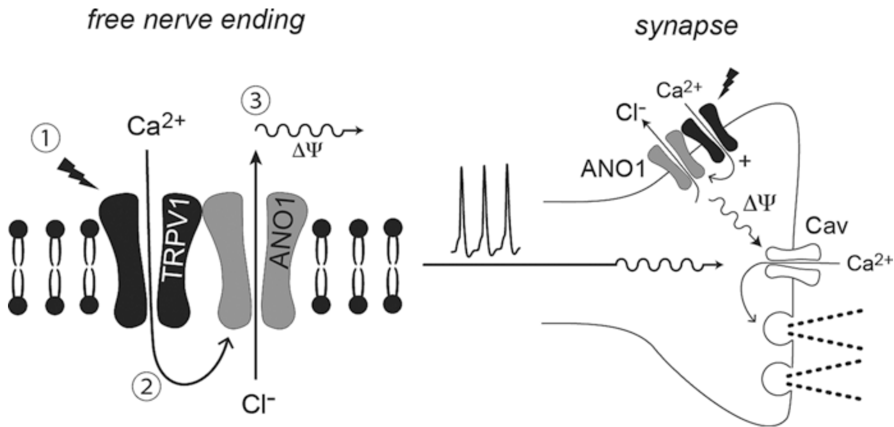

Fig. 3.1 Schematic model of interactions between TRPV1 and ANO1. TRPV1 interacts with ANO1 on both free nerve endings and synapses of DRG neurons. TRPV1 is initially activated and ANO1 is also immediately activated in calcium nano-domains. The ANO1 activation enhances action potential generation ($\Delta\Psi$). TRPV1 also interacts with ANO1 on the central side, and the depolarization activates voltage-gated calcium channels. These two pathways are involved in neurotransmitter release from presynaptic regions in secondary neurons of the spinal cord

reduction, we analyzed the effect of T16Ainh-A01 on capsaicin-induced pain-related behaviors in mice. We found that pain-related behaviors were significantly ameliorated by concomitant administration of T16Ainh-A01. Thus, the TRPV1 and ANO1 interaction appears to be involved in pain enhancement, and TRPV1 and ANO1 behave as irritant detector and signal amplifier, respectively, although ANO1 could act as a suppressor in some DRG neurons (Fig. 3.1).

3.4 Analgesic Agents to Target TRPV1-ANO1 Interactions

The specificity of channel antagonists might not always be an important property in pain reduction because selective drugs often have strong side effects that discourage their use in vivo. Moreover, complete reduction of pain is not always desirable in clinical applications because pain pathways can have a protective effect in certain situations, such as avoiding bone destruction in *Candida* infection [21]. An alternative strategy would be to identify an agent that can inhibit several ion channels involved in pain sensation in peripheral regions. For instance, TRPV4 is also thought to be involved in pain sensation, and the weak-specific antagonist, compound 16-8, is more effective at reducing pain than the TRPV4-specific antagonist GSK205 [14]. While investigating the interaction between TRPM8 and ANO1, we fortuitously found that menthol inhibits ANO1 [31]. Although in that study we were unable to characterize the physiological role of the TRPM8-ANO1 interaction, the menthol-related findings were nonetheless interesting because menthol can also

inhibit the TRPV1 activation [28]. However, the ability of menthol to inhibit both ANO1 and TRPV1 is puzzling given the differences in the structures of these channels. TRPV1 and ANO1 have six and ten transmembrane regions, respectively, and TRPV1 is a tetramer, whereas ANO1 is a dimer [5, 17, 23]. We first assessed the effects of other menthol analogues, including menthone, 1,4-cineole, and 1,8-cineole, on ANO1 currents. In whole-cell patch-clamp recordings of HEK293T cells expressing ANO1, only 1,8-cineole lacked a strong inhibitory effect on the ANO1 current induced by high free calcium concentration. Because the chemical structure of 1,8-cinaole is the most divergent among the three analogues tested, we surmised that potential menthol-based agents should contain a critical minimum structure. Therefore, we next investigated the separate moieties comprising menthol. From these studies we showed that isopropylcyclohexane is the core structure needed to completely inhibit ANO1 currents. Since the kinetics of current reduction by isopropylcyclohexane were slower than that for menthol, we focused on 4-isopropylcyclohexanol (4-iPr-CyH-OH), which has greater hydrophilicity, which could be valuable if the affinity site lies in the intracellular domain of the ion channel. According to our expectations, 4-iPr-CyH-OH showed rapid inhibition that was similar to that of menthol. Interestingly, 4-iPr-CyH-OH also inhibits TRPV1, TRPA1, TRPV4, and TRPM8 activity. Thus, 4-iPr-CyH-OH could have inhibitory effects on many different irritation pathways. The half inhibition concentration (IC_{50}) of 4-iPr-CyH-OH for mouse TRPA1, TRPV1, and ANO1 was 0.23, 0.73, and 1.09 mM, respectively (Fig. 3.2). IC_{50} of 4-iPr-CyH-OH in TRPV1 current induced by 100 nM capsaicin was lower than that of ANO1 current. However, the capsaicin at the concentration does not fully activate TRPV1, whereas 500 nM intracellular free calcium strongly activates ANO1 in our experiments. Three hundred micrometer allyl isothiocyanate (AITC) also induces the almost saturated TRPA1 activation. Thus, 4-iPr-CyH-OH could have a lower inhibitory effect toward TRPV1. Furthermore, we investigated the effects of 4-iPr-CyH-OH on capsaicin-evoked action potential in isolated small DRG neurons and capsaicin-induced pain-related behaviors in mice. In these experiments, 4-iPr-CyH-OH completely inhibited capsaicin-evoked action potentials with strong suppression of depolarization, and

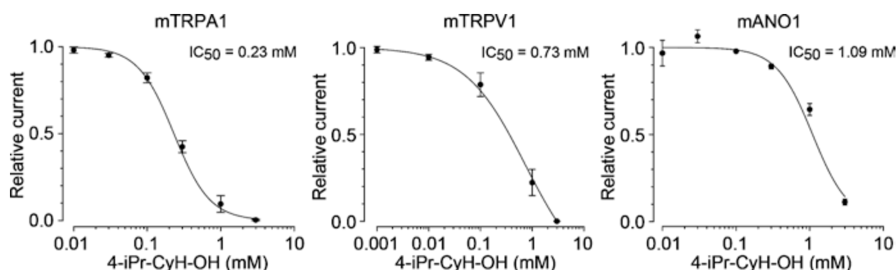

Fig. 3.2 Dose-response curves of 4-isopropylcyclohexanol (4-iPr-CyH-OH) at −60 mV. Mouse TRPA1, TRPV1, and ANO1 expressed in HEK293T cells were activated by 300 μM AITC, 100 nM capsaicin, and 500 nM free calcium, respectively

pain-related behaviors were significantly diminished with concomitant administration of 4-iPr-CyH-OH.

Although 4-iPr-CyH-OH is currently used as a food additive in Japan, the pharmacological understanding of its effects beyond those we found for pain sensation is limited [10, 19]. Thus, 4-iPr-CyH-OH could have potential as a basis for the development of novel drugs that target ion channels, particularly ANO1 and TRP channels.

3.5 Conclusion

TRP-ANO1 interactions are involved in several physiological mechanisms. For instance, TRPC2-ANO1 interaction could be involved in iodide homeostasis in thyroid cells and vomeronasal transduction [7, 33], and TRPC6-ANO1 interaction reportedly enhances vasoconstriction [35]. In addition, our findings indicated that ANO1 activation could generate sufficient depolarization to induce exocytosis in synapses between primary sensory neurons and secondary neurons in the spinal cord (Fig. 3.1). In fact, ANO1-dependent membrane potential changes could accelerate insulin secretion from pancreatic β-cells [4, 36]. Not only ANO1, targeting TRP-ANO interactions could be also a promising approach because ANOs are expressed in the whole body [8, 16, 25, 27], and ANOs have three functions, including chloride channel, scramblase, and internalization [24, 27]. Thus, additional physiological phenomena could be better explained by future investigations that focus on TRP-ANO interactions.

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