

Anoctamin 1 Mediates Thermal Pain as a Heat Sensor

Hawon Cho¹ and Uhtaek Oh^{1,2,*}

¹Sensory Research Center, CRI, College of Pharmacy, Seoul National University, ²Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology, Seoul National University, Seoul 151-742, Republic of Korea

Abstract: Vertebrates can sense and avoid noxious heat that evokes pain. Many thermoTRP channels are associated with temperature sensation. TRPV1 is a representative ion channel that is activated by noxious heat. Anoctamin 1 (ANO1) is a Cl⁻ channel activated by calcium that is highly expressed in small sensory neurons, colocalized with markers for nociceptors, and most surprisingly, activated by noxious heat over 44°C. Although ANO1 is a Cl⁻ channel, opening of this channel leads to depolarization of sensory neurons, suggesting a role in nociception. Indeed, the functional deletion of ANO1 in sensory neurons triggers the reduction in thermal pain sensation. Thus, it seems clear that ANO1 is a heat sensor in a nociceptive pathway. Since ANO1 modulators are developed for the purpose of treating chronic diseases such as cystic fibrosis, this finding is likely to predict unwanted effects and provide a guide for better developmental strategy.

Keywords: ANO1, CaCC, pain, heat, thermo TRP, sensory neurons.

INTRODUCTION

Temperature sensation of noxious heat is critical for the survival and maintenance of life for all animals [1-3]. These thermal stimuli are mediated by specialized classes of sensory neurons that have cell bodies in dorsal root ganglia (DRG) and innervate to peripheral tissues [1, 3, 4]. Many ion channels are present in DRG neurons as molecular sensors that transduce noxious or non-noxious thermal stimuli into electrical signals.

The molecular mechanism of heat sensation originated from the cloning and characterization of TRPV1 [5] that belongs to the transient receptor potential (TRP) channels family [6]. TRP channel family is composed of non-selective cation channels that are permeable to cations such as Ca²⁺ and predicted to have six transmembrane domains [7]. In the last decade, numerous reports have demonstrated that a subset of TRP channels family responds to temperature ranging from cold to heat, which are now called as a thermoTRP channels [1, 3]. They have a distinct range of activation threshold for temperature changes. In addition to thermal sensation, these thermoTRP channels also have diverse physiological or pathological functions including chemosensation [8-14]. One best known example of thermoTRPs is the TRPV1, since it is activated by heat over 43°C, a threshold for thermal pain [5, 15]. Furthermore, mice lacking TRPV1 display reduced responses to noxious heat [5, 15]. Thus, TRPV1 is considered as a heat sensor. Although the TRPV1 is considered to play an important part in heat sensation, several lines of evidences support

the existence of additional heat sensors [3, 16, 17]. Recently, Cho and colleagues suggested an additional heat sensor, anoctamin 1 (ANO1); it is a Cl⁻ channel, not cation channel [18]. Thus, the present chapter reviews the historic background of thermoTRPs. In addition, the physiological role of ANO1 as a heat sensor is further discussed.

THERMAL PAIN SENSATION AND TRP CHANNELS

Among thermoTRPs, TRPV1 was firstly identified as a temperature sensitive channel because it is activated at heat over 43°C [5, 8, 15, 19]. After the discovery of TRPV1, a large number of reports have provided substantial evidence claiming that many other TRP channels, such as TRPV2-TRPV4, TRPA1, and TRPM8 are responsible for the detection of temperature change from cold to heat [5, 10, 11, 14, 15, 20]. These thermoTRPs are largely divided into innocuous and noxious thermo sensors. Whereas innocuous thermo sensors mediate mild responses, such as warm or cool, noxious thermo sensors mediate extreme sensation, such as cold or hot [1, 21, 22]. These channels with distinct temperature thresholds therefore make us perceive diverse ranges of temperature change.

TRPV1 is expressed mainly in small DRG neurons and activated by capsaicin, a major ingredient of hot pepper [5]. TRPV1 is the first TRP channel that is known to be activated by heat. Recently, it is further confirmed by Julius group that TRPV1 is activated by heat even when it is reconstituted in liposomes [23]. In addition, TRPV1 is also activated by extracellular acidity [8]. Thus, TRPV1 is a multimodal sensor in nociceptors that respond to various pain causing stimuli. Although it is clear that TRPV1 is activated by heat, its role in mediating acute thermal pain is somewhat controversial; Caterina and colleagues reported that TRPV1-deficient mice have demonstrated reduced behavioral

*Address correspondence to this author at the Sensory Research Center, CRI, Seoul National University, College of Pharmacy, Kwanak, Shinlim 9-dong, Seoul 151-742, Korea; Tel: 82-2-880-7854; Fax: 82-2-872-0596; E-mail: utoh@snu.ac.kr

responses to heat whereas TRPV1-deficient mice from Davis group failed to show the reduced responses to heat [16, 24]. Unequivocal agreement over the role of TRPV1 on heat sensation, however, is its mediation of inflammatory thermal pain because thermal hyperalgesia after inflammation is significantly reduced in TRPV1-deficient mice obtained by both groups [16, 24]. Even though there is no doubt that TRPV1 mediates thermal pain, the presence of additional heat sensors was suggested due to the fact that TRPV1 knock-out mice still exhibited residual nocifensive behaviors to noxious thermal stimuli [16, 24]. Additionally, DRG neurons collected from mice lacking TRPV1 responded to heat [17]. Therefore, TRPV1-independent mechanisms which are responsible for the detection of noxious heat have been suggested [3, 17]. In this regard, TRPV2 has been considered as a candidate gene for a heat sensor [15]. TRPV2, a close homolog of TRPV1, is activated under extreme thermal condition, over 52°C and expressed in a subpopulation of DRG neurons, medium to large-diameter neurons that are sensitive to temperature with high threshold [15]. However, its role as a heat sensor *in vivo* has been questioned. TRPV2 deficient mice showed normal behavioral responses to noxious heat [25]. Furthermore, TRPV1/TRPV2 double knock-out mice also exhibited comparable latencies to heat to that of the TRPV1-deficient mice [25]. These results lead to the question regarding the TRPV2's role in terms of noxious thermal sensation.

TRPV3 is activated by warm temperature with threshold of 33°C [11, 12, 20]. Repeated heat or chemical sensitized activities of TRPV3. Interestingly, TRPV3 is less abundant in sensory neurons but mainly expressed in keratinocytes of the skin. TRPV3-deficient mice alter warmth sensation [26]. TRPV4, as known for responding to hypotonic swelling and various chemicals, is also activated by warm temperature with range of 25-34°C [9, 13, 27, 28]. TRPV4 is present in keratinocytes and DRG neurons [9]. However, its role in detecting thermal change *in vivo* is somewhat intriguing. Although the inflammatory thermal hyperalgesia is reduced, the detection of warmth is still unclear in TRPV4-deficient mice [29]. For instance, while wild-type mice fail to discriminate temperature change between 30 and 34°C, TRPV4-deficient mice prefer a floor of 34°C to that of 30°C [30]. However, the roles of TRPV3 and TRPV4 in heat sensing were challenged because TRPV3 and TRPV4 double knock-out mice showed similar heat nociceptive response or thermal preference behaviors as those of wild type mice [31]. More recently, TRPM3, a member of melastatin subfamily of TRP channel, is implicated in detection of noxious heat [32]. TRPM3 is expressed in small-diameter DRG neurons and activated by heat. Indeed, TRPM3 deficient mice show the impairment of avoidance response to noxious heat and reduced temperature preference as well. In the same context, DRG neurons cultured from TRPM3 knock-out mice exhibited substantial thermal response after blocking TRPV1 by its inhibitor, AMG9810.

Cl⁻ REGULATION IN THE PAIN PATHWAY

The peripheral endings of primary sensory neurons detect many kinds of stimuli including noxious stimuli that

can evoke damage or injury to tissue [33]. Particularly, nociceptive neurons (nociceptor) that are sensitive to noxious stimuli express many different channels that conduct diverse monovalent or divalent cations [34]. It is relatively unknown whether Cl⁻ channels are involved in nociception. However, chloride-based depolarization also contributes to the generation of nociceptive signaling in peripheral sensory ending [35, 36].

The most well-known example that can explain this phenomenon is GABA_A receptor mediated depolarization due to the efflux of Cl⁻ ion through GABA_A receptors [37, 38]. GABA-induced depolarization could evoke excitatory or inhibitory responses [39, 40]. For example, it has been reported that GABA depolarizes and excites central neurons during embryonic and early postnatal development states [41]. In contrast, GABA evokes hyperpolarizing and inhibitory responses in mature central neurons [36, 42, 43]. Unlike central neurons, however, primary sensory neurons are depolarized by GABA through adulthood [44]. GABA mediated depolarization of central terminals of primary sensory neurons, as termed primary afferent depolarization (PAD), is a major factor that regulates presynaptic inhibition of primary afferent fibers in the dorsal horn [41, 45]. GABA_A mediated PAD and presynaptic inhibition are influenced by the magnitude of the driving force for Cl⁻ that is maintained by transport mechanism [42, 46, 47]. This Cl⁻ gradient measured by difference of E_{Cl} and E_m determines the amplitude of PAD and the strength of presynaptic inhibition [43]. The intracellular Cl⁻ gradient could be determined by two Cl⁻ transport proteins, such as NKCC1 (sodium-potassium-chloride co-transporter) and KCC2 (potassium-chloride co-transporter) [35]. NKCC1 is a transporter protein that plays a key role for active Cl⁻ uptake. Cl⁻ uptake by NKCC1 evokes Cl⁻ accumulation in the cell that maintains above the electrochemical equilibrium [42, 48, 49]. This induces the efflux of Cl⁻ that produces depolarization. For example, the spinal application of bumetanide, a NKCC1 blocker, inhibits dorsal-root reflex in capsaicin-induced neurogenic inflammation [50]. In contrast, KCC2 that is a neuronal specific form of K⁺-Cl⁻ cotransporter extrudes Cl⁻, maintaining below the electrochemical equilibrium causing hyperpolarizing inhibition. Therefore, KCC2 regulates intracellular chloride concentration by counteracting Cl⁻ uptake mediated by NKCC1 [51]. Consequently, the condition that can determine intracellular Cl⁻ concentration is dependent on the expression of NKCC1 or KCC2 in various tissues. However, whereas KCC2 expression was reported in adult central neurons [47, 52, 53], this protein is not expressed in primary sensory neurons [49, 54-57]. Yet, the depolarization by Cl⁻ efflux is not confined to central terminals of DRG neurons in the spinal cord [58, 59]. The depolarization by Cl⁻ efflux also has an excitatory effect on peripheral sensory endings of DRG neurons.

Excitatory Cl⁻ Effect in DRG Neurons

The regulation of intracellular chloride in DRG neurons plays a role in the transmission of nociceptive signals or mediating in condition of inflammatory hyperalgesia [36, 49]. Early studies on the measurements of intracellular Cl⁻ concentration ([Cl⁻]_i) support that Cl⁻ accumulation in the

cytoplasm is one possible cause. $[Cl^-]_i$ is maintained above electrochemical equilibrium, which is ~ 23.6 mM in DRG neurons isolated from adult frog [48]. In the same context, Cl^- concentrations in DRG neurons of adult cats and chick embryo are ~ 53 mM [60] and ~ 31 mM, respectively when measured with the reversal voltage of GABA_A receptor-mediated currents [61]. When $[Cl^-]_i$ was measured using a Cl^- sensitive fluorescent dye, $[Cl^-]_i$ was 31 mM in rat DRG neurons [62]. By means of fluorescence imaging microscopy to measure $[Cl^-]_i$, Alvarez-Leefmans and colleagues found that $[Cl^-]_i$ in rat DRG neurons is maintained ~ 44.2 mM [36]. With these Cl^- concentrations, the E_{Cl} in DRG neurons of various species ranges from -26 to -46 mV. Since DRG cells have the resting membrane potential (E_m) of $-60 \sim -55$ mV, the E_{Cl} is more positive than the E_m .

As noted, high internal Cl^- concentration in DRG neurons is maintained by NKCC1 [42, 49]. A number of studies explain the correlation between Cl^- accumulation by co-transporters and pain behaviors. For example, significant nociceptive effects by several NKCC blockers such as bumetanide, piretanide, and furosemide, were observed in the formalin-induced tissue injury model [63]. In this model, peripheral administration of bumetanide diminishes phase I and phase II behavioral responses evoked by formalin [63]. Moreover, intrathecal administration of bumetanide eliminates formalin induced phase II behavioral responses [63]. In the same fashion, bumetanide and furosemide decrease itch and flare responses to histamine in human skin [64]. In particular, NKCC1 knock-out mice showed increased withdrawal latency from noxious thermal stimuli in hot-plate [65] and tail-flick tests [66]. NKCC1 is expressed in all DRG cells irrespective of their size or sensory modalities [36, 65, 67]. GABA-induced depolarization in persistent inflammation, however, is due to mechanisms other than NKCC1 activity change. This is because GABA_A currents are increased after inflammation without change in NKCC1 or phospho-NKCC1 protein levels [68].

Before molecular identities of Ca^{2+} -activated chloride channels (CaCCs), many researchers proposed a hypothesis claiming that chloride-based amplification of sensory signals in sensory systems. For example, when Ca^{2+} permeable channels such as TRP channels or voltage-gated Ca^{2+} channels are activated by noxious stimuli, the rise of $[Ca^{2+}]_i$ through Ca^{2+} permeable transduction channels open CaCCs. Activation of CaCCs depolarizes DRG neurons due to Cl^- efflux, thereby resulting in the amplification of excitatory responses [69-72]. These speculations remained elusive until candidate genes for CaCCs were cloned.

Ca^{2+} -ACTIVATED CHLORIDE CHANNELS

CaCCs are a class of anion channels that are activated by intracellular Ca^{2+} [73]. CaCCs are permeable to various anions, including F^- , Cl^- , Br^- , I^- , and other anions. CaCC currents have a unique property. CaCCs are activated by voltage and their currents that are outwardly rectifying. The kinetic of activation by depolarization is dependent on voltage. Namely, under relatively low Ca^{2+} condition, CaCCs are slowly activated by depolarization with their outwardly-rectifying current voltage ($I-V$) relationship. At high Ca^{2+} ,

however, CaCCs are quickly activated by depolarization and their $I-V$ curves are linear. As its title suggests, CaCCs are activated by intracellular Ca^{2+} , which is also voltage sensitive. For example, CaCCs are activated by much lower $[Ca^{2+}]_i$ at depolarization than that activated by hyperpolarization. Because of its activation by intracellular Ca^{2+} , many of the important physiological G-protein coupled receptor (GPCR) ligands, such as ATP, endothelin 1, and angiotensin II, are known to activate CaCCs [74-77].

CaCCs are present in almost all types of cells, including transport epithelia [73, 78]. CaCCs are thought to mediate fluid secretion in salivary glands, the airway, intestinal epithelium, and pancreas [79-81]. It is also known that CaCCs regulate smooth muscle contraction and neuronal and cardiac excitability. In the nervous system, CaCCs also regulate sensory transduction for vision, taste, smell, and somatic sensations [78]. In the case of vision, CaCCs are expressed in retina, specifically inner segments of rods and cones. Although the roles of CaCCs in rods are still unknown, it has been suggested that the role of CaCCs in cones is to modulate lateral inhibition [82, 83]. CaCCs also play a critical role in olfactory transduction because currents of CaCC are found in vertebrate olfactory receptor neurons. The CaCCs in olfactory system are thought to amplify odorant signals [84-86].

Candidate Genes for CaCCs Proposed Earlier

Despite the physiological importance of CaCCs, the molecular identity of CaCCs remained unknown for fairly long time. Some proteins, like CLCA and bestrophins, have been proposed earlier as CaCC candidates [87-89]. Although CLCA which was cloned from bovine trachea induced Ca^{2+} -dependent currents, there were some differences between CLCA and endogenous CaCCs channels in biophysical, biochemical or pharmacological properties [73, 90, 91]. CLCA currents are activated by depolarization without rise of intracellular Ca^{2+} [87, 92] and exhibit linear $I-V$. Moreover, the CLCA current is not blocked by niflumic acid, which is an inhibitor of endogenous CaCCs. Most importantly, the expression pattern of CLCA differs from cells where CaCC currents are found. Thus, CLCA is no longer considered as a candidate gene for CaCCs [91, 93]. Bestrophin, another candidate for CaCC, was identified as a gene which was associated with vitelliform macular dystrophy, also known as Best disease [94]. Several studies revealed that bestrophins, expressed in oocyte or HEK 293T cells, confer Cl^- currents activated by voltage [88, 95]. Bestrophins are activated by Ca^{2+} in a submicromolar range [96, 97]. Yet, whether bestrophins represent CaCCs is somewhat controversial because best1-deficient mice failed to affect currents of endogenous CaCCs in retinal pigment epithelium where best1 is highly expressed [98]. CaCCs in vascular smooth muscle cells are not down-regulated as well by knock-down of best3 [99]. Thus, the candidate genes for CaCCs remained elusive.

Cloning of ANO1 and its Characterization

Three groups of scientists cloned and identified TMEM16A (also known as anoctamin 1, ANO1) with a different cloning strategy. Yang and his colleagues found TMEM16A using

bioinformatic analysis for searching channel- or transporter-like genes with multiple transmembrane [100]. Gallietta and his colleagues cloned TMEM16A by microarray analysis of lung epithelial cells that had upregulated the expression of CaCC currents after IL-4 treatment [101]. Lastly, the Jan group in UCSF cloned TMEM16A by expression cloning approach using two different species of amphibians with differential expression of CaCCs [102]. Since this protein has eight transmembrane domains and conduct anion currents, Yang and colleagues renamed this protein as anoctamin 1; it is an anion channel with 8 (octa) transmembranes. The sequence similarity and phylogenetic analysis now demonstrate that there are 10 members in Anoctamin family in the vertebrates [100-102].

Properties of ANO1 as a CaCC

It is not obscure to accept that ANO1 is a candidate for a CaCC because it possesses typical biophysical and pharmacological property profile very similar to those found in endogenous CaCCs. When ANO1 is overexpressed heterologously, intracellular application of Ca^{2+} activates Cl^- currents with similar half-maximal concentration (EC_{50}) observed with native CaCCs [100, 103]. ANO1 is also activated by voltage pulses. Similar to the $I-V$ curves of native CaCCs, the $I-V$ relationship of ANO1 is outwardly rectifying. As observed with native CaCCs [104, 105], single-channel conductance of ANO1 is small [100]. Additionally, the Ca^{2+} sensitivity of ANO1 is voltage dependent, which is another unique feature of the property of endogenous CaCC's [103, 106, 107]. For example, the EC_{50} of ANO1 is 2.6 μM at -60mV holding potential whereas at +60mV the EC_{50} is 0.4 μM , indicating a greater sensitivity to Ca^{2+} at depolarization [100]. Pharmacological profile of ANO1 is also similar to that of native CaCCs [78, 108]. ANO1 activities are markedly blocked by several pharmacological blockers such as 4,4-diisothiocyanatostilbene-2,2-disulphonic acid (DIDS), 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), and niflumic acid that are reported to be a classical CaCC blockers [100-102, 106]. Lastly, ANO1 is activated *via* the PLC/ IP_3 pathway by various physiological ligands such as endothelin 1, angiotensin II, histamine, ATP, and acetylcholine as these GPCR ligands activate native CaCCs [100, 102]. Moreover, silencing ANO1 by the siRNA application reduced I^- flux induced by purinergic receptor

stimulation and transepithelial short-circuit currents [101]. Collectively, these results indicate that ANO1 is a candidate gene for a CaCC.

ANO1 PLAYS A ROLE IN PERCEPTION OF THERMAL PAIN

Roles of CaCCs in Neuronal Cells

As stated above, CaCCs are now known to play various physiological roles in various organs or tissues [73]. Among these, CaCC currents are reported in nerve tissues such as DRG neurons, spinal cord neurons, and autonomic neurons where they are thought to modulate neuronal excitability [109-112]. Specifically, a rise of intracellular Ca^{2+} concentration induces large anion conductance, being responsible for after-depolarization following depolarization in rat DRG neurons [109]. CaCC currents are also observed in a subpopulation of DRG neurons [113] and the spinal cord neurons where they would repolarize membrane during action potentials [114]. Furthermore, activation of CaCCs in rabbit parasympathetic neurons causes the after-depolarization [115]. Interestingly, high ANO1 immunoreactivity is detected in most of DRG neurons in mouse [100]. Smaller neurons are stained more densely than larger DRG ones, suggesting that ANO1 may participate in the transduction of nociceptive signals.

Activation of ANO1 by Noxious Temperature

ANO1 expression in DRG neurons indicates its possible role in the somatosensory system. Thus, our group casually tested whether ANO1 responds to changes in ambient temperature as many thermoTRP channels do. Surprisingly, ANO1-expressing HEK cells showed robust inward currents when the temperature of bath solution rose over 44°C [18] (Fig. 1). The temperature sensitivity of channels can be obtained with temperature coefficient (Q_{10}). The Q_{10} of ANO1 is 19.4 [18]. The range of Q_{10} value of TRP family is between 6 and 25 [1]. Since ANO1 is activated directly by intracellular Ca^{2+} , this leads to the possibility that heat-induced activation of ANO1 might be an indirect response to Ca^{2+} release from intracellular store. However, ANO1 activation by heat is less likely mediated by Ca^{2+} because heat evokes ANO1 currents even when intracellular Ca^{2+} is chelated with BAPTA. Furthermore, noxious heat also induces ANO1 currents in isolated inside-out membrane patches.

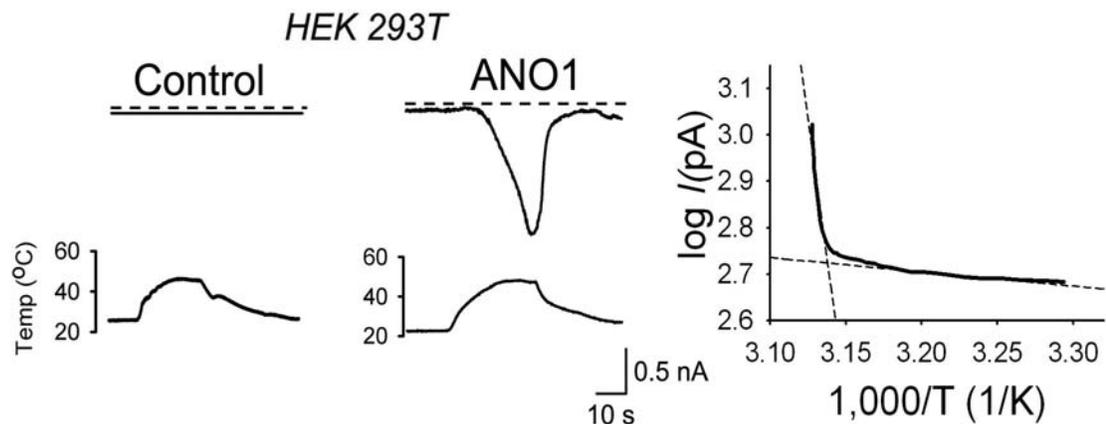


Fig. (1). Heat-evoked robust inward currents were induced in ANO1 transfected HEK293T cells (Cho et al., *Nature Neurosci*, 2012).

Synergistic Effect of ANO1 Activation by Heat, Voltage and Ca^{2+}

Many thermoTRP channels are activated by various stimuli, such as voltage or their ligands beside temperature change. In most cases, these stimuli are synergistic to each other. For example, TRPV1 displays synergistic effects of noxious heat, acid (proton), and capsaicin [8]. Moderate acidity, such as pH 6.4 that normally does not evoke any current potentiates heat and capsaicin-induced responses of TRPV1. The moderate acidity decreases the temperature threshold of TRPV1 and causes the activation of TRPV1 even at physiological body temperature (37°C) [8]. Similar to TRPV1, TRPV3 which is sensitive to warm temperature is opened by camphor and 2-aminoethoxyphenyl borate [26, 116]. TRPV4 activation by hypotonic stimuli at room temperature is augmented at 37°C of temperature [9]. In addition, the activation threshold of TRPM8, a cold sensor, is increased by sub-threshold concentration of menthol, a cooling compound [10, 117]. TRPM3 channel is also activated by noxious heat and pregnenolone sulfate, a neuroactive steroid. Recently, it was reported that heat and pregnenolone sulfate have a strong synergistic effect on TRPM3. Pregnenolone sulfate shifts the temperature-response curve of TRPM3 to the left. In contrast, 37°C of temperature augments the TRPM3 response to submicromolar pregnenolone sulfate [32].

Similar to thermoTRPs, the heat-induced activity of ANO1 is markedly enhanced by an endogenous ligand, Ca^{2+} . The rise of intracellular Ca^{2+} augments heat-induced ANO1 currents and decreases the threshold of ANO1 activation by heat [18]. In addition, ANO1 displays much greater currents during co-application of both thermal stimuli and Ca^{2+} than the application of Ca^{2+} alone. ANO1 is activated by near body temperature under condition of intracellular Ca^{2+} greater than 0.5 μ M [18]. Therefore, we speculate that a slight increase in intracellular Ca^{2+} during a pathological condition such as inflammation can open ANO1 at the body temperature [18].

Expression of ANO1 in DRG Neurons

Substantial studies have divided pain-conducting nociceptive neurons into two major classes, A δ fibers that convey acute and fast pain and C-fibers that mediate slow-conducting pain [3, 118]. C-fibers are unmyelinated with small diameter, dividing into several groups according to molecular and anatomical characterization [119]. Although most C-fibers are polymodal receptors that respond to heat, mechanical and chemical stimuli, but C-fibers are less sensitive to mechanical stimuli compared to chemical or thermal stimuli [120]. C-nociceptors are divided into peptidergic neurons containing substance-P or calcitonin gene-related peptide (CGRP) and non-peptidergic neurons lacking substance-P or CGRP [3, 121]. It has been proposed that a large percent of non-peptidergic neurons bind isolectin B4 and express c-Ret neurotrophin. TRPV1, first identified heat sensitive channel, is expressed exclusively in small-sized nociceptive neurons including A δ - and C-nociceptors [5, 8]. Therefore, TRPV1 is often considered as a marker for nociceptors because capsaicin injection causes pain and no other major sensory sensation.

ANO1 immunoreactivity is found in DRG neurons, but not in satellite cells [100]. ANO1 is expressed in small diameter DRG neurons that also express TRPV1. About 78% of ANO1-positive DRG neurons are also positive to TRPV1. In addition, ~58% and 31% of ANO1-positive neurons are positive with isolectin B4 and CGRP, markers for non-peptidergic and peptidergic neurons, respectively [3, 121]. ANO1 immunoreactivity is also observed in large myelinated neurons as well since some portions (~25%) of ANO1-positive neurons are co-localized with neurofilament M, marker for myelinated neurons [18].

Heat Induces Cl^- Currents in Sensory Neurons

Since ANO1 is expressed in the majority of small DRG neurons and activated by heat [18], it is unknown whether heat actually induces Cl^- currents in DRG neurons. Indeed, under the symmetrical NMDG-Cl solution conditions, heat ramps evoked inward Cl^- currents. Like ANO1 expressed in HEK cells, the temperature threshold of the heat-induced Cl^- currents in DRG neurons was ~44°C. Since TRPV1 is activated by heat, heat evoked-currents in DRG neurons would be of those evoked by TRPV1. Therefore, the heat-evoked response was examined in DRG neurons cultured from TRPV1^{-/-} mice to exclude the possibility of heat-induced TRPV1 currents. The application of heat still causes inward Cl^- currents in TRPV1^{-/-} mice with similar amplitude compared to those observed in wild type mice [18].

One big question regarding the role of Cl^- channels in sensory neurons is whether the activation of these chloride channels can depolarize or hyperpolarize sensory neurons. Determination of membrane potentials of sensory neurons depends on $[Cl^-]_i$ [36]. As mentioned above, the measurement of $[Cl^-]_i$ in DRG neurons has been obtained using Cl^- -selective microelectrodes [48], evaluating the reversal voltage of GABA receptors [60, 61], or the use of Cl^- -sensitive fluorescent dyes [36]. Most recent measurement of $[Cl^-]_i$ in isolated soma of DRG neurons reveals that it is about 44 mM, which sets the E_{Cl} at -27 mV [36]. Since the E_{Cl} of DRG neurons is more positive than the resting membrane potential of DRG neurons that ranges from -60 ~ -50 mV, the opening of Cl^- channel causes the efflux of Cl^- , and therefore bringing about the depolarization of DRG neurons in consequence [49]. Indeed, the application of noxious heat evokes the depolarization in DRG neurons from WT as well as TRPV1-deficient mice at the 30 mM $[Cl^-]$ of pipette solution [18].

Thermal Nociception after ANO1 Down-regulation

Physiological relevance of ANO1 as a heat sensor in nociception was determined in behavioral tests using pharmacological blockers or gene knock-down and knock-out methods [18]. Firstly, administration of mefloquine that completely blocks the heat-induced ANO1 currents significantly increases the withdrawal latency from radiant heat in tail flick assay. The analgesic effect of mefloquine is also effective in the inflammatory pain model. When inflammation is induced by the injection of carrageenan to the hind paw, mefloquine-treated rats show slower withdrawal response from the radiant heat than control rats. On the other hand, mefloquine injection failed to change responses to

mechanical stimuli in von Frey or Randal Selitto tests. The physiological role in nociception is also proven in the ANO1 knock-down experiment using small interfering RNA (siRNA) treatment. When tails of mice are immersed in hot water of range of 50 to 54°C, the tail withdrawal latency of ANO1 siRNA-treated mice increases significantly compared to that of scrambled siRNA treated or vehicle treated mice. Indeed, ANO1 siRNA treated mice also showed increased withdrawal latency to radiant heat with higher intensity [18].

Thermal Nociception in ANO1 Conditional Knock-out Mice

More reliable evidence of the role of ANO1 in nociception came from the knock-out experiments. The systemic knock-out of ANO1, however, brings lethal defects so that the neonates cannot survive in early neonatal periods. Poor growth and respiratory defects are major causes of early death [122]. In this regard, tissue-specific ANO1 disruption is needed to validate a more direct and clear function of ANO1 in thermal pain sensation. ANO1 conditional knock-out mice were generated using Advillin^{cre} transgenic mice that express Cre recombinase under the control of the *Avil* (advillin) promoter (*Avil^{cre}*) [18]. Advillin is an actin-binding protein that belongs to the gelsolin superfamily [123]. Hasegawa and colleagues studied the expression pattern of Advillin in the mouse nervous system and they found that Advillin is exclusively expressed in peripheral sensory neurons [124]. Thus, *Avil^{cre}* is a suitable tool for studying the specific function of ANO1 in sensory neurons. Consistent with the results observed in ANO1-siRNA treatment, tissue specific deletion of ANO1 in DRG neurons results in marked reduction in pain sensing like behavior to heat stimuli. For example, when tails of mice are immersed into hot water where temperature ranges from 50

to 54°C, ANO1 conditional knock-out (CKO) mice have longer tail withdrawal latencies compared to those of control mice. Similar antinociceptive reactions are also observed in ANO1 CKO mice in Hargreaves tests. Thus, these behavioral test results clearly suggest that ANO1 plays an important role in mediating acute thermal pain, presumably as a heat sensor (Fig. 2).

Other Anoctamins and their Responses to Heat

As discussed above, several channels that belong to the TRP channel family are temperature sensitive with distinct temperature thresholds. Therefore, it is particularly interesting to know which members of the anoctamin family are sensitive to heat. Among these families, ANO2 (TMEM16B) shares the highest sequence homology with ANO1 [100]. ANO2 displays Ca²⁺-induced currents in whole cell or inside-out patch configuration [125-127]. However, Ca²⁺ sensitivities of the two ANOs are different. For example, the half-maximal Ca²⁺ concentration of ANO2 at -60mV and +60mV was 4.0 and 5.1 μM, respectively, indicating the lower Ca²⁺ sensitivity than those of ANO1. A rundown effect of ANO2 was also observed under the condition of high concentration of Ca²⁺, suggesting that ANO2 may have different inactivation kinetics [125]. Moreover, ANO2 displays 1.2 pS of single channel conductance that is much smaller than the conductance of ANO1 [125]. Because ANO2 is expressed in cilia of olfactory receptor neurons and vomeronasal neurons, many studies suggest its role in the amplification of olfactory signals [126, 128, 129]. However, its physiological role in olfaction appears to be minimal because ANO2-deficient mice fail to show any change in olfaction [130]. In addition, ANO2 is expressed in presynaptic terminals of photoreceptors in retina, expecting some role in vision [127]. The

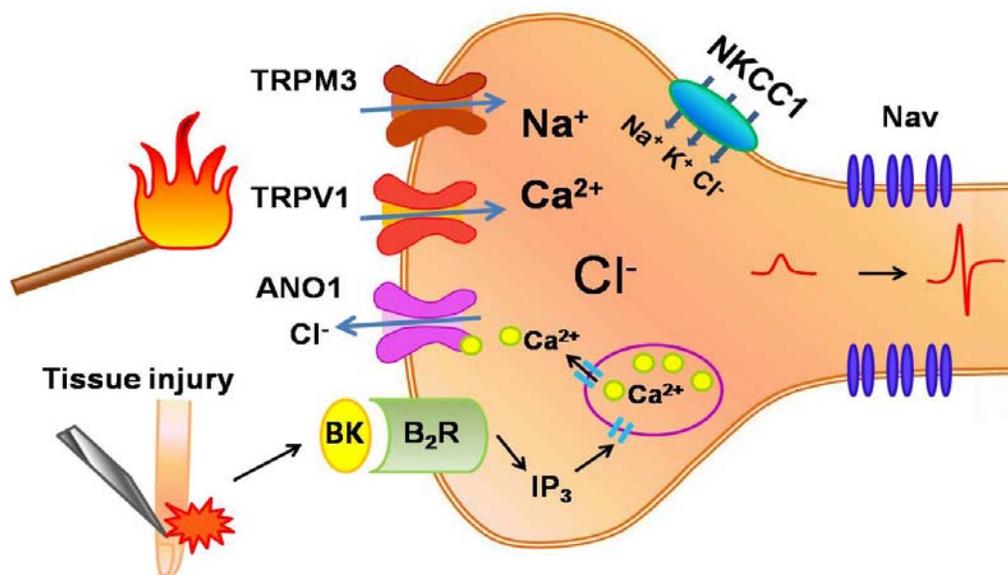


Fig. (2). Schematic representation of ion channels which are identified as a heat sensors in nociceptive neurons. The Ca²⁺ activated Cl⁻ channel, ANO1 is also activated by noxious heat. The depolarization of nociceptor membrane result from the Na⁺, Ca²⁺ influx which enter through activation of TRPV1 or TRPM3 by thermal stimuli or Cl⁻ efflux mediated by opening of ANO1, thereby open voltage gated sodium channels (Nav) and generate excitation of nociceptors for painful sensation. A depolarizing Cl⁻ efflux through activation of ANO1 is due to accumulation of intracellular Cl⁻ supplied by Na⁺, K⁺, 2Cl⁻ cotransporter (NKCC1). ANO1 also contributes to inflammatory nociception by bradykinin, mediated through B₂R-IP₃-Ca²⁺ signaling cascade.

physiological roles of ANO2 in phototransduction, however, are still in question. Recently, Liu *et al.* suggested that ANO2 is also expressed in DRG neurons after their quantitative RT-PCR analysis. The transcript level, however, is low compared to ANO1 [131]. Despite their scarcity in sensory neurons, ANO2 is sensitive to heat with the temperature threshold comparable to that of ANO1. Although ANO2 is activated by heat, its physiological implication as a heat sensor remains to be determined.

ANO4 and ANO5 are known to be associated with human diseases [132-134]. Mutations in ANO5 result in musculoskeletal disorders such as gnathodiphyseal dysplasia, which is a rare autosomal skeletal syndrome. Gnathodiphyseal dysplasia is characterized by bone mineralization and fragility which is caused by the mutation of cysteine residue in the first extracellular loop of ANO5 [135]. Recent studies also reported that patients with muscular disease, like proximal limb-girdle muscular dystrophy or distal nondysferlin Miyoshi myopathy, have recessive mutations of ANO5 that presumably cause defective skeletal membrane repair [136, 137]. In addition, two groups of researchers reported that the dysfunction of ANO6 is associated with Scott syndrome, a rare bleeding disorder [138]. Suzuki and colleagues firstly reported that ANO6 is essential for Ca^{2+} dependent scramblase activity, which redistributes phosphatidylserine and phosphatidylethanolamine from extracellular surface of the plasma membrane in platelets, a necessary procedure for blood clotting of platelets [138]. Recently, the Jan's group in UCSF confirmed that ANO6-deficient mice exhibit impaired blood-clot homeostasis and phosphatidylserine exposure [139]. Duran and colleagues claimed that ANO3 - ANO7 are present in intracellular organelles [140]. In contrast, the expressions of ANO8, ANO9 and ANO10 are observed mostly in the cytosol whereas ANO4, ANO6, and ANO7 are expressed in the plasma membrane [141].

With the exception of ANO1 and ANO2, other members in anoctamin family are not activated by intracellular Ca^{2+} at physiological concentration [100, 133]. Interestingly, when the heat sensitivity of ANO family members was examined, only ANO1 and ANO2 were activated by heat [18].

ANO1 is Involved in Acute Nociception by Bradykinin

Bradykinin (BK) is a potent algogenic substance that is produced from sites of inflammation or injury [142, 143]. Thus, BK is considered as an inflammatory mediator for nocifensive responses [144]. Pain-producing effect of BK is mediated by the activation of B_2 receptors, which is a G-protein coupled receptor [145, 146]. BK has two effects on pain. Spontaneous pain is one effect which is induced by the direct activation of sensory nerve by BK [143]. The other effect of BK is the sensitization of the sensory nerve, which reduces the threshold for pain evoked by other noxious or non-noxious stimuli [22, 145]. The mechanism of BK-induced hyperalgesia or allodynia is relatively understood better than that of the BK-induced acute pain. For example, B_2 receptor activation by BK causes the sensitization to TRPV1 [147, 148] or TRPA1 [149]. However, the mechanism of acute nociceptive response induced by BK has not been well understood. A study by Liu *et al.* revealed that acute

pain response by BK is mediated both by the inhibition of M-type K^+ channel and the activation of ANO1 [131]. It is crystal clear that the stimulation of B_2 receptors by BK activates phospholipase C, which in turn hydrolyzes phosphatidylinositol-4,5-bisphosphate to inositol 1,4,5-triphosphate [150]. The latter triggers the release of Ca^{2+} from endoplasmic reticulum [145]. Thus, since BK can increase $[Ca^{2+}]_i$, it is conceivable that BK would activate ANO1 in sensory neurons. Indeed, Liu and colleagues confirmed the signaling cascade leading to increase in $[Ca^{2+}]_i$ by BK in small DRG neurons. Furthermore, they found that BK-induced inward currents are not blocked by ruthenium red, a TRP channel blocker. However, low concentration of intracellular Cl^- solution dramatically reduces inward currents evoked by BK. Treatment of Cl^- channel blockers, including 4,4-diisothiocyanatostilbene-2,2-disulphonic acid, 5-nitro-2-(3-phenylpropylamino) benzoic acid, and niflumic acid, abolish the BK-induced currents. More importantly, the application of siRNA of ANO1 to DRG neurons attenuates the BK-induced currents. Moreover, BK-induced nocifensive behavior in rats is also attenuated by the intraplantar injection of CaCC blockers [131]. Thus, the involvement of ANO1 in the BK-evoked pain signal is much consistent with the perspective of the role of ANO1 in mediating pain in nociceptors.

CONCLUDING REMARKS

In the long search for the molecular mechanism responsible for TRPV1-independent heat sensation, ANO1 might be one candidate for an additional heat sensor. Several lines of evidence now suggest that ANO1 is a molecular sensor that transduces noxious thermal pain. First, ANO1 is exclusively expressed in small nociceptive DRG neurons. Second, heat over 44°C evokes currents in ANO1-transfected cells that have similar biophysical and pharmacological properties with those of Ca^{2+} -evoked currents. Third, ANO1 depolarizes DRG neurons under physiological chloride concentration. Finally, functional deletion of ANO1 in DRG neurons elicits significant loss of thermal pain.

Although these results clearly suggest that ANO1 plays an important role in mediating thermal pain as a heat sensor, there are a few questions that still remain unclear: First, which part of ANO1 mediates heat sensing? Second, is ANO1 implicated in thermal hyperalgesia evoked by chronic tissue injury? Third, are other ANO family members involved in temperature sensation? In order to elucidate the thermal sensitivity of other ANO members, further study may be required. In fact, since Ca^{2+} acting site of ANO1 is unknown, answers to these questions may help understanding the gating mechanisms of ANO1 by thermal stimuli. More importantly, because ANO1 agonists are considered possible cystic fibrosis targeting drug candidates, the understanding of the role of ANO1 in pain may support safer drug development.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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