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A Study of TRPA1 Activation via Covalent Electrophilic Modification of Cysteines

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A Study of TRPA1 Activation via Covalent Electrophilic Modification of Cysteines

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
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Abstract

A subset of sensory nerves called nociceptors are stimulated by noxious stimuli and evoke nocifensive responses in different organ systems. Transient Receptor Potential Ankyrin 1 (TRPA1) is a tetrameric, nonselective, cation channel that initiates the generator potential that evokes afferent signaling in nociceptive nerves. TRPA1 is activated by many harmful irritants, such as food chemicals, environmental pollutants, reactive oxygen species and other endogenous mediators. Most TRPA1 agonists have electrophilic properties, which covalently modify cysteine residues on the cytosolic side of the channel. Biochemical studies in our lab have identified four cysteines (C273, C621, C665, C1085) that rapidly bound iodoacetamide (irreversible electrophile); C621 bound at a rate that was 6,000-fold greater than nonreactive cysteines. Electrophilic adduction of cysteines can result in TRPA1 activation, but the detailed mechanism underlying this process is poorly understood. We hypothesize that electrophilic modification of C621 and C665 independently contributes to TRPA1 activation. In this study, we have utilized live-cell calcium imaging, whole-cell patch clamp and single-channel patch clamp to determine the role of highly reactive cysteine residues in TRPA1 activation via electrophiles. We determined that the highly reactive C621 is critical for TRPA1 and the local environment surrounding C621 contributes to its reactivity. C665 is not required to initiate TRPA1 activation but could contribute to the transition from partial to full activation. We determined that depletion of cytosolic glutathione in whole-cell recordings decreases electrophile-evoked currents. Lastly, in single-channel recordings we identified two sequential activation profiles for TRPA1 which were differentially regulated by C621 and C665, suggesting that TRPA1 has multiple open states. Our novel data
provide novel functional insights consistent with recently published structural data of TRPA1 activation.
Chapter 1

Introduction

Sensory Nerves

Organisms constantly receive local cues that allow them to adapt to their environment. There are various types of stimuli such as light, sound, and temperature that come from the environment and is detected by the organism’s sensory systems. Afferent sensory nerves respond to stimuli, innervate organ systems throughout the body (via the peripheral nervous system (PNS)) and propagate signals to the central nervous system (CNS)(e.g. the spinal lamina and the brainstem medulla) (Costanzo, 2014). Afferent nerves are heterogenous with respect to anatomy and gene expression (Kupari et al., 2019; Perry & Lawson, 1998; Usoskin et al., 2015). Aα-fibers are highly myelinated afferent nerves that innervate muscles/tendons and detect proprioceptive body movements (Bear et al., 2007; Costanzo, 2014). Aβ-fibers are myelinated nerves that innervate cutaneous tissue and detect low-threshold mechanical stimuli (Bear et al., 2007; Costanzo, 2014). Other afferent sensory nerves detect noxious stimuli, which drive nocifensive reflexes and behaviors. Nociceptive Aδ-fibers are myelinated afferent nerves that detect sharp pain and cool and nociceptive C-fibers are unmyelinated nerves that are detect dull pain, noxious heat/cold and chemical (Djouhri et al., 1998; Konietzny et al., 1981; Ochoa & Torebjörk, 1989). The soma of afferent sensory nerves resides in anatomical ganglia such as the dorsal root ganglia (DRG), vagal (VG), nodose (NG), jugular (JG), trigeminal (TG), glossopharyngeal (GG), etc. (Vermeiren et al., 2020). These ganglia contain heterogeneous
sensory neurons that express various types of receptors which are activated by a plethora of stimuli.

When activated, receptors that are expressed in sensory neurons of nociceptive C-fibers evoke a generator potential current. If the generator potential is higher than the threshold for voltage-gated sodium channel (e.g., Na\textsubscript{V}1.7, Na\textsubscript{V}1.8, Na\textsubscript{V}1.9) activation, these channels will activate and evoke membrane depolarization (Rush et al., 2007). This Na\textsubscript{V}-mediated self-renewing depolarization will trigger action potential propagation towards the central synapses (e.g., the laminae in the medullary dorsal horn of the spinal cord for DRG afferents) (Watson, 1981). Afferent signals can continue transmission through to postsynaptic nerves in the dorsal horn. C-fibers can release neurotransmitters like glutamate (De Biasi & Rustioni, 1988), serotonin (5-HT), substance P (SP) (De Biasi & Rustioni, 1988), calcitonin-gene related peptide (CGRP) (Abbadie et al., 1997) that will activate the postsynaptic secondary afferent nerve. The signals produced by the secondary afferent nerves will propagate to the spinothalamic & spinoreticulothalamic tracts, and finish in the somatosensory cortex where the pain signals are processed (Willis & Coggeshall, 2004). Furthermore, nociceptive afferent signals can be processed in the amygdala, which results in emotional and adverse responses to pain (Petrov et al., 1993; Zald, 2003). The cortex may produce efferent signals, which are transmitted to muscles and glands to elicit motor or autonomic reflex responses. In addition, local efferent nerves in the dorsal horn may also be activated independently of the CNS by the initial afferent signals.

Chronic exposure to noxious stimuli causes increased c-fiber activation, local inflammation, and sensitization at the site of injury or at the synapse (Woolf, 1983). Neurogenic inflammation, which is caused by neuropeptide release from the peripheral terminals of c-fibers, produces an “inflammatory soup” of immune cells, cytokines, and proinflammatory mediators that lowers the threshold for action potentials, sensitizes the nerve, and produces an amplified response to pain (Herbert & Holzer, 2002). The sensitization of c-fibers can result in allodynia (normally innocuous sensations that cause pain) (Willis, 1993) or hyperalgesia (noxious
sensations that cause increased pain) (LaMotte et al., 1992), which can prove to be unbearable for patients that suffer from these conditions.

As previously mentioned, polymodal nociceptive c-fibers express ion channels that are activated by harmful mechanical, thermal, and chemical stimuli which stimulates afferent signaling. For example, mechanically activated Piezo channels (Piezo1 and Piezo2) are Ca\textsuperscript{2+}-permeable non-selective cation channels that are expressed in sensory nerves that innervate the bladder, kidneys, vascular endothelial cells and lungs (Coste et al., 2010). Touch or harmful pressure stimuli creates tension in the cellular membrane, where Piezo channels are expressed. Acid-sensing ion channels (ASIC) are part of the voltage-insensitive degenerin/epithelial sodium ion channel family (DEG/ENaC) that are activated by extracellular protons in conditions of acidosis during tissue injury and inflammation (Waldmann et al., 1997). There are six subtypes of ASIC that may also be sensitive to noxious mechanical stimuli (Waldmann et al., 1997). The purinoreceptor P2X (P2X\textsubscript{2} & P2X\textsubscript{3}) is a nonselective cation channel that is activated by extracellular ATP (released during tissue injury), which initiates action potentials in afferent nerves (Lewis et al., 1995). When ATP binds to the extracellular binding pockets between subunits, P2X gating is initiated.

Transient Receptor Potential (TRP) channels, a large group of multimodal, non-selective cation channels, play a major role in sensory systems. In 1969, TRP channels were first identified within a mutant Drosophila strain that showed blindness (Cosens & Manning, 1969). TRP channels consist of four subunits, six transmembrane domains, a pore-forming hydrophilic loop between the fifth and six transmembrane domains and cytosolic N- and C-termini with ankyrin repeats. Six subtypes of TRP channels in mammals that have been identified: TRP classical or canonical (TRPC), melastatin (TRPM), polycystin, (TRPP), mucolipin (TRPML), vanilloid (TRPV) and ankyrin (TRPA) (Montell et al., 2002). When TRP channels are activated, they lead to cation fluxes that evoke generator potentials. TRPM8 is a well-researched TRP channel which is activated by cool and noxious cold (10-23°C), and chemicals that cause a cold sensation with
analgesic properties (e.g., menthol and eucalyptol) (Peier et al., 2002). Cold sensitization is stimulated by neurotrophic factors that interact with receptors that are co-expresssed TRPM8 (Ciobanu et al., 2009). Also, studies show that phosphatidylinositol 4,5-bisphosphate (PIP$_2$) modulates TRPM8 cold sensitization via the protein kinase C (PKC) pathway and can activate TRPM8 (Rohács et al., 2005). The noxious heat (≥42°C), extracellular acid (pH ≤6.0) pH sensitive TRPV1 is another well-characterized polymodal TRP channel (Caterina et al., 1997). Vanilloids such as capsaicin, the pungent chemical from chili peppers, activate TRPV1 which triggers the release of pro-inflammatory mediators and cytokines (Bhave et al., 2002; Premkumar & Ahern, 2000; Prescott & Julius, 2003). TRPV1 is highly expressed in small diameter nociceptive sensory neurons. TRPV1 can undergo phosphorylation due to inflammatory mediators activating protein kinase pathways (e.g., protein kinase A (PKA) (Bhave et al, 2002), PKC (Bhave et al., 2003), etc.), which can increase channel activity and pain signaling.

**TRPA1**

In sensory neurons, TRPV1 is not the only TRP channel that has a major contribution on chemically sensitive nociception signaling. Transient Receptor Potential Ankyrin 1 (TRPA1), originally known ANKTM1, was cloned from cultured lung fibroblasts and consists of 14 (mTRPA1) or 16 (hTRPA1) ankyrin repeats (Story et al., 2003). TRPA1 is a tetrameric, nonselective cation channel with six transmembrane domains, a cytosolic N- and C-termini, and a pore loop between the 5$^{th}$ and 6$^{th}$ transmembrane domains (Story et al., 2003). Prior studies show evidence of TRPA1 expression throughout different species as a temperature sensor. In *Drosophila* (dTRPA1) (Kang et al., 2010), snakes (Gracheva et al., 2010), frog (Saito et al., 2012), chicken (Saito et al., 2014), and zebrafish, TRPA1 is used as at heat sensor, but TRPA1 is sensitive to cold temperatures in rat (rTRPA1) and mouse (mTRPA1) (Chen et al., 2013). In pit vipers, TRPA1 is expressed in the pit organ and is used as a thermal detector to sense other organisms (Panzano et al., 2010). In addition to temperature sensing, TRPA1 is a ligand-gated
channel. It is activated by allyl isothiocyanate (AITC, a pungent ingredient in wasabi and mustard oil) and many other agents (see below) (Jordt et al., 2004). The N-terminal ankyrin domains are potentially involved in protein-to-protein interactions but replacement of hTRPA1 ankyrin repeats with rattlesnake TRPA1 (which is insensitive to AITC) (rsTRPA1) chimeras reduces AITC sensitivity and confers heat sensitivity (Cordero-Morales et al., 2011; H. Wang et al., 2013). Native TRPA1 is expressed as a homo-tetrameric channel but evidence suggests that TRPA1 and TRPV1 subunits could form heteromeric channels that are sensitive to both capsaicin and mustard oil (Akopian et al., 2007; Fischer et al., 2014; Staruschenko et al., 2010).

The pore of TRPA1 contains glutamic acid residues (E920, E924, E930) surrounding the outer portion which attracts cations (Christensen & Corey, 2016; Paulsen et al., 2015). In addition, the TRPA1 pore consists of an outer gate with a selectivity filter (composed of L913, G914, and D915 residues), and the lower gate which remains in a partially closed state (Paulsen et al., 2015; Christensen et al., 2016). Within the selectivity filter, the D915 residue on each subunit form a negatively charged ring that promotes permeability of Ca^{2+}, K^+, and Na^+. The lower gate is formed by I957 and V961, which creates two hydrophobic seals that binds one rehydrated Ca^{2+} (Paulsen et al., 2015). Extracellular Ca^{2+} has a modulatory effect on TRPA1 activation. TRPA1 currents can be potentiated by 12-50µM concentrations of extracellular Ca^{2+} but millimolar concentrations of Ca^{2+} can quickly inactivate TRPA1 currents (Wang et al., 2008). Inorganic polyphosphates also have a modulatory effect on TRPA1 activity by preventing rundown of currents in excised patch clamp techniques (whole-cell and inside out patch), although this mechanism has yet to be determined (Kim & Cavanaugh, 2007).

There is evidence of g-protein coupled receptor signaling pathways modulating TRPA1 activation via the bradykinin 2 receptor and OAG, which is a cell permeable analog of DAG (a product of phosphatidylinositol 4,5-bisphosphate (PIP_2) due to cleavage by phospholipase C (PLC)) (Bandell et al., 2004). Studies also show that phospholipase C (PLC) inhibitors, which prevents the production of IP_3 and DAG, reduce AITC-induced TRPA1 currents and PLC and PKA
activators potentiate AITC-induced currents (Wang et al., 2008). PIP$_2$, the precursor to IP$_3$ and DAG, inhibits TRPA1 activation and a reduction of PIP$_2$ synthesis via phenylarsine oxide (PAO) rescues TRPA1 activation (Karashima et al., 2008; Kim et al., 2008).

The TRPA1 pore has a diameter of 7-8Å but can undergo reversible dilation during activation of up to 1-3 Å (J Chen et al., 2009), which makes the channel permeable to divalent ions (Bobkov et al., 2011; Karashima et al., 2010). Such pore dilation may be the cause of the variation of the recorded TRPA1 unitary conductances, which range from 60pS (Zhou et al., 2011) to 173pS (Bobkov et al., 2011).

TRPA1 is expressed in nociceptive Aδ- and C-fibers derived from sensory neurons of the JG, NG, TG and DRG (Anand et al., 2008; Hsu & Lee, 2015; Y. S. Kim et al., 2010; Kobayashi et al., 2005; Nagata et al., 2005; Story et al., 2003) Previous studies also identified TRPA1 expression in non-neuronal tissue such as basal keratinocytes, respiratory airways and small intestine (Anand et al., 2008; Caceres et al., 2009; Stokes et al., 2006). Initially, studies suggested a role of TRPA1 in mouse ear hair cell transduction, but TRPA1 knockout mice (TRPA1 KO) revealed no auditory deficiencies (Bautista et al., 2006; Corey et al., 2004; Kwan et al., 2006; Nagata et al., 2005). Activation of neuronal TRPA1 produces generator potentials that contributes to the initiation of action potentials, which evokes afferent signaling in nociceptive C-fibers. Because afferent C-fibers innervate organ systems throughout the body, excessive TRPA1 activation can play a major role in a multitude of diseases and proves to be appealing target for treatments. For example, inhibition or knockout of TRPA1 prevents hypersensitivity of mechanical stimuli in diabetic and non-diabetic mice (Kerstein et al., 2009; Wei et al., 2010, 2009); and HC-030031 decreased airway inflammation in an ovalbumin mice model of asthma and TRPA1 KO mice showed a reduction in airway hyperreactivity (Caceres et al., 2009). In humans, an autosomal-dominant mode of inheritance of the TRPA1 gene causes Familial Episodic Pain Syndrome, where patients have random periods of extreme pain that is triggered by illness, cold temperature and physical exertion (Kremeyer et al., 2010).
Activators of TRPA1

TRPA1 is a promiscuous channel that can be activated by a diverse number of agonists such as pungent food chemicals, irritants and endogenous mediators (Holzer, 2011). Initial studies showed evidence that noxious cold (≤17°C) evokes activation of TRPA1, but there are additional studies that fail to observe cold-initiated TRPA1 activation in TG sensory neurons and heterologous-expressed HEK293 cells (Story et al., 2003; Jordt et al. 2004; Nagata et al. 2005; Bautista et al. 2006). These studies inferred that cold-induced activation of TRPA1 was contributed by noxious cold temperatures or menthol-induced activation of TRPA1 (Jordt et al. 2004; Nagata et al. 2005; Bautista et al. 2006). However, there is substantial evidence of TRPA1 activation by low temperature in vitro and in vivo in rats and mice without contribution from TRPM8 (Bandell et al., 2004; Fajardo et al., 2008; Karashima et al., 2009; Sawada et al., 2007).

Many irritants and pollutants activate TRPA1. Mustard oil causes TRPA1 activation in calcium imaging experiments with dissociated TG neurons (Jordt et al., 2004; Bautista et al., 2006) and wildtype mice display increased nocifensive behaviors (e.g., flinch and licking) in response to injection of mustard oil in the paw, but these pain responses are ablated in TRPA1 KO mice (Bautista et al., 2006). Cinnamaldehyde (pungent chemical in cinnamon) (Bandell et al., 2004), allicin (garlic) (Bautista et al., 2006; Macpherson et al., 2005), gingerol (ginger) (Bandell et al., 2004) and thymol (thyme) (S. P. Lee et al., 2008) also trigger TRPA1 activation in sensory neurons and Xenopus oocytes. Toxic chemicals in environmental pollutants (e.g., hypochlorite) (Bessac et al., 2008), chemical irritants (e.g., isocyanates) (Bessac et al., 2009; Thomas E. Taylor-Clark et al., 2009) and cigarette smoke (e.g., acrolein and crotonaldehyde) (Andrè et al., 2008; Bautista et al., 2006; Lynch et al., 2020) can evoke TRPA1 activation. Consistent with their ability to activate TRPA1, the inhalation of acrolein, AITC, and cinnamaldehyde elicit cough responses in guinea pigs (Andre et al., 2009; Birrell et al., 2009). Lastly, general anesthetics (e.g., isoflurane, propofol) (Fischer et al., 2010; Matta et al., 2008; Nishimoto et al., 2015), local
anesthetics (lidocaine) (Leffler et al., 2011) activate hTRPA1 and mTRPA1 expressing cells in live-cell calcium imaging and electrophysiological studies.

In addition to exogenous irritants, endogenous products that are produced in the event of cellular damage, oxidative stress and inflammation can activate TRPA1. Oxidative and nitrative stress produces reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as hydrogen peroxide (H$_2$O$_2$) (Andersson et al., 2008; Bessac et al., 2008; Sawada et al., 2008), nitric oxide (NO) (Miyamoto et al., 2009) and nitrooleic acid (9-OA-NO$_2$) (Taylor-Clark et al., 2009) that evoke TRPA1 and C-fiber activation in extracellular recordings. ROS cause lipid peroxidation, leading to the production of 4-hydroxy-2-nonenal (4-HNE) (Andersson et al., 2008; Trevisani et al., 2007) and 4-oxo-2-nonenal (4-ONE) (Graepel et al., 2011), which evoke nocifensive behaviors via TRPA1 activation. Tissue injury and inflammation can also promote the formation of prostaglandins (PG), some of which (e.g. 15dPGJ$_2$) activate TRPA1 (Taylor-Clark et al., 2008).

**Electrophilic Activation of TRPA1**

As previously mentioned, TRPA1 is activated by a plethora of agonists. However, the mechanism underlying activation of TRPA1 is poorly understood. Many TRPA1 agonists are oxidants, isothiocyanates, methanethiosulfonates, or $\alpha$, $\beta$-unsaturated aldehyde/carbonyl compounds that have electrophilic properties. Electrophiles accept electrons from nucleophiles (electron donors) and adduct via covalent bonds (Rudolph & Freeman, 2009). Thiol groups, including cysteines, have nucleophilic properties which allows them to donate electrons to electrophiles. Biochemical studies show that isothiocyanates covalently modify glutathione (GSH) to form dithiocarbamates, but in an unstable or reversible manner (Karlsson et al., 2016; Petri et al., 2020). Evidence showed that electrophilic isothiocyanates (e.g., AITC and benzyl isothiocyanate (BITC)) activate TPRA1, but non-electrophilic compounds that are isosteric with isothiocyanates (e.g., thiocyanates) fail to activate TRPA1 (Hinman et al., 2006). Also, AITC-induced TRPA1 activation can be washed out over time, suggesting that direct covalent
modification by AITC is reversible. Endogenous (i.e., 4-HNE and 4-ONE) (Doom & Petersen, 2002) and exogenous (i.e., acrolein) (Uchida et al., 1998) TRPA1 ligands that have α, β-unsaturated aldehyde/carbonyl compounds covalently modify nucleophilic cysteines via stable, irreversible Michael addition reactions. N-methylmaleimide (NMM), another irreversible electrophile, activates TRPA1 in an irreversible manner and these responses were ablated by Ruthenium Red, a TRP channel pore blocker (Hinman et al., 2006). The nucleophilic cysteines involved in TRPA1 activation are located on the cytosolic side of the channel – this was indicated by TRPA1 activation by MTSEA (membrane permeable) but not by MTSET (a membrane impermeable analog) when exogenously applied (Hinman et al., 2006; Macpherson et al., 2007).

In addition, a role for electrophilicity in TRPA1 activation is also demonstrated by a study of prostaglandin TRPA1 activation: the electrophiles 15dPGJ2 and PGA2 activated TRPA1 unlike the non-electrophilic precursors PGD2 and PGE2 (Taylor-Clark et al., 2008). Importantly PGB2, which has a sterically hindered electrophilic group that cannot undergo thiol modification, failed to activate TRPA1.

The initial point mutation study identified three evolutionarily conserved cysteines that prevented electrophilic-induced TRPA1 activation: C621, C641 and C665 (Fig. 1.1). Thus, a TRPA1 channel that contained mutations at the three cysteine sites (TRPA1-3C) failed to activate during NMM treatment but surprisingly maintained some sensitivity to AITC (Hinman et al., 2006). In addition to their thiol reactivity, isothiocyanates have the ability to modify amine groups and one study saw that mutation of a lysine residue (K708Q) (Fig. 1.1) along with TRPA1-3C (TRPA1-3C/K-Q)
abolished AITC-induced activation (Hinman et al., 2006). Another study identified that mutations at three cysteine residues (C415, C422 and C622) on the mTRPA1 channel that lost sensitivity to mustard oil, which further suggests that covalent modification of cysteines is a mechanism for TRPA1 activation (Macpherson et al., 2007). Although these key cysteines were required for TRPA1 activation by electrophiles, it was unclear if the cysteines played a direct role in electrophilic binding or played an allosteric role in the channel activation. Our lab determined that electrophiles adduct to reactive cysteines on TRPA1 at a rate that is substantially quicker than reactions of electrophiles to conventional cysteines like GSH (Bahia et al., 2016). 2-minute treatment of the electrophile iodoacetamide (IA) identified four cysteine residues (C273, C621, C665, and C1085) that were rapidly modified in binding assays (Fig. 1.2). Specifically, C621 was modified by IA at a reaction rate that is 6000-fold greater than unreactive cysteines (e.g., C540). This evidence suggests that C621 is highly reactive to electrophiles and could play a critical role in electrophilic-induced TRPA1 activation (Bahia et al., 2016). At physiological pH (~7.4 pH), the side group on canonical cysteines (GSH) are mainly in the thiol form due to the sulfhydryl group’s pKa (~8.5) (Peng et al., 2013). However, the local amino acid environment surrounding cysteines in protein structures can lower the pKa of sulfhydryl side groups, which will deprotonate the thiol and form a reactive thiolate anion side group (Wilson et al., 1977). Therefore, the local environment surrounding C621 could be contributing to its high reactivity to electrophiles. Cryo-EM studies revealed modeling evidence of modification of C621 by electrophiles within a binding pocket on the TRPA1 channel (Paulsen et al., 2015; Suo et al., 2020; Zhao et al., 2020). These studies also suggested some degree of electrophilic modification...
of C665 upon electrophile exposure, and predicted that modification of C621 could decrease the pKa of the C665 sulfhydryl group.

There is sufficient evidence suggesting that cysteine modification of electrophiles evokes activation of TRPA1. However, the link between electrophilic binding of cysteines and activation of TRPA1 is poorly understood. Overall, our goal is to determine the mechanism underlying TRPA1 activation. We want to identify how many cysteine modification events are required for TRPA1 gating, and we wish to characterize the activation profile for the TRPA1 channel and determine how each TRPA1 subunit contributes to channel activation. We hypothesize that TRPA1 activation is a stepwise activation that requires independent cysteine modification events (per subunit) to occur before the channel reaches an open state.
Chapter 2

Methods

HEK293 cell culture, TRPA1 constructs and expression

The full length human TRPA1 (hTRPA1) genes were provided by David Julius (University of California, San Francisco, San Francisco, CA). hTRPA1 was subcloned into pcDNA3.1 V5-His-TOPO® (Life Technologies; Grand Island, NY) using primers (Biosynthesis, Lewisville, TX) containing restriction sites allowing ligation into the vector as described previously (Bahia et al., 2016). Point mutations were made using site-directed mutagenesis via PCR. The hTRPA1-K3C mutations (K620A, C621A, C641A and C665A) were introduced by synthesizing a 1722 bp fragment (Genscript, NJ, USA) and ligating into hTRPA1-V5-His. hTRPA1-K3C was subcloned into pIRES2-EGFP (Takara Bio, Mountain View, CA) using In-Fusion PCR (Takara Bio, Mountain View, CA). hTRPA1 WT and mutant channels were expressed in HEK293 cells (cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin) using Lipofectamine 2000 (Thermo Fisher Scientific).

Live-cell Ca2+ imaging

Live cell Ca2+ imaging measured changes in intracellular Ca2+ in non-transfected, transiently expressed human TRPA1 (hTRPA1) or hTRPA1-mutant HEK 293 cells. HEK 293 cell-covered coverslips were incubated with Fura-2AM (4µM) for 30-60 minutes. Coverslips were perfused with heated (33-34-F) HEPES buffer (composed of (mM): 145 NaCl, 10 HEPES, 5.6
dextrose, 4.7 KCl, 1.2 MgCl2, 2.5 CaCl2; pH adjusted to 7.4 with NaOH) and equilibrated for 10 minutes prior to experimentation. Changes in intracellular Ca
were monitored using sequential excitation at 340nm and 380nm (510 nm emission) using microscopy (C13440-20CU; Hamamatsu, Bridgewater, NJ) and Nikon Elements (Nikon, Melville, NY). Cells were treated with vehicle (0.1% ethanol) and then either iodoacetamide (IA, 30µM), N-ethylmaleimide (NEM, 30µM) or allyl isothiocyanate (AITC, 30µM) for 3 minutes. Cells were also treated with the non-electrophilic TRPA1 agonist thymol (200µM). In studies observing the effects of non-electrophilic agonists on TRPA1, cells were treated with thymol (200µM) for 1 minute and 2APB (non-electrophilic TRPA1 agonist) for 2 minutes following a 3-minute washout period. Maximal intracellular Ca
responses were determined with ionomycin (5µM). In preliminary studies and published binding studies (Bahia et al., 2016), all TRPA1 constructs displayed significant responses to thymol (compared to non-transfected HEK293, (data not shown), indicating the presence of functional channels. K3C-EGFP expression was identified by eGFP expression at 470nm (488nm excitation, 509nm emission). Cells with mean GFP intensity above background mean intensity were considered positive. Ca
imaging data is presented as Δ340/380 ratio: (R- Rbi), where R is the ratio at a given time point and Rbi is the mean baseline ratio response period for the 1st minute.

**Electrophysiology**

Recordings from hTRPA1-expressing or non-transfected HEK293 cells were performed at room temperature (20-22°C) using whole-cell, perforated or cell-attached single channel recording techniques. Patch pipettes were fabricated from 1.5-mm o.d., 1.1-mm i.d. borosilicate glass (Sutter Instrument Co., CA) and fire-polished. Cell membrane currents were recorded and analyzed using a MultiClamp 700B amplifier, Digidata 1440A and pClamp 10 acquisition software (Molecular Devices, Sunnyvale, CA).
Whole-Cell Patch Clamp

Whole-cell recordings used a sampling rate of 100kHz with a 2kHz single pole RC lowpass filter. Whole cell pipettes (3–9MΩ) were filled with solution composed of (mM): 130 CsCl, 1 CaCl2, 2 MgCl2, 10 HEPES, 10 dextrose, 11 EGTA, and 5 Na-triphosphate; adjusted to pH 7.2 with NaOH. 5mM Na-triphosphate was used to prevent rundown of TRPA1 currents (D Kim & Cavanaugh, 2007). Pipette [Ca2+]i, was calculated to be ~25µM, which maximizes TRPA1 currents while limiting channel inactivation (Y. Y. Wang et al., 2008). Cells were clamped at 0 mV and 500 millisecond voltage ramps from -70mV to +70mV were applied every second. After an initial two-minute calibration period, NEM (3, 10 & 30µM) was applied to the cells for 3 minutes. Following a two-minute washout period, AITC (30µM) or Thymol (200µM) was applied for one minute. Ruthenium Red (RR, 30µM), a TRP channel pore blocker, was applied immediately following AITC treatment for confirmation of TRPA1 responses. Thymol (200µM) replaced AITC in whole-cell recordings where 3µM NEM was used. 5mM glutathione (GSH) or 5mM ophthalmic acid (OPT) was added to the pipette buffer in some recordings.

Perforated Patch Clamp

Perforated patch recordings used a sampling rate of 100kHz with a 2kHz single pole RC lowpass filter. Perforated patch was achieved using pipettes (1-5MΩ) filled with the same pipette solution as whole-cell studies supplemented with 25-50 µg/ml gramicidin. Gramicidin creates pores in the membrane that are permeable to univalent ions and keeps large intracellular components in the cell (Andersen, 1984). In both cases cells were perfused with buffer composed of (mM): 145 NaCl, 10 HEPES, 5.6 dextrose, 4.7 KCl, 1.2 MgCl2, 0.01 CaCl2, 4.7 KCl; pH adjusted to 7.4. 5mM Na-triphosphate was used to prevent rundown of TRPA1 currents (Kim & Cavanaugh, 2007). Currents were measured in response to NEM (3, 10 & 30µM), the reversible electrophilic agonist allyl isothiocyanate (AITC, 30µM) and ruthenium red (RR, 30µM), a TRP pore blocker. Thymol (200µM) replaced AITC in whole-cell recordings where 3µM NEM was used. The
rate of activation was calculated using current density over time (pA/pS/s). The experimental protocol is previously mentioned in the whole-cell methods.

Single Channel Patch Clamp

Single channel recordings used a sampling rate of 20kHz with a 2kHz single pole RC lowpass filter, supplemented with a post-acquisition 1000Hz 8-pole Bessel low pass filter. Cell-attached patches used pipettes that were fabricated from 1.5-mm o.d., 0.75-mm i.d. borosilicate glass (7-50MΩ, coated in Sylgard 184) and filled with HEPES buffer composed of (mM): 145 NaCl, 10 HEPES, 5.6 dextrose, 4.7 KCl, 1.2 MgCl2, 4.7 KCl, 5 EGTA, 5 Na-triphosphate; pH adjusted to 7.4. Cells were perfused with the same HEPES buffer. In inside-out patch recordings, we begin in the cell-attached position and excise the patch away from the cell. However, the protocol in inside-out recordings is the same as the cell-attached recordings. The patch was held at 0 mV, then the presence of TRPA1 channels was confirmed by spontaneous conductances observed in voltage steps: 100 milliseconds from −80mV to +80mV in 20mV steps. Cell-attached patches were then held at +40mV, and individual channel responses were observed in response to NEM (30µM, 3 minutes) then thymol (200µM). Half-amplitude threshold analysis (Colquhoun & Sigworth, 1995) used to idealize single-channel events using Clampfit. Open and closed dwell times were analyzed as square root of events on a logarithmic histogram (Sigworth & Sine, 1987). Filter dead time was calculated as \( T_d = 0.179/f_c \) (Colquhoun & Sigworth, 1995). With \( f_c = 1kHz \), \( T_d = 179\mu s \), thus all events <400µs were excluded. Dwell times were fitted to sums of exponential components to determine time constants (\( \tau \)) (Colquhoun & Sigworth, 1995). Currents were fitted to a Gaussian function, with the peak corresponding to unitary amplitude. The open probability (NPO) for each channel was determined for 1s bins. NEM-evoked channel activity (NPO) was separated into 3 phases: P1/F1 – no measurable change in spontaneous activity; P2/F2 – the phase following NPO > threshold NPO (i.e., 0.02); and (for only some channels) F3 – the phase with sustained (>5s) and full activation (NPO>0.9). In some recordings, two channels were
detected in a single patch. Although this did not prevent a qualitative identification of partial vs. full activation or the determination of current amplitude, these channels were omitted from the NPo and dwell time analyses.

**Statistical analysis**

Data were analyzed using GraphPad software, and Microsoft Excel. A p-value less than 0.05 was considered as significant. A two-way ANOVA was used to analyze the relationships of the rate of change with NEM-evoked responses compared to control in whole-cell and perforated patch clamp. A one-way ANOVA with a Tukey’s post hoc test was used to analyze the relationships of conventional whole-cell (WC), WC + GSH and WC + OPT rate of change and WC, GSH and OPT baseline. One-way ANOVA with Tukey’s post hoc test was used to analyze the relationship between NT, WT, K3C-EGFP and 1:1 WT/K3C-EGFP mean Δ NEM response. Chi-square analysis was used to determine the relationship for the percentage of single channels that reach full activation between WT and C665S. Unpaired student’s t-test analysis was used to determine the relationships between Full vs. Partial and WT vs. C665S. Paired student’s t-test analysis was used to determine the relationship between baseline (BL) and P2. All data was expressed as mean ± S.E.M. unless otherwise noted.

**Chemicals**

NEM, ethanol and DMSO were purchased from Thermo Fisher Scientific (Waltham, MA). Fura-2AM was purchased from TEFLabs (Austin, TX). Ionomycin was purchased from LKT Laboratories (St. Paul, MN). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).
Chapter 3
Investigation into the Role of Specific Cysteines in the Activation of TRPA1 by Electrophiles in Live-cell Calcium Imaging Studies

Introduction

The detection of noxious stimuli through primary nociceptive nerves is important for an organism’s ability to sense danger in the environment (Price & Dubner, 1977). Polymodal nociceptive nerves express receptors that are activated by different types of noxious stimuli (Julius, 2001). TRPA1 is a non-selective tetrameric cation channel whose activation initiates generator potentials (and downstream action potentials) in nociceptive sensory nerves. TRPA1 can be activated by various noxious chemicals like food chemicals, environmental irritants, and oxidative stress that is produced during cellular damage (Bandell et al., 2004; Jordt et al., 2004). Prior evidence has revealed that many TRPA1 agonists have electrophilic chemical properties that covalently modify nucleophilic cysteines residues (Hinman et al., 2006; Macpherson et al., 2007). Biochemical assays determined that some electrophiles (allyl isothiocyanate (AITC)) bind reversibly to cysteines and cause activation of TRPA1 that washes out over time. Other electrophiles bind irreversibly to cysteines and elicit continued TRPA1 activation that can only be ablated by Ruthenium Red (Hinman et al., 2006). Irreversible electrophiles like iodoacetamide (IA) bind covalently to cysteines in biochemical assays and evoke rapid TRPA1 activation in functional assays (Macpherson et al., 2007). Cysteines can also undergo oxidation (Rhee et al.,
and activation of TRPA1 by hydrogen peroxide (H$_2$O$_2$) could take place through thiol oxidation (Sawada et al., 2008).

The initial investigations of cysteines involved in TRPA1 activation identified a triple mutation of C621S, C641S and C665S residues (TRPA1-3C) that showed no response to irreversible electrophiles like N-methylmaleimide (NMM) (Hinman et al., 2006). In mouse TRPA1 (mTRPA1), single point mutations of C415S, C422S and C622S in mTRPA1 channels were less responsive to cinnamaldehyde and mustard oil (Macpherson et al., 2007). Binding studies from our lab have identified four cysteine residues (C273, C621, C665, C1085) which rapidly react with the irreversible electrophile iodoacetamide (IA). In particular, C621 binds IA at a rate that is >6,000 fold greater than non-reactive cysteines (Bahia et al., 2016). The reactivity of cysteines to electrophiles is in part dependent on lowering the pKa of the thiol side group to a more reactive thiolate form (Rhee et al., 2000). Positively charged amino acids (e.g., lysine) can lower the pKa of neighboring cysteines which contributes to reactive thiolate anion groups (Rhee et al., 2000). Our lab determined that mutation of K620 (which is next to C621) significantly reduces BODIPY-IA (B-IA) binding on the TRPA1 channel (Bahia et al., 2016). In addition, there is evidence that proline residues can contribute to deprotonation of cysteines near the N-termini in protein structures (Kortemme & Creighton, 1995), which prompted our investigation of the neighboring P622 residue. There is a gap in our knowledge of (1) which cysteines are important for TRPA1 activation and (2) why C621 is so reactive.

In this study, we performed live-cell calcium imaging studies (which will indirectly measure channel activation) to determine the role of electrophilic modifications of cysteines in the activation of TRPA1. Here, we found that mutation of C621 prevents TRPA1 activation but the role of C665 in TRPA1 activation is dependent on the size of the electrophile. In addition, we found that amino acids adjacent to C621 are also important for electrophile-evoked TRPA1 activation. Lastly, activation of co-transfected wildtype (WT) and mutant TRPA1 channels suggest that homomeric WT TRPA1 subunits are not necessary for activation.
Results

We hypothesize that highly reactive cysteines prone to covalent modification by electrophiles play a critical role in the activation of TRPA1. Here, we measured increases in Ca\(^{2+}\) flux in hTRPA1 and hTRPA1 mutant-expressing HEK293 cells during electrophile treatment in live-cell Ca\(^{2+}\) imaging. TRPA1 is a Ca\(^{2+}\)-permeable cation channel, whose activation will induce Ca\(^{2+}\) fluxes. In initial calcium imaging studies, we observed that 10\(\mu\)M NEM was enough to produce a maximal response with WT TRPA1-expressing cells (Fig. 3.1). Also, we observed that 10\(\mu\)M NEM was enough to prevent ensuing activation with 100\(\mu\)M NEM, which suggests that the preceding NEM treatment caused desensitization of TRPA1, or all of the critical cysteines needed for activation are already modified. During three-minute IA (30\(\mu\)M) treatment, wildtype (WT) TRPA1-expressing cells exhibited rapid and robust increases in Ca\(^{2+}\) fluxes compared to non-transfected (NT) cells (Fig. 3.2A). IA-evoked TRPA1 activation was ablated in C621A and C665L TRPA1 channels but nonetheless the non-electrophile thymol produced substantial activation, indicating that the mutations did not significantly impair global channel function (Figure 3.2A). Mutation of C641 in the TRPA1-3C mutant failed to be activated by electrophiles (Hinman et al., 2006), but here we saw that NEM evoked rapid and robust activation in the single C641A mutant that is similar to WT responses (Figure 3.2A). To determine how the local amino acid
residue environment around C621 affects TRPA1 activation, we observed IA-evoked TRPA1 activation with mutations at K620 and P622. K620A exhibited reduced IA-induced TRPA1 activation and P622A ablated TRPA1 activation (Fig. 3.2B). With three-minute treatment of NEM (30µM), we observed rapid and robust activation of WT and C641A TRPA1, which was similar to

Figure 3.2: Mutation of key cysteines reduces electrophile induced Ca²⁺ fluxes, as well as K620 and P622. A-D. Mean ± S.E.M. Ca²⁺ responses in non-transfected HEK293 (NT) and HEK293 expressing TRPA1 constructs. A. Responses to 0.1% ethanol vehicle (Veh), IA (30µM) and Thymol (200µM) in NT (n=200), hTRPA1 wildtype (WT, n=351), C621A (n=73), C641A (n=167) and C665L (n=29) constructs. B. Responses to vehicle, IA (30µM) and Thymol (200µM) in NT (n=200), WT (n=351), K620A (n=159), and P622A (n=40) constructs. C. Responses to vehicle, NEM (30µM) and Thymol (200µM) in NT (n=237), WT (n=831), C621A (n=139), C641A (n=154) and C665L (n=80) constructs. D. Responses to vehicle, H₂O₂ (100µM), AITC (100µM), and Thymol (200µM) in WT (n=148), C621A (n=34) and K620A (n=132) constructs.
IA-induced WT and C641A responses (Fig. 3.2C). Likewise, NEM-evoked TRPA1 activation was ablated in C621A and C665L mutant channels, although thymol activated these constructs (Fig. 3.2C). In WT-expressing cells, 100µM H₂O₂ elicited a rapid response, and we observed a more robust response to 100µM AITC following a 2-minute washout period (Fig. 3.2D). With C621A and C665L mutants, we observed no response to 100µM H₂O₂ compared to WT (Fig. 3.2D). However, K620A and C621A were partially sensitive to application of 100µM AITC (Fig. 3.2D).

Our findings suggest that oxidation of the highly reactive C621 via H₂O₂ will activate TRPA1 in a rapid, yet reversible manner. It should be noted that we upgraded our camera in the midst of our calcium imaging studies. The initial studies with NEM, IA and H₂O₂ were performed on one camera, while the NEM studies with the TRPA1 mutants were performed on a newer camera with a lower signal-to-noise ratio. This explains why studies with NEM had a lower ratiometric response than studies with IA, despite NEM being a more potent TRPA1 agonist.

Recent studies showed that C665S TRPA1 showed no response to small electrophiles (IA) but was robustly activated by large electrophiles (B-IA) (Zhao et al., 2020). We therefore investigated IA- and NEM-evoked responses in the C665S mutant. During three-minute NEM
treatment, we observed rapid and robust activation of C665S and WT TRPA1 (Fig. 3.3A). Nevertheless, there was a significant decrease in the Ca2+ flux in the C665S mutant (Fig. 3.3C). Interestingly, IA (30µM) produced no activation of C665S (Fig. 3.3B&C), which nonetheless responded robustly to the non-electrophile thymol (not shown, mean± S.E.M. thymol response of 0.19±0.004 and 0.18±0.003 for WT and C665S, respectively; p>0.05). Based on our findings, the role of C665 in TRPA1 activation depends on the activating electrophile. We also noted that activation of TRPA1-WT by thymol was greatly reduced after NEM treatment compared with IA treatment. Importantly, in additional control studies (Fig. 3.4A) the magnitude and the speed of

Electrophilic modifications of C621 and C665 could occur on any of the four subunits in the homotetrameric channel. In lieu of studies of concatemers (which we were unable to synthesize), we attempted to investigate the role of individual subunits in electrophile-evoked TRPA1 activation using co-transfection of WT and K3C-EGFP channels (mutations of K620, C621, C641, C665). 30µM AITC rapidly activated WT but failed to activate K3C-EGFP mutant channels (Fig 3.5A). We observed a response to thymol (200µM) in K3C-EGFP cells, which
confirmed that the TRPA-null mutant channel is functional. During NEM (30µM) treatment, we saw similar rapid and robust activation with WT that we observed with AITC, but we saw no activation with K3C-EGFP cells (Fig. 3.5B). Our K3C-EGFP results are consistent with the previously described role of C621, C665 and K620. If WT channels and null mutants can associate into heteromeric channels, then it is predicted that homomeric WT would make up only 6.25% of the available channels when WT and K3C-EGFP are co-expressed in a 1:1 ratio. We saw rapid activation in 1:1 WT/K3C-EGFP expressing cells, but the magnitude of responses was reduced compared to WT (Fig. 3.5B). After 90 seconds of NEM treatment, we saw a significant decrease in mean response rate in 1:1 WT/K3C-EGFP (0.1705 ± 0.0101) compared to WT (0.4185 ± 0.006) (Fig. 3.5C). Nevertheless, the magnitude of the NEM-evoked response in the 1:1 WT/K3C-EGFP co-transfection was greater than 6.25% of the WT response.

Discussion

Here, we have shown that point mutations of key cysteine residues prevent activation of TRPA1 via irreversible electrophiles in live-cell calcium imaging. Prior evidence showed that
triple mutant TRPA1 channel of C621, C641, and C665 (TRPA1-3C) is insensitive to N-methylmaleimide treatment in functional assays (Hinman et al., 2006). Previous studies identified C641 as one of three cysteines (C621 and C665) that ablated activation in the TRPA1-3C mutant (Hinman et al., 2006), but evidence suggests that (1) C641 lacked reactivity to electrophiles (Bahia et al., 2016) and (2) single mutations of C641 were activated by IA (Zhao et al., 2020). Here, IA and NEM activated C641A channels, indicating that C641 is not involved in electrophilic activation of TRPA1. In our lab, binding assays showed that IA reacted with C621 at a rate that was >6,000 fold greater than conventional cysteines (Bahia et al., 2016). Here, we observed that mutation of C621 ablated activation of TRPA1 during NEM and IA treatment. Also, H_{2}O_{2}-induced activation was also ablated in the C621A construct. Thus, we suggest that electrophilic modification of C621 is essential for TRPA1 activation by most electrophiles, with the exception of isothiocyanates such as AITC, which can produce weak TRPA1 activation independently of C621 via the modification of K708 (Hinman et al., 2006).

The reactivity of cysteines is dependent on the acid dissociation constant (pK_{a}) of the thiol side group and the local environment surrounding the thiol group. Lowering of the pK_{a} from physiological pH deprotonates the thiol group and produces a more nucleophilic, highly reactive thiolate group that is more sensitive to electrophilic modification and oxidation by peroxides (LoPachin et al., 2007; Sue et al., 2005; Wood et al., 2002). Due to proximity to the basic side group, lysine residues are able to lower the pKa of sulfhydryl side groups on cysteines, which shifts thiols to a deprotonated and reactive thiolate form (Kojima et al., 1976). Cryo-EM models of TRPA1 show that the basic NH_{4}^{+} side chain of K620 is in close proximity to C621’s side group (Paulsen et al., 2015), which could contribute to the reactivity of C621 (Bahia et al., 2016). In binding assays, our lab observed a reduction in total B-IA binding with mutant K620 (K620A) TRPA1 channels compared to WT TRPA1, suggesting that K620 aids in the reactivity of C621 (Bahia et al., 2016). As such, our observation of ablated K620A activation by IA and H_{2}O_{2} is consistent with the critical role of C621 in these responses. As shown in Fig 1.1, there is a
conserved lysine or arginine adjacent to C621 in the 620 position in almost all mammalian (i.e., sensitive to electrophiles) TRPA1 orthologs.

Interestingly, rattlesnake TRPA1 (rsTRPA1) is neither bound or activated by electrophiles despite the fact that it possesses both a K620 and a C621 (Fig 3.6) (Bahia et al., 2016; Cordero-Morales et al., 2011). The major difference between mammalian TRPA1 sequences in the C621 region and rsTRPA1 is that mammalian TRPA1 have a conserved P622 (Fig 1.1, 3.6), whereas this is absent in the rsTRPA1 (Fig 3.6). Our calcium imaging studies showed that mutation of P622 (P622A) in hTRPA1 produced a channel that was insensitive to the electrophile IA (yet responded to thymol), suggesting that this proline may contribute indirectly to C621-mediated TRPA1 activation. In protein structures, prolines create kinks in α-helices (Gunasekaran et al., 1998). Based on the structure of hTRPA1, the P622 residue creates a kink that pushes K620 in close proximity of C621 (Paulsen et al., 2015). Using RaptorX-Property analysis of the hTRPA1 secondary structure, we predict that C621 is located at the N-terminus of the α-helix (Fig. 3.6) (Wang et al., 2016). The α-helix creates a dipole moment with the N-termini, which could decrease the pKa of the sulfhydryl group of C621, thus increasing its reactivity (Kortemme & Creighton, 1995). The electrophile-insensitive rsTRPA1 lacks the equivalent of P622 and RaptorX-Property analysis suggests that the α-helix is significantly extended, so that...
the equivalent of C621 is now located in the middle of the α-helix, with a much-reduced dipole moment (Kortemme et al., 1995). Interestingly, the RaptorX-Property analysis predicts that mutation of hTRPA1 to P622A would similarly extend the α-helix, indicating the importance of the proline in determining the α-helix structure. Our observation that the P622A mutant is insensitive to IA is consistent with this modeling. Thus, we conclude that the location of C621 in the α-helix structure is as important as the presence of K620 in determining the high reactivity of C621 (and its role in TRPA1 activation).

Based on our results, the role of C665 in electrophile-evoked TRPA1 activation is more complex than the role of C621. Previously we had shown that C665 was rapidly modified by IA (Bahia et al., 2016). Functionally, the C665S mutation was part of the ablated responses to N-methylmaleimide (NMM) in the original triple mutant (Hinman et al., 2006), whereas prostaglandin 15d-PGJ$_2$, an endogenous irreversible electrophilic TRPA1 agonist, elicited responses to C665S that were comparable to WT responses (Takahashi et al., 2008). The C665L mutant was modified by B-IA to the same level as WT in binding assays, which suggests that the majority of electrophilic cysteine modification is independent of C665 (Bahia et al., 2016). C665L was insensitive to IA and NEM in our calcium imaging studies; C665S was insensitive to IA but was activated by NEM (although this was reduced compared to WT), which is consistent to other studies (Suo et al., 2020; Zhao et al., 2020). Our findings suggest that large electrophiles activate TRPA1 via modification of C621 alone, but small electrophiles need to modify C665 to trigger channel activation. Based on the chemical structure, NEM is larger than IA but smaller than JT010, which was used in Cryo-EM mapping (Suo et al., 2020). We hypothesize that the C665L mutant prevents the conformational change evoked by C621 modification that regulates the A-loop and the downstream gating mechanism (Zhao et al., 2020).

Our data indicates that C621 and C665 are involved in TRPA1 activation, but each individual modification event likely occurs on all four subunits of TRPA1 channel. So, there are 8 electrophilic cysteine binding events that are potentially needed to activation TRPA1. It is
unknown exactly how many modification events are needed for TRPA1 activation, but we
determined that 1:1 co-transfection of WT and K3C-EGFP TRPA1 produces a ~45% less
response to NEM compared to WT. Based on probability, there is a 6.25% chance of expressing
homotetrameric WT TRPA1 channels with 1:1 WT/K3C-EGFP co-transfection. Thus, our data
shows that all four subunits may not be needed for TRPA1 activation. Previous studies show that
concatemers that express two functional TRPA1 subunits are still prone to electrophile induced
TRPA1 activation (Fischer et al., 2014; Ye et al., 2018). Functional studies of concatemers with
specific subunit cysteine mutants must be performed before any conclusions can be made about
the role of each subunit in TRPA1 activation.
Chapter 4

Dialysis of Unknown Cytosolic Components reduces NEM-induced TRPA1 Activation, which is Rescued by Supplementation of GSH in Conventional Whole-Cell Recordings

Introduction

Studies show that irreversible electrophiles can evoke increases in Ca\textsuperscript{2+} fluxes in cells expressing hTRPA1 within seconds (Hinman et al., 2006). Site-directed mutagenesis studies indicate that electrophile-induced TRPA1 activation is mediated by key cysteines (Hinman et al., 2006; Macpherson et al., 2007) (See Chapter 3). Binding studies in our lab have identified cysteine residues that can bind to electrophiles at rates that are significantly greater than conventional cysteines (i.e., glutathione (GSH)) (Bahia et al., 2016). The highly reactive cysteines on TRPA1 out compete 5mM cytosolic GSH to bind to electrophiles, which makes electrophilic activation of TRPA1 a very efficient mechanism.

In our calcium imaging studies, we measured rapid increases in Ca\textsuperscript{2+} fluxes within HEK293 cells by using the Fura-2AM ester. Fura-2AM is a membrane-permeable, ratiometric calcium ion indicator that binds Ca\textsuperscript{2+}, changing its fluorescence Fura-2AM detects all Ca\textsuperscript{2+} fluxes in cells (including Ca\textsuperscript{2+} released from intracellular stores and Ca\textsuperscript{2+} influx through plasma membrane channels/transporters), and as such is only an indirect measure of channel function. It is therefore difficult to directly observe the biophysical properties of the TRPA1 channel using the calcium...
imaging technique. Thus, we utilized whole-cell patch clamp technique to directly measure ion current flowing through TRPA1 channels in a single cell.

In our whole-cell recordings, we observed gradual, partial IA and NEM-induced TRPA1 activation which contrasts the rapid and robust TRPA1 activation that we witnessed in calcium imaging experiments with intact cells. Perforated patch clamp recordings revealed rapid and robust TRPA1 activation with exposure to NEM, suggesting that preventing cell dialysis prevented the loss of NEM-evoked TRPA1 activation. Finally, we found that supplementation of the pipette solution with GSH rescued NEM-evoked TRPA1 activation in whole-cell patch clamp studies.

Results

To observe TRPA1 activation via irreversible electrophiles, we utilized whole-cell patch clamp (WC) to directly measure electrophile-evoked currents in TRPA1-expressing HEK293 cells. Surprisingly, we found that a sub-maximal application of IA (50μM) evoked only a partial and slow increase in TRPA1 current density at -65mV and +65mV (Fig. 4.1). As expected, AITC (30μM) treatment caused a rapid and robust increase in TRPA1 current density. The currents then were ablated by Ruthenium Red (RR, 30μM), which indicates that they were dependent on TRPA1 activation (Fig. 4.1A&B). The slow, partial IA-induced TRPA1 activation in whole-cell recordings was dramatically different to the rapid and robust TRPA1 activation that occurred in live-cell calcium imaging studies during IA (30μM) treatment (Fig. 4.1D)
To investigate the observed reduction in electrophile-induced TRPA1 activation in whole cell patch clamp studies, we performed additional studies with N-ethylmaleimide (NEM, 10μM). Again, we witnessed slow and diminished NEM-induced TRPA1 activation (Fig. 4.2A&B). NEM failed to evoke currents in non-transfected HEK293 (mean ± S.E.M. response 0.24±0.6 pA/pF, n=4, not shown). A major impact of whole-cell recording is the dialysis of cytosolic factors. To test the impact of this, we compared whole-cell patch clamp studies with the perforated patch clamp (PP) technique. During a three-minute NEM (10μM) treatment, we observed a quick and large
increase in TRPA1 current density within seconds in perforated patch clamp studies (Fig. 4.2A&C). Two-way ANOVA analysis determined that using the perforated patch clamp method contributed a significant increase in the rate of change in perforated patch clamp experiments compared to control WC experiments at -65mV and +65mV during NEM treatment (p<0.05) (Fig 4.3A&B). Linear regression analysis showed no significant relationship between access
resistance and TRPA1 currents (data not shown), indicating the reduced responses in whole-cell recordings were not due to differences in access resistance.

Given that TRPA1 is a redox-sensitive channel, we hypothesized that the dialysis of cytosolic GSH was responsible for the reduced TRPA1 activation in whole-cell experiments. Therefore, we added 5mM GSH to the pipette solution in whole-cell patch clamp studies to determine if we could rescue TRPA1 activation. During NEM (10µM) treatment, we observed a rapid and robust increase in TRPA1 current density within seconds in whole-cell experiments with 5mM GSH (Fig 4.4A). Thymol (200µM), the non-electrophilic agonist for TRPA1, elicited a quick increase in TRPA1 current density and then the currents were promptly ablated by RR (30µM),

Figure 4.4: Decreased NEM-evoked TRPA1-mediated whole cell currents are rescued by pipette solution GSH. A-B. Representative current density (+65mV and -65mV) of whole-cell recordings with 5mM glutathione (GSH) (A) or 5mM ophthalmic acid (OPT) (B) in the pipette solution during NEM (10µM), Thymol (200µM), and RR (30µM). C-D. Mean ± S.E.M. Rate of change of 10µM NEM-induced TRPA1-mediated currents in whole-cell recordings without 5mM GSH (control, n=10), with 5mM GSH (n=10) and with 5mM OPT (n=9) at +65mV (C) and -65mV (D). *, p<0.05 vs baseline; #, p<0.05 vs control + NEM; $, p<0.05 vs GSH + NEM.
as expected. Hence, our analysis indicates that an addition of GSH rescues robust TRPA1 activation in whole-cell patch clamp experiments.

GSH has nucleophilic properties that can prevent oxidation in the cytosol in times of cellular damage. To determine if this property was required for the observed effect on NEM-evoked TRPA1 activation we performed whole-cell patch clamp experiments with 5mM ophthalmic acid (OPT), a non-nucleophilic analog of GSH, supplemented into the pipette solution. During NEM treatment, we observed only slow, gradual increases in TRPA1 current density compared to whole-cell studies with GSH (Fig. 4.4B). Overall, we saw a significant increase in NEM-evoked mean current density rate of change in whole-cell + GSH studies compared to control at -65mV and +65mV (p<0.05), and compared to the OPT supplemented studies (p<0.05) (Fig 4.4C&D). We also analyzed the time taken for NEM to initiate TRPA1 activation, by assessing the time for the current density to rise about a threshold set at 5pA/pF. Although there was a trend for GSH-supplemented currents to exceed the threshold quicker (9.4±1.6s) than the control (14.4±4.5s, p=0.16) and OPT studies (15.7±3.6s, p=0.06), this did not reach significance (not shown).

Discussion

In this study, we treated transiently expressing HEK293 cells with IA to observe TRPA1 activation in whole-cell patch clamp recordings. During exposure to IA, we observe a slight increase in current density at -65mV and +65mV. Although there is evidence of partial and limited activation of TRPA1 with cinnamaldehyde and prostaglandin J2 in whole-cell recordings (Bandell et al., 2004; Hynkova et al., 2016; T E Taylor-Clark et al., 2009, 2008), rapid and robust TRPA1 is typically observed in calcium imaging studies. Similar to the whole-cell studies with IA treatment, we saw slow, gradual TRPA1 activation with NEM. As expected, in live-cell calcium imaging experiments, we observed a rapid and robust increase in Ca$^{2+}$ influx in TRPA1-expressing HEK293 cells during IA treatment.
Whole-cell patch clamp technique causes disruption of the cell membrane to gain electrical access to the cell, which can result in run-down of ion channel currents during the recording (Belles et al., 1988). Prior studies confirmed that the whole-cell technique can result in dialysis of large intracellular components like GFP (Kim and Cavanaugh, 2007). Also, dialysis of cytosolic cofactors can contribute to run-down of TRPA1 currents in whole-cell recordings, which can be prevented with supplementation of inorganic polyphosphates in the pipette solution (Kim and Cavanaugh, 2007). Even with Na+-triphosphate supplementation in the pipette solution, we still observed slow and reduced electrophile-induced TRPA1 activation in our whole-cell recordings. To investigate the role of cell dialysis in slow TRPA1 activation during conventional whole-cell recordings, we measured NEM-induced TRPA1 currents in perforated whole cell patch-clamp experiments, which have no significant cell membrane disruption and dialysis of cytosolic cofactors (Strauss et al., 2001). We observed a significant increase in NEM-evoked TRPA1 rate of change with treatment of NEM compared to the conventional whole-cell recordings. Thus, the prevention of cell dialysis in perforated patch-clamp recordings rescued rapid TRPA1 activation.

To rescue rapid TRPA1 activation, we performed conventional whole-cell patch clamp recordings with 5mM GSH added to the pipette solution. Cytosolic GSH prevents indiscriminate oxidation and electrophilic modification of cytosolic thiols (cysteines) and cells that are depleted of GSH cells are more susceptible to oxidative stress (Gul et al., 2000; Keen & Jakoby, 1978). In addition, TRPA1 can be activated by oxidative stress (e.g., H₂O₂) (Andersson et al., 2008; Sawada et al., 2008) and products of oxidative stress (e.g., 4-HNE, 4-ONE) (Graepel et al., 2011; Takahashi et al., 2008; Trevisani et al., 2007). NEM-evoked current density with GSH supplementation was significantly greater than control recordings. Supplementation of 5mM OPT, a non-nucleophilic analog of GSH, displayed a significant reduction in NEM-induced activation rate compared to recordings with GSH supplementation, indicating that the nucleophilic nature of GSH was critical for its observed effects of NEM-evoked TRPA1 activation.
Our results revealed that “going whole-cell” induces oxidative stress (and cysteine modification) that makes the channel less sensitive to subsequent electrophile treatment. H$_2$O$_2$ can oxidize thiolate side groups on cysteines and form sulfenic acid, which are reversible by glutathione (Berndt et al., 2007). Further oxidation of sulfenic acid via H$_2$O$_2$ produces sulfenic acid and sulfonic acid, which are irreversible forms of thiol oxidation that can lead to cellular damage (Berndt et al., 2007). Although H$_2$O$_2$ can activate TRPA1, the threshold of cysteine oxidation that can lead to TRPA1 activation or cause reduced TRPA1 activation is not known.

It should be noted that AITC-evoked responses appeared unaffected by the whole cell patch technique: AITC produced quicker and larger increases in TRPA1 current compared to IA-induced TRPA1 currents. AITC-evoked TRPA1 activation differs from NEM-evoked activation in two ways: firstly, AITC forms reversible bounds with cysteines, unlike the irreversible adductions by NEM; secondly, in addition to activating TRPA1 via the modification of key cysteines, AITC can also activate TRPA1 via the modification of the K708 residue (Hinman et al., 2006). It is presently not clear which of these factors protects AITC-evoked TRPA1 activation from the effect of cytosolic dialysis. Future experiments with a K708A mutant would provide insight into this unresolved issue.
Chapter 5

Identification of Multiple Activation States of TRPA1 in Single-Channel Recordings

Introduction

Originally, site-directed mutagenesis identified three cysteines (C621, C641, and C665) that were required for TRPA1 activation in functional assays (Hinman et al., 2006). Our biochemical assays then identified four cysteines (C273, C621, C665, C1085) that are rapidly bound by IA (Bahia et al., 2016). Specifically, IA reacted with C621 at a rate that is 6,000-fold quicker than less reactive cysteines; C665 reacted to IA at a much slower rate compared to C621 (Bahia et al., 2016). Recently, cryo-EM models have revealed a distinct binding site that contains C621 and C665, which is surrounded by four aromatic residues (F612, H614, F669, and Y680) that make up the binding pocket (Suo et al., 2020; Zhao et al., 2020). The adduction of C621 by electrophiles causes machinery in the upper part of the binding pocket (A-loop) to move upward in a stabilized conformation (Zhao et al., 2020). The upward A-loop conformation rotates C665 within the pocket and lowers its pKa, which makes it reactive to electrophiles (Zhao et al., 2020).

Despite the developments on the conformation of TRPA1 following electrophilic modification of C621 in cryo-EM studies (Suo et al., 2020; Zhao et al., 2020), there is little understanding about the underlying channel events that lead to TRPA1 activation via electrophiles. Indeed, in both recent cryo-EM studies some structures were resolved where C621 was adducted but the pore remained closed (Suo et al., 2020; Zhao et al., 2020). We propose
that TRPA1 activation could manifest as different profiles based on the modification of different cysteines. TRPA1 activation could be an all-or-none activation, which is typically seen in voltage-gated sodium channels (Wisedchaisri et al., 2020), where each electrophilic cysteine modification event can trigger complete activation of the channel. Or TRPA1 activation could exhibit as a stepwise activation where each electrophilic cysteine modification event independently contributes to the gating of the channel in multiple open states, which is observed with TRPV1 (Canul-Sánchez et al., 2018; Hui et al., 2003). In chapter 4, we observed TRPA1 activation using whole-cell patch clamp methods. In order to characterize TRPA1 gating and biophysical channel properties, we must observe stochastic channel events using a single-channel patch clamp approach.

Here, we determined in cell-attached single-channel recordings that electrophilic-evoked responses demonstrate two distinct and sequential TRPA1 activation phases. We suggest that these phases are the product of multiple activation states caused by consecutive modification events of C621 and C665 by electrophiles.

**Results**

To characterize electrophilic-induced TRPA1 activation, we used single-channel patch clamp techniques to record stochastic TRPA1 gating events during treatment with NEM (30µM). During inside-out single-channel recordings, open probability analysis showed a slight increase in TRPA1 gating above baseline during NEM treatment (Fig. 5.1A). During NEM (30µM) treatment, we observed an initial delay in channel events followed by a quick increase in Po with an average below 0.05 (0.029 ± 0.013) and an average maximum Po just below 0.2 (0.193 ± 0.057) in inside-out recordings (n=4) (Fig. 5.1B). As such, these inside-out recordings in excised patches exhibited relatively underwhelming increases in TRPA1 NPo, which is consistent with the limited electrophilic TRPA1 activation observed in our whole-cell recordings in Chapter 4. There, we were able to increase TRPA1 activation by using perforated patch clamp to prevent cell
membrane disruption. So, we switched to the cell-attached patch clamp technique which will prevent cell dialysis for the remained of the single channel studies.

Open probability (N\text{Po}) analysis in cell-attached recordings revealed two different NEM-evoked activation profiles: in some recordings (n=10 recordings) we observed an NEM-evoked partial activation (Fig. 5.2A), with only transient increases of N\text{Po} above threshold levels (P2 phase); and full activation (n=13), with a sustained (>5s) N\text{Po}>0.9 (F3 phase) following an initial increase in N\text{Po} above threshold levels (F2 phase) (Fig. 5.2B). Unsurprisingly, there was a significant difference in the mean N\text{Po} (0.9149 ± 0.021 (Full) vs. 0.103 ± 0.033 (Partial), p<0.05) and maximum N\text{Po} (0.9998 ± 0.0001 (Full) vs. 0.4661 ± 0.097 (Partial), p<0.05) evoked by NEM in the full and partial activation states and these were both greater than the almost absent channel activity noted in patches from non-transfected cells (p<0.05) (Fig. 5.2C). However, there was no significant difference between the current amplitudes of open channel events in full activation F2 phase and partial activation P2 phase (P2: 5.5 ± 0.63pA vs. F2: 4.1 ± 0.4pA; unpaired, p>0.05) and between F2 and F3 phases (F2: 4.1 ± 0.4pA vs. F3: 4.2 ± 0.55pA; paired, p>0.05) (Fig. 5.3A-C).
Figure 5.2: NEM induces distinct TRPA1 activation phases in cell-attached single channel recordings. A and B, Representative open probability (NPo) analysis of single TRPA1 channels induced by NEM (30µM) into partial (A) and full (B) activation phases. P1/F1 denotes prior to activation, P2/F2 denotes phase following initiation of activation, F3 denotes full activation phase. C, maximum and mean NPo analysis for full (F, n=13) and partial (P, n=10) TRPA1 activation recordings and non-transfected HEK293 (Nt, n=5). #, p<0.05 vs non-transfected; *, p<0.05 vs full.

Figure 5.3: No differences between currents evoked in partial and full activation profiles. A and B, amplitude histograms for partial (A) and full (B) activation shown in Fig. 5A and B. C, mean ± S.E.M. current for channels induced into partial and full (separated into paired F2 and F3 phases) activation.
Fitting of the open time distribution demonstrated 3 exponents in the partial activation profiles (Fig. 5.4A) and 4 exponents in the full activation profiles (Fig. 5.4B). There were no differences in the $\tau$ of the first 3 components between partial and full activation profiles, but less time was spent during Exp$_2$ and more time was spent during Exp$_4$ in full activation profiles ($p<0.05$) (Fig. 5.4C & D). Closed times in both profiles had 4 exponents, but the $\tau$ for Exp$_2$, Exp$_3$ and Exp$_4$ were significantly shorter in full activation profiles (Fig. 5.5A-D). We also analyzed the dwell times of F2 and F3 phases separately and found that the exponents for F2 phase (e.g., 3 open time exponents) were not significantly different from the partial activation profiles ($p>0.05$) (Fig. 5.6A-B).

**Figure 5.4:** Open time histogram exponential fit of partial and full activation profiles. A and B, histogram of open time distribution for partial (A) and full (B) activation shown in Fig. 5.2A and B; fitted by the sum of exponentials. C and D, mean ± S.E.M. of open time exponential $\tau$ (C) and proportion (D) for partial and full activation channels. *, $p<0.05$ vs partial.

**Figure 5.5:** Closed time histogram exponential fit of partial and full activation profiles. A and B, histogram of closed time distribution for partial (A) and full (B) activation shown in Fig. 5.2A and B; fitted by the sum of exponentials. C and D, mean ± S.E.M. of closed time exponential $\tau$ (C) and proportion (D) for partial and full activation channels. *, $p<0.05$ vs partial.
In all cases of NEM-evoked activation, there was an initial delay in activation initiation (time to threshold), likely due to the time taken for the initial electrophile modification of TRPA1 cysteines. Importantly, there was no difference in the time to threshold between partial (50.3±13.9s) and full (95±18.6s) activation profiles (p>0.05) (Fig. 5.7A). Furthermore, when we analyzed the timing of the F2 and F3 phases in the full activation profiles, we found that there was no correlation between the time to threshold and the Δ time to full activation (Fig. 5.7.B).

Our Ca\textsuperscript{2+} imaging studies suggest that C665 partially contributes to NEM-evoked TRPA1 activation. In cell-attached recordings, we noted that NEM-evoked increases in NPo were delayed.
in C665S channels compared to WT (Fig. 5.8A), while open channel amplitudes were no different (p>0.05) (Fig. 5.8B). A major cause of C665S’s effect on NPo was the significant reduction in the percentage of channels progressing to full activation (p<0.05) (Fig. 5.8C).

![Figure 5.8: C665S channels have a lower mean NPo and are less likely to reach full activation compared to WT. A, mean NPo for all WT (n=31) and C665S (n=19) recordings (combining profiles) during NEM treatment (30µM). B, mean ± S.E.M. current for WT and C665S channels. C, percentage of TRPA1 channels that reach full activation for WT (13/49) and C665S (3/43) ($, p<0.05). D, mean ± S.E.M. time to threshold for all individually-recorded WT (n=23) and C665S (n=12) channels.](image)

Importantly, there was no difference in the time to threshold between WT and C665S channels (p>0.05) (Fig. 5.8D). These data suggest that C665 is involved with the NEM-evoked progression into the full activation state, but not in the *initiation* of TRPA1 activation. There were few significant differences between the dwell time exponents in the P2 phase of C665S compared to WT (Figs. 5.9A-D): like WT, the P2 phase of C665S channels only had 3 open time exponents.

![Figure 5.9: Comparison of open and closed time exponents for partial activation profiles in C665S and WT channels. A-D, mean ± S.E.M. of open (A, B) and closed time (C, D) exponential τ (A, C) and proportion (B, D) for partial activation (P2) in WT and C665S channels. $, p<0.05 vs WT.](image)
Nevertheless, C665S P2 phase occupied the longest closed time exponential (Exp_4) for longer than WT (Fig. 5.9D), and this likely contributed to the significantly reduced mean NPo for the P2 phase of C665S (WT P2: 0.09±0.02; C665S P2: 0.04±0.02, p<0.05) (Fig. 5.10).

![Mean NPo](image)

**Figure 5.10: C665S mean NPo is significantly reduced compared to WT.** Mean NPo analysis for partial activation (P2) in WT and C665S channels. *, p<0.05 vs baseline (paired); $, p<0.05 vs WT (unpaired).

**Discussion**

In this study, we utilized single-channel patch clamp recordings to characterize the mechanism underlying TRPA1 activation by measuring TRPA1 channel gating events. Inside-out recording demonstrated reduced NEM-evoked increases in NPo compared to cell-attached recordings. Importantly, NEM-evoked TRPA1 gating fail to reach full activation in inside-out recordings. These data are consistent with the minimal Po observed during iodoacetamide (IA) treatment in inside-out patch recordings in a recent paper (Zhao et al., 2020). As we identified in Chapter 4, cytosolic dialysis in whole-cell recordings reduced electrophilic-induced TRPA1 currents. It is likely that a similar process occurs in inside-out recordings. Whether GSH supplementation would “rescue” NEM-evoked TRPA1 gating in inside-out recordings was not assessed here.

In cell-attached single-channel recordings of NEM-evoked TRPA1 activation we clearly identified two distinct TRPA1 activation profiles. Upon NEM exposure and following a random
delay of onset in activity, we observed an initial partial activation, which in some channels was followed by a sudden switch to full activation, that was irreversible. There was some variation in TRPA1 conductance across patches, likely due to the effect of variable resting membrane potential of HEK293 (Chemin et al., 2000) on cell-attached patch voltage during the +40 mV step (Fischmeister et al., 1986; Perkins, 2006). Consistent with sequential modification of cysteines with differential reactivities (Bahia et al., 2016), we present three lines of evidence that the partial and ‘irreversible’ full activation phases represent distinct and potentially sequential TRPA1 activation states. Firstly, analysis of WT open times indicated that the full activation profile had the same 3 exponents as the partial profile with an additional large $\tau$ exponent. The time spent in this fourth exponential was grossly responsible for the dramatic increase in open probability in full activation profiles. The identification of multiple exponentials suggests that TRPA1 has multiple activation states, consistent with other studies of TRP channels (Benedikt et al., 2009; Canul-Sánchez et al., 2018; Hui et al., 2003). Secondly, the times taken for NEM to initiate threshold activation and to then induce full activation in WT channels were not correlated. Lastly, while there was no effect of C665S mutation on channel conductance or the time taken for NEM-evoked initiation of activation, this mutation significantly decreased the percentage of channels that reached full activation. It is likely that C665S does not affect the initial NEM-evoked activation states but rather selectively inhibits the state/states associated with higher open probability and longer open times. Importantly, the C665S mutation had little effect on TRPA1 activation by non-electrophiles, and this lack of global channel inhibition is consistent with other studies (Suo et al., 2020).

Based on our single-channel analysis, we determined that TRPA1 activation is neither a stepwise or all-or-none process, but occurs in different states. Our findings revealed an initial increase in NPo (partial activation profile) and is followed by spontaneous and rapid increase to near 100% NPo (full activation profile). C621 is covalently modified by electrophiles in binding assays (Bahia et al., 2016) and in cryo-EM models before any adduction of C665 occurs (Suo et
Thus, the initial activation phase (P2 or F2) in our activation profiles is likely triggered by modification of C621. The ability for NEM-treated TRPA1 channels to proceed into full activation (F3 phase) could be dependent on C665 but it is not essential, which was confirmed by the rare full activation events with the C665S mutant. Mutation of C621 abolishes all TRPA1 responses (Chapter 3), so C621 is important for both initiation and proceeding to full activation. This is consistent with C621 modulation of C665 reactivity, but the importance of C665 is based upon the size of the electrophile (Suo et al., 2020; Zhao et al., 2020). Binding of large electrophiles triggers the A-loop into an upward conformation, which activates the TRP helix due to dipole moments with K671 (Zhao et al., 2020). This conformational change produces repulsion between subunits and expansion of S6 domains that form the lower gate, which causes an open state conformation (Zhao et al., 2020). Cryo-EM studies show that to stabilize the A-loop machinery and maintain the open confirmation during exposure of small electrophiles, C621 and C665 must be adducted (Zhao et al., 2020). Our single-channel studies consist of only NEM, so further study with larger (e.g., JT010, B-IA) and smaller electrophiles (e.g., IA) would provide more information of how C665 contributes to TRPA1 activation. In chapter 3, we observed that co-transfection of WT and K3C-EGFP (TRPA1-null) channels evoke rapid activation of TRPA1, albeit at a lower magnitude compared to WT. Nevertheless, our findings show that all four subunits are not needed to contribute to gating and activation of TRPA1.

We are currently unable to model NEM-evoked TRPA1 activation, principally due to a lack of understanding of the individual contributions of each subunit in the homotetramer (Fischer et al., 2014; Ye et al., 2018). Nevertheless, we conclude that the initial activation is C665-independent and is likely due to electrophilic modification of C621, and that the time taken for NEM-treated channels to proceed into full activation depends on C665 (but it is not essential). This suggests that C665 modification increases the probability for NEM-treated channels of undergoing the irreversible switch to the full state. How many activation states exist, and how many are sensitive to mutation of C665 is unclear. How these states relate to the position and
stability of the A-loop (Zhao et al., 2020) is also unknown. Future single-channel recordings with reversible agonists (e.g., AITC) may offer a clearer model for TRPA1 activation.
Chapter 6

Conclusion

Various studies have identified that electrophilic modification of cysteines will lead to activation of TRPA1, but a thorough understanding of the events underlying this mechanism is currently lacking. Initial TRPA1 studies determined that mutations of C621, C641 and C665 prevented activation of TRPA1 in the presence of NMM (Hinman et al., 2006). Our laboratory showed that IA rapidly binds to C273, C621, C665 and C1085 (Bahia et al., 2016). In calcium imaging studies, we have confirmed that a single point mutation of C621 ablates TRPA1 activation during IA, NEM and H₂O₂, which indicates that modification of C621 is the primary mechanism for activation. The reactivity of C621 is dependent on the local amino acid environment and proximity to α-helices, which lowers the pKa of thiol side groups to a thiolate anion form (Kojima et al., 1976; Kortemme & Creighton, 1995). We hypothesized that the basic side group of K620 contributes to the reactivity of C621; and indeed, mutation of K620 reduced TRPA1 activation via IA and NEM in calcium imaging experiments. We predicted that P622 creates a kink in the structure of TRPA1 that brings C621 closer to K620 and the N-cap of the α-helix, which makes C621 highly reactive to electrophiles. When mutated to an alanine residue, P622A prevented activation via IA, which is further confirmation that the high reactivity of C621 is critical for electrophile-evoked TRPA1 activation. The C665 residue seems to play a secondary/modulatory role in TRPA1 activation. When C665 is substituted to a leucine, activation is ablated during IA and NEM treatment. When C665 is mutated to a serine, IA-evoked activation is ablated but NEM rapidly activates the
channel. Our analysis suggests that C665 activation is not required for TRPA1 activation although this is dependent on the chemical structure and size of the electrophile. Concerning the C273 and C1085 residues, the role they play in TRPA1 activation is still unknown. Although these residues are not located in the electrophilic binding pocket that was recently identified in cryo-EM studies (Suo et al., 2020; Zhao et al., 2020), future calcium imaging experiments with single point mutation at C273 and C1085 should determine how these residues impact TRPA1 activation. Furthermore, our data suggests that not all four subunits were not required for TRPA1 activation: NEM activated HEK293 cells that were co-transfected with WT and TRPA1-null (K3C-EGFP) in calcium imaging studies. However, we cannot specifically identify the combination of WT and K3C-EGFP subunits that are activated by electrophiles. Thus, future studies with WT/TRPA1-null concatemers will allow us to directly determine which combination of subunits are needed to activate TRPA1.

In whole-cell recordings, we identified that irreversible electrophilic-evoked activation of TRPA1 was reduced compared to rapid TRPA1 activation in live-cell calcium imaging studies. Perforated patch clamp recordings rescued rapid NEM-evoked TRPA1 activation which indicated that whole-cell access to the cell causes dialysis of cytosolic components that are needed for TRPA1 activation. Since cellular membrane disruption can cause depletion of GSH, we supplemented cytosolic GSH in whole-cell recordings, which rescued NEM-evoked TRPA1 activation. OPT failed to rescue rapid NEM-evoked TRPA1 activation, suggesting that the nucleophilic properties of GSH are preventing random oxidation of cysteines during oxidative stress and promotes activation. Reactive oxygen species can modify cysteine residues, but this is typically thought to evoke TRPA1 activation. There seems to be a threshold where ROS can either activate or inhibit TRPA1, and this may be mediated by distinct cysteines. We should note that our whole-cell currents were measured after the whole-cell access had stabilized. It is thus possible that the oxidative stress caused by whole-cell access has caused TRPA1 activation then TRPA1 desensitization prior to our current recordings. It is possible TRPA1 proteomics or cryo-EM modeling with H\textsubscript{2}O\textsubscript{2} would provide insight on how much oxidation of specific cysteines is
required to activate TRPA1. Our findings are important for the interpretation of studies that use excised patch electrophysiology to observe TRPA1 activation via electrophiles. Whole-cell and inside-out patch clamp are widely used methods to measure ion channel activity, so supplementation with GSH in these studies may yield more physiological TRPA1 activation states.

We hypothesized that TRPA1 activation profile would be a stepwise process, where each cysteine modification event would individually contribute to channel gating. Instead, our single-channel analysis determined that TRPA1 activation has two distinct profiles. NEM evoked an initial increase in open probability, which we identified as the partial activation profile. Partial activation is often (but not always) followed by an irreversible increase to near 100% NPo, which is what we identify as full activation. There was no difference in the open time analysis in the initial activation phase in partial (P2) and full (F2) activation but the F3 phase has a 4th exponent, which suggests that the two profiles are sequential. C665S channels were less likely to reach full activation. Based on C621 mutant studies in Chapter 3 and our single-channel analysis, we propose that C621 adduction is the trigger to initiate TRPA1 activation, in addition to being necessary for all TRPA1 activation states. C665 is not required to initiate TRPA1 activation but may assist in the transition from partial to full activation. Our findings provide new insights into a possible TRPA1 activation model. The irreversible nature of NEM-cysteine adducts provides an analysis of the sequential role of cysteine modification in TRPA1 activation, which has allowed us to determine the two sequential activation profiles in our single-channel studies. To determine a kinetic model of the multiple activation states of TRPA1, further studies with a reversible ligand (so as to approach equilibrium) would be necessary. Thus, future single-channel experiments with AITC could create a possible TRPA1 activation model with more clearly-defined open states.

Modification of C621 by small electrophiles evokes a conformational change that shifts the A-loop upward and exposes C665 to possible adduction by electrophiles (Zhao et al., 2020). But, the impact of subsequent C665 adduction on the A-loop (and the downstream pore gating) is not understood. Single-channel analysis with WT and C665S TRPA1 channels during treatment of a
larger electrophile (i.e., JT010) could provide a clearer role of C665’s contribution to the A-loop machinery and adduction to electrophiles after modification of C621. We would expect to see more C665S channels reach full activation with JT010 treatment. Lastly, single-channel recordings of concatemers with different combinations of subunits would yield more definitive information regarding the number of subunits required to activate the channel and the relationship between the subunits in TRPA1 activation.

In summary, our study presents novel insights into our understanding of TRPA1 activation. TRPA1 is activated by oxidative stress, which is an indicator of cellular damage. TRPA1 also plays an underlying role in various diseases, so it is a target for potential treatments. TRPA1 is a polymodal channel which likely plays a role in multiple physiological and pathophysiological processes in addition to its role in nociception. Studies have shown that TRPA1-KO mice have increased learning, memory and cognition, as well as decreased less anxiety-related behavior (Lee et al., 2017). Also, blocking TRPA1 activation has antidepressant and anxiolytic effects in mice (de Moura et al., 2014). Therefore, complete inhibition of TRPA1 may have some undesirable and unintended effects. If possible, development of modal-specific block of reactive oxygen species that activate TRPA1 could alleviate symptoms of diseases that would otherwise be debilitating for patients. A detailed understanding of modal-specific TRPA1 activation would therefore provide a rational basis for the development of modal-specific block of TRPA1.
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Appendices
Appendix A: A Note to Reader

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