Characterization of human TRPA1 and TRPV1 channels in response to naturally occurring defensive compounds

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Characterization of human TRPA1 and TRPV1 channels in response to naturally occurring defensive compounds

Abstract

The transient receptor potential channels, ankyrin 1 (TRPA1) and vanilloid 1 (TRPV1), are non-selective cation-permeable channels that have retained their function as chemical sensors since their first appearance in metazoan species several hundred million years ago. In vertebrates, TRP channels have evolved multiple functions which make it difficult to understand exactly how they transmit signals to the brain that are interpreted very differently. For example, TRPA1 and TRPV1 are sensitive to various chemicals and activation of these channels produce sensations with opposing effects. Pain is felt when TRPV1 is activated by spider toxins, but activation by plant cannabidiol results in a pain-relieving sensation. Similarly, TRPA1 activation by delta-tetrahydrocannabinol is reported to relieve symptoms of pain, but TRPA1 activation by the active ingredient in wasabi results in a repulsive or noxious sensation. Much of what we know about TRPA1 and TRPV1 comes from the use of plant products or exposure to substances that cause or alleviate pain and inflammation. In this study, whole-cell voltage clamp recordings of heterologously expressed human TRPA1 and human TRPV1 were tested for sensitivity to a hallucinogenic plant compound, salvinorin A and an arthropod-defensive compound, para-benzoquinone. Neither compound has yet been reported to activate TRP channels but both are known to be involved in pain and inflammation signaling in humans. I show that the arthropod compound,
para-benzoquinone, activates and desensitizes TRPA1 in a cysteine-dependent manner, but activation of TRPV1 is not dependent on cysteine reactivity. Although salvinorin A is known to be a potent agonist of the kappa-opioid and cannabinoid receptors, here I show that it also acts as a highly potent agonist of both TRPA1 and TRPV1. Its interaction with TRP channels may contribute to its antinociceptive effects in behavioral studies with animals that are reported to be independent of opioid signaling.
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Introduction

Chemosensation is a common trait among multi-cellular organisms. The emergence and evolution of diverse, multifunctional, ligand-activated ion channels and other receptors that activate channels through second messengers enabled all animals to detect and distinguish if chemicals are harmful. Animal behavior studies have revealed that exposure to certain chemical compounds result in an aversive reaction in some animals, but appear to be harmless to others (e.g. capsaicin laced birdseeds to deter squirrels; Fitzgerald et al., 1995). Interestingly, ion channels expressed in modern mammals still retain sensitivity to insect and plant compounds. Toxins from snakes can activate or inhibit acid sensing channels (ASICs) causing or relieving pain (Bohlen et al., 2011; Diochot et al, 2011); the mammalian voltage-gated sodium channel is blocked by the puffer fish tetrodotoxin (TTX), but the Drosophila sodium channel homologue is insensitive to TTX (Zhang et al., 2011); several plant compounds used as food flavoring are also agonists of various TRP channels while also containing antimicrobial and insecticidal properties (Park et al., 2008). A handful of mammalian sensory ion channels are known to be involved in the detection of thousands of chemicals, which make it difficult to pinpoint exactly how an ion channel can “sense” the difference between harmless and harmful.

Much of pharmacological ion channel research involves the use of naturally produced plant and animal products, such as: capsaicin and resiniferatoxin (plants), tetrodotoxin and conotoxins (sea animals), double-knot toxin and myotoxin (insects). Many of these natural compounds are dangerous to humans, but other natural products that are not perceived as harmful include: menthol, cinnamon, oregano (carvacrol) and
rosemary (cineole). Some plant compounds have been used in naturopathic settings for centuries and are known to have antinociceptive and anti-inflammatory effects, such as cannabis (Δ-tetrahydrocannabinol) (Langford et al., 2012; Izzo et al., 2009). The natural plant compounds that we associate with having an aromatic or naturopathic property come from a family of chemicals known as terpenoids or isoprenoids. These compounds are made up of at least one isoprene unit, \( \text{CH}_2=\text{C(CH}_3\text{)}\text{CH=CH}_2 \), but more moieties can be built upon this core to make more complicated structures. Examples of plant terpenoids are shown below in Figure 0.1.

![Figure 0.1: Naturally occurring plant terpenoids. A large tetraterpene, beta-carotene and smaller monoterpene, carvacrol (oregano). (Figures copied from www.internetchemie.info).](image)

Plant terpenoids are involved in the metabolism and development of plants but are also emitted into the environment for survival purposes (Langenheim, 1994). They often have a pleasant aromatic component and beneficial effects on humans, but another function of terpenoids outside of the plant structure is to act as repellants for small mammals, insects, and microbes (Gershenzon and Dudareva, 2007). For example, cinnamon and carvacrol both have antimicrobial and insecticide properties.
and are sometimes used to treat inflammation in humans (Park et al., 2008; Tung et al., 2008; Cheng et al., 2009; Guimaraes et al., 2012; Sleha et al., 2012).

Insects and marine animals also produce and emit terpenes as a defensive strategy and these terpenes are chemically similar to the plant compounds (M. Gordaliza, 2010; Conner et al., 2007). However, it is not apparent that there are any beneficial effects of insect-derived compounds on humans other than for antimicrobial purposes. Most of what is known about insect terpenes is that they function as protective substances, which indicates that these compounds are reactive and used to cause harm (Roth and Eisner 1962; Gershenzon and Dudareva, 2007). Plant and insect terpenes contain chemical groups that are unsaturated (have double bonds) and can easily form covalent bonds by sharing electrons with nucleophiles, which makes them electrophilic (see Figure 0.2; Alarie et al., 1998).

![Figure 0.2: Examples of reactive defensive quinones produced and emitted from animals. On the left is the large nakijiquinone, isolated from marine sponges native to Okinawa, Japan (M. Gordaliza, 2010). On the right is a small methyl 1, 4 – benzoquinone that is produced by the flour beetle (Uhruh et al., 1998).](image-url)
Chemical detection of electrophilic compounds is a sensory modality that has been conserved for over 500 million years, from invertebrates to vertebrates and it is known that some of the insects’ detection mechanisms are mediated by TRP channels \((\text{Cosens and Manning, 1969}; \text{Matsuura et al., 2009})\). More specifically, in \textit{Drosophila}, the TRPA channel is responsible for the detection of electrophiles and necessary for avoidance behavior; the mammalian TRPA homologue has retained its sensitivity to electrophiles throughout evolution \((\text{Tracey et al., 2003}; \text{Kang et al., 2010})\). The detection of sound in \textit{Drosophila} involves ion channels that are related to the TRPV1 family \((\text{Kim et al., 2003})\), but this channel evolved in mammals as one of the key receptors involved in pain signaling.

Mammalian TRPA1 and TRPV1 are known to be target molecules of reactive naturally occurring electrophiles \((\text{Jordt and Julius, 2002}; \text{Xu et al., 2006}; \text{Taylor-Clark et al., 2008}; \text{Vriens et al., 2008}, \text{Riera et al., 2009}; \text{Kang et al., 2010}; \text{Bohlen et al., 2010}; \text{DePetrocellis et al., 2011})\). However, it is still unclear how these channels become activated by electrophiles. Hinman and colleagues \((2006)\) proposed that the human TRPA1 channel contains three specific cysteine residues, located on the channels’ amino terminus that are targeted by electrophiles and cause channel opening. Macpherson and colleagues \((2007)\) showed similar results in the mouse TRPA1 homologue. Since 2006, numerous groups have shown that TRPA1 cysteines are important for gating and activation by multiple types of agonists. TRPA1 is unique among mammalian TRP channels because it contains a large cysteine-rich amino terminus with 17 ankyrin repeat domains. Other mammalian TRP channels contain between 4- 6 ankyrin repeats. It is thought that these repeat domains are involved in
protein-protein interactions, gating and maintaining the structural integrity of the protein (Mosavi et al., 2004; Sotomayor et al., 2005; R. Gaudet, 2008). Similar to TRPA1, the TRPV1 channel also has numerous electrophilic agonists but there is less agreement about the existence of a common mechanism of activation by naturally occurring electrophiles. Two groups suggest a mechanism of activation of TRPV1 that involve molecules tethering or binding at the pore domain by insect (tarantula toxin) and plant (camphor) compounds (Bohlen et al., 2010; Marsakova et al., 2012).

Various forms of benzoquinones are commonly produced and emitted for defensive purposes by arthropods and marine organisms (Gordaliza, 2010; DeCapitani et al., 2011). Benzoquinone generally does not result in the death of the predator but somehow causes a strong aversive and possibly a mildly painful response, presumably through TRPA1 and/or TRPV1. A defensive plant terpene, salvinorin A, was originally used for alleviating mild pain and inflammation by indigenous tribes in Mexico (Seibert, 1994). The salvinorin A compound is a potent hallucinogen that targets kappa-opioid and cannabinoid receptors (Roth et al., 2002; Braida et al., 2007). Since the vertebrate TRPA1 and TRPV1 channels have been known to act as targets of naturally occurring plant and insect compounds, we set out to test whether benzoquinone and salvinorin A were agonists of these ion channels. Whole-cell current recordings of cells over-expressing human TRPA1 and TRPV1 revealed that both defensive compounds were potent agonists of these sensory TRP channels.
Materials and Methods

Cells and heterologous expression: HEK-293 FlpIn Trex cells (Life Technologies, Carlsbad, CA) stably expressed the tet repressor, either alone (parental), or with an integrated wild-type human TRPA1 coding sequence. Cells were cultured in DMEM/F-12 (1:1) supplemented with 5-10% FCS and 15 µg/ml blasticidin at 37°C in a 5% CO₂ incubator. To induce expression of wild-type TRPA1, 0.1 µg/mL doxycycline was added 18-24 h before recordings. For transient expression of human TRPA1 mutant (C856S or C421S) and TRPA1 triple mutant (C619S, C639S and C663S) channels, parental 293 FlpIn Trex cells were transfected for 3 h using Lipofectamine 2000 (Life Technologies). Human TRPA1 C856S mutants were generated with primer sequences:

5' TATCTTCAAGATTTGAAAATTCTGGAATTTTTATTGTTATGTTG (forward),
5' CAACATAACAATAAAAATTCCTCTATTTTCAAATCTTTGAAGATA (reverse). Site-directed mutagenesis was completed using the GeneArt kit purchased from Life Technologies. The cDNA of mutant C421S in pCI-neo vector was a gift from Dr. Yasuo Mori (Kyoto University). 1.5-2 µg TRPA1 mutant cDNA was transfected with the transfection marker, eGFP-C1 plasmid (0.1 µg) or mCherry (0.3 µg). Cells were seeded onto 12 mm glass coverslips and recorded 18-24 h after transfection.

Electrophysiology: Cells were voltage-clamped with an Axopatch 200B amplifier controlled with pClamp 9 (Molecular Devices, Union City, CA). Currents were filtered at 2 kHz and acquired at 4 kHz with a Digidata 1320A or 1322. Capacity current was reduced as much as possible using amplifier circuitry, and series resistance was corrected to 75-85%. Cells were held at –60 mV, and currents were elicited by a 40 ms
step to −100 mV, followed by a 200 ms ramp to +100 mV, and held at +100 mV for 40 ms. This protocol was applied every 2 s. Pipettes were pulled from borosilicate glass (WPI, Sarasota, Florida) to resistances ranging from 2.0 to 5.5 MΩ and filled with an internal solution containing (in mM): 122 Cs-methanesulfonate, 1.8 MgCl₂, 9 EGTA, 14 Tris-creatine phosphate, 4 MgATP, 0.3 NaGTP, 10 HEPES (pH 7.20 with CsOH). The extracellular solution had no added calcium and consisted of (in mM): 150 NaCl, 4 KCl, 3 MgCl₂, 10 glucose, 10 HEPES, pH 7.40 (with NaOH). Solutions were applied after lifting the cell off the coverslip and placing it in front of an array of ~200 μm diameter quartz tubes; complete solution exchange required less than 1 s. Currents were imported with Data Access (Bruxton, Seattle, WA) and analyzed with Igor Pro 5.5 software (Wavemetrics, Lake Oswego, OR). Currents were averaged over 5 ms at the end of steps to ±100 mV (before and after the ramp), or for 0.4 ms around ±60 mV (±60.2 to ±59.8 mV) during the ramp.

Reagents: HC-030031 was from Tocris Bioscience (Bristol, United Kingdom). MTSET was from Toronto Research Chemicals (Toronto, Ontario, Canada). All other reagents were from Sigma-Aldrich (St. Louis, MO). Stock solutions of HC-030031, carvacrol and salvinorin A were prepared in DMSO. AITC stock solution was prepared in ethanol. All other reagents were prepared and diluted to working concentrations in external or internal solutions immediately prior to the experiment (pBQN was initially dissolved in ethanol prior to dilution into working concentrations). Control solutions containing DMSO or ethanol did not elicit current activation.
Chapter 1

Benzoquinone reveals a cysteine-dependent mechanism of desensitization in TRPA1

Attributions: This chapter was written in collaboration with Dr. Nathanial Blair. All text and figures were generated by Yessenia Ibarra with the exception of figures 1.6C and 1.6D, including all text in the results and discussion related to figures 1.6C and 1.6D.

Introduction

Organisms in their natural environment face the constant threat of predation. As a consequence, several strategies for protection evolved, including crypsis, behavioral adaptations, and the production of defensive chemicals. Insects from the Phylum Arthropoda are incredibly diverse and are a source of a large number of defensive compounds, including venoms for injection and irritants for contact spray and secretion (L. Karalliedde, 1995; T. Eisner, 2005). One striking example of an arthropod defensive spray is from the bombardier beetle from the Family Carabidae. This spray can be aimed with great accuracy and consists largely of benzoquinone, produced by oxidation of hydroquinone, and is expelled at ~100 °C (Aneshansley et al., 1969). The beetle’s defense is quite effective, deterring predators such as other insects, frogs, birds and small mammals, yet the underlying sensing mechanism in this case is poorly understood. The use of quinones in defensive secretions is widespread, occurring in many groups of arthropods (Conner et al., 2007).
Many chemical deterrents produced by plants activate members of the transient receptor potential (TRP) superfamily of ion channels, which are calcium-permeable nonselective cation channels expressed in many tissues (Patapoutian et al., 2009; Wu et al., 2010). Perhaps the best known example is the activation of TRPV1 by capsaicin, the active ingredient in ‘hot’ peppers. Other plant-derived chemicals activate a variety of TRP channels: menthol from mint (TRPM8; Peier et al., 2002), carvacrol from oregano, eugenol from cloves, citral from lemon (TRPV3; Xu et al., 2006; Stotz et al., 2008). TRPA1 is another common target of plant compounds and is activated by allyl isothiocyanate (AITC) from mustard seeds, cinnamaldehyde from cinnamon, and allicin from garlic (Bandell et al., 2004; Bautista et al., 2005; Macpherson et al., 2005). TRPA1 is primarily expressed in vertebrate sensory neurons, where its activation excites nociceptive sensory neurons. TRPA1 also contributes to hyperalgesia during inflammatory states in peripheral tissues, as well as contributing to cough and airway inflammation (Bessac and Jordt, 2008; Nilius et al., 2011; Bautista et al., 2012).

TRPA1 activation occurs via two described mechanisms: First, electrophilic molecules (including AITC, cinnamaldehyde, allicin and N-methylmaleimide) react with free sulfhydryl groups on cysteine and lysine residues of the amino-terminus, which leads to channel opening (Cebi and Koert, 2007; Macpherson et al., 2007; Hinman et al., 2006). Mutagenesis studies show that residues C421, C619, C639, C663, and K704 (numbered residues refer to the human sequence) are crucial for activation by electrophiles (Hinman et al., 2006; Macpherson et al., 2007a). Second, non-electrophile agonists of TRPA1, such as carvacrol, 2-aminoethoxydiphenyl borate (2-APB), Δ-THC, nicotine and menthol, presumably bind in other channel locations to cause opening (Xu
et al., 2006; Hinman et al., 2006; Jordt et al., 2004; Talavera et al., 2009; Karashima et al., 2007).

The major component of bombardier beetle spray, para-benzoquinone (pBQN), is an electrophile that reacts with cysteines and lysines in multiple proteins (Mason and Liebler, 2000, Lame et al., 2003; Dayon et al., 2005; Diedrich and Julian, 2010). In addition to being behaviorally aversive, pBQN is toxic to mammalian cells (Tian et al., 2011; Fisher et al. 2011; Sarma et al., 2011; Crespo et al., 2011). In humans, prolonged exposure to insect secretions of benzoquinone results in ulcers, blisters or discolored skin lesions with a local burning sensation (de Capitani et al., 2011), but the potential involvement of TRPA1 in these symptoms is unknown. Interestingly, Andersson and colleagues (2011) found that pBQN can activate TRPA1, and yet when injected into the spinal cord of mice produces an antinociceptive effect. In this case, pBQN may mimic the effects of the chemically similar break down products of acetaminophen.

Although much of the attention on TRPA1 channels has focused on their activation properties by various agonists, less is known about how desensitization of TRPA1 occurs. TRPA1 is known to be desensitized by Ca\(^{2+}\) entering through the channel, although where it binds and its precise mechanism is unknown (Nagata et al., 2004; Wang et al., 2008). Recently, Raisinghani and colleagues (2011) showed that continuous N-methyl maleimide (NMM) application can desensitize TRPA1 channels in the absence of Ca\(^{2+}\) entry, suggesting a new type of desensitization. Here we show that pBQN acts as an electrophilic TRPA1 agonist, activating channels at low nanomolar concentrations. Activation did not require the cysteines at positions 619, 639, or 663. We also show that higher concentrations of pBQN led to complete desensitization of
TRPA1, abolishing responses to both electrophilic and non-electrophilic agonists. pBQN induced desensitization also occurs in the absence of an apparent activation when excess cysteine is added to the intracellular solution. TRPA1 desensitization by pBQN results from modification of cysteine residues that are distinct from intracellular cysteines involved in activation.

Results

Figure 1.1 shows the activation of current by 30 nM pBQN applied to a HEK cell expressing human TRPA1 channels. The current activation began with a lag of 60 s, and reached a steady-state after 6 min in pBQN (Figure 1.1C). (In 6 cells, the delay ranged from 50 to 90 s, and steady-state was reached after 4 to 8 min). The currents activated by pBQN had a reversal potential at -3 mV and were slightly outward rectifying (Figure 1.1B), typical of TRPA1 channels (Story et al., 2003; Jordt et al., 2004). In all cells tested, washing off pBQN did not reverse the activated currents (n=13). However, applying the TRPA1 selective blocker, HC-030031 (50 µM), reduced the pBQN activated currents by 95 ± 5% at +100 mV (McNamara et al., 2007). pBQN (at 1 µM) did not elicit any change in current when tested in 3 HEK cells with no TRPA1 expression (i.e., not induced by doxycycline).

We next tested the effect of higher pBQN concentrations on TRPA1 channels. Figure 1.2A shows the activation of TRPA1 current with [pBQN] ranging from 10 nM to 10 µM. With 10 nM pBQN, the currents reached a steady-state after a 6 min application (similar to 30 nM, Figure 1.1B). As the concentration of pBQN increased, two observations were apparent. First, the delay time before channel activation was
Figure 1.1: p-Benzquinone (pBQN) activates heterologously expressed human TRPA1. A) Currents evoked by a voltage protocol consisting of a 40 ms step to –100 mV followed by a 200 ms ramp to +100 mV, followed by 40 ms at +100 ms (holding potential –73 mV). Baseline currents (red), steady-state current in response to 30 nM pBQN (green) and currents after block by 50 μM HC-030031 (black). B) Current-voltage curves from sweeps above. C) Activation time-course of current by 30 nM pBQN and inhibition by 50 μM HC-030031. Symbols plot the current averages at ±100 mV. The dashed line indicates zero current.
Figure 1.1 (Continued)
reduced: 100 nM pBQN activated currents that peaked in 110 ± 11 s, whereas 1 μM and 10 μM pBQN activated currents peaked in 20 and 5 s, respectively. Second, the current no longer remained at a steady-state level, but instead began to decline after reaching its peak. In 100 nM pBQN, the current declined by 45% after 300 s, and in 1 and 10 μM pBQN the decline was faster (215 and 100 s, respectively) and essentially complete (95 and 100%, respectively). Figure 1.2B shows the concentration response of TRPA1 current as a function of pBQN concentration. Maximal currents were elicited by 300 nM pBQN and currents activated by larger concentrations were smaller. The decline in peak current for concentrations above 300 nM seems to be correlated with the faster decay of the current seen in the time course records with higher concentrations of pBQN. The concentration dependence of the decay rate is plotted in Figure 1.2C. Decay of TRPA1 currents by pBQN is observed only at concentrations of 100 nM or higher and these were fitted with a single exponential function. At 100 nM pBQN, currents declined with an average time constant of 254 ± 167 s, or when converted to a decay rate (rate = 1/τ), gives 0.00393 ± 0.0006 s⁻¹ (n=9). Exposure to higher concentrations of pBQN (3-10 μM) resulted in faster decay, with a time constant of ~35 s (rate ~0.032±0.003 s⁻¹).

Andersson and colleagues (2011) recently found that pBQN activates human and mouse isoforms of TRPA1, but they did not observe a net reduction in peak current amplitude of TRPA1 at micromolar pBQN concentrations. This might be due to the presence of external calcium in their recording solutions, which both potentiates and desensitizes TRPA1 (Nagata et al., 2005; Zurborg et al., 2007; Doerner et al., 2007; Wang et al., 2008). One possibility is that calcium potentiation enhanced TRPA1 current at higher pBQN concentrations more rapidly than desensitization occurred. The decline
**Figure 1.2:** Dose-dependence of pBQN activation and desensitization of human TRPA1. **A)** TRPA1 currents (measured at +60 mV) elicited by extracellular application of pBQN at concentrations from 10 nM to 10 μM. The dashed line indicates the zero current level. **B)** TRPA1 current amplitude as a function of pBQN concentration. Current densities were calculated using the peak or steady state currents at ±60 mV (each data point \(n=4-9\); bars represent ±SEM). Steady-state currents were measured for concentrations up to 30 nM and peak current for higher concentrations. **C)** Rate of TRPA1 desensitization by pBQN is concentration dependent (each data point \(n=4-5\); bars represent ±SEM).
Figure 1.2 (Continued)
in TRPA1 current we observe is not likely to result from this process, as we omitted calcium from the external solution and tightly buffered intracellular calcium with 9 mM EGTA in the pipette solution. Furthermore, we were able to record long-lasting, non-desensitizing currents in response to other agonists, including N-methylmaleimide (NMM), 2-trimethylammonium ethyl methanethiosulfonate (MTSET) and iodoacetamide (IA) (see below). This suggests that TRPA1 channels have a calcium-independent form of desensitization when strongly activated by electrophilic agonists. A similar desensitization in response to NMM and allyl isothiocyanate (AITC) was recently noted by Raisinghani and colleagues (2011).

Does the decay in current with larger pBQN concentrations represent desensitization? If so, it might result in TRPA1 insensitivity to other agonists. To test this, we applied commonly used TRPA1 agonists to the channel after pBQN induced a decline in currents. We first used two electrophilic agonists, allyl isothiocyanate (AITC, 100 μM) and N-methylmaleimide (NMM, 50 μM). These substances bind to a series of cysteine side chains in the amino terminus of TRPA1 to activate the channel (Hinman et al., 2006; Macpherson et al., 2007). pBQN is itself an electrophile, which can covalently bind to thiol groups on cysteine side chains and lysine side chains in many proteins (Mason and Liebler, 2000; Lame et al., 2003; Fisher et al., 2007). Figure 1.3A and 1.3B shows that application of 1 μM pBQN causes TRPA1 currents to activate and then decline, but neither 100 μM AITC nor 50 μM NMM was able to subsequently increase the current (representative of 3 and 6 cells, respectively). This shows that TRPA1 channels treated with high concentrations of pBQN for ~2 min are unable to respond to a 2nd application of an electrophilic agonist. Short applications of both NMM and AITC
**Figure 1.3:** TRPA1 is unresponsive to electrophilic agonists after desensitization.

**A, B)** 1 μM pBQN activates and desensitizes the TRPA1 channel. The TRPA1 agonists, allyl isothiocyanate (AITC, 100 μM) and N-methylmaleimide (NMM, 50 μM) do not elicit current activation. **C,** After 50 μM NMM activates and desensitizes TRPA1, the channel is unresponsive to 1 μM pBQN. The dotted line indicates the zero current level. All currents were average at ±100 mV before and after ramps.
Figure 1.3 (Continued)
activated TRPA1 current when given prior to pBQN application but the currents remained at elevated levels and occasionally resulted in desensitization or unstable pipette seal despite washout. **Figure 1.3C** shows that TRPA1 channels activated by 50 µM NMM become desensitized after prolonged application. TRPA1 current initially increased and then began to decline, very similar to the response to pBQN alone. The major difference was that 50 µM NMM desensitization was about twice as slow as 1 µM pBQN desensitization, requiring 6-8 min to reach a steady-state (τ=94 s, n=4). When 1 µM pBQN was added after the current declined, there was no response.

TRPA1 can also be activated by a number of non-electrophilic substances that are able to activate the channel independent of the amino terminal cysteines, including 2-aminoethoxydiphenyl borate (2-APB, 500 µM) and carvacrol (300 µM) (Hinman et al., 2006; Xu et al., 2006). **Figure 1.4** shows that both 2-APB and carvacrol can reversibly activate TRPA1 channels. Following 2-APB or carvacrol, the application of 1 µM pBQN resulted in the typical activation and subsequent complete decline in the current. After removal of the pBQN-containing solution, neither 2-APB nor carvacrol was able to activate TRPA1 current (2-APB, n=3; carvacrol, n=4). Even after removal of pBQN, at concentrations of 100 nM or higher, desensitization of TRPA1 by pBQN appears to be irreversible for the duration of our recordings (up to 30 min in some cells). Clearly, TRPA1 channels desensitized by pBQN application are unresponsive to both electrophilic and non-electrophilic agonists. Both AITC and NMM were unable to activate TRPA1 current after pBQN-induced desensitization, suggesting that pBQN may be acting through its ability to bind to cysteine residues. If so, then eliminating this ability should prevent activation. To test whether pBQN activation of TRPA1 depends on
Figure 1.4: TRPA1 is unresponsive to non-electrophilic agonists after desensitization.

**A, B)** The TRPA1 agonists, 2-aminophenylborate (2-APB, 500 μM, n=3) and carvacrol (300 μM, n=4) reversibly activate TRPA1. Neither agonist elicits a second response after desensitization by 1 μM pBQN. All currents were average at ±100 mV before and after ramps.
cysteine reactivity, we preincubated 1 μM pBQN with 5 μM L-cysteine before applying it to the cell. This reaction results in a complex mixture of products, which lack much of the electrophilic character of pure benzoquinone (Crescenzi et al., 1988). Addition of pBQN preincubated with L-cysteine did not activate TRPA1 currents (Figure 1.5A) (n=3). In the same experiment, subsequent application of 1 μM pBQN alone activated TRPA1 normally and these currents were blocked by 50 μM HC-030031.

In other experiments we noticed that a shorter exposure to 50 μM NMM activated TRPA1 but did not cause desensitization. Are there unreacted cysteines remaining on the channel if no desensitization has been produced? We tested this by activating TRPA1 with a 20 s exposure to NMM, presumably allowing only some cysteines to bind to NMM. As expected for a covalent action, currents activated by 50 μM NMM remained unchanged during a wash with extracellular solution (Figure 1.5B). Application of the pBQN – L-cysteine mixture had no effect on the NMM-activated currents. Subsequent application of 1 μM pBQN alone also did not produce any additional activation, suggesting that NMM had already reacted with all available cysteines that are involved in channel activation. However, the application of pBQN did desensitize the channel and reduced the current by >60% (n=3). The ability of pBQN to induce desensitization without additional activation suggests that pBQN modifies cysteine residues that are important for desensitization and they are not the same residues required for activation by either NMM or pBQN. The failure of pBQN to produce additional activation after NMM activation, also suggests that pBQN induced activation does not depend on its ability to modify other amino acid residues, such as lysine (Fisher et al., 2011). However, because we did not test if pBQN is able to react with other amino acid
**Figure 1.5**: pBQN activation involves cysteine reactivity.  

**A)** A 20 s exposure to 5 μM L-cysteine alone or L-cysteine preincubated with 1 μM pBQN has no effect on TRPA1. A subsequent application of 1μM pBQN alone activates TRPA1 and is blocked by 50 μM HC-030031.  

**B)** A 60 s application of 5 μM cysteine followed by a 20 s bath application of 50 μM NMM activates TRPA1. The activated currents remain unchanged during washout and during a 60 s bath application of preincubated cysteine plus pBQN. Application of 1 μM pBQN alone quickly reduces the NMM activated currents.  

Currents in part **A** were averaged at ±60 mV due to amplifier saturation at ±100 mV. Part **B** is averaged at ±100 mV before and after ramps.
residues we cannot exclude the possibility that pBQN can desensitize the channel by another method.

Our data, so far, suggests that pBQN activates and desensitizes TRPA1 by reacting with cysteine, but we have not determined where those cysteines are. The human isoform of TRPA1 contains 28 cysteine residues: 20 in the amino terminus, 5 in the transmembrane region, and 3 in the cytosolic terminus (Cvetkov et al., 2011). Because most of the cysteine residues on the TRPA1 channel are located on intracellular residues, we asked if pBQN targets cytosolic cysteines to activate TRPA1. To test this, we wanted to manipulate the cytosolic activity of pBQN by applying the preincubated mixture of 5 μM L-cysteine and 1 μM pBQN intracellularly. Similar to the results in Figure 1.5, the preincubated mixture added to the intracellular solution did not activate TRPA1 (Figure 1.6A). However, when additional 1 μM pBQN alone was applied extracellularly it was able to activate and desensitize the TRPA1 currents (n=3). These experiments show that pBQN must maintain its electrophilic character or maintain its chemical structure to both activate and desensitize TRPA1 channels.

In another experiment, we added 20 mM L-cysteine to the pipette solution and tested whether pBQN activation would be impeded by excess amounts of cysteine in the cytosol (Figure 1.6B). Free L-cysteine might react more readily with pBQN as it enters the cell, out-competing accessible cysteines of TRPA1. L-cysteine has limited membrane permeability and should not prevent pBQN from accessing cysteines located on the TRPA1 transmembrane region or in other regions inaccessible to aqueous solvent. Intrapipette cysteine was allowed to diffuse into the cell for 3 min after break-in and prior to the addition of agonists. We first applied the non-cysteine-reactive TRPA1
Figure 1.6: pBQN activation, but not desensitization, requires cytosolically-accessible cysteines. A) Intrapipette 5 μM cysteine and 1 μM pBQN has no effect on TRPA1 currents after 180 s of recording. Extracellularly applied 1 μM pBQN induces activation and desensitization. B) 300 μM carvacrol activates TRPA1 in the presence of 20 mM intrapipette cysteine. 1 μM pBQN is applied for 360 s and has minimal effects on activation. The channel is unresponsive to a second application of carvacrol. C) Intrapipette 2-trimethylammonium ethyl methanethiosulfonate (MTSET, 100 μM) elicits sustained TRPA1 currents. A short application of 50 μM HC-030031 blocks the currents reversibly. A subsequent application of extracellular 10 μM pBQN rapidly desensitizes the channel. D) Application of 100 μM iodoacetamide elicits sustained TRPA1 current activation. After 4 minutes in iodoacetamide, addition of 10 μM pBQN initially increases TRPA1 current, followed by rapid and complete desensitization. The pipette contained the typical Cs-methanesulfonate solution. Dashed lines indicate zero current. All currents were averaged at ±60 mV.
Figure 1.6 (Continued)

A: Cs-Mes internal with 1 μM pBQN + 5 μM L-cysteine

B: Cs-Mes internal with 20 mM L-cysteine

C: Cs-Mes internal with 100 μM MTSET+

D: 100 μM iodoacetamide 10 μM pBQN
agonist, carvacrol (300 μM), to ensure the channel was still functional. Next, we applied 1 μM pBQN externally and noticed a delay before minimal current activation ($n=4$). Normally, 1 μM pBQN will elicit immediate and robust activation (e.g., Figure 1.2A), yet in these cells, more than 40 s elapsed before we observed slight increases in both inward and outward current followed by a slow and subtle decay. We verified that this decay was actually desensitization when a second application of 300 μM carvacrol failed to activate any current. This shows that pBQN requires contact with cytosolically-accessible reactive sites to robustly activate TRPA1.

Because desensitization still occurs in the absence of an obvious activation response these two processes might require different groups of cysteines. Perhaps pBQN desensitization requires access to transmembrane cysteines or cysteines located on the proteins’ interior core which are not equally accessible to all thiol-reactive compounds. To test this we added a thiol-reactive agent, 2-trimethylammonium ethyl methanethiosulfonate (MTSET), to the pipette solution. MTSET is a charged molecule that forms disulfide bonds with free thiol groups, but cannot diffuse through the lipid bilayer. When applied extracellularly, MTSET does not activate TRPA1 (Hinman et al., 2006). However, when added to the intracellular solution, TRPA1 began to activate within 1-2 min and reached maximum activation plateau by 10-15 min (Figure 1.6C). Once the plateau was reached, these large currents did not decline. A short application of 50 μM HC-030031 inhibited the current by 89%, demonstrating that the currents were mediated by the TRPA1 channel. At this point we applied 10 μM pBQN externally to test whether TRPA1 channels could be desensitized after activation by intracellular MTSET. Indeed, addition of extracellular pBQN desensitized the channel by $>99\%$ (tau = 56.7 ±
14.4 s, \( n=3 \)). This indicates that pBQN is able to desensitize the channel by binding to sites that are not accessible to intracellular MTSET.

The ability of thiol-reactive compounds to modify different cysteine side chains in native proteins depends upon a number of factors, including steric hindrance and the reactivity of cysteines (resulting from differences in the chemical microenvironment within the polypeptide chain). We tested whether iodoacetamide (IA), another commonly used cysteine modifying reagent, could activate and/or desensitize TRPA1.

Iodoacetamide alkylates free thiol groups via \( S_N2 \) nucleophilic attack, slightly different than the Michael addition describing benzoquinone and NMM reactions with thiols (Dennehy et al., 2006). Previous results show that NMM and iodoacetamide modify different groups of cysteines on TRPA1 (Wang et al., 2012; Macpherson et al., 2007a). When we applied 100 \( \mu \)M IA externally, TRPA1 current activated immediately and continued to increase over 4 minutes until a steady-state was reached (range 4-8 min, \( n=4 \) cells; Figure 1.6D). In contrast to pBQN or NMM, IA did not cause desensitization in any of the 4 recorded cells (recorded up to 20 min). In 3 cells, 400 \( \mu \)M IA was applied for 120-840 s. TRPA1 current transiently increased in 400 \( \mu \)M IA before declining by 19 ± 3%. Both the transient increase and the sustained reduction were reversible.

Once the steady-state had been reached, we applied 10 \( \mu \)M pBQN to test whether IA might prevent pBQN desensitization. When pBQN was first applied, the TRPA1 current increased 2.3 ± 0.7 fold (\( n=3 \)) until it reached a peak and began to desensitize, ultimately declining by 97 ± 1% with a time constant of 49.1 ± 27.8 s. The additional activation induced by pBQN suggests that IA is a partial agonist. This
indicates that pBQN activates and desensitizes TRPA1 through sites that are inaccessible to IA.

A human TRPA1 triple cysteine mutant ion channel (TRPA1-3C) was created by HInman et al. (2006). The cysteines at positions 619, 639, and 663 were replaced with serine residues and the mutant TRPA1 was no longer activated by NMM but is still responsive to non-electrophilic agonists (Hinman et al., 2006). We expected that the cysteines required for NMM activation were probably also the targets of pBQN. Surprisingly, we found that the TRPA1−3C channel had robust activation by 1 μM pBQN. Just as in wild-type channels, pBQN activation in the triple mutant channel was followed by desensitization over several min (Figure 1.7A). To test if pBQN desensitized the mutant channel to other agonists, as it does in wild-type channels, we applied 300 μM carvacrol or 500 μM 2-APB (not shown) to the cell after the currents had decayed. In all cells tested, both carvacrol (n=6) and 2-APB (n=3) activated the mutant channel even after pBQN desensitization (distinct from wild-type channels, which failed to respond to carvacrol and 2-APB, see Figure 1.4). The absence of these three cysteines does not prevent pBQN activation, but does prevent the channel’s ability to become desensitized to non-electrophiles.

Although pBQN activated the TRPA1−3C channels just as robustly as it did the wild-type, we noticed that the current-voltage relationship was different for the triple mutant. The TRPA1−3C mutants were more outwardly rectifying than the wild type channels, due to a relative reduction of inward current (Figure 1.7B). The inset shows a representative I-V curve of a typical response of the wild type TRPA1 to 1 μM pBQN. We quantified the degree of rectification as the ratio of TRPA1-induced current at +60
Figure 1.7: pBQN activates TRPA1 triple cysteine mutant (C619S, C639S, and C663S).  

A) The TRPA1 triple cysteine mutant activates in response to 1 μM pBQN and 300 μM carvacrol. B) A representative current-voltage trace illustrates the pronounced outward rectification of TRPA1 mutant in response to 1 μM pBQN. Inset shows a wild TRPA1 response to 1 μM pBQN. C) Rectification index of the triple mutant compared to wild type. The dotted line indicates the level where no rectification would occur (n=9 for both mutant and WT, bars represent ± SEM). All currents were averaged at ± 60 mV.
Figure 1.7 (Continued)

Triple cysteine mutant (C619S, C639S, C663S)

A

1 µM pBQN

300 µM carv

2 nA

30 s

B

WT

10 nA

-100 mV

C

rectification index (+60/-60 mV)

3C mutant

WT
mv and -60 mV (Figure 1.7C). The triple mutant channels were far more outwardly rectifying (rectification ratio of 3.8±1, n=9) than wild type (1.4±0.1, n=9) when activated by 1 µM pBQN.

Our data suggests that the TRPA1 channel has two separate groups of target cysteines that are independently responsible for activation and desensitization by pBQN. Hinman and colleagues found that the triple cysteine mutant is not activated by NMM. We asked if NMM would still desensitize the mutant channel, indicating that cysteines other than C619, C639, and C663 are involved in TRPA1 desensitization to electrophilic compounds. First, we exposed the mutant channel to 50 µM NMM for 10 s, which did not activate current (Figure 1.8A). Next, we tested whether 2-APB and pBQN could still activate the channel after a short NMM exposure. Both 2-APB and pBQN effectively activated the channel, indicating that a short exposure to NMM neither activated current on its own, nor prevented activation by either 10 µM pBQN or by 2-APB (n=4).

We then repeated the same experiment but with a longer (60 s) exposure to NMM (Figure 1.8B). A longer application of NMM might result in modification of additional cysteines involved in the desensitization process, as suggested by the results with the wild-type channels (see Figure 1.3C). After a 60 s exposure to NMM, 500 µM 2-APB was applied to the cell and induced an outwardly rectifying current. However, there was no activation by 10 µM pBQN after NMM (n=4), similar to what we observed in wild type channels. Although we do not observe an NMM-induced activation response here, a 60 s exposure (or longer) to NMM does produce an evident desensitization to pBQN. However, unlike the wild-type channels, the TRPA1-3C mutant is still responsive
Figure 1.8: TRPA1 triple cysteine mutant can activate in response to non-electrophilic agonists following pBQN desensitization. A) 50 μM NMM is applied to the TRPA1 triple cysteine mutant for 10 s followed by 500 μM 2-APB. The triple mutant channel responds to a 30 s exposure to 10 μM pBQN. B) A similar experiment as in A, but with a 60 s exposure to 50 μM NMM. C) The mutant channel responds to a 10 s exposure to 50 μM NMM followed by additional activation with 1 μM pBQN. After pBQN desensitization, 300 μM carvacrol induces activation. D) A similar experiment as in C, but with a 120 s application of 50 μM NMM followed by a 60 s exposure to 1 μM pBQN and 5 s of 300 μM carvacrol. All currents were averages at ±100 mV.
to the non-electrophilic agonists, carvacrol and 2-APB, after desensitization by NMM or pBQN.

When repeating the experiments with short versus long exposure to NMM on the TRPA1-3C mutant, we noticed that in ~40% of our cells, NMM activated a small (<1 nA), but detectable current, predominantly at positive potentials. NMM-induced currents were seen in some cells with both short (Figure 1.8C) and long NMM exposure (Figure 1.8D), but this did not change the mutant channel's subsequent response to pBQN. Just as we show in Figure 1.8A and 1.8C, a short NMM exposure did not prevent activation by pBQN (n=4), whereas a long exposure did (n=4). In both cases, the channels responded robustly to 300 μM carvacrol. These experiments show that TRPA1-3C mutation does not completely abolish the ability of NMM to activate the channel. Regardless of whether NMM activated any current, the subsequent response to pBQN was the same: robust activation after a 10 second NMM exposure, but no activation after a 60 s or longer NMM exposure.

The TRPA1 cysteine located at position 421 is one of five cysteines that has been conserved throughout evolution and has maintained its sensitivity to exogenous electrophiles (Kang et al., 2010). Takahashi et al. (2008) found that TRPA1 is activated by an endogenous electrophilic prostaglandin, 15d-PGJ₂, which can covalently bind to cysteines 421 and 621 (C621 is labeled C619 by Hinman et al.). Takahashi and colleagues showed that a serine substitution of C421 caused a significant decrease in calcium flux in response to 15d-PGJ₂ but retained its sensitivity to 100 μM AITC. We wanted to test if the C421S TRPA1 mutant would respond to pBQN. First, we tested the mutant channel with 300 μM carvacrol. The response was robust and reversible as it is
Figure 1.9: pBQN does not require reactivity with cysteine 421 for activation or desensitization of TRPA1. **A)** 300 μM carvacrol reversibly activates the C421S TRPA1 mutant. A second response is evoked by 300 μM carvacrol. **B)** 1 μM pBQN activates and desensitizes the C421S mutant. After pBQN desensitization the mutant is unresponsive to 100 μM AITC and 300 μM carvacrol. All currents were averages at ±100 mV.
in wild-type channels. A second application of carvacrol after a 5 min wash also resulted in immediate activation, although some cells had a reduced maximum response compared to the first application (Figure 1.9A). In another cell we applied 1 μM pBQN and observed activation of both inward and outward currents. After 4 minutes, the channel became desensitized to pBQN. Once the currents remained at a steady state we applied 100 μM AITC followed by 300 μM carvacrol and observed no significant change in current. The mutant was no longer sensitive to AITC or carvacrol. Because these results are similar to what we observe with wild type TRPA1 channels we believe that C421 is not involved in the activation or desensitization process induced by pBQN.

In another study by Takahashi and colleagues (2011), the TRPA1 channel was found to be activated by hyperoxic conditions (solutions containing 86% oxygen; normoxic solutions contains 20% oxygen). The activation of TRPA1 by the hyperoxic solution was severely diminished when the cysteine at position 856 was replaced with serine. Even though the reactivity of an electrophile is not identical to that of oxygen (oxygen will remove an electron from the cysteine as opposed to sharing it), the diminished response after a serine substitution at position 856 indicates that this cysteine is important in TRPA1 gating. This is not surprising since the amino acid position at 856 is located in the transmembrane space between S4 and S5, near the putative pore-forming region of S6. In another study, an asparagine residue at position 855 has been implicated in TRPA1 channel gating (Kremeyer et al., 2010). A point mutation at this site, from asparagine to serine (N855S), is the cause of familial episodic pain syndrome. This mutation causes a 5-fold increase of inward current that ultimately results in sensations of pain that would not normally exist. Since C856 is the closest
**Figure 1.10:** pBQN does not require reactivity with cysteine 856 for activation or desensitization of TRPA1.  

**A)** 1 μM pBQN activates and desensitizes the TRPA1 C856S mutant.  

**B)** The C856S mutant is activated by 300 μM carvacrol. 1 μM pBQN activates and desensitizes the mutant channel. After desensitization, 100 μM AITC does not elicit any currents but 300 μM carvacrol does.  

**C)** 1 μM pBQN induces an inwardly rectifying current followed by desensitization.  

**D)** A representative current-voltage trace from the cell in C. All currents were averages at ±100 mV.
cysteine residue near the putative pore region (S6), we wanted to test if this cysteine was involved in the channel's sensitivity to pBQN. With site-directed mutagenesis, we replaced cysteine, at position 856, with serine. We applied 1 μM pBQN to the C856S mutant for five and a half minutes and observed a typical activation and desensitization response (Figure 1.10A). The current profile appeared to be similar to the wild-type TRPA1 current, with an immediate activation that was more outwardly rectifying followed by desensitization.

In the next cell, we wanted to test if the channels would respond to 300 μM carvacrol and 100 μM AITC after pBQN desensitization (Figure 1.10B). First we tested C856S in its response to carvacrol. This cell had a typical response to carvacrol, with immediate activation of both inward and outward current followed by an immediate return to the baseline current once carvacrol was removed. Next, we applied 1 μM pBQN until the channel desensitized. The cell was not responsive to AITC but still retained its sensitivity to carvacrol. This is similar to what we observe in the triple cysteine mutants, where carvacrol still produced a response after pBQN desensitization. When attempting to repeat this experiment on a different cell, we noticed that the rectification of the current was significantly more inward rectifying, to the point of saturation (Figure 1.10C). Although this result was unexpected, it was not surprising since Kremeyer and colleagues showed that enhanced inward currents were induced in N855S mutants. The current-voltage relationship of this cell is shown in Figure 1.10D. In this example the TRPA1 C856S mutant has an unusual decline in the outward current when reaching voltages higher than +60 mV and the reversal potential shifted slightly to the left. This could be due to a gating malfunction induced by the mutation.
**Figure 1.11:** The non-electrophile, carvacrol, desensitizes TRPA1. The wild-type TRPA1 is desensitized by 300 μM carvacrol when applied for 4 minutes. After a 3 minute wash with extracellular solution, the channels remained unresponsive to a 3.5 minute application of 1 μM pBQN. Currents were averages at ± 100 mV.
The larger inward current was more linear than usual but this was also a feature of the N855S mutant that was reported by the Kremeyer group. It seems reasonable to assume that the cysteine mutation in this region of the channel had some affect on the gating process because of its proximity to the putative pore region and its location next to N855. However, since we only have one example of this particular inward rectifying activity it is difficult to determine if this was an actual result of the mutation or if this is an artifact. If this was an actual consequence of the mutation perhaps the rectification change was the result of indirectly disrupting the activity of the neighboring asparagine residue.

Although we attempted to record from dozens of HEK cells transfected with C856S, we were unsuccessful in recording another cell that was expressing a functional mutant channel. We do not know if the channel was expressed but not transported to the membrane, or in the membrane but non-functional, or possibly transported to the membrane but later endocytosed. With the three cells that we were able to record from, we show results that are similar to what we find in the triple cysteine mutant. C856 is not a key residue involved in pBQN activation or desensitization to pBQN but it is involved in the channel's sensitivity to non-electrophiles.

Our focus in this study was on the electrophilic induced (and calcium independent) desensitization of TRPA1. But we cannot ignore the possibility of an electrophilic independent mechanism for desensitization. In one study by Xu et al. (2006), carvacrol was thought to contribute to skin sensitization by activating TRPA1. In this study they determined that 250 μM carvacrol quickly activated TRPA1, but when carvacrol was applied for longer than 30 seconds, the channel became desensitized.
Figure 1.12: A comparison of desensitization rates of TRPA1. Rates of desensitization from 1 μM pBQN are compared between wild-type TRPA1, TRPA1-3C mutant, C421S mutant, C856S mutant and wild-type TRPA1 desensitized by 50 μM NMM and wild-type TRPA1 desensitized by 300 μM carvacrol. The number of cells used to calculate the value of each bar are in parentheses above each bar. The inlayed values represent the average rate of desensitization. The p-value of 0.003, above the bar representing the carvacrol desensitized channels, indicates that 0.041 is significantly faster than the desensitization rates of the other conditions (error bars represent ± SEM). All averages were generated using currents measured at ± 100 mV.
Because physiological calcium concentrations were used both intracellularly and extracellularly it is not clear whether or not this desensitization was induced by carvacrol or if it was induced by calcium. We tested our wild-type TRPA1 channels for a carvacrol induced desensitization in the absence of added calcium. We find that TRPA1 can desensitize with a continuous application of 300 μM carvacrol with a time constant (τ) of 26 seconds (a rate of 1/τ = 0.041 s⁻¹) (Figure 1.11). Xu and colleagues showed that a second carvacrol application and subsequent AITC application was able to elicit activation, after carvacrol induced desensitization. In contrast, we were unable to elicit a response to a second application of 300 μM carvacrol or 1 μM pBQN after the carvacrol induced desensitization (n=4). In fact, the carvacrol induced desensitization was irreversible for the duration of our recording (~10 minutes). It may be possible that TRPA1 simply becomes desensitized to any agonist if exposed to it long enough or if it is applied at a high concentration.

Throughout this project we noticed that the desensitization rates of the WT, triple cysteine mutant, C856S or C421S mutant TRPA1 channels appeared to have a similar decay rate in response to 1 μM pBQN that was fitted to a single exponential function \( y = y_0 + A \exp\left(-\frac{(x-x_0)}{\tau}\right) \). We calculated the mean desensitization rate (1/τ) induced by 1 μM pBQN for each of the mutant channels and compared them to the desensitization rate of the WT channel (see Figure 1.12), individual rates are embedded within each column and number of cells are in parentheses above the column). There was no significant difference between any of the TRPA1 channels in response to 1 μM pBQN (p>0.05). This suggests that pBQN could be inducing the same mechanism of desensitization in the TRPA1 channel regardless of the serine substitutions. Is this
desensitization process specific to pBQN or is this specific to any electrophile? If
TRPA1 has a mechanism for electrophile induced desensitization then the rate of NMM
induced desensitization should fall within the same range as pBQN. However, it might
not be fair to compare 1 μM pBQN to 50 μM NMM since we do not know what
concentration of NMM will induce maximum TRPA1 current. Regardless, we still wanted
to get an idea of the kinetics of the TRPA1 response to NMM. Our results show that 50
μM NMM induces a rate of desensitization (1/τ = 0.011, p ≥ 0.05) that is not statistically
different from the group of pBQN treated TRPA1 channels, but it is slower than the WT
channel (p ≤ 0.05) (Figure 1.12). Why is the rate of TRPA1 desensitization faster with
300 μM carvacrol than with NMM or pBQN (p ≤ 0.05)? Without a TRPA1 concentration
response curve to carvacrol, we are not certain if the rate is faster because 300 μM is a
more potent agonist than 1 μM pBQN or 50 μM NMM and has a faster desensitization
rate, or if this is a non-electrophilic induced mechanism that happens to be faster than
desensitization induced by electrophiles. There are very few studies that have examined
TRPA1 desensitization in response to non-electrophilic compounds and it is not certain
how carvacrol interacts with TRPA1. It may be possible that TRPA1 has more than one
mechanism of desensitization since more than one mechanism of activation exists.

Discussion

We show that para-benzoquinone (pBQN), a naturally-occurring compound in the
defensive spray of many arthropods, activates the human TRPA1 channel at low
nanomolar concentrations. pBQN activated TRPA1 quickly, with a delay of tens of
seconds at 3-30 nM pBQN and <1 second with 1 μM pBQN. At higher concentrations,
the pBQN-evoked current reached a peak followed by desensitization, which was rapid and complete at concentrations of 1 µM and above. At these concentrations desensitization overlaps with activation, and the maximal evoked current begins to decline.

pBQN is an electrophile, and known to modify cysteines (Mason and Leibler., 2000; Lame et al., 2003). It is similar to other electrophilic agonists including AITC and NMM, which activate TRPA1 by covalently modifying free thiol groups in cysteine side chains (Dennehy et al., 2006; Cebi and Koert, 2007). The human TRPA1 channel contains 28 cysteines residues. Mutating just three of the cysteines (C619S, C639S, C663S) prevents most activation by NMM and dramatically reduces sensitivity to other electrophilic compounds (Hinman et al., 2006; Macpherson et al., 2007b; Trevisani et al., 2007). To our surprise, pBQN was still able to robustly activate the triple mutant, with the same rapid kinetics as the wild-type channel. The identical responses of wild-type and TRPA1-3C channels might reflect the ability of pBQN to modify the lysine at position 708, which underlies the residual AITC activation of the 3C mutant. Our extensive attempts to express and record TRPA1 channels with all three cysteines plus the lysine at 708 mutated to glutamine, failed. Alternatively, we found that in ~40% of the TRPA1-3C expressing cells, NMM still produced a slight but measurable amount of activation (Figure 8D). Because NMM largely labels only cysteine side chains, this suggests a mode of TRPA1 activation not completely eliminated by the triple cysteine mutations.

Although pBQN activation did not depend on cysteines 619, 639 or 663, our experiments suggested that pBQN activation does depend on its reactive, electrophilic
nature. First, pre-treating pBQN with free excess L-cysteine before applying it to the cell prevented its action. Second, pre-exposure to NMM, modifying all accessible cysteines, prevented any subsequent activation by pBQN. Third, pBQN action was prevented when cells contained an intracellular solution with 20 mM cysteine, as the excess cysteine competes with TRPA1 cysteine residues for pBQN bonding. Fourth, the membrane-impermeant cysteine reagent MTSET, applied inside the cell, activated TRPA1 and prevented any additional activation by pBQN. Taken together, these results suggest that pBQN must react with TRPA1 amino acid residues that are accessible from the cytosolic surface, but not limited to the three cysteine residues whose loss severely diminishes the action of NMM or AITC. It did not appear that pBQN action on other cellular components was an issue since non-TRPA1 expressing cells (control cells) had no response to pBQN.

Many TRPA1 agonists have a bimodal effect on the channel, with activation at low concentrations and reversible inhibition at higher concentrations (e.g., menthol, citral, nicotine; Karashima et al., 2007; Stotz et al., 2008; Talavera et al., 2009). The desensitization of TRPA1 channels by pBQN, which caused the reduction in peak currents at micromolar concentrations, is quite different. The effect was long lasting, with no reversal over >30 min. Desensitization by pBQN prevented subsequent activation by other electrophilic agonists, but also by non-electrophiles such as carvacrol and 2-APB. In our study we eliminated the possibility of a calcium-dependent desensitization by omitting calcium from our recording solutions.

Our results suggest that pBQN produces desensitization by also reacting with cysteine residues and that the critical cysteine residues for desensitization are distinct
from those involved in activation. In cells where a short exposure to NMM was used to activate TRPA1 currents, pretreated pBQN (with free L-cysteine) was unable to activate or desensitize the channel (Figure 1.5). This suggests that pBQN cysteine reactivity is critical for both activation and desensitization of TRPA1. However, while pBQN activation involves cysteine residues accessible from the channel’s cytosolic surface, desensitization by pBQN instead involves cysteines that are likely to reside in regions inaccessible to aqueous solvent, perhaps the transmembrane regions or other hydrophobic domains. First, 20 mM L-cysteine in the pipette was able to prevent activation of TRPA1 by extracellular pBQN, but did not prevent desensitization (Figure 1.6B). Second, intracellular application of MTSET activated TRPA1 currents, presumably by binding to cytosolically accessible cysteines. When pBQN was applied, the channel immediately underwent desensitization without additional activation (Figure 1.6C). Thus, TRPA1 channels can be activated by cysteine modification without being desensitized, and can desensitize without being activated.

The inability of iodoacetamide (100 or 400 µM) to cause desensitization is interesting in light of the mass spectrometry analysis that Macpherson and colleagues (2007a) performed after labeling live cells with iodoacetamide. Upon treating cells over-expressing TRPA1, with 100 or 400 µM iodoacetamide, they found 14 cysteine residues labeled on the amino terminus of TRPA1, with some residues labeled by the higher concentration (the remaining 17 cysteines were not detected in any of the TRPA1 peptide fragments by mass spectrometry). This suggests that at least one of the remaining, unlabeled cysteines is responsible for desensitization.
Wang and colleagues (2011) recently examined the disulfide binding of cysteines within TRPA1, as well as the ability of two NMM concentrations to modify cysteines, using mass spectrometry. Their results suggest that TRPA1 may have four different disulfide bonds within each subunit: C663-C619, C663-C460, C663-C190 and C619-C606 (numbering based on human TRPA1). Takahashi and colleagues (2011) also suggest that C663 can form a disulfide bond with C856. It is noteworthy that the triple mutant replaces cysteines at positions 663 and 619 with serine, which could have eliminated the ability of the channel to form disulfide bonds and affected its stability and/or function (such a change might contribute to the greater outward rectification in the triple mutant). In addition to acquiring new traits, cysteine substitution mutations at sites that might involve disulfide bonding may have caused the channel to redistribute the location of many of the remaining reactive cysteines. Therefore, it is difficult to know whether an electrophilic agonist, like NMM or pBQN, fails to activate TRPA1 with mutated cysteines because its target cysteine residues are removed or because the removal of cysteine changes the channel conformation such that the target residues become inaccessible to electrophiles.

Wang and colleagues also showed that 30 of the 31 mouse TRPA1 cysteines were modified by NMM, yet some had greater labeling at higher NMM concentrations (e.g., C214, C259, C609, C705, C729). Modification of those cysteines might simply have been slow, requiring long incubations to allow modifications. Alternatively, the conformational state of TRPA1 might be changing as NMM continually modifies the channel, exposing previously inaccessible cysteines. If higher NMM concentrations are generating channels in increasing desensitized states, the greater labeling of these
residues might reflect conformational changes related to cysteine-dependent desensitization.

One surprising observation is that the cross-desensitization between electrophilic and non-electrophilic compounds is absent in the TRPA1-3C mutant. Why does the removal of cysteine residues, which are involved in channel activation by electrophiles, allow carvacrol and 2-APB to open the channel after long pBQN exposures? Wang and colleagues found that in wild-type TRPA1, C619 showed less labeling by long applications of high [NMM]. This might reflect developing desensitization. If this region is undergoing conformational changes during desensitization, cysteine mutations anywhere throughout the protein might affect the process.
Chapter 2

TRPV1 is activated by para-benzoquinone

Many studies have shown that TRPV1 is a molecular target for numerous natural plant compounds such as allicin, capsaicin and resiniferatoxin (Salazar et al., 2008; Macpherson et al., 2005; Caterina et al., 1997; Seabrook et al., 2002). Many of these compounds are known to cause sensations of pain, discomfort or pungency when eaten or when they come into contact with the skin. Although TRPV1 is known to be involved in pain signaling, little is known about its involvement as a molecular target of insect-derived compounds. Two groups have identified compounds produced and secreted in spider venom that activate TRPV1 channels. One group showed that the peptide, vanilla toxin 3 (VaTx3), from a tarantula native to the West Indies, targets TRPV1 on the extracellular surface (Siemens et al., 2006). Bohlen and colleagues (2010) discovered that the Earth Tiger tarantula produces the “double-knot” toxin (DkTx) that also targets the extracellular side of TRPV1, locking the channel in an open state. Both spider toxins contain multiple cysteine residues that form disulfide bonds, most likely to keep the peptides in a stable conformational state.

Cysteine residues are clearly important to the stability and function of proteins. TRPV1 contains 18 cysteine residues, three of which are located in the putative pore region between transmembrane 5 and 6 at positions 616, 621 and 634 (Vyklicky et al., 2002). Recently, one group found that the plant-derived TRPV1 agonist, camphor, relies on the presence of a threonine residue at position 633 because its absence significantly reduced TRPV1 activation (Marsakova et al., 2012). Were currents reduced because
threonine was targeted by camphor or did the removal of a residue next to C634 affect the stability of the pore region and resulted in less sensitivity to camphor? Although TRPV1 is a known exogenous chemical detector, not much is known about how its cysteine residues are involved in electrophilic detection. Most studies on TRP channel activation with electrophilic compounds are centered on TRPA1. It is possible that TRPV1 could have similar mechanisms of activation to TRPA1, especially since both channels respond to the same agonists, 4-oxononenal (an endogenous electrophile), and citral (an exogenous plant compound) (Taylor-Clark et al., 2008; Stotz et al., 2008).

In this study, we wanted to test if TRPV1 responded to the electrophilic insect defensive compound, para-benzoquinone (pBQN) in the same cysteine-dependent manner that we observe in TRPA1.

Results/Discussion

The human isoform of TRPV1 is activated with 10 nM pBQN (Figure 2.1A). In this example, the TRPV1 currents began to activate after a 50 second lag once pBQN was applied. Activation with 10 nM pBQN gradually increased until a plateau was reached in 100 seconds. When the pBQN solution was removed, the currents declined, but did not return to the original baseline that was recorded prior to the addition of the pBQN solution. This could indicate that some channels were somehow bound to pBQN and still remained activated or perhaps there are two modes of activation, with one being easily reversible. This is unlike the behavior that is observed with 10 nM pBQN on TRPA1 (Figure 2.1D). In HEK cells expressing TRPA1, the currents took much longer to reach steady state and when pBQN was washed off, the currents remained elevated.
Figure 2.1: TRPV1 is activated by pBQN. A) TRPV1 activates in response to 10 nM pBQN. The currents are reduced when pBQN is removed. B) The current-voltage relationship of the pBQN activated TRPV1 shows an outward rectification. TRPV1 currents were averaged at ±100 mV. C) 1 μM pBQN activates TRPV1 and activated currents do not decline during washout. The channel is able to respond to a second application of 1 μM capsaicin. D) An example of TRPA1 activation by 10 nM pBQN, showing the persistent elevated currents after removal of pBQN.
TRPV1 current is typically more outward rectifying and is increasingly activated with increasing voltage (Caterina et al., 1997; Matta and Ahern, 2007). **Figure 2.1B** shows the current-voltage relationship of 10 nM pBQN. The reversal potential is at -5 mV for both the baseline current and the activated current in response to pBQN. In this example, the application of 10 nM pBQN produced more outward current than inward. With a higher concentration of pBQN (1 μM) the TRPV1 activated currents remain elevated during washout and slightly more inward current is activated, although most cells did not show significant increases in inward current at higher concentrations. Unlike TRPA1 activation, TRPV1 did not become desensitized with a prolonged exposure to 1 μM pBQN. The channels were able to respond to 1 μM capsaicin even though the channel did not recover from pBQN activation. This suggests that pBQN and capsaicin do not share binding sites.

TRPV1 contains cysteine residues that are conserved in several species (Salazar et al., 2008; Boukalova et al., 2010). One group found that TRPV1 becomes sensitized when exposed to cysteine reactive compounds that convert neighboring cysteines into disulfides (Chuang and Lin, 2009). The activation of TRPV1 by allicin, an electrophilic compound produced by garlic and onion, was dependent on one intracellular cysteine located at position 157 (Salazar et al., 2008). In our experiments with TRPA1, pBQN seemed to be activating the channel by reacting with intracellular cysteines. We first determined this by pretreating pBQN with free L-cysteine before applying it to our cells. pBQN with L-cysteine did not activate the TRPA1 channel. This was either because pBQN reactivity was removed by binding to L-cysteine and/or because pBQN bound to cysteine was not able to pass through the membrane. We
Figure 2.2: TRPV1 activation by pBQN could involve an extracellular and intracellular reaction. A) A solution containing a preincubated mixture of 3 μM pBQN and 15 μM L-cysteine reversibly activated outward rectifying TRPV1 currents. Exposure to 3 μM pBQN alone irreversibly activated TRPV1 currents. B) The current-voltage relationship of TRPV1 currents activated by 1 μM capsaicin (red), 3 μM pBQN + 15 μM L-cysteine (blue), 3 μM pBQN alone (green) and the baseline current (black). All currents were averaged at ± 100 mV.
wanted to repeat this experiment with TRPV1 to test if pBQN activates TRPV1 through reactivity with intracellular cysteines. First we applied 1 μM capsaicin for 5 seconds to make sure functioning channels were present (Figure 2.2). Capsaicin induced a slightly outward rectifying current and the currents were reduced when capsaicin was washed off, which is a typical response to capsaicin (Caterina et al., 1997; Seabrook et al., 2002). A 15 μM L-cysteine vehicle control solution was added and we observed no response. Next, a combination of 3 μM pBQN and 15 μM L-cysteine was applied to the cell and the solution induced an outward rectifying current that was completely reversed upon removal of the solution. This was unexpected since we assumed pBQN and L-cysteine would not elicit any activation, as we show with TRPA1 (see Figure 1.5). If activation of TRPV1 by the pBQN + L-cysteine solution occurred because of an incomplete reaction between pBQN and L-cysteine molecules, this would result in free pBQN passing through the membrane and covalently binding to intracellular cysteines, resulting in an irreversible activation. But this is not the case, since removing the solution resulted in currents declining back to the baseline during washout. This then suggests that the activation was due to a non-covalent process, perhaps an extracellular reaction. However, if pBQN does not pass through the bilayer because it is attached to L-cysteine, then this suggests the pBQN + L-cysteine somehow retains some reactivity, but we are not certain how or with which amino acids. TRPV1 contains at least three cysteines (C616, C621 and C634) that may be accessible from the extracellular side (Vyklicky et al., 2002). Vyklicky and colleagues showed that when C616, C621 and C634 were in a reduced state (available to act as nucleophiles) it increased the amount of inward and outward current induced by heat and capsaicin.
Assuming that these extracellular cysteines are constantly fluctuating between an oxidized and reduced state on each subunit, it could be possible that pBQN+L-cysteine may have been able to activate TRPV1 through a transient bond to these cysteines or perhaps the combined product was able to elicit currents through an unknown non-covalent mechanism. If these cysteines are more stable in an oxidized state through disulfide bonds with neighboring cysteines then this could explain the reversibility of the activation.

The size of the TRPV1 currents increased with increasing concentrations of pBQN until maximum current was reached, resulting in a typical sigmoid concentration response curve (Figure 2.3). This could suggest a cooperative pBQN binding mechanism, but further tests will be required to verify this. We applied pBQN to the cell, starting at 3 nM, and recorded an average current of 5 ± 2pA/pF (n=3). The channels began to open approximately 30 seconds after the cells were exposed to 3 nM pBQN. The currents reached a steady state by 60 seconds and once the wash solution was applied, the currents decreased but did not return to the baseline. Currents reached half the maximum current, 216 ± 54 pA/pF, with 1 μM pBQN (EC_{50} = 1 μM) (n=4). Maximum current amplitudes were reached with 3 μM pBQN, 391 ± 86 pA/pF (n=5). At pBQN concentrations in the micromolar range, TRPV1 currents were activated immediately until a steady state was reached. We did not observe a peak of activation induced by pBQN in cells expressing TRPV1 nor did we observe any decline or desensitization in response to pBQN as cells were still responsive to capsaicin after exposure to pBQN.

There were several differences between TRPA1 and TRPV1 in response to pBQN. Although both showed a concentration-dependent activation by pBQN, TRPV1
Figure 2.3: TRPV1 concentration response to pBQN. TRPV1 currents (measured at +100 mV) elicited by extracellular application of pBQN at concentrations from 30 nM to 100 μM. The numbers of cells used to generate the averaged current densities are in parentheses above each point. Bars represent ± SEM.
does not become desensitized after prolonged exposure to pBQN and is still able to respond to capsaicin. TRPV1 activation was partially reversible at lower concentrations during washout, but most of the TRPA1 currents remained elevated throughout the remainder of the recordings. pBQN is a more potent agonist of TRPA1: the EC$_{50}$ of pBQN with TRPV1 is 1 μM and with TRPA1 it is 100 nM. Interestingly, the ability of TRPV1 to activate reversibly in response to the pBQN + cysteine solution suggests that TRPV1 contains an extracellular site of activation. However, it is not certain how the pBQN+ cysteine does this. pBQN alone may activate TRPV1 through both covalent and non-covalent action on its cysteine or other residues but until further experiments on cysteine mutant TRPV1 can be completed, we do not know if this is an interaction with extracellularly accessible cysteines or intracellular cysteines.
Chapter 3

Plant hallucinogen, salvinorin A, activates TRPA1 and TRPV1

Many cultures around the world have been using the essential oils of plants as medicine for centuries. Many of the herbal remedies are used to alleviate symptoms of pain and inflammation such as peppermint for irritable bowels, clove oil for toothaches, ginger for headaches and thyme for bronchitis. Besides the pain relieving properties of plant products and their obvious use as food flavoring, many cultures use herbs for ritualistic purposes such as energy cleansing (sage and marijuana) or spiritual ceremonies to communicate with the gods (peyote and ayahuasca). Over the last two decades, research has revealed that many of the terpenoid compounds produced by medicinal plants are agonists of ion channels and some terpenoids have more than one ion channel target (Vriens et al., 2008; Pertwee et al., 2010). This can explain why some plants can be used for more than one purpose, such as marijuana. The chemical components of marijuana, including the well known diterpene, \( \Delta^2 \)-tetrahydrocannabinol (THC), was extracted and purified from the plant in the late 1960s (Turk et al., 1969). Its effects on human attention, motor skills, hear rate, and working memory were determined to have dose-dependent detrimental effects (Schaefer et al., 1977; Bocker et al., 2010). In the 1990s, one of the ion channel targets of THC was found to be the newly cloned cannabinoid receptor type 1, CB1 (Gerard et al., 1991). CB1 is expressed in various parts of the brain, spinal cord and various organs including the lungs. Another target of THC is the cannabinoid type 2 receptor, CB2, which is expressed in cells of the immune system, tissues in the gastrointestinal tract, peripheral neurons, and in the brain.
In 2004, Jordt and colleagues found that THC also targets the TRPA1 channel and induced nociceptor excitation. Another diterpene compound isolated from the marijuana plant, cannabidiol, was shown to activate and desensitize the TRPV1 channel (Bisogno et al., 2001). Activation and desensitization of TRPA1 and TRPV1 through inhalation of the marijuana plant may explain why it is often used to alleviate symptoms of pain and inflammation.

Another plant that has very potent psychoactive effects is *Salvia divinorum*, also known as “ska Maria Pastora” or “Mexican mint”. This plant is indigenous to a region of Mexico currently known as Oaxaca. Indigenous tribes of Oaxaca (Mazatecs) chewed the leaves or crushed the leaves and ingested it as a tea to cure various ailments, such as stomach inflammation, headache, pain from rheumatism and also to stop diarrhea (Valdes et al., 1983). Another use of the plant (still used today) by the “curandero” or shaman of the tribe is to smoke the dried leaves to induce hallucinations or visions that enable the curandero to speak with the gods (similar to peyote and ayahuasca). The active ingredient known to cause the hallucinogenic effects is the diterpene, salvinorin A (SA). It is reported to be the most potent naturally occurring hallucinogen so far (D.Seibert, 1994). The first molecular target of SA was discovered to be the kappa-opioid receptor (KOR) (Roth et al., 2002, Chavkin et al., 2004), which is known to have a function in pain, mood, consciousness and addiction. Salvinorin A was shown to also activate the CB1 receptor of zebrafish and mice (Braida et al. 2007; Fichna et al., 2009). Many other studies show that SA also has an antinociceptive effect through KOR activation, but when the KORs were blocked *in vivo*, in mice and primates, the animals still showed antinociceptive behavior. It was thought that SA may have been affecting
Figure 3.1: Chemical structures of the plant diterpenes, salvinorin A (left), the TRPA1 agonist, Δ-tetrahydrocannabinol (center), and the TRPV1 agonist, cannabidiol (right). Images were copied from es.wikipeida.org.
downstream pathways of KOR activation or SA may have had an unknown interaction with vehicle solutions, DMSO and saline (Butelman et al., 2004; Ansonoff et al., 2006; McCurdy et al., 2006). The ability of SA to alleviate some symptoms of pain and inflammation that is independent of KOR signaling could indicate that SA has additional molecular targets other than KOR and CB1. In this study, we show that SA is also an agonist of both TRPA1 and TRPV1.

Results/Discussion

We added 10 pM salvinorin A (SA) to HEK cells heterologously expressing human TRPA1 and recorded small activating currents. In Figure 3.2A we show that a 60 second application of 10 pM SA activated both inward and outward currents. The currents remained activated until SA was removed and did not cause desensitization. Although the activation appeared to be reversible, both inward and outward currents did not decrease to the level of the baseline current before the SA solution was applied. In this example the outward and inward currents continued to increase gradually. We applied 50 μM of the TRPA1 selective blocker, HC-030031, to test if the increase in current was due to activated channels or ‘leak’ current. The HC-030031 blocked the outward currents but not the inward component. When HC-030031 was removed, the outward currents continued to gradually increase. A second application of 10 pM SA appeared to elicit additional currents that were equally robust as the first application, inducing activation of at least 1 nA of current in both directions.

Interestingly, many recreational users of SA report more intense psychoactive effects after a second dose of SA when taken minutes after the first dose (information
**Figure 3.2**: Salvinorin A activates TRPA1. **A)** TRPA1 currents remained activated for the duration of a 60 second application of 10 pM salvinorin A. During washout, the currents were reduced but did not return to the original baseline current and slowly began to increase during washout. A 10 second application of the TRPA1 blocker, HC-030031, was able to block the salvinorin A activated currents. A second application of 10 pM salvinorin A activated additional currents and when washed off, currents declined. Dashed line indicates zero current level. **B)** A representative current-voltage relationship trace of TRPA1 in response to salvinorin A (green) and the baseline current (black). The reversal was near -10 mV. All currents were averaged at ± 60 mV.
from various *Salvia divinorum* website forums). When the second application of SA was removed, the current did not return to the same steady state level that it reached prior to the addition of SA. This may be due to a slow reversible interaction between the channel and SA or perhaps this was due to accumulation of SA within hydrophobic regions of the channel. **Figure 3.2B** shows the current-voltage relationship of the TRPA1 channel at the initial baseline (black) and during the first and second SA application (green). The activated currents appear to have the typical, slightly outward rectifying shape with the reversal near -10 mV (*Story et al.*, 2003).

Raisinghani and colleagues (2011) showed that the TRPA1 agonists, AITC and NMM, cause desensitization when applied for long periods of time. We tested whether a continuous exposure to SA would also induce desensitization of TRPA1. In **Figure 3.3A** we show that a 6 minute application of 50 μM SA did not induce desensitization but the activated currents remained at a steady state level until 500 μM AITC was added. Once AITC was added there was an additional increase in current amplitude followed by desensitization. The desensitized currents decayed to the same level that was induced by SA. The channels that were previously activated by SA remained activated despite the presence of AITC, suggesting that SA does not completely interfere with ATIC binding. Why are currents still activated after AITC desensitization? Based on these preliminary experiments alone, it is not certain if the SA activated currents would have eventually declined, but so far it appears SA, at 50 μM, has a slow off-rate. The inability of 50 μM SA to desensitize TRPA1 was not likely due to 50 μM being too low of a dose because the concentration response curve (see **Figure 3.4** ) indicates that the current density induced by 50 μM SA fell within the same range as 10 pM SA.
Figure 3.3: Salvinorin does not desensitize TRPA1. A) A prolonged application of 50 μM salvinorin A activated TRPA1 currents. TRPA1 currents remained at a steady state level until 500 μM AITC was applied. AITC induced additional activation of TRPA1 followed by desensitization. B) A representative current-voltage trace shows that TRPA1 currents induced by 50 μM salvinorin A still retained the characteristic TRPA1 profile with a reversal near -10 mV.
The interaction of SA with the ion channel is not likely to involve a covalent action that results in permanent or major conformational shifting. This is because the AITC response was still present after the long exposure to SA, suggesting that SA, even if still bound to the channel, did not physically block AITC from its binding site or that SA did not significantly affect the channel morphology, allowing AITC access to its target region. However, as we show in Figure 3.2A, the SA activated currents were reduced when the cell was washed with extracellular solution, but the currents did not completely return to the baseline. One possible explanation for this is that some SA molecules may have remained bound to unopened channels and only with time, this interaction caused a delayed activation. Another interesting observation was that HC-030031 only blocked the outward SA activated currents (Figure3.2). Typically, this antagonist readily blocks both inward and outward currents, as we show in Figures 1.1C, 1.5A, 1.6C. Could SA be interfering with the channel’s gating mechanism? Again this could be a result of a delayed and slow reversible interaction. Figure 3.3B shows the current voltage relationship of the channel at the initial baseline (black), in response to 50 μM SA (green) and in response to 500 μM AITC.

The concentration response curve is shown in Figure 3.4. We tested SA at concentrations ranging from 1 fM up to 50 μM. In this study, the maximum current density was recorded with 1 nM SA and reached 144 ± 36 pA/pF (n=6). At 1 fM, the average current density in our HEK cells is 11 ± 4 pA/pF (n=3) with an EC$_{50}$ near 5 pM. The potency of SA as a kappa-opioid receptor agonist has been reported to be extremely high, with activation beginning in the low pM range and an EC$_{50}$ near 1 nM, in vitro (Roth et al., 2002; Chavkin et al., 2003; Aviello et al., 2011). There has not yet
Figure 3.4: TRPA1 concentration response to salvinorin A. TRPA1 currents (measured at + 60 mV) elicited by extracellular application of salvinorin A at concentrations from 0.001pM to 50 μM. The numbers of cells used to generate the averaged current densities are in parentheses above each point. Bars represent ±SEM. Hash marks on the horizontal axis denote where concentrations are no longer at a log scale.
been a report of an exogenous TRPA1 agonist that has the potency of SA. Although the concentration response curve is incomplete, the data at this point indicates that SA has a very low effective concentration, beginning in the low pM (1fM) range.

Salvinorin A (SA) was also tested on HEK cells expressing TRPV1. We found that 1 μM SA was able to activate TRPV1 immediately upon application (n=3, Figure 3.5). The currents continued to rise for approximately 130 seconds until washout. When the SA solution was removed, the currents quickly began to decline to a new steady state. A second application of 1 μM SA activated TRPV1 currents at a slow steady rate, but after 160 seconds of exposure, there was a sudden increase of approximately 2 nA. The cell was exposed to the SA solution until it reached a steady state. Once the SA was removed, the currents slowly began to decline. Unlike the activity that was observed with TRPA1, the TRPV1 currents appeared to recover faster in the absence of SA and the activated currents were outwardly rectifying. The current-voltage trace reveals that both applications of SA results in a typical TRPV1 response (Figure 3.5B). The current was largely outward rectifying and the reversal potential remained at -5 mV for the 1st and 2nd SA applications (green), including the vehicle control trace (black).

We applied various concentrations of salvinorin A to our cells to generate a dose response curve. We discovered that 1 fM SA was still able to elicit an activation response in our cells (Figure 3.6). In this example, 1 fM SA was applied to the cell for three minutes. Currents began to rise slowly once the cell was exposed to SA, which was similar to what was observed with 1 μM SA. The current continued to rise until it reached a plateau in 180 seconds. In two other cells, the plateau was reached in 80 or140 seconds after the start of application of 1 fM SA. Once the plateau was reached,
**Figure 3.5:** Salvinorin A activates TRPV1. **A)** 1 μM SA induces a reversible outward rectifying TRPV1 current. A longer, second application of SA induces more TRPV1 activation. **B)** A representative current-voltage trace of SA induced TRPV1 currents. The first and second response to 1 μM SA is shown in green and the baseline current in black. Currents were averaged at ± 100 mV.
the SA solution was removed and cells were washed with extracellular solution. The currents declined to a steady state but did not return to the original baseline level. A second application of 1 fM SA was applied and just as we observed in the first exposure, there was no lag time for onset of current activation. The currents reached a plateau at nearly the same level as the first application, near 2.7 nA. When washed with vehicle solution, again the currents began to decline. Unlike the TRPA1 activation by SA, a second application of SA on TRPV1 did not generally result in additional current activation. Also, TRPV1 currents declined more rapidly during washout than TRPA1.

The current – voltage trace of TRPV1 in response to two application of 1 fM SA shows that SA induced outward rectifying currents and does not shift the reversal potential in any direction, but remains steady near -12 mV with both SA applications, including the baseline current with vehicle solution.

The response of TRPV1 to increasing concentrations of SA is plotted in Figure 3.7. A range of SA concentrations were tested on HEK cells expressing TRPV1, starting at 1 fM to 1mM. With 1 fM SA, the average current density was 64 ± 34 pA/pF (n=3). Preliminary tests show that a maximal current was induced by 1 μM SA, 189 ± 30 pA/pF (n=3). However, it may be possible that the maximum current could have been induced by a concentration of SA that is lower than 1 μM. At 1 mM SA, the TRPV1 current density fell within the range of current densities induced by 1 μM SA. Therefore, it is reasonable to assume that the EC\(_{50}\) of SA on TRPV1 is slightly less than 0.1 pM, which makes SA a more potent agonist of TRPV1 than TRPA1 (EC\(_{50}\) ~ 5 pM).

Although we do not understand the mechanism of activation elicited SA on either TRP channel, we do demonstrate that SA is able to activate both channels at extremely
Figure 3.6: TRPV1 is highly sensitive to salvinorin A. A, B) A low concentration of salvinorin A can reversibly activate outward rectifying TRPV1 currents. Currents were averaged at ± 100 mV.
Figure 3.7: TRPV1 concentration response to salvinorin A. TRPV1 currents (measured at + 100 mV) elicited by extracellular application of salvinorin A at concentrations from 0.001 pM to 1 mM. The numbers of cells used to generate the averaged current densities are in parentheses above each point. Bars represent ±SEM. Hash marks on the horizontal axis denote where concentrations are no longer at a log scale.
low concentrations. It is possible that SA may be eliciting its effects not as a reactive chemical agonist but by accumulation within the membrane or at hydrophobic regions within the channels, similar to what is reported with THC. Regardless of the mechanism of activation, we have evidence to support a KOR and CB receptor independent mechanism that could contribute to the anti-inflammatory and antinociceptive effects of SA.
Conclusion

Spraying of pBQN by the bombardier beetle repels many potential predators of distinct phyla including frogs, birds, rodents, and other insects. TRPA1 is a sensor of numerous noxious chemicals and has conserved its electrophilic sensitivity throughout vertebrate evolution (Kang et al., 2010). A wide range of insects produces multiple forms of benzoquinone and other electrophilic compounds that are strongly aversive to their natural predators. Our results show that the human TRPA1 channel is potently activated and desensitized by pBQN. However, it is not likely that human contact with insect pBQN results in serious health effects. One case study reports that humans exposed to insect secretions of benzoquinone causes mild pain, localized skin surface irritation (De Capitani et al., 2011) and also has a deterrent effect if inhaled or even tasted by humans (T. Eisner, 2005). Based on results presented here, these symptoms may be the result of TRPV1 and TRPA1 activation in sensory neurons.

In contrast to exposure to insect pBQN, there are serious health effects when pBQN is produced from benzene (Westphal et al., 2009). Westphal and colleagues showed that pBQN, an intermediate of benzene oxidation, is toxic to human cultured cells. Benzene is produced from petroleum, which is formed naturally over millions of years from fossilized marine plants and animals. Interestingly, benzoquinones are also produced by many marine organisms (M. Gordaliza, 2010). In humans, exposure to sources of benzene, such as cigarette smoke and industrial pollutants, is quite common. The Center for Disease Control reports that the severity of the benzene induced health effects, such as cancer or neurological disorders, is dependent on the dose of benzene exposure as well as duration of exposure over time.
TRPA1 and TRPV1 ion channels are expressed in non-myelinated nociceptors that innervate tissues in the airway. Many inhaled natural products have been proven to induce airway inflammation by activating and sensitizing TRPV1 and TRPA1 (Bessac and Jordt, 2008). In this report, I show that salvinorin A potently activates both TRPA1 and TRPV1 in a dose-dependent manner. It is still unclear whether or not the interaction of the salvinorin A molecule to the TRP channels involves any covalent interaction or perhaps finds a hydrophobic pocket. Follow-up experiments should test whether or not prolonged exposure causes desensitization. In some studies salvinorin A is reported to alleviate symptoms of inflammation and pain behavior in rodents, non-human primates as well as humans, and anti-nociception seems to be independent of opioid and cannabinoid receptor activation (Valdes et al., 1983; McCurdy et al., 2006; Fichna et al., 2009; Butelman et al., 2010). It may be possible that the unexplained reduction in pain behavior observed in some animal studies is due to salvinorin A agonist activity on TRP channels.

I find it interesting that TRPV1 is not desensitized by pBQN in this study. pBQN also activates regardless of the removal of pBQN cysteine reactivity. Unlike TRPA1, the activation of TRPV1 is reversed when pBQN is washed off. This could indicate that the site of pBQN agonist activity could be located on or near the channel’s extracellular side, as was determined for the spider toxins. pBQN acts as a partial agonist of TRPV1 since additional current was recorded when capsaicin was added after pBQN. This also suggests that pBQN and capsaicin do not compete for the same binding site. Insect pBQN does not generally cause harsh, acute pain, only very mild pain and local skin inflammation, which could be attributed to TRPA1 activation. What would be the
evolutionary benefit of pBQN activation of the human TRPV1 isoform? Perhaps it is not activation of TRPV1 alone that would induce a behavioral response. It is possible that multiple chemosensors must work together to transmit a pain inducing signal.

How agonists of TRPA1 and TRPV1 are interpreted as anti-inflammatory or pro-inflammatory at the cellular level is unknown. Is the message translated immediately during receptor-agonist binding or is it downstream? TRPA1 is the target of numerous naturally produced electrophiles and other unrelated exogenous and endogenous chemical compounds that contribute to sensations of pain and inflammation (Bautista et al., 2006; Macpherson et al., 2007; Trevisani et al., 2007, Bessac and Jordt, 2008, Andersson et al., 2008; Takahashi et al., 2011; Cordero-Morales et al., 2011). TRPA1 desensitization has been recorded by many groups over the past several years (Xu et al., 2006; Raisinghani et al., 2011; Wang et al., 2011). However, an electrophile-induced mechanism for desensitization has not been studied thoroughly. Although I propose such a mechanism in this study, it does not reveal if desensitization will occur in vivo. TRP channels might not behave exactly the same way as we observe them in an in vitro environment. The desensitization rates will certainly change since room temperature recordings will slow down protein kinetics. Any protein containing multiple ankyrin repeats is certainly capable of multiple functions, depending on where it is expressed. Will human TRPA1 still desensitize when expressed in a neuron or intestinal tissue? If it does, is it a protective effect or the initiation of an inflammatory response?

Many questions remain unanswered. The significance of this study was to reveal two novel and potent, naturally occurring agonists of the human TRPA1 and human TRPV1 ion channels. Both agonists are used for survival of the bombardier beetle and
Salvia divinorum plant, but when humans are exposed to these defensive compounds they have opposing experiences. The observations reported here can serve as a guide for future experiments that test pBQN and SA on TRPA1 and TRPV1 in their native environments, such as peripheral nerve cells.
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