Benzoquinone Reveals a Cysteine-Dependent Desensitization Mechanism of TRPA1

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ABSTRACT

The transient receptor potential ankyrin 1 (TRPA1) nonselective cation channel has a conserved function as a noxious chemical sensor throughout much of *Metazoa*. Electrophilic chemicals activate both insect and vertebrate TRPA1 via covalent modification of cysteine residues in the amino-terminal region. Although naturally occurring electrophilic plant compounds, such as mustard oil and cinnamaldehyde, are TRPA1 agonists, it is unknown whether arthropod-produced electrophiles activate mammalian TRPA1. We characterized the effects of the electrophilic arthropod defensive compound *para*-benzoquinone (pBQN) on the human TRPA1 channel. We used whole-cell recordings of human embryonic kidney cells heterologously expressing either wild-type TRPA1 or TRPA1 with three serine-substituted cysteines crucial for electrophile activation (C621S, C641S, C665S). We found that pBQN activates TRPA1 starting at 10 nM and peaking at 300 nM; higher concentrations caused

rapid activation followed by a fast decline. Activation by pBQN required reactivity with cysteine residues, but ones that are distinct from those previously reported to be the key targets of electrophiles. The current reduction we found at higher pBQN concentrations was a cysteine-dependent desensitization of TRPA1, and did not require prior activation. The cysteines required for desensitization are not accessible to all electrophiles as iodoacetamide and internally applied 2-(trimethylammonium) ethyl methanesulfonate failed to cause desensitization (despite large activation). Interestingly, following pBQN desensitization, wild-type TRPA1 had dramatically reduced response to the nonelectrophile agonist carvacrol, whereas the triple cysteine mutant TRPA1 retained its full response. Our results suggest that modification of multiple cysteine residues by electrophilic compounds can generate both activation and desensitization of the TRPA1 channel.

Introduction

Organisms in their natural environment face the constant threat of predation. As a consequence, myriad strategies for protection evolved, including crypsis, behavioral adaptations, and the production of defensive chemicals. The incredibly diverse Arthropods are a source of numerous defensive compounds, including venoms for injection and irritants for contact spray and secretion (Karalliedde, 1995; Eisner, 2005). One striking example of an arthropod defensive spray is from the bombardier beetle, from the Family Carabidae. This spray is 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 is poorly understood. The use of quinones in defensive secretions is widespread, occurring in many arthropod groups (Conner et al., 2007).

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Many plant derived chemical deterrents 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 transient receptor potential vanilloid subtype 1 (TRPV1) by capsaicin, the active ingredient in "hot" peppers, but other plant-derived chemicals activate a variety of TRP channels: transient receptor potential melastatin subtype 8 (TRPM8; e.g., menthol from mint; Peier et al., 2002), TRPV3 (e.g., carvacrol from oregano, eugenol from cloves, citral from lemon; Xu et al., 2006; Stotz et al., 2008). TRP ankyrin subtype 1 (TRPA1) 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 neurons. TRPA1 also contributes to hyperalgesia during inflammation in peripheral tissues, as well as contributing to cough and airway inflammation (Bessac and Jordt, 2008; Bautista et al., 2013; Nilius et al., 2012).

The activation of TRPA1 occurs via two described mechanisms: First, electrophilic molecules (including AITC,

ABBREVIATIONS: AITC, allyl isothiocyanate; 2-APB, 2-aminoethoxydiphenyl borate; HC-030031, 2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-purin-7-yl)-N-(4-isopropyl-phenyl)-acetamide; HEK, human embryonic kidney; IA, iodoacetamide; MTSET, 2-(trimethylammonium)ethyl methanesulfonate; NaGTP, guanosine 5'-triphosphate, sodium; NMM, *N*-methylmaleimide; pBQN, *para*-benzoquinone; TRPA1, transient receptor potential ankyrin subtype 1; TRPA1-3C, TRPA1 triple cysteine mutant (C621S, C641S, C665S).

cinnamaldehyde, allicin and *N*-methylmaleimide) react with free sulfhydryl groups on cysteine and the primary amine of lysine residues in the amino terminus, which leads to channel opening (Hinman et al., 2006; Cebi and Koert, 2007; Macpherson et al., 2007a). Mutagenesis studies show that residues Cys422, Cys621, Cys641, Cys665, and Lys710 (Hinman et al., 2006; Macpherson et al., 2007a) are crucial for activation by electrophiles. Second, nonelectrophile 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 (Jordt et al., 2004; Hinman et al., 2006; Xu et al., 2006; Karashima et al., 2007; Talavera et al., 2009).

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., 2006; Diedrich and Julian, 2010). In addition to being behaviorally aversive, pBQN is toxic to mammalian cells (Crespo et al., 2011; Fisher et al., 2011; Sarma et al., 2011; Tian et al., 2012). 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 et al. (2011) found that pBQN can activate TRPA1, but when injected into the spinal cord of mice it produces an antinociceptive effect. In this case, pBQN may mimic the effects of chemically similar breakdown products of acetaminophen.

Although much of the attention on TRPA1 channels has focused on its activation properties by various agonists, less is known about how desensitization of TRPA1 occurs. TRPA1 is desensitized by Ca^{2+} entering the channel, although where it binds and its precise mechanism is unknown (Nagata et al., 2005; Wang et al., 2008). Recently, Raisinghani et al. (2011) showed that continuous *N*-methylmaleimide (NMM) applications 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 621, 641, or 665. We also show that higher concentrations of pBQN led to complete desensitization of TRPA1, abolishing responses to both electrophilic and nonelectrophilic agonists. TRPA1 desensitization could occur in the absence of activation, similar to when excess cysteine was added to the intracellular solution or when NMM was applied to the TRPA1 triple cysteine mutant (C621S, C641S, C665S) (TRPA1-3C mutant). This suggests that this desensitization results from modification of cysteine residues distinct from intracellular cysteines involved in electrophilic activation.

Materials and Methods

Cells and Heterologous Expression. Human embryonic kidney (HEK)293 FlpIn Trex cells (Life Technologies, Carlsbad, CA) stably expressed the *tet* repressor, either alone (parental) or with wild-type human TRPA1 coding sequence integrated. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 (1:1) supplemented with 5–10% fetal calf serum (FCS) and 15 $\mu\text{g}/\text{ml}$ blasticidin at 37°C in a 5%- CO_2 incubator. To induce expression of wild-type TRPA1, 0.1 $\mu\text{g}/\text{ml}$ doxycycline was added 18–24 hours before recordings. For transient expression of human TRPA1 triple mutant (C621S, C641S, C665S; numbering from sequence at GenBank accession number NP_015628.2) channels, parental 293 FlpIn Trex cells were

transfected for 3 hours using LipofectAMINE 2000 (Life Technologies). TRPA1 mutant cDNA (1.5–2 μg) 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 hours later.

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 20 kHz with a Digidata 1320A or 1322. Capacity current was reduced as much as possible using amplifier circuitry, and series resistance was compensated 75–85%. Cells were held at -68 mV, and currents were elicited by a 40-millisecond step to -95 mV, followed by a 200-millisecond ramp to $+105$ mV, and held at $+105$ mV for 40 milliseconds. This protocol was applied every 2 seconds. Pipettes were pulled from borosilicate glass (WPI, Sarasota, FL) to resistances ranging from 2.0 to 5.5 M Ω and filled with an internal solution containing 122 mM Cs-methanesulfonate, 1.8 mM MgCl_2 , 9 mM EGTA, 14 mM Tris-creatine phosphate, 4 μg of ATP, 0.3 mM NaGTP, and 10 mM HEPES (pH 7.20 with CsOH). The typical extracellular solution had no added calcium and consisted of 150 mM NaCl, 4 mM KCl, 3 mM MgCl_2 , 10 mM glucose, and 10 mM HEPES, pH 7.40 (with NaOH). In the experiments shown in Fig. 3, an extracellular solution with the 3 mM MgCl_2 replaced by 2 mM CaCl_2 , and 1 mM MgCl_2 (with all other components the same) was used as indicated. 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 second. Currents were imported with Data Access (Bruyton, Seattle, WA) and analyzed with Igor Pro 5.5 software (Wavemetrics, Lake Oswego, OR). Currents were averaged over 0.4 milliseconds around ± 60 mV (± 60.2 to ± 59.8 mV) during the ramp. Because 2-APB-elicited currents were relatively small, in Figs. 5 and 9, they were averaged over 5 milliseconds at the end of steps to -95 and $+105$ mV (i.e., before and after the ramp). All recordings were performed at room temperature (22–25°C).

Reagents. HC-030031 [2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydropurin-7-yl)-*N*-(4-isopropyl-phenyl)-acetamide] was purchased from Tocris Bioscience (Bristol, UK). 2-(Trimethylammonium)ethyl methanethiosulfonate (MTSET) was purchased from Toronto Research Chemicals (Toronto, ON, Canada). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of HC-030031 and carvacrol were prepared in dimethylsulfoxide. AITC and pBQN stock solution was prepared in ethanol. The dimethylsulfoxide and ethanol vehicle solutions did not elicit current changes in TRPA1-expressing HEK cells. All other reagents were prepared and diluted to working concentrations in external or internal solutions immediately before the experiment (pBQN was initially dissolved in ethanol before addition to solutions).

Results

Figure 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 seconds, and reached a steady-state after 6 minutes in pBQN (Fig. 1C). (In six cells, the average delay was 24 ± 7 seconds, and steady-state was reached after 445 ± 205 seconds). The currents activated by pBQN had a reversal potential at -3 mV and were slightly outwardly rectifying (Fig. 1B), typical of TRPA1 channels (Story et al., 2003; Jordt et al., 2004). However, applying the TRPA1 selective blocker HC-030031 (50 μM), reduced the pBQN-activated currents by $79 \pm 3\%$ at $+60$ mV ($n = 3$; McNamara et al., 2007). pBQN (at 1 μM) did not elicit any change in current when tested in three HEK cells with no TRPA1 expression (i.e., not induced by doxycycline).

After reaching steady-state, the pBQN-activated currents remained at an elevated level during washout. After 100 seconds in control solution, current was $101 \pm 0.1\%$, $n = 4$,

Fig. 1. p-Benzoquinone (pBQN) activates heterologously expressed human TRPA1. (A) Currents evoked by a voltage protocol consisting of a 40-millisecond step to -95 mV followed by a 200-millisecond ramp to $+105$ mV, followed by 40 milliseconds at $+105$ mV (holding potential -68 mV). Baseline currents (red), steady-state current in response to 30 nM pBQN (green) and currents after block by 50 μ M HC-030031 (black) are shown. (B) Current-voltage curves from sweeps in (A). (C) Activation time-course of current by 30 nM pBQN and inhibition by 50 μ M HC-030031. The dashed line indicates zero current. Symbols plot the average currents at ± 60 mV.

and in two cells, an additional 9 minutes of washout led to no change in current amplitude.

We next tested the effect of higher pBQN concentrations on TRPA1 channels. Figure 2A shows the activation of TRPA1 current with [pBQN] ranging from 10 nM to 10 μ M. With 10

nM pBQN, the currents reached a steady state after a 6-minute application (similar to 30 nM, Fig. 1B). As the concentration of pBQN was increased, two observations were apparent. First, the delay time before channel activation was reduced: 100 nM pBQN-activated currents that peaked in 110 ± 11 seconds ($n = 10$), whereas 1 and 10 μ M pBQN-activated currents peaked in 20 ± 2 and 6 ± 1 seconds, respectively ($n = 5$ and 6). 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 $57.2 \pm 5.3\%$ after 300 seconds ($n = 7$), and in 1 and 10 μ M pBQN, the decline was faster (215 and 100 seconds, respectively) and essentially complete (89.7 ± 2.6 and $95.2 \pm 1.7\%$, respectively).

Figure 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 Fig. 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 (τ) of 254 ± 167 seconds, or converting to a decay rate (rate = $1/\tau$) gives 0.00393 ± 0.0006 second^{-1} ($n = 9$). Exposure to higher concentrations of pBQN (3–10 μ M) resulted in smaller current amplitudes and a faster decay, with a time constant of ~ 35 seconds (rate $\sim 0.032 \pm 0.003$ second^{-1} at 3 μ M, $n = 4$ and 0.035 ± 0.005 at 10 μ M, $n = 6$).

Andersson et al. (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 Ca^{2+} in their recording solutions, which both potentiates and desensitizes TRPA1 (Nagata et al., 2005; Doerner et al., 2007; Zurborg et al., 2007; Wang et al., 2008; Sura et al., 2012). One possibility is that Ca^{2+} -potentiation enhanced TRPA1 current at higher pBQN concentrations more rapidly than desensitization occurred. The decline in TRPA1 current we observe is not likely to result from this process, as we omitted Ca^{2+} from the external solution and tightly buffered intracellular Ca^{2+} with 9 mM EGTA in the pipette solution.

Although omitting Ca^{2+} from the external solution helps minimize the confounding effect of Ca^{2+} -mediated desensitization, this is not the normal physiologic situation during pBQN exposure when used as a deterrent. We tested whether external Ca^{2+} alters the activation and desensitization of TRPA1 channels. Figure 3A shows that application of 30 nM pBQN (in 2 mM external Ca^{2+}) elicited a slow increase in TRPA1 current that rapidly accelerated 76 seconds after application. The current peaked at 5104 pA at $+60$ mV, before declining to a steady-state level of 243 pA. The current did not change after the removal of pBQN. Presumably, the rapid increase and decrease reflect the opposing Ca^{2+} -dependent potentiation and desensitization. From three cells recorded this way, the peak pBQN elicited currents were 208 ± 76 pA/pF at $+60$ mV and -110 ± 39 pA/pF at -60 mV. After reaching the peak, the current declined by $95.2 \pm 0.7\%$. Clearly, TRPA1

Fig. 2. Dose dependence of pBQN activation and desensitization of human TRPA1. (A) TRPA1 currents elicited by extracellular application of pBQN at concentrations from 10 nM to 10 μM. The dashed line indicates the zero current level. Currents are the average at +60 mV. (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 $n = 4-9$; bar indicates $\pm$ S.E.M.). 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 indicate $\pm$ S.E.M.).

channels are able to respond to 30 nM pBQN when external Ca^{2+} is present, but Ca^{2+} also caused desensitization. Desensitization did not occur when 30 nM pBQN was added in the absence of Ca^{2+} (Fig. 1). We next tested whether any TRPA1 channels were available to respond to 100 μM AITC after 3 minutes in pBQN-free external solution. 100 μM AITC evoked a small current after ~20 seconds (net 270 pA current at +60 mV), which began to desensitize as the exposure continued. We tested whether a second 30 nM pBQN application would activate any TRPA1 channels. There was a small amount of current activated during the extended pBQN application, but after removal of pBQN the current increased, slowly at first, and then more rapidly. We observed a similar post-pBQN response in two of the three cells we recorded (average peak current density was 1322 ± 449 and -1071 ± 301 pA/pF at ± 60 mV). This response was unexpected; perhaps the low pBQN concentration and relatively short application led to incomplete activation of TRPA1 channels during the first 30-nM pBQN exposure. Some fraction of TRPA1 channels may have remained

available to respond to the second, brief pBQN application. As these channels were slowly activated by pBQN, Ca^{2+} began to enter, which would further potentiate TRPA1 channels. But at some point sufficient Ca^{2+} entered to cause Ca^{2+} -desensitization came into play, reducing the current amplitude after it peaked.

We next tested whether a higher concentration of pBQN (10 μM) applied in 2 mM external Ca^{2+} would activate larger and faster TRPA1 currents, similar to Fig. 2A. Indeed, 10 μM pBQN activated large TRPA1 currents immediately, which peaked in 4–6 seconds. In two of the three cells, currents were large at ± 60 -mV amplitudes >20 nA, but these amplitude currents only lasted for ~4 seconds at most (a value of 20 nA was used for statistics). Interestingly, the TRPA1 current amplitudes recorded in the presence of external calcium were larger than those recorded in its absence (1794 ± 186 pA/pF at +60 mV, $n = 3$ in Ca^{2+} external, compared with 231 ± 77 pA/pF, $n = 6$ without Ca^{2+}). Ca^{2+} potentiation may be contributing during the initial activation, enhancing the current

Fig. 3. pBQN activates and desensitizes TRPA1 in the presence of external calcium. (A) 30 nM pBQN was applied in external solution containing 2 mM Ca^{2+} . After activation and desensitization of TRPA1 current, 100 μM AITC activated only a small current. A second application of 30 nM pBQN slowly activated the current, which increased before again desensitizing (after pBQN removal). A third application of BQN failed to activate any current. (B) 10 μM pBQN applied in 2 mM external Ca^{2+} activated large TRPA1 currents that desensitized rapidly and completely. A subsequent application of 300 μM carvacrol (carv) failed to activate any current. (C) A 10-second application of 10 μM pBQN, in external solution with zero added Ca^{2+} and containing 3 mM Mg^{2+} , led to sustained TRPA1 currents up to 5 minutes following removal. Addition of 2 mM Ca^{2+} external solution increased current initially, followed by a substantial desensitization. Addition of 300 μM carvacrol did not increase TRPA1 current. A final, long application of 10 μM pBQN decreased the remaining small current. Dashed lines indicate zero current level. Currents in all panels are the average at ± 60 mV.

amplitude. After peaking, the current declined rapidly and completely. The decline was well fitted by a single exponential (time constant = 18.3 ± 1.5 seconds) and was complete ($99.0 \pm 0.2\%$, $n = 3$). This desensitization rate was faster with external Ca^{2+} present, 0.055 ± 0.007 second $^{-1}$ compared to 0.033 ± 0.005 second $^{-1}$ without. Addition of external Ca^{2+} appears to speed the desensitization of the pBQN-activated TRPA1 current. Finally, we tested whether 300 μM carvacrol, which activates TRPA1 in a noncovalent, reversible manner, would activate any current. In all three cells, carvacrol failed to activate significant current (net 3.6 ± 10.6 pA/pF at +60 mV), suggesting that TRPA1 channels were fully desensitized.

The desensitization of TRPA1 channels by external Ca^{2+} can also result from entry of other divalent cations, including Mg^{2+} (Wang et al., 2008). Wang et al. (2008) showed that 2 mM external Mg^{2+} led to near complete desensitization of TRPA1 channels within ~ 2 minutes. In our experiments shown in Fig. 3, A and B, pBQN was applied in the presence of external Ca^{2+} , and we presume that desensitization was attributed to Ca^{2+} . However, the desensitization of TRPA1 channels resulting from pBQN in Fig. 2 was recorded with zero added Ca^{2+} and 3 mM Mg^{2+} . We tested whether short application pBQN with 3 mM Mg^{2+} present would lead to sustained or completely desensitized currents (Fig. 3C). We applied 10 μM pBQN for 4–10 seconds, activating large TRPA1 currents (1132 ± 209 pA/pF, $n = 5$). The current did begin to decrease during, and slightly after, pBQN was present. Yet the current did not completely desensitize, and $59.7 \pm 14.7\%$ of the peak remained activated even 5 minutes after pBQN removal ($n = 5$). This suggests that the complete desensitization of the current in Fig. 2A resulted from the prolonged application of 10 μM pBQN, and not due to Mg^{2+} entry through activated channels. Similar large sustained currents were recorded in response to short NMM, internal MTSET, and external iodoacetamide (IA) application (Fig. 7).

We subsequently added 2 mM external Ca^{2+} to cells with sustained pBQN activated TRPA1 channels. The TRPA1 currents initially increased when Ca^{2+} was added (241 ± 121 pA/pF, or $\sim 61\%$; $n = 5$), before beginning to decrease rapidly (average single exponential time constants at +60 mV was 11.1 ± 3.5 seconds). Ca^{2+} -dependent potentiation appears to precede desensitization, as in Fig. 3A. The desensitization was substantial, but not always complete, reaching a maximal value of $93.8 \pm 2.0\%$. In fact, some channels appeared to recover slightly, as shown by the whole-cell recording in Fig. 3C. We tested whether TRPA1 channels had recovered by applying 300 μM carvacrol (in zero added Ca^{2+} solution). Only small currents were activated, reaching just 15.9 ± 4.1 pA/pF, consistent with a very small population of available TRPA1 channels. These open channels were desensitized by a long application of 10 μM pBQN in three of five cells ($91.8 \pm 1.3\%$ reduction; the other two cells showed no change in current).

The results in Fig. 3 suggest that TRPA1 channels have a Ca^{2+} -independent form of desensitization when activated by high pBQN concentrations. Does this desensitization cause 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 AITC (100 μM) and NMM (50 μM). These substances activate the TRPA1 channel by binding to a series of cysteine side chains in the amino terminus (Hinman et al., 2006; Macpherson et al., 2007a). pBQN is itself an electrophile that

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 4, A and B, shows that application of 1 μ M pBQN causes TRPA1 currents to activate and then decline, but neither 100 μ M AITC nor 50 μ M NMM was able to subsequently increase the current (current density changed -5 ± 4 pA/pF, $n = 3$, and 1 ± 1 pA/pF, $n = 3$, respectively). This shows that TRPA1 channels treated with high concentrations of pBQN for ~ 2 minutes are unable to respond to a second application of an electrophilic agonist. Both brief NMM and AITC activated TRPA1 current when given prior to pBQN application but the currents remained at elevated levels despite washout. A similar Ca^{2+} -independent desensitization in response to NMM and AITC was recently noted by Raisinghani et al. (2011).

Figure 4C shows that TRPA1 channels activated by 50 μ M NMM become desensitized after prolonged application. TRPA1 current initially increased and then began to decline, very similarly to the response to pBQN alone. The major difference was that 50 μ M NMM desensitization was about twice as slow as 1 μ M pBQN desensitization, requiring 6–8 minutes to reach a steady-state ($\tau = 49.9 \pm 12.8$ seconds, $n = 4$). When 1 μ M pBQN was added after the current declined, there was no response (current actually declined a further 6 ± 2 pA/pF, or $\sim 8\%$, over the ~ 60 seconds in pBQN).

TRPA1 can also be activated by a number of nonelectrophilic substances that activate the channel independently of the amino-terminal cysteines, including 2-APB (500 μ M) and carvacrol (300 μ M) (Hinman et al., 2006; Xu et al., 2006). Figure 5 shows that both 2-APB and carvacrol can reversibly activate TRPA1 channels (2-APB-elicited currents were smaller, and little or no inward current was observed; whether this reflects simply a lower potency of 2-APB activation or a more complicated pharmacology is not clear). Following 2-APB or carvacrol, the application of 1 μ M pBQN resulted in the typical activation and subsequent $93.4 \pm 0.5\%$ decline in the current ($n = 7$). After removal of the pBQN-containing solution, 2-APB was now completely unable to activate TRPA1 current (current declined 3.6 ± 1.5 pA/pF during 2-APB application, $n = 3$). When carvacrol was used to test available TRPA1 channels, a small net current was observed (average current density at +105 mV, 20.7 ± 8.7 pA/pF, $n = 4$). The peak pBQN-activated currents in these cells were very large, with an average density of 1096 ± 215 pA/pF, so this response may represent the presence of a small fraction of nondesensitized channels. Desensitization of TRPA1 by pBQN appeared to be irreversible for the duration of our recordings (up to 30 minutes). Clearly, TRPA1 channels desensitized by pBQN application have greatly reduced responses to electrophilic and nonelectrophilic 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 cysteine reactivity, we preincubated 1 μ M pBQN with 5 μ M L-cysteine before applying it to the cell. This reaction results in a complex mixture of products that 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 (Fig. 6A) (net 10.2 ± 11.4 pA/pF at +60 mV, $n = 3$). In the same cells, subsequent application

of 1 μ M pBQN alone activated TRPA1 normally (1337 ± 340 pA/pF).

In other experiments, we noticed that a shorter exposure to 50 μ M 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-second exposure to 50 μ M NMM, presumably allowing only some cysteines to bind to NMM. As expected for a covalent action, currents activated by

Fig. 4. TRPA1 is unresponsive to electrophilic agonists after pBQN desensitization. (A and B) Application of 1 μ M pBQN activates and desensitizes TRPA1 channels. After pBQN application, the TRPA1 agonists AITC (100 μ M) and *N*-methylmaleimide (NMM; 50 μ M) do not elicit current activation. (C) After 50 μ M NMM activates and desensitizes TRPA1, the channel is unresponsive to 1 μ M pBQN. The dashed line indicates the zero current level. The transient spike in the -60 mV current is a vibration artifact. Currents in all panels are the average at ± 60 mV.

Fig. 5. TRPA1 is unresponsive to nonelectrophilic agonists after desensitization. (A and B) The TRPA1 agonists, 2-aminoethoxydiphenyl borate (2-APB; 500 μ M) and carvacrol (carv; 300 μ M), reversibly activate TRPA1. Neither agonist elicits a second response after desensitization by 1 μ M pBQN. Currents were averaged at -95 and $+105$ mV.

brief NMM remained unchanged during a wash with extracellular solution (Fig. 6B). Application of the pBQN/L-cysteine mixture had no effect on the NMM-activated currents. Subsequent application of 1 μ M pBQN alone also did not produce any additional activation, suggesting that NMM had already reacted with all available cysteines that were involved in channel activation. However, the application of pBQN did desensitize the channel and reduced the current at $+60$ mV by $87 \pm 10\%$ ($n = 3$). 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).

Thus far, our data suggest that pBQN activates and desensitizes TRPA1 by reacting with cysteine, but we have not determined where those cysteines are located. The human isoform of TRPA1 contains 28 cysteine residues as follows: 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 whether pBQN targets cytosolic cysteines to activate TRPA1. To test this, we manipulated the cytosolic activity of pBQN by applying the preincubated mixture of 5 μ M L-cysteine and 1 μ M pBQN intracellularly. Similar to the results in Fig. 6, the preincubated mixture added to the intracellular solution did not activate TRPA1 (Fig. 7A). However, when additional 1 μ M pBQN alone was applied extracellularly, it was able to activate and desensitize the TRPA1 currents (peak current density at $+60$ mV reached 466 ± 134 pA/pF, before desensitizing $91 \pm 4\%$, $n = 3$). These experiments show that pBQN must maintain its electrophilic character 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 (Fig. 7B). 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 minutes after break-in and before

Fig. 6. pBQN activation involves cysteine reactivity. (A) A 20-second exposure to 5 μ M L-cysteine alone or 5 μ M L-cysteine preincubated with 1 μ M pBQN has no effect on TRPA1. A subsequent application of 1 μ M pBQN alone activates TRPA1, which is blocked by 50 μ M HC-030031. (B) An initial 60-second application of 5 μ M cysteine does not activate TRPA1; however, a subsequent 20-second bath application of 50 μ M NMM activates a sustained TRPA1. The current remained activated during washout and during a 60-second bath application of preincubated L-cysteine and pBQN. Application of 1 μ M pBQN alone quickly desensitized NMM activated currents. Currents in all panels are the average at ± 60 mV.

Fig. 7. pBQN activation, but not desensitization, requires cytosolically accessible cysteines. (A) 1 μ M pBQN and 5 μ M cysteine added to the pipette solution has no effect on TRPA1 currents during 180-seconds of recording. Extracellularly applied 1 μ M pBQN induces activation and desensitization. (B) 300 μ M carvacrol activates TRPA1 in the presence of 20 mM intrapipette cysteine. pBQN, 1 μ M, is applied for 360 seconds and has minimal effects on activation. The channel is unresponsive to a second application of carvacrol. (C) Intrapipette 2-(trimethylammonium)ethyl methanethiosulfonate (MTSET; 100 μ M) elicits sustained TRPA1 currents. A short application of 50 μ M HC-030031 blocks the currents reversibly. A subsequent application of 10 μ M extracellular pBQN rapidly desensitizes the channel. (D) Application of 100 μ M iodoacetamide elicits sustained TRPA1 current activation. After 4 minutes in iodoacetamide, addition of 10 μ M pBQN initially increases TRPA1 current, followed by rapid and complete desensitization. The pipette contained the typical Cs-methanesulfonate (Cs-Mes) solution. Dashed lines indicate zero current. Currents in all panels are the average at ± 60 mV.

the addition of agonists. In two cells, we first applied the noncysteine-reactive TRPA1 agonist, carvacrol (300 μ M), to ensure the channel was still functional (176 ± 12 pA/pF was activated). Next, we applied 1 μ M pBQN externally and noticed a delay before minimal current activation (net 90 ± 35 pA/pF activation, $n = 4$). Normally, 1 μ M pBQN will elicit immediate and robust activation (e.g., Fig. 2A), yet in these cells, more than 40 seconds elapsed before we observed just 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 μ M carvacrol failed to activate any current (1 ± 2 pA/pF from three cells). This shows that pBQN requires readily accessible cytosolic cysteines 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 cores, which are not equally accessible to all thiol-reactive compounds. To test this, we added a thiol-reactive agent 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 minutes and reached a peak by 10–15 minutes (Fig. 7C). Once the peak was reached, these large currents did not decline (average current density at +60 mV was 357 ± 89 pA/pF, $n = 4$). A short application of 50 μ M HC-030031 inhibited the current by an average of $82 \pm 2\%$, demonstrating that the currents were mediated by TRPA1. At this point, we applied 10 μ M pBQN externally to test whether TRPA1 channels could be desensitized after activation by intracellular MTSET. Indeed, addition of extracellular pBQN desensitized the channel by $99.5 \pm 0.1\%$ ($\tau = 56.7 \pm 14.4$ seconds, $n = 4$). This indicates that pBQN is able to desensitize the channel by reacting with cysteines that are not accessible to intracellular MTSET.

The ability of thiol-reactive compounds to modify different cysteine side chains in native proteins depends on 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 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 from the Michael addition describing benzoquinone and NMM

reactions with thiols (Dennehy et al., 2006). Distinct populations of proteins are modified by these chemicals, and previous results show that NMM and iodoacetamide modify different groups of cysteines on TRPA1 (Macpherson et al., 2007a; Wang et al., 2012). When we applied 100 μ M IA externally, TRPA1 current activated immediately and continued to increase over 4 minutes until a steady state was reached (average current density 347 ± 67 pA/pF reached in 260 ± 53 seconds, $n = 4$ cells; Fig. 7D). In contrast to pBQN or NMM, IA did not cause desensitization in any of the four recorded cells (recorded up to 20 minutes). In three cells, 400 μ M IA was applied for a duration of 120–840 seconds. TRPA1 current transiently increased in 400 μ M IA before declining by $19 \pm 3\%$. Both the transient increase and the sustained reduction were reversible. Once the steady state had been reached, we applied 10 μ 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. The current ultimately declined by $97 \pm 1\%$, with a time constant of 49.1 ± 27.8 seconds. The additional activation induced by pBQN suggests that IA is a partial agonist. This also indicates that pBQN activates TRPA1 by modifying cysteines that are inaccessible to IA.

The human TRPA1 triple cysteine mutant channel (amino-terminal C621S, C641S, C665S, i.e., TRPA1-3C) is no longer activated by NMM but is still responsive to nonelectrophilic 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 μ M pBQN. Just as in wild-type channels, pBQN activation in the triple-mutant channel was followed by desensitization over several minutes (Fig. 8A). The rate of desensitization was 0.021 ± 0.003 second⁻¹ ($n = 9$), similar to rates in wild-type channels (0.022 ± 0.001 second⁻¹, $n = 4$). To test whether pBQN desensitized the mutant channel to other agonists, as it does in wild-type channels, we applied 300 μ M carvacrol or 500 μ M 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. The average peak current elicited by carvacrol was actually 1.22 ± 0.19 -fold larger than the pBQN-elicited current, whereas it was 0.53 ± 0.44 for 2-APB-activated current. The difference presumably results from the extent of activation caused by carvacrol and 2-APB and suggests that they have distinct potencies and/or efficacies on TRPA1-3C channels.

This behavior is quite distinct from wild-type channels. In those cases, carvacrol and 2-APB elicited small or no current after pBQN desensitization had occurred (Fig. 5). The absence of these three cysteines does not prevent pBQN activation, but does dramatically reduce the ability of the channel to become desensitized to nonelectrophiles.

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 (Fig. 8B). The inset shows a representative I-V curve of a typical response of the wild-type TRPA1 to 1 μ M pBQN. We quantified the degree of rectification as the ratio of TRPA1-induced current at +60 mV and -60 mV (Fig. 8C). The triple

mutant channels were far more outwardly rectifying (rectification ratio of 3.8 ± 1.0 , $n = 9$) than wild-type (1.4 ± 0.1 , $n = 9$) when activated by 1 μ M pBQN.

It is interesting to note that when TRPA1 wild-type and 3C mutant channels were activated by 300 μ M carvacrol (before any pBQN exposure), the resulting rectification was slightly different. The rectification ratio of 3C mutant channels was 2.5 ± 0.5 ($n = 4$), whereas for wild-type 1.8 ± 0.3 ($n = 5$). Thus,

Fig. 8. pBQN activates TRPA1 triple cysteine mutant (C621S, C641S, C665S). (A) The TRPA1-3C mutant activates in response to 1 μ M pBQN and 300 μ M carvacrol. (B) A representative current-voltage trace illustrates the pronounced outward rectification of TRPA1-3C in response to 1 μ M pBQN. Inset shows a wild-type TRPA1 response to 1 μ M pBQN. (C) Rectification index of the triple mutant compared with wild type. The dashed line indicates the level where no rectification would occur ($n = 9$ for both mutant and WT, bars indicate $\pm$ S.E.M.). Currents in all panels are the average at ± 60 mV.

TRPA1-3C channels activated by carvacrol were still more outwardly rectifying than wild type, but to a lesser degree than when activated by pBQN. The degree of rectification in these experiments is likely a complex result of how the particular agonist and its concentration affect the voltage-dependent gating of the channel, which is itself apparently different in the TRPA1-3C channel.

Our data suggest that the TRPA1 channel has two separate groups of target cysteines that are independently responsible for activation and desensitization by pBQN. Hinman et al. (2006) found that the triple cysteine mutant is not activated by NMM. We asked whether NMM would still desensitize the mutant channel, indicating that cysteines other than Cys621, Cys641, and Cys665 are involved in TRPA1 desensitization to electrophilic compounds. First, we exposed the mutant channel to 50 μ M NMM for 10 seconds, which did not activate current (Fig. 9A). 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 (average current densities at +105 mV were 288 ± 83 pA/pF, $n = 3$, for 2-APB and 365 ± 145 pA/pF, $n = 4$, for pBQN). This indicates that a short exposure to NMM neither activated current on its own, nor prevented activation by either 2-APB or by 10 μ M pBQN.

We then repeated the same experiment but with a longer (60-second) exposure to NMM (Fig. 9B). 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 Fig. 4C). After a 60-second exposure to NMM, 500 μ M 2-APB was applied to the cell and induced an outwardly rectifying current (current density of 227 ± 107 pA/pF, $n = 4$). However, there was no activation by 10 μ M 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-second exposure (or longer) to NMM does produce evident desensitization to pBQN. However, unlike the wild-type channels, the TRPA1-3C mutant is still responsive to the nonelectrophilic agonist 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. From all cells, the average net current density at +105 mV after NMM was 31 ± 10 pA/pF. In contrast, for these same cells the inward current at -95 mV was just -1 ± 1 pA/pF. NMM-induced currents were seen in some cells with both short (Fig. 9C) and long NMM exposures (Fig. 9D), but this did not change the mutant channel's subsequent response to pBQN. Just as we show in Fig. 9, A and C, 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 μ M carvacrol (241 ± 21 pA/pF current at +105 mV activated in four cells tested). These experiments show that TRPA1-3C mutation does not completely

Fig. 9. TRPA1 triple cysteine mutant can activate in response to nonelectrophilic agonists following pBQN desensitization. (A) 50 μ M NMM is applied to the TRPA1 triple cysteine mutant for 10 seconds followed by 500 μ M 2-APB. The triple mutant channel responds to a 30-second exposure to 10 μ M pBQN. (B) A similar experiment as in (A) but with a 60-second exposure to 50 μ M NMM. (C) The mutant channel responds to a 10-second exposure to 50 μ M NMM followed by additional activation with 1 μ M pBQN. After pBQN desensitization, 300 μ M carvacrol induces activation. (D) A similar experiment as in (C), but with a 120-second application of 50 μ M NMM followed by a 60-second exposure to 1 μ M pBQN and 5 seconds of 300 μ M carvacrol. Currents were averaged at -95 and +105 mV.

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-second or longer NMM exposure.

Discussion

We show that pBQN, a naturally occurring compound in the defensive spray of many arthropods, activates the human TRPA1 channel at low nanomolar concentrations. pBQN quickly activated TRPA1, with a delay of tens of seconds at 3–30 nM pBQN and <1 second with 1 μ M pBQN. At higher concentrations, the pBQN-evoked current reached a peak followed by desensitization, which was rapid and complete at concentrations of 1 μ M 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 Liebler, 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 cysteine residues. Mutating just three of the cysteines (C621S, C641S, C665S) 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 (Fig. 9D). 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 621, 641, or 665, 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 is not limited to the three cysteine residues whose loss severely diminishes the action of NMM or AITC.

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 minutes. Desensitization by pBQN prevented subsequent activation by other electrophilic agonists, and also reduced activation by nonelectrophiles such as carvacrol and 2-APB. In our study we eliminated the possibility of a Ca^{2+} -dependent desensitization by omitting Ca^{2+} 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 (Fig. 6). This suggests that pBQN cysteine reactivity is critical for both activation and desensitization of TRPA1. However, although 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 (Fig. 7B). 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 (Fig. 7C). 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 μ M) to cause desensitization is interesting in light of the mass spectrometry analysis that Macpherson et al. (2007a) performed after labeling live cells with iodoacetamide. Upon treating TRPA1-expressing cells with 100 or 400 μ M iodoacetamide, they found 15 cysteine residues labeled, with some residues labeled by the higher concentration (the remaining 16 cysteines were not detected in any peptides by mass spectrometry). This suggests that perhaps one of the other cysteines is responsible for desensitization.

Wang et al. (2012) 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: Cys665-Cys621, Cys665-Cys462, Cys665-Cys192, and Cys621-Cys608 (human TRPA1 numbering). Takahashi et al. (2011) also suggest that Cys665 can form a disulfide bond with Cys856. It is noteworthy that the triple mutant replaces cysteines at positions 665 and 621 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 now become inaccessible to electrophiles.

Wang et al. (2012) 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., Cys213, Cys258, Cys608, Cys703, Cys727; human TRPA1 numbering). 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 nonelectrophilic compounds is dramatically less in the TRPA1-3C mutant. Why does the removal of cysteine residues that are involved in channel activation by electrophiles allow carvacrol and 2-APB to robustly open the channel after long, desensitizing pBQN exposures? Wang et al. (2012) found that in wild-type TRPA1, C621 showed less labeling by long applications of high [NMM]. This might reflect developing desensitization. If this region is undergoing conformational changes during desensitization, mutations anywhere throughout the protein might affect the process. It is also possible that both agonist activation and desensitization may affect channel opening in a voltage-dependent manner, each of which could couple to the channel's gating mechanism. Testing the concentration-dependence of electrophilic and nonelectrophilic agonists at varying potentials, before and after desensitization, in both wild-type and 3C mutant channels, will help address this possibility.

Spraying of pBQN by the bombardier beetle repels many potential predators in 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 produce 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 human exposure to insect secretions of pBQN causes skin surface irritation (De Capitani et al., 2011) and also has a deterrent effect if inhaled or even tasted by humans (Eisner, 2005).

In contrast to exposure to insect pBQN, there are serious health effects when pBQN is produced from benzene (Westphal et al., 2009). In humans, exposure to sources of benzene, such as cigarette smoke and industrial pollutants, is quite common. TRPA1 activation can induce airway inflammation (Bessac and Jordt, 2008) and it is possible that TRPA1 activation by even low [pBQN], or related compounds, would occur during prolonged exposure to cigarette smoke or other industrial air pollutants.

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Authorship Contributions

Participated in research design: Ibarra, Blair.

Conducted experiments: Ibarra, Blair.

Performed data analysis: Ibarra, Blair.

Wrote or contributed to the writing of the manuscript: Ibarra, Blair.

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