Human TRPA1 is intrinsically cold- and chemosensitive with and without its N-terminal ankyrin repeat domain

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We have purified and reconstituted human transient receptor potential (TRP) subtype A1 (hTRPA1) into lipid bilayers and recorded single-channel currents to understand its inherent thermo- and chemosensory properties as well as the role of the ankyrin repeat domain (ARD) of the N terminus in channel behavior. We report that hTRPA1 with and without its N-terminal ARD (Δ1–688 hTRPA1) is intrinsically cold-sensitive, and thus, cold-sensing properties of hTRPA1 reside outside the N-terminal ARD. We show activation of hTRPA1 by the thiol oxidant 2-((biotinoyl)amino)ethyl methanethiosulfonate (MTSEA-biotin) and that electrophilic compounds activate hTRPA1 in the presence and absence of the N-terminal ARD. The nonelectrophilic compounds menthol and the cannabinoid Δ^9-tetrahydrocannabiorcol (C16) directly activate hTRPA1 at different sites independent of the N-terminal ARD. The TRPA1 antagonist HC030031 inhibited cold and chemical activation of hTRPA1 and Δ1–688 hTRPA1, supporting a direct interaction with hTRPA1 outside the N-terminal ARD. These findings show that hTRPA1 is an intrinsically cold- and chemosensitive ion channel. Thus, second messengers, including Ca^{2+}, or accessory proteins are not needed for hTRPA1 responses to cold or chemical activators. We suggest that conformational changes outside the N-terminal ARD by cold, electrophiles, and nonelectrophiles are important in hTRPA1 channel gating and that targeting chemical interaction sites outside the N-terminal ARD provides possibilities to fine tune TRPA1-based drug therapies (e.g., for treatment of pain associated with cold hypersensitivity and cardiovascular disease).

cold sensing | irritants | pain | sensory neuron | TRP channels

A number of vertebrate and invertebrate transient receptor potential (TRP) ion channels have been implicated in temperature sensation (1–3), but only the rat menthol receptor TRP subtype M8 (TRPM8) and the rat capsaicin receptor TRP subtype V1 (TRPV1) have been shown to possess intrinsic thermosensitivity (4, 5). In 2003, Story et al. (6) proposed that the mouse TRPA1 is a noxious cold sensor. Story et al. (6) showed that TRPA1 was present in nociceptive primary sensory neurons and that CHO cells heterologously expressing the mouse TRPA1 displayed cold sensitivity. Most subsequent studies of cold responses in heterologous TRPA1 expression systems, isolated primary sensory neurons, and whole animals have provided evidence in support of mouse and rat TRPA1 being involved in noxious cold transduction (7). Interestingly, a familial episodic pain syndrome triggered by cold is caused by a gain-of-function mutation in the TRPA1 gene, indicating that TRPA1 may have a key role in human noxious cold sensation (8). Thus, human TRPA1 (hTRPA1) may be a relevant drug target for treatment of this condition and other pathological conditions, such as inflammation, nerve injury, and chemotherapy-induced neuropathy, that are characterized by TRPA1-dependent cold allodynia or hypersensitivity (7). However, in vitro studies of the expressed hTRPA1 have generated conflicting findings (8–15), and no study has provided evidence that mammalian TRPA1 channels are, indeed. intrinsically cold-sensitive proteins, which would require examination of the purified protein in a defined membrane environment.

Heterologous expression studies of chimeric or mutated TRPA1 channels have proposed that the N-terminal region plays an important role in thermal and chemical sensitivity of both mammalian and insect TRPA1 (14, 16–19). Initial studies indicated that mammalian TRPA1 is activated by cysteine-reactive electrophilic compounds and oxidants, such as diallyl disulfide in garlic (9, 10, 20, 21). Targeted gene mutations have identified cysteines present in the N terminus of TRPA1 as important for electrophilic and oxidative TRPA1 channel activation (22, 23). Because several of these cysteines are involved in protein disulfide formation (24–26), it is not unlikely that such mutations will have pronounced effects on the overall TRPA1 channel structure and function (7). Electrophilic compounds can also covalently bind to cysteines in the transmembrane segments and the C-terminal domain of mammalian TRPA1 (23, 26), and the electrophiles *p*-benzoquinone, isovelleral, and polygodial robustly activate the heterologously expressed triple mutant hTRPA1-3C (27, 28) that was initially used to identify certain N-terminal cysteine residues in hTRPA1 as key targets for electrophiles (22). However, it is yet to be shown that covalent binding sites outside the N-terminal

Significance

The ability of an organism to detect and avoid noxious temperatures is crucial for survival. It is, therefore, of great interest that several transient receptor potential (TRP) ion channels have been proposed as temperature sensors. However, to date, only the menthol receptor (TRP subtype M8) and the chili pepper receptor (TRP subtype V1) have been shown to be intrinsically temperature-sensitive proteins in mammals. In this study, we show that the purified wasabi receptor (TRP subtype A1) is a cold sensor. Thus, mammals have at least two cold sensors that, together, cover pleasant (TRP subtype M8) and unpleasant (TRP subtype A1) cold temperatures. Our findings add to the understanding of how the temperature sense is organized and its role in pain associated with cold hypersensitivity.

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ankyrin repeat domain (ARD) contribute to the regulation of channel gating.

Another key feature of mammalian TRPA1 is the responsiveness to nonelectrophilic compounds with very different chemical structures, such as menthol and the cannabinoids Δ^9-tetrahydrocannabinol (Δ^9-THC) and Δ^9-tetrahydrocannabiorcol (C16) (7). However, if nonelectrophilic compounds activate TRPA1 directly and at the same site on TRPA1 is not known. The site of action of nonelectrophilic TRPA1 activators is important to clarify, because some TRPA1 activators are antinoiceptive (29, 30), and nontissue-damaging TRPA1 activators may be used clinically for pharmacological regulation of TRPA1 channel activity (29).

Here, we have purified and inserted hTRPA1 with and without its N-terminal ARD (Δ1–688 hTRPA1) into lipid bilayers for functional studies using patch-clamp electrophysiology to explore the inherent thermo- and chemosensitivity of hTRPA1. Because of the great potential of TRPA1 as a drug target for treatment of human pain and the existence of mammalian TRPA1 species differences (7), the human variant of TRPA1 was chosen for these studies. We addressed the role of the N-terminal ARD in cold and chemical sensitivity by deleting the N-terminal ARD, something that cannot be studied in cells heterologously expressing TRPA1, because the N-terminal ARD is needed for insertion of the ion channel into the plasma membrane (31). Our findings consolidate hTRPA1 as a multimodal nocisensor responding to cold and chemicals. It is suggested that conformational changes outside the N-terminal ARD by cold, electrophiles, and nonelectrophilic compounds are important in hTRPA1 channel gating. Targeting chemical interaction sites outside the N-terminal ARD may provide possibilities to fine tune TRPA1-based drug therapies [e.g., for treatment of pain associated with cold hypersensitivity (7) and cardiovascular disease (32)].

Results

Purification of Functional hTRPA1. To purify hTRPA1 and Δ1–688 hTRPA1, we used a *Pichia pastoris* expression system previously proven to be successful in the purification of other integral membrane proteins (33, 34). Initial screening of various detergents identified a series of fos-choline detergents as particularly effective for extraction of hTRPA1 and Δ1–688 hTRPA1 from *P. pastoris* cell membranes. Out of this screening, we chose fos-choline-14 for the purification and functional studies of the hTRPA1 channels. Intact hTRPA1 and Δ1–688 hTRPA1 with N-terminal decahistidine tags, which did not compromise the functionality of hTRPA1 when expressed in HEK293 cells (Fig. S1 *A–D*), were purified by Nickel affinity chromatography (Fig. 1); Δ1–688 hTRPA1 was obtained at a higher yield (3 mg/10 g cells) and purity than hTRPA1 (0.3 mg/10 g cells) using a one-step purification procedure. Based on Image Quant analysis, the purities of hTRPA1 and Δ1–688 hTRPA1 were estimated to be 50% and 95%, respectively.

In contrast to the heterologously expressed hTRPA1, there is no information on the functional properties and folding of Δ1–688 hTRPA1. Therefore, gel filtration and circular dichroism (CD) spectroscopy were implemented to evaluate the consequences of the removal of the N terminus on tetramerization and folding of the protein. As shown by the chromatogram, Δ1–688 hTRPA1 eluted mainly as a tetramer (Fig. 1*C*), and the far UV CD spectra showed that Δ1–688 hTRPA1 has the characteristics of a predominately α-helical structure with minima at 208 and 222 nm (Fig. 1*D*). The secondary structure composition was estimated by the Dicroweb software using CDSSTR, SELCON3, and CONTINLL algorithms, suggesting that Δ1–688 hTRPA1 contains 35–45% of α-helices, 15–20% of β-strands, 20–25% of turns, and 20–25% of unordered structure. The expected minima of α-helical content based on prediction of transmembrane domains (transmembrane hidden Markov models) and sequence alignment of Δ1–688 hTRPA1 with the potassium channel (Kv1.2) with

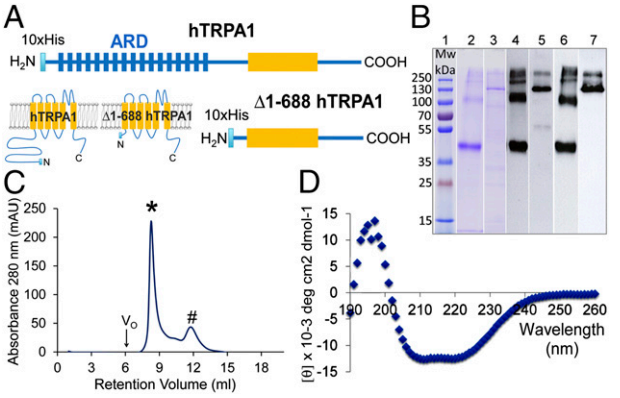

Fig. 1. Purification of hTRPA1 with and without the N-terminal ARD. (*A*) hTRPA1 was expressed either intact (1–1,119 aa) or without the ARD of the N terminus (Δ1–688 hTRPA1; 689–1,119 aa) with N-terminal decahistidine tags (10×His). (*B*) Affinity-purified hTRPA1 (lanes 3, 5, and 7) and Δ1–688 hTRPA1 (lanes 2, 4, and 6) visualized by Coomassie staining (lanes 2 and 3) and Western blotting (lanes 4–7) using either tetrahistidine antibody (lanes 4 and 5) or hTRPA1 antibody (lanes 6 and 7). The amounts of protein were 5 μg for Coomassie staining and 200 ng hTRPA1 and 40 ng Δ1–688 hTRPA1 for Western blotting. (*C*) As shown by the chromatogram, using a Superdex 200 size exclusion column, Δ1–688 hTRPA1 eluted mainly as a tetramer (*). V_o and # indicate void volume and monomer, respectively. (*D*) CD spectroscopy analysis disclosed typical characteristics of Δ1–688 hTRPA1 as a folded protein of high α-helical content. Mw, molecular mass.

known structure were 27% and 36%, respectively, which are in good agreement with the experimental data. Both the CD spectral data and the tetrameric oligomeric state of the purified protein suggest that it is correctly folded and functionally intact. In line with the general view that functional TRP channels are tetrameric protein complexes (2, 24), initial electrophysiological experiments measuring ramp currents (−100 to + 100 mV in 2 s) showed that hTRPA1 and Δ1–688 hTRPA1 reconstituted into planar lipid bilayers are functional proteins, because they responded to allyl isothiocyanate (AITC) and menthol, respectively, with single-channel currents at both negative and positive test potentials (Fig. S1*E*). Because AITC is supposed to activate TRPA1 by binding to the N terminus (22, 23), menthol was used to assess the functionality of Δ1–688 hTRPA1, because this nonelectrophilic compound presumably interacts with the S5 transmembrane domain (35).

Activation of hTRPA1 by Cold. TRPA1 activity was rarely observed at room temperature (22 °C), whereas cooling consistently activated hTRPA1 and Δ1–688 hTRPA1 at both positive and negative test potentials (Fig. 2, Figs. S2*B* and S3 *A* and *D*, and Table S1). Bilayers without proteins did not respond to cooling (Fig. S2*C*). Exposure to the TRPA1 blocker HC030031 inhibited cold-induced activation of hTRPA1 and Δ1–688 hTRPA1 by 71% and 76%, respectively (Fig. 2 *C* and *D*). Cooling from 22 °C to 10 °C dramatically increased single-channel open probability (P_o) (Fig. 2 *A*, *B*, and *E*). Our experimental setup did not allow us to obtain stable temperatures below 10 °C, which is why P_o values between 22 °C and 10 °C were used to calculate Q10 values of 0.025 and 0.018 from Arrhenius plots for hTRPA1 and Δ1–688 hTRPA1, respectively (Fig. 2*E*). A similar temperature dependence of the heterologously expressed mouse TRPA1 was observed in cell-attached and inside-out patches (36, 37) and with single-channel conductance (G_s) values at 10 °C, comparable with what we found for hTRPA1 (G_s = 50 ± 4 pS, n = 4). The G_s values at 10 °C differed between hTRPA1 and Δ1–688 hTRPA1 ($P < 0.05$, one-way ANOVA followed by Bonferroni's multiple comparison test) (Table S1). Notably, the cold-induced activity was reversible, and the G_s, but not P_o, decreased by 49% ± 10% (n = 3;

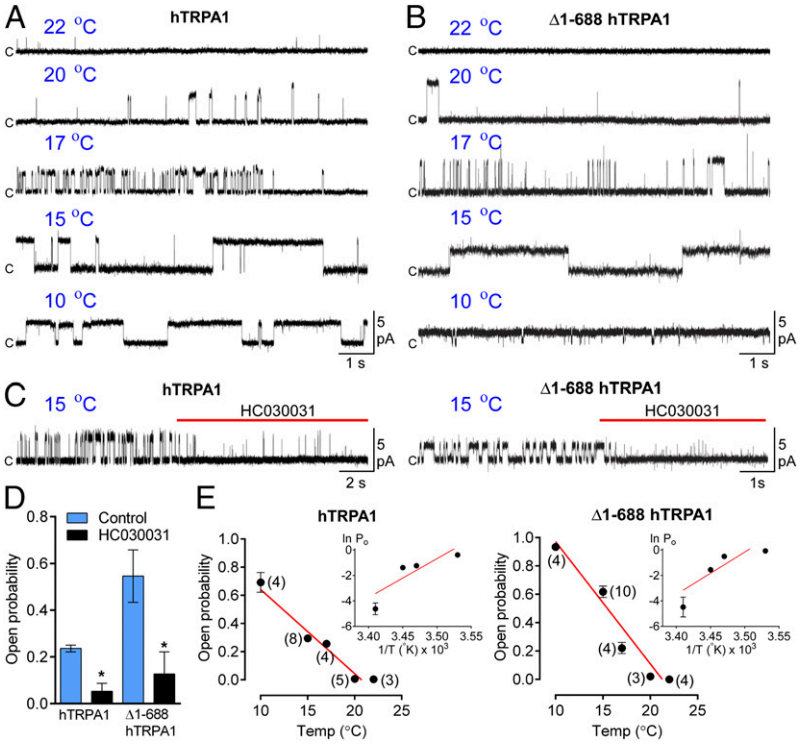

Fig. 2. The hTRPA1 is intrinsically cold-sensitive with and without the N-terminal ARD. Purified hTRPA1 and Δ1–688 hTRPA1 were inserted into planar lipid bilayers. (*A–C*) As shown by representative traces, cooling evoked substantial hTRPA1 and Δ1–688 hTRPA1 single-channel activity at a test potential of +60 mV (upward deflection shows open-channel state, and c shows closed-channel state). Amplitude histograms corresponding to each trace are shown in Fig. S2*A*. (*C*) Traces showing inhibition of cold responses of hTRPA1 and Δ1–688 hTRPA1 by the TRPA1 antagonist HC030031 (100 μM). (*D*) Graph with P_o values calculated from a time period of 1 min before and a time period of 1 min after treatment of hTRPA1 ($n = 3$) and Δ1–688 hTRPA1 ($n = 4$) with HC030031 at 15 °C. Complete inhibition was achieved after 1 min. Data are represented as means ± SEMs. $*P < 0.05$ indicates statistically significant differences using the Student's paired *t* test. (*E*) Plots summarize the P_o at various temperatures (number of experiments within parentheses) for (*Left*) hTRPA1and (*Right*) Δ1–688 hTRPA1. *Insets* show Arrhenius plots of the same data, with P_o values as the natural logarithm (ln) and temperature (T) as reciprocal Kelvin. Single-channel currents were recorded with the patch-clamp technique in a symmetrical K^+ solution.

$P < 0.05$, one-sample *t* test) when hTRPA1 was repeatedly exposed to 15 °C (Fig. S3*A*). This decline in G_s may reflect an inherent calcium-independent change in pore size, but it should not affect our analysis of cold-induced responses, because the analysis was based only on a first exposure to the indicated temperatures.

Activation of hTRPA1 by Electrophiles in the Absence of the N-Terminal ARD. Having established that the N-terminal ARD of hTRPA1 is not required for activation by cold, we asked if electrophiles can activate hTRPA1 in the absence of its N-terminal ARD at room temperature. Single-channel currents were rarely observed at test potentials of −60 and +60 mV or in voltage ramp recordings from −100 to +100 mV in the absence of TRPA1 activators (Fig. 2 *A*, *B*, and *E*, and Figs. S1*E* and S3*A*). However, AITC, cinnamaldehyde (CA), and *N*-methylmaleimide (NMM) but not the vehicle (1% ethanol) produced activation of both hTRPA1 and Δ1–688 hTRPA1 at −60 and +60 mV, whereas no activity was observed in the presence of activators on empty bilayers (Fig. 3*A* and Figs. S5, S6*B*, and S7). As shown for cinnamaldehyde, the effect was reversible (Fig. S3*B*). The activities of hTRPA1 and Δ1–688 hTRPA1 were blocked by HC030031 in a reversible manner (Fig. 3*B* and Fig. S3*C*): $P_o = 0.56 \pm 0.09$ and $P_o = 0.03 \pm 0.02$ as calculated for 1 min before and 1 min after the addition of HC030031, respectively, for hTRPA1 ($n = 4$). Whereas the G_s values for hTRPA1 and Δ1–688 hTRPA1 at −60 mV were similar in the presence for all electrophilic compounds, the G_s values at +60 mV differed between hTRPA1 and Δ1–688 hTRPA1 in the presence of AITC and NMM (Fig. 4*A* and Table S1). Comparison of P_o values revealed differences in ligand activation between hTRPA1 and Δ1–688 hTRPA1 at both positive and negative test potentials (Fig. 4*B* and Table S1). Additional calculations of the rectification index (+60/−60 mV) for G_s and P_o showed clear differences between hTRPA1 and Δ1–688 hTRPA1 when exposed to electrophiles (Fig. 4*C*), indicating that the N terminus modified hTRPA1 channel behavior in a voltage-dependent manner.

The ability of the hydrophilic thiol oxidant 2-((biotinoyl) amino)ethyl methanethiosulfonate-biotin (MTSEA-biotin; Fig. S4), which is active on TRPA1 from the intracellular side (23), to activate hTRPA1 ($G_s = 128 \pm 2$ pS and $P_o = 0.71 \pm 0.02$ at +60 mV; $n = 5$) only when applied in the bath solution (Fig. S4) indicates a uniform orientation of the protein in the lipid bilayer. Based on our findings that NMM activated the N-terminal ARD-deleted hTRPA1 and its ability to bind cysteine residues outside the N-terminal ARD (23, 26), we used a hydrophilic maleimide-biotin derivative (Fig. S4) for studies of Δ1–688 hTRPA1. Our data suggest a similar uniform orientation for Δ1–688 hTRPA1 in the lipid bilayer, because maleimide-biotin only activated this protein when applied to the bath solution (Fig. S4). The Gs and P_o values for maleimide-biotin ($G_s = 46 \pm 6$ pS and $P_o = 0.41 \pm 0.03$ at +60 mV, $n = 4$) are similar to those obtained with NMM at a test potential of +60 mV (Fig. 4 *A* and *B* Table S1).

Activation of hTRPA1 by Nonelectrophilic Compounds. In addition to being a chemosensor of thiol-reactive electrophiles and oxidants, TRPA1 is also activated by nonelectrophilic compounds, including Δ^9-THC, C16, menthol, carvacrol, clotrimazole, and dihydropyridines (7). A few heterologous expression studies using site-directed mutagenesis, chimeric channel constructs, or isolated

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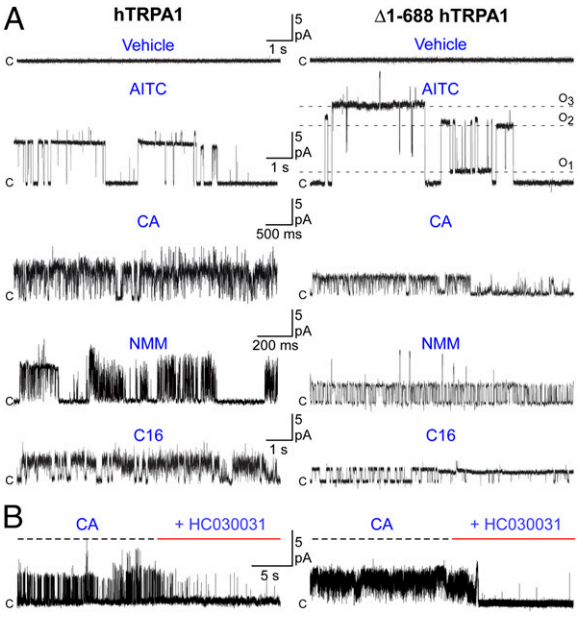

Fig. 3. Electrophilic and nonelectrophilic compounds activate purified hTRPA1 with and without the N-terminal ARD. (*A*) Representative traces show single-channel activity (upward deflection shows open-channel state, and c shows closed-channel state) for (*Left*) hTRPA1 and (*Right*) Δ1–688 hTRPA1 without the N-terminal ARD when inserted into planar lipid bilayers and exposed to the electrophilic compounds AITC (100 μM), cinnamaldehyde (CA; 100 μM), and NMM (100 μM) and the nonelectrophilic compound C16 (100 μM). Multiple distinct open-channel levels (dotted lines) were observed for AITC in Δ1–688 hTRPA1, of which the main level (O_3) was used for calculations of the G_s values presented in Fig. 4 and Table S1 (Fig. S5 presents responses at −60 mV). As shown by representative traces, the vehicle (ethanol at 1%) used for all test compounds evoked no activation of either hTRPA1 variant ($n = 3$). Amplitude histograms corresponding to each trace in *A* are shown in Fig. S7. (*B*) The TRPA1 antagonist HC030031 (100 μM) blocked CA-induced hTRPA1 ($n = 4$) and Δ1–688 hTRPA1 channel activity ($n = 2$). Channel currents were recorded with the patch-clamp technique in a symmetrical K^+ solution at a test potential of +60 mV.

inside-out patches suggest that nonelectrophilic compounds, including menthol and Δ^9-THC, directly interact with TRPA1 (35, 38). In this study using the purified channel, we provide final proof that Δ^9-THC, C16, and menthol directly activate hTRPA1 without the involvement of other cellular proteins or second messengers, including inositol triphosphate and Ca^{2+} (Figs. 3*A*, 4, and 5 and Figs. S1*E* and S5–S7). As with electrophilic compounds, menthol and C16 also activated hTRPA1 without the N-terminal ARD (Figs. 3*A*, 4, and 5 and Figs. S5 and S7). Importantly, the very lipophilic and potent cannabinoid receptor agonist CP55940, which in contrast to Δ^9-THC and C16, does not activate hTRPA1 expressed in HEK293 cells (29), did not trigger hTRPA1 channel activity, whereas Δ^9-THC produced activation when subsequently applied to the same bilayer (Fig. S6*A*); G_s for Δ^9-THC at +60 mV was 87 ± 7 pS ($n = 5$). This pharmacological profile together with our finding that electrophilic and nonelectrophilic TRPA1 activators are without effect on lipid bilayers in the absence of protein (Fig. S6*B*) support a specific interaction with hTRPA1.

Analysis of single-channel behavior revealed differences in the action of menthol and C16 on hTRPA1 and Δ1–688 hTRPA1 (Fig. 4 and Table S1). At +60 mV, G_s was larger and P_o was smaller for Δ1–688 hTRPA1 than hTRPA1 in the presence of menthol but not C16, whereas at −60 mV, P_o was larger for hTRPA1 than Δ1–688 hTRPA1 in the presence of C16 but not menthol (Fig. 4 *A* and *B* and Table S1). The rectification index (+60/−60 mV) for G_s and P_o showed clear differences in single-channel behavior between hTRPA1 and Δ1–688 hTRPA1 when exposed to nonelectrophilic compounds (Fig. 4*C*). Furthermore, C16 activated the menthol-insensitive chimera *Drosophila* transmembrane segment 5 (dTM5) -hTRPA1 between *D. melanogaster* (dTM5) and hTRPA1 (Fig. S8). Taken together, these findings are consistent with distinct binding sites for menthol and C16 and indicate that the N terminus modifies hTRPA1 channel responses to nonelectrophilic compounds in a voltage-dependent manner.

To further explore the influence of the N terminus on single-channel behavior, we used menthol as an hTRPA1 activator at room temperature. This ligand is assumed to bind to the transmembrane region of the channel protein and therefore, should not interfere with the integrity of the N terminus (35). As revealed by comparing single-channel currents and P_o between hTRPA1 and Δ1–688 hTRPA1 at different test potentials, the presence of the N-terminal ARD increased P_o at positive test potentials, while decreasing P_o at negative test potentials (Fig. 5). In contrast, the presence of the N-terminal ARD decreased G_s at positive test potentials, while leaving G_s unaffected at negative test potentials (Fig. 5).

Discussion

The ability to detect and avoid noxious temperatures is crucial for organism survival, but the underlying mechanisms of this beneficial property may also contribute to thermal allodynia or hypersensitivity, hallmarks of many chronic pain conditions in humans. Understanding the molecular mechanisms behind

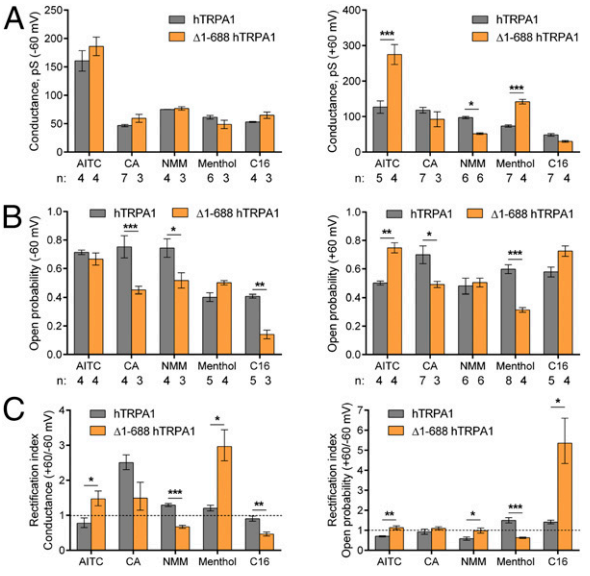

Fig. 4. The N-terminal ARD influences the channel behavior of the purified hTRPA1 when exposed to electrophilic and nonelectrophilic compounds. (*A* and *B*) Bar graphs summarize the mean G_s and P_o values for the TRPA1 activators AITC (100 μM), cinnamaldehyde (CA; 100 μM), NMM (100 μM), menthol (500 μM), and C16 (100 μM) at a test potential of either −60 or +60 mV. Multiple distinct open-channel levels were observed for AITC in Δ1–688 hTRPA1 (Fig. 3*A* and Fig. S5), of which the main levels (O_3 at +60 mV and O_1 at −60 mV) were used for calculations of G_s values. Single-channel currents of purified hTRPA1 and Δ1–688 hTRPA1 inserted into planar lipid bilayers were recorded with the patch-clamp technique in a symmetrical K^+ solution. (*C*) Analysis of the rectification index (+60/−60 mV) using the G_s and P_o data shown in *A* and *B* (Table S1). The dotted line indicates the level where no rectification would occur. Data are represented as means ± SEMs. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate statistically significant differences between the hTRPA1 variants using (*A* and *B*) one-way ANOVA followed by Bonferroni's multiple comparison test or (*C*) a priori contrast analysis with adjustment for the degrees of freedom in the *F* test according to the Welch–Satterthwaite procedure.

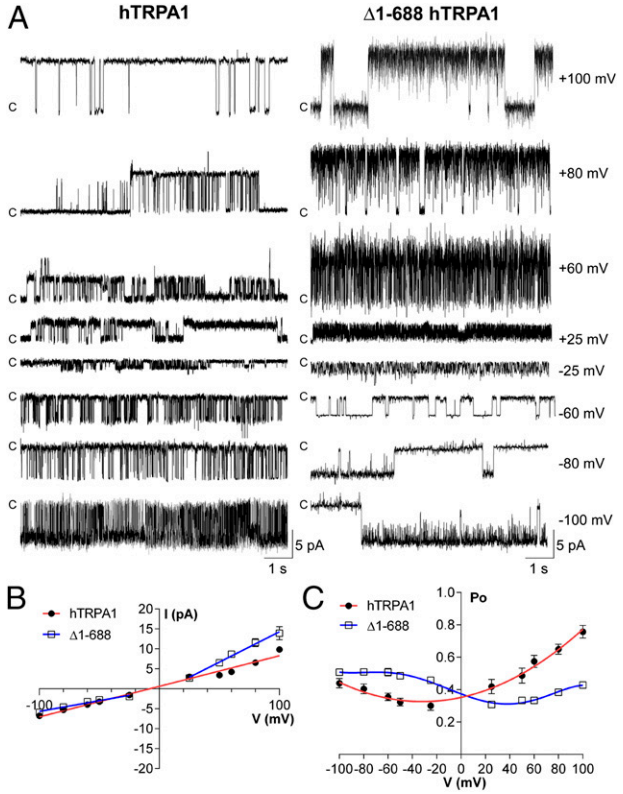

Fig. 5. The N-terminal ARD modifies hTRPA1 activity induced by menthol in a voltage-dependent manner. Purified hTRPA1 and Δ1–688 hTRPA1 were inserted into planar lipid bilayers. (*A*) As shown by representative traces, menthol (500 μM) evoked (*Left*) hTRPA1 and (*Right*) Δ1–688 hTRPA1 single-channel activity (upward deflection shows open-channel state at positive test potential, downward deflection shows open-channel state at negative test potential, and c shows closed-channel state). (*B*) Single-channel current–voltage (I–V) relationship and (*C*) single-channel P_o–voltage (P_o–V) relationship of the hTRPA1 and Δ1–688 hTRPA1 when activated by menthol (500 μM). The calculated mean slope G_s values were 77 ± 4 pS for hTRPA1 and 54 ± 6 pS (−100 to −25 mV) and 152 ± 7 pS (+25 to +100 mV) for Δ1–688 hTRPA1. These values are in good agreement with G_s values obtained at −60 and +60 mV in separate experiments (Fig. 4 and Table S1). Single-channel currents were recorded with the patch-clamp technique in a symmetrical K^+ solution at the various test potentials indicated on the right in *A*. Data are represented as means ± SEMs of four separate experiments. (*B*) $P < 0.01$ (+50 mV) and $P < 0.001$ (+60, +80, and +100 mV), and (*C*) $P < 0.05$ (+25 mV), $P < 0.01$ (−60 and +50 mV), and $P < 0.001$ (−50, −25, +60, +80, and +100 mV) indicate statistically significant differences at each test potential between the hTRPA1 channels using one-way ANOVA followed by Bonferroni's multiple comparison test.

thermosensation is, therefore, of importance from both biological and medical perspectives (1, 39). Several TRP channels have recently been proposed as thermosensors, of which the majority is involved in warm sensation (1–3, 39). However, to define a TRP channel as a thermosensor would require examination of the purified channel protein in a defined membrane environment, and to date, only rat TRPM8 and rat TRPV1 have been shown to be intrinsically thermosensitive proteins (4, 5). Here, we show for the first time, to our knowledge, that the purified hTRPA1 inserted into artificial lipid bilayers is an inherently cold-sensitive protein. Thus, the mammalian TRP channel family consists of an additional cold sensor—TRPA1—that, together with TRPM8, covers noxious to pleasant cold temperatures.

Mutational and chimeric strategies have been used to suggest specific thermosensitive regions and drug binding sites in TRP channels (8, 11, 13, 14, 16, 17, 19, 40). In this study, using the purified N-terminal ARD-deleted hTRPA1 to avoid potential artifacts in TRPA1 function caused by mutations or the creation of xenogeneic channels (7), we clearly show that the cold sensitivity and the binding site for HC030031, an inhibitor of rodent TRPA1 and hTRPA1 (7, 41), are located outside the N-terminal ARD. The search for a specific cold-sensitive region in mammalian TRPA1 channels should, thus, be directed toward the transmembrane and the C-terminal domains of TRPA1.

As shown for cold, electrophilic compounds also evoked robust hTRPA1 activity in the absence of the N-terminal ARD, providing a more complex picture of electrophile activation of hTRPA1 than generally believed. By comparing G_s and P_o values at +60 and −60 mV, we found voltage-dependent differences in channel behavior between the two hTRPA1 variants when exposed to electrophilic compounds. Interestingly, a triple cysteine mutation of the hTRPA1 N-terminal region (hTRPA1-3C) changed the voltage-dependent electrophilic activation of hTRPA1 by *p*-benzoquinone, an acetaminophen (paracetamol) metabolite (28, 29). Furthermore, the N-terminal region may suppress hTRPA1 channel gating, because the response to NMM was intact in the N-terminal ARD-deleted hTRPA1 (this study) but lost in the N-terminal triple cysteine mutant (22), having a greatly reduced capacity to form disulfide bonds (25). The response of hTRPA1 to the nonelectrophilic compounds menthol and C16 also did not require the presence of the N-terminal ARD, which however, modified hTRPA1 channel behavior in a voltage-dependent manner.

Although our data clearly show that electrophilic and nonelectrophilic compounds activate hTRPA1 outside the N-terminal ARD, random protein orientation in the artificial lipid bilayer and the existence of multiple levels of channel opening could complicate the analysis of the voltage-dependent channel behavior. A uniform protein insertion was not confirmed in each single experiment, but separate experiments showed the ability of the biotin derivatives of 2-((biotinoyl)amino)ethyl methanethiosulfonate and maleimide to activate hTRPA1 and the N-terminal ARD-deleted hTRPA1, respectively, only when applied to the bath solution, supporting a consistent asymmetric orientation of both proteins in the lipid bilayers. For comparison of G_s between the hTRPA1 variants at different voltages, we analyzed the current magnitude only for the main open-channel level. Because distinct multiple open-channel levels were frequently observed for AITC in the N-terminal ARD-deleted hTRPA1, G_s values and the rectification index obtained with AITC in hTRPA1 without the N terminus should be interpreted with caution. Interestingly, the hTRPA1 single-channel G_s values for electrophilic and nonelectrophilic compounds vary greatly, although within the wide range of values reported for mammalian TRPA1 (7). Whether this ligand-dependent variation in single-channel G_s is caused by pore dilation and modified by the N terminus warrant additional investigations. Based on our electrophysiological data, it is difficult to provide a simple biophysical fingerprint of hTRPA1, which may not be surprising considering the great variety of chemical interactions between ligands and TRPA1 (7). Nevertheless, our data raise the possibility that the N terminus modifies hTRPA1 channel behavior in a voltage-dependent manner.

The ability of TRPA1 to respond to nonelectrophilic compounds is intriguing but could indicate that endogenous TRPA1 modulators with similar chemical structures or properties exist (7). In this study, we show that menthol and the cannabinoids C16 and Δ^9-THC directly activate hTRPA1 without the need for cytoplasmic (e.g., Ca^{2+}) or cell membrane-associated factors. We found that the menthol-insensitive dTM5–hTRPA1 chimera (35) expressed in HEK293 cells was activated by C16, indicating that menthol and cannabinoids interact with distinct binding sites. Future studies identifying the binding sites for C16 and other cannabinoids may help us to understand nonelectrophilic regulation of TRPA1 and the potential of these binding sites as targets for

analgesics, including nontissue-damaging TRPA1 activators (29). Perhaps such TRPA1 activators can also be used to modulate aging in humans, because the TRPA1 activator AITC extended lifespan in transgenic *Caenorhabditis elegans* expressing hTRPA1 (42).

Although this study clearly shows that hTRPA1 is an inherently cold-activated ion channel, it does not resolve why studies of the expressed hTRPA1 have generated such conflicting findings regarding its cold sensitivity (8–15). However, we know that the regulation of TRPA1 is complex and that the channel sensitivity to ligands is dependent on the cellular context, including the redox state in the cell, the phosphoinositide composition of the cell membrane, and the intracellular activities of proline hydroxylase and protein kinase/phosphatase enzymes (7). Many of these factors are probably dependent on the cell expression system and the experimental conditions, and their future disclosure may pinpoint novel drug targets other than TRPA1 for treatment of clinical conditions characterized by TRPA1-dependent cold allodynia or hypersensitivity (7).

In conclusion, we show that hTRPA1 is an intrinsically cold-activated ion channel that constitutes a molecular thermosensor explaining TRPA1-dependent behavioral responses to noxious cold (7). The N-terminal ARD is not needed for activation of hTRPA1 by cold, electrophiles, and nonelectrophilic compounds or inhibition of hTRPA1 by HC030031. However, the N-terminal ARD may modify hTRPA1 channel behavior in a voltage-dependent manner. Targeting chemical interaction sites outside the TRPA1 N-terminal ARD may offer possibilities to fine tune TRPA1-based drug therapies [e.g., for treatment of pain associated with cold hypersensitivity (7) and cardiovascular disease (32)].

Materials and Methods

P. pastoris cell membranes, containing hTRPA1 or Δ1–688 hTRPA1, were collected and solubilized with fos-choline-14 detergent (Anatrace). Both proteins were purified using Ni-nitrilotriacetic acid affinity chromatography; Δ1–688 hTRPA1 was also subjected to size exclusion chromatography. Purified hTRPA1 (after Ni-nitrilotriacetic acid purification) and Δ1–688 hTRPA1 (tetrameric fraction from size exclusion chromatography) were reconstituted into preformed planar lipid bilayers or giant unilamellar vesicles. The vesicles were formed by electroformation using Vesicle Prep Pro Station (Nanion Technologies). Lipid stock was made by dissolving 10 mM 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine:cholesterol (9:1) in trichloromethane. All lipid bilayer recordings were done on a Port-a-Patch planar patch-clamp system (Nanion Technologies) in a symmetrical K^+ solution at room temperature (22 °C) or below. Group data are expressed as means ± SEMs from *n* independent experiments (lipid bilayers or cell transfections). *SI Materials and Methods* has full details.

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