JBC RESEARCH ARTICLE

Selective activation of TRPA1 ion channels by nitrobenzene skin sensitizers DNFB and DNCB

Received for publication, August 7, 2021, and in revised form, December 17, 2021 Published, Papers in Press, December 30, 2021, https://doi.org/10.1016/j.jbc.2021.101555

Han Wu[1], Canyang Niu[1], Yaxuan Qu[1], Xiaoying Sun[1,2,*], and KeWei Wang[1,2,*]
From the [1]Department of Pharmacology, School of Pharmacy, and [2]Institute of Innovative Drugs, Qingdao University, Qingdao, China

Edited by Mike Shipston

2, 4-dinitrofluorobenzene (DNFB) and 2, 4-dinitrochlorobenzene (DNCB) are well known as skin sensitizers that can cause dermatitis. DNFB has shown to more potently sensitize skin; however, how DNFB and DNCB cause skin inflammation at a molecular level and why this difference in their sensitization ability is observed remain unknown. In this study, we aimed to identify the molecular targets and mechanisms on which DNFB and DNCB act. We used a fluorescent calcium imaging plate reader in an initial screening assay before patch-clamp recordings for validation. Molecular docking in combination with site-directed mutagenesis was then carried out to investigate DNFB and DNCB binding sites in the TRPA1 ion channel that may be selectively activated by these tow sensitizers. We found that DNFB and DNCB selectively activated TRPA1 channel with EC_{50} values of 2.3 ± 0.7 μM and 42.4 ± 20.9 μM, respectively. Single-channel recordings revealed that DNFB and DNCB increase the probability of channel opening and act on three residues (C621, E625, and Y658) critical for TRPA1 activation. Our findings may not only help explain the molecular mechanism underlying the dermatitis and pruritus caused by chemicals such as DNFB and DNCB, but also provide a molecular tool 7.5-fold more potent than the current TRPA1 activator allyl isothiocyanate (AITC) used for investigating TRPA1 channel pharmacology and pathology.

Nitrobenzene compounds such as 2, 4-dinitrofluorobenzene (DNFB) and 2, 4-dinitrochlorobenzene (DNCB) have long been known to cause skin irritation and sensitization, and they are used for establishment of skin inflammatory models in rodents (1–3). Topical applications of DNFB and DNCB can mediate contact hypersensitivity and induce allergic contact dermatitis (ACD) (4, 5). DNFB, commonly called Sanger's reagent used for protein sequencing, also causes colitis in mice (6, 7). Chemical DNFB as an allergen additionally elicits immune reactions by inducing mast cell degranulation and releases of histamine (3), interleukin-1 (IL-1) and prostaglandin E2 (PGE2) (8). It is of interest that both DNFB and DNCB share similar structures, but the skin is more sensitive to DNFB-induced irritation than DNCB (9, 10).

TRPA1 is a temperature-sensitive and calcium-permeable cation channel with a fourfold symmetry around the central ion conductance pathway and proximal cytoplasmic regions involved in electrophile detection (11–13). TRPA1 serves as a sensor for chemical irritants, such as mustard oil (MO) (14), acrolein, cinnamaldehyde (CA) (15) and allyl isothiocyanate (AITC) (16). TRPA1 is also activated by harmful electrophiles that are recognized by the channel *via* covalent modifications of specific cysteine residues located in the cytoplasmic C domain (17). Nitrobenzene compound 2, 4, 6-trinitrobenzene sulfonic acid (TNBS) causes colitis by activating TRPA1 (18).

A growing number of evidences indicate that activation of transient receptor potential ankryn 1 (TRPA1) ion channels is involved in skin inflammation (19). TRPA1 is robustly expressed in primary sensory nerve terminals (20) and numerous nonneuronal cell types of the skin (21) and CD4+ T lymphocytes that play a central role in the adaptive immune response (22, 23). Activation of TRPA1 by icilin in keratinocytes leads to an elevation of proinflammatory cytokine interleukin-1 (IL-1), which suggests a role of TRPA1 in promoting cutaneous inflammation (21). Pharmacological inhibition or deficiency of TRPA1 alleviates inflammation of atopic dermatitis (AD) (24, 25). Conversely, specific activation of TRPA1 by agonist MO leads to severe colitis, which is inhibited by HC-030031 or reduced in $TRPA1^{-/-}$ mice (18).

DNFB or DNCB induces Ca^{2+} flux response in HEK293 cells expressing TRPA1 (26, 27). Although nitrobenzene compounds have been shown to cause calcium influx through TRPA1 channels, activation on TRPA1 with different sensitivities at a molecular level remains unknown.

In this study, we show that chemicals DNFB and DNCB specifically activate TRPA1 channels through binding to three key residues critical for electrophile irritant sensing in the channel coupling domain using patch-clamp recordings, site-directed mutagenesis, and molecular docking. Our findings not only help explain the mechanistic insights into nitrobenzene compounds causing contact skin irritation or dermatitis, but also provide a molecular tool for further understanding of TRPA1 channel pharmacology in skin-related diseases.

* For correspondence: KeWei Wang, wangkw@qdu.edu.cn; Xiaoying Sun, xiaoyingsun@qdu.edu.cn.

Results

Selective activation of TRPA1 channels by chemicals DNFB and DNCB in calcium fluorescent assay and patch-clamp recordings

To test the effect of DNFB (Fig. 1*A*, left) and DNCB (Fig. 1*A*, right) on TRPA1 and other thermo-TRPs, we started using the calcium fluorescent imaging of HEK293 cells expressing several TRP channels in FlexStation3 microplate reader assay. Adding different concentrations (3–300 μM) of DNFB and DNCB caused a dose-dependent increase of intracellular Ca^{2+} level, as compared with TRPA1 agonist AITC (300 μM), which was used as a positive control (Fig. 1*B*). In contrast, there was a lack of detectable signals from TRPV1 (Fig. 1*C*), TRPV3 (Fig. 1*D*) and TRPV4 (Fig. 1*E*) channels in response to DNFB and DNCB in the same range of concentration (3–300 μM). These results suggest that DNFB and DNCB selectively activate TRPA1 over the other tested members of the TRP channel family.

To confirm the activation of TRPA1 channels by DNFB, we recorded the whole-cell currents of TRPA1, TRPV1, TRPV3, and TRPV4 channels expressed in HEK293 cells in the presence of 5 μM DNFB and 300 μM DNCB. As shown in Figure 2, DNFB-mediated and DNCB-mediated activations of TRPA1 currents were blocked by the TRPA1 inhibitor A-967079 (A-96) at 10 μM (Fig. 2*A*). In contrast, TRPV1 (Fig. 2*B*), TRPV3 (Fig. 2*C*), and TRPV4 (Fig. 2*D*) channels were not responsive to DNFB (5 μM) and DNCB (300 μM), although these channels were activated by their agonists such as 1 μM capsaicin for TRPV1, 50 μM 2-APB for TRPV3 and 0.1 μM GSK101 for TRPV4. Consistent with the data from calcium fluorescent assay, these results confirm that DNFB and DNCB are selective agonists of TRPA1.

To determine the potency of DNFB and DNCB on TRPA1 activation, we made the whole-cell recordings of TRPA1 currents in the presence of different concentration of DNFB (0.1–100 μM) or DNCB (3–3000 μM) and observed a dose-dependent activation of TRPA1 currents with EC_{50} values of 2.3 ± 0.7 μM (DNFB) (Fig. 3, *A* and *D*) and 42.4 ± 20.9 μM (DNCB) (Fig. 3, *B* and *D*). As a control, TRPA1 agonist AITC (3–3000 μM) also elicited a dose-dependent activation of the channel current with an EC_{50} value of 17.8 ± 12.3 μM (Fig. 3, *C* and *D*), which is consistent with a previous report (28). These results indicate that DNFB activates TRPA1 currents in dose-dependent manner with approximately 7.5-fold potency better than AITC.

Direct targeting of single TRPA1 channels by DNFB and DNCB

To further confirm DNFB and DNCB directly acting on single TRPA1 channels, we performed the single-channel recordings in an inside-out patch configuration. As a control, application of TRPA1 agonist 300 μM AITC increased the channel opening levels from level 1 to level 2 with the single channel conductance about 148.37 ± 0.38 pS and also the channel open probability to 0.26 ± 0.27 from 0.01 ± 0.01 of basal control (n = 9–11, $p < 0.05$) (Fig. 4, *A–C*). Adding DNFB at 300 μM resulted in a significant increase of the channel opening from level 1 to level 4 with single channel conductance about 153.5 ± 0.05 pS and channel open probability to 0.33 ± 0.20 from 0.01 ± 0.01 (n = 6–14, $p < 0.001$) (Fig. 4, *A–C*). Similarly, adding DNCB at 300 μM resulted in a significant increase of the channel opening from level 1 to level 4 with single channel conductance about 148.37 ± 0.1 pS and channel open probability to 0.32 ± 0.27 from 0.01 ± 0.01 (n = 6–14, $p < 0.001$) (Fig. 4, *A–C*). Conversely, adding A-96, a selective antagonist of TRPA1, reduced the channel open probability to 0.01 ± 0.02 from 0.32 ± 0.27 (Fig. 4, *A* and *C*). DNFB caused higher open frequency than DNCB or AITC (Fig. 4*D*). These results show that DNFB activates TRPA1 currents by directly acting on single channels through increase of channel open frequency.

Identification of TRPA1 channel residues critical for DNFB and DNCB binding

To identify residues critical for DNFB and DNCB binding to TRPA1, we carried out the molecular docking of DNFB and DNCB onto the cryo-EM structure of human TRPA1 (PDB: 3J9P) using the Rosetta modeling. As shown in Figure 5, *A* and *B*, the docking reveals that DNFB and DNCB are confined near the short helixes (H2, H4, and H5) in the coupling domain that is involved in electrophile irritant sensing (17). The orthonitro groups of DNFB and DNCB are both bound through hydrogen bonds to Cys621 at the end of short helix H2 and Tyr658 in the loop between β1.2 and H4 (Fig. 5, *C* and *D*). In particular, in the binding mode of DNFB, the fluorine atoms unique in DNFB interact with Thr684 through halogen bond, thus fixing the orientation of paranitro pointing to H2, which causes the hydrogen bond between the paranitro and Glu625, the π-Alkyl interaction with Ala688, and the π-sulfur interaction with Cys621 to maintain the conformation and position of the ligand (Fig. 5*C*). In the binding mode of DNCB, the absence of fluorine leads to the loss of these intermolecular interactions that help to stabilize the conformation (Fig. 5, *C–E*). There are also some aromatic amino acids, such as Tyr681, interacting with DNFB through weak van der Waals forces, which appears to be inessential for the binding of DNFB to TRPA1.

To confirm that residues Cys621 and Tyr658 in hTRPA1 are required for the DNFB and DNCB binding, we introduced glycine or alanine mutations of C621G, Y658A, E681A, and T684A. As shown in Figure 6, application of 10 μM DNFB or 300 μM DNCB was unable to elicit detectable currents of TRPA1C621G mutant (Fig. 6, *B* and *H*) and TRPA1Y658A mutant (Fig. 6, *D* and *I*). In contrast, DNFB at 10 μM elicited robust E681A currents (Fig. 6*E*). Interestingly, the 10 μM DNFB could not evoke the current of TRPA1E625A, suggesting that Glu625 is also essential for activation of TRPA1 by DNFB (Fig. 6*C*). As shown in Fig. 6*F*, 10 μM DNFB could not evoke T684A current, but DNCB at 300 μM was still able to activate T684A currents. The residue T684 that forms halogen bonds with fluorine is important for DNFB activity, but not DNCB that cannot form halogen bonds with T684. This difference may explain the discrepancy in sensitization ability

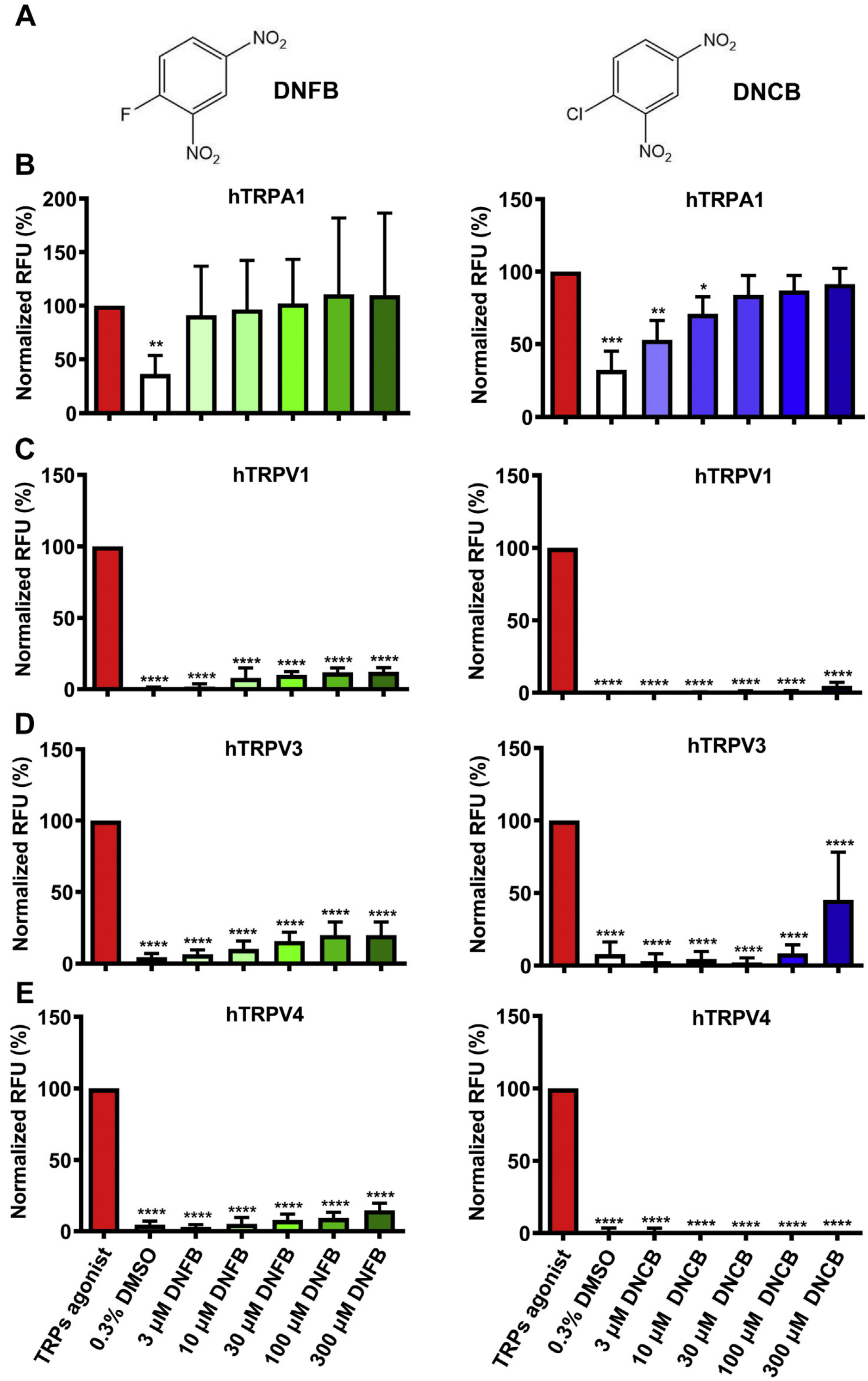

Figure 1. Activation of TRPA1 channels by chemical DNFB and DNCB in calcium fluorescent assay. *A*, chemical structures of DNFB and DNCB. *B–E*, normalized increase of intracellular Ca^{2+} levels in response to different concentrations of DNFB or DNCB (3–300 μM) compared with channel agonists of AITC (300 μM) for TRPA1 (*B*), capsaicin (1 μM) for TRPV1 (*C*), 2-APB (200 μM) for TRPV3 (*D*), and GSK101 (0.1 μM) for TRPV4 (*E*) (n = 6, six parallel experiments). The negative control is 0.3% DMSO. Data are shown as the mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, by one-way ANOVA with post-hoc Sidak's multiple comparison test. 2-APB, 2-aminoethoxydiphenyl borate; AITC, allyl isothiocyanate; Cap, capsaicin; DNCB, 2, 4-dinitrochlorobenzene; DNFB, 2, 4-dinitrofluorobenzene; GSK101, GSK1016790A; TRP, transient receptor potential.

between DNFB and DNCB. These results demonstrate that the three residues C621, E625, and Y658 are critical for DNFB and DNCB binding to the TRPA1 channel coupling domain and sensing electrophile irritants.

Discussion

In this study, we identified TRPA1 as a molecular target for irritant chemicals nitrobenzene DNFB and DNCB that can selectively activate the channels. DNFB is about 7.5 times more

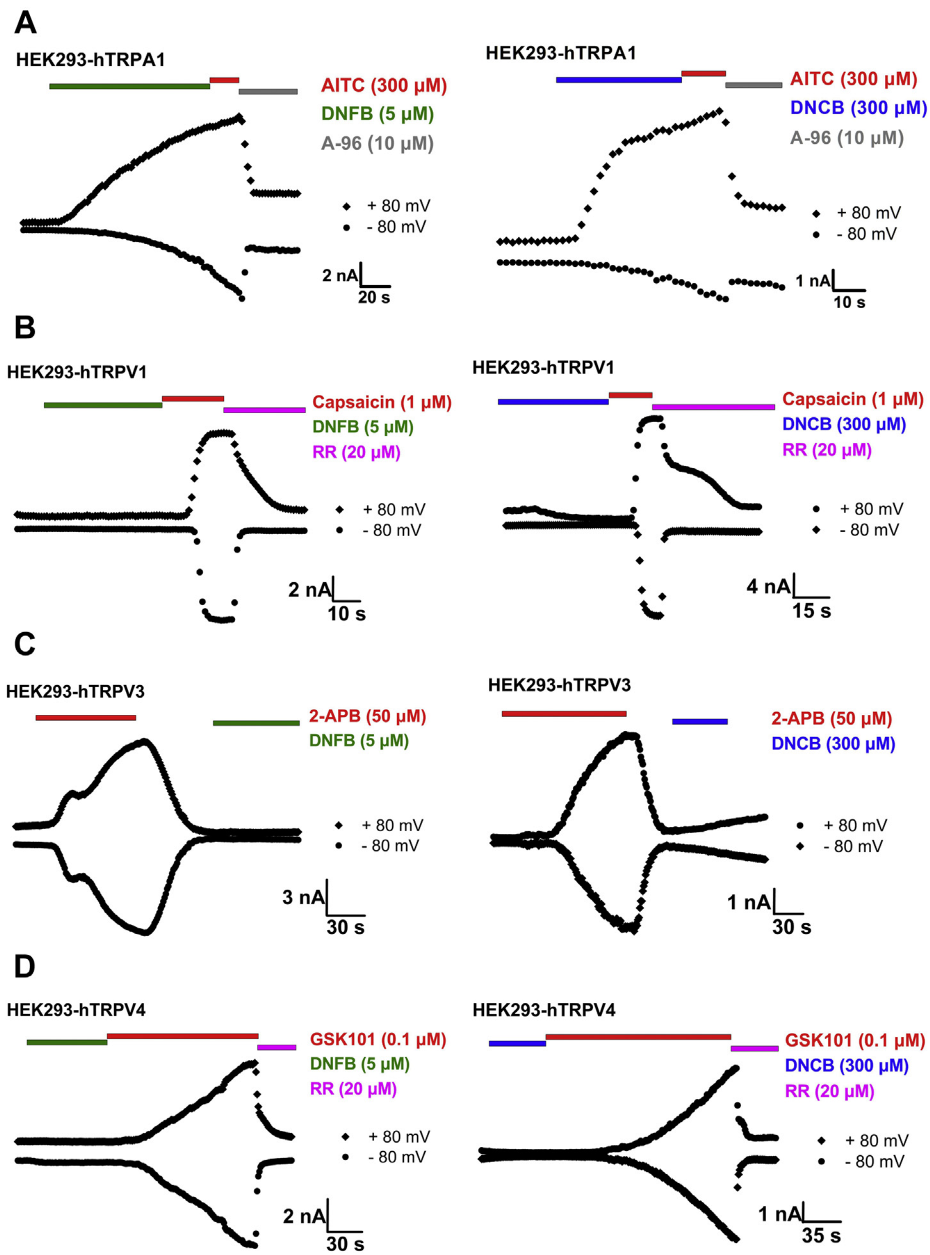

Figure 2. Selectivity activation of TRPA1 currents by DNFB and DNCB in whole-cell recordings. *A*, whole-cell TRPA1 currents were activated by 5 μM DNFB (*green bar*), 300 μM DNCB (*blue bar*), or 300 μM AITC (*red bar*) before inhibition by TRPA1 inhibitor 20 μM A-96 (*gray bar*) (n = 5). *B*, TRPV1 currents in response to 5 μM DNFB (*green bar*) and 300 μM DNCB (*blue bar*) or 1 μM TRPV1 agonist capsaicin (*red bar*) before inhibition by 20 μM RR, a nonselective inhibitor of TRP channels (*pink bar*), DNFB and DNCB displaying no activation on TRPV1 (n = 5). *C*, TRPV3 currents evoked by 50 μM TRPV3 agonist 2-APB (*red bar*) or 5 μM DNFB (*green bar*) and 300 μM DNCB (*blue bar*), DNFB and DNCB showing no activation (n = 5). *D*, TRPV4 currents were activated by 5 μM DNFB (*green bar*) and 300 μM DNCB (*blue bar*) or 1 μM TRPV4 agonist 0.1 μM GSK101 (*red bar*) before inhibition by 20 μM RR (*pink bar*), DNFB and DNCB showing no activation (n = 5). (A-96, A-967079). AITC, allyl isothiocyanate; DNCB, 2, 4-dinitrochlorobenzene; DNFB, 2, 4-dinitrofluorobenzene.

potent than AITC that is one of the most commonly used agonist of TRPA1. At the single-channel level, DNFB and DNCB can increase the channel open probability through binding to three key residues C621, E625, and Y658 in the channel coupling domain that functions to sense electrophile irritants (17). Our identification of DNFB and DNCB as selective TRPA1 agonists not only provides a powerful tool for further understanding of the channel pharmacology and pathology, but also demonstrates how nitrobenzene compounds activate TRPA1 that is a potential therapeutic target for allergic contact dermatitis (24).

DNFB has long been reported to cause skin sensitization that is dependent on immune activation (29), leaving the underlying molecular mechanism largely unaddressed. DNFB as an allergen binds to self-protein in the dermis, which produces antibodies and produces inflammation (1, 3). It is of interest to note that DNFB-induced allergic response is lack of stronger inflammatory response on the second exposure of DNFB

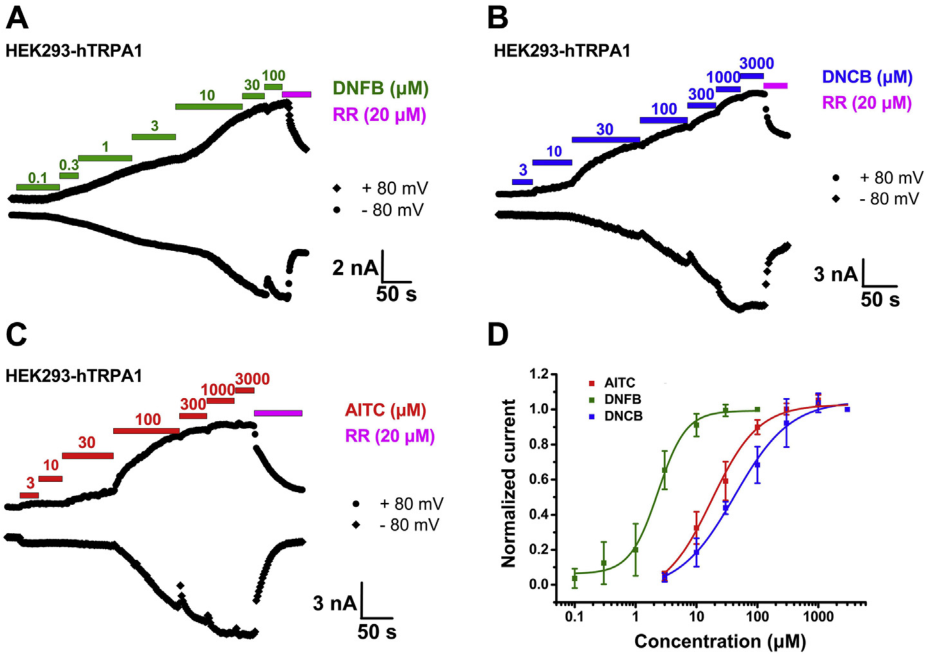

Figure 3. Concentration-dependent activation of TRPA1 currents by DNFB, DNCB and AITC. Concentration-dependent activation of TRPA1 currents by different concentrations of DNFB (0.1 μM to 100 μM) (*A*), DNCB (3 μM to 3000 μM) (*B*), and AITC (3 μM to 3000 μM) (*C*). *D*, fitting curves by Hill equation for comparing concentration-dependent activation of hTRPA1 currents by DNFB with EC_{50} value of 2.3 ± 0.7 μM (n = 6, *green*), DNCB with EC_{50} value of 42.4 ± 20.9 μM (n = 6, *blue*), and AITC with EC_{50} value of 17.8 ± 12.3 μM (n = 6, *red*), respectively. AITC, allyl isothiocyanate; DNCB, 2, 4-dinitrochlorobenzene; DNFB, 2, 4-dinitrofluorobenzene.

hapten challenge that cannot exacerbate the inflammatory response (30, 31). These observations suggest that DNFB and DNCB may also cause skin inflammation through other mechanisms, and there might be a specific target responsible for DNFB-induced skin sensitization and inflammation. TRPA1 as a sensor of chemical irritants and transducer of allergen responses can cause multiple types of inflammation. TRPA1 is expressed in sensory nerve fibers in the skin and also cutaneous keratinocytes, mast cells, and endothelial cells, and it is involved in chronic pruritus (1, 32). TRPA1 is activated by inflammatory agents from nonneuronal cells and is also required for the release of inflammatory neuropeptides and neurogenic inflammation, which serves as a detector and instigator of inflammatory agents (33). Irritants, such as acrolein and crotonaldehyde, can directly activate TRPA1 stimulating vagal neurons and inducing airway plasma extravasation (34, 35). In addition, it has been reported that skin inflammation induced by nitrobenzene derivative in mice can be effectively improved by TRPA1 knockout and pharmacological blockade (25, 27), which further suggests that nitrobenzene can directly cause dermatitis by activating TRPA1 in mice.

Previous studies have shown that several TRP channels, such as TRPA1, TRPV1, TRPV3, and TRPV4, are implicated in skin physiology and pathology including skin inflammation (19, 36–40). Our selectivity evaluations indicate that DNFB and DNCB specifically active TRPA1 that is featured in a unique electrophilic sensing pocket located in the channel C-terminal coupling domain (17). The electrophilic sensing pocket is highly rich in reactive cysteines such as C621 and C655 (17, 41–43), and the electrophile sensing region is surrounded by a number of nucleophilic aromatic amino acids for facilitating the entry of electrophiles (17). In contrast, the cryo-electron microscopy structures of TRPV1 (44), TRPV3 (45) and TRPV4 (46) reveal that these three channels lack the electrophile sensing coupling domain that serves to sense electrophilic irritants (12), which can explain the selective activation of TRPA1 by chemicals DNFB and DNCB (27).

Chemicals DNFB and DNCB as electrophilic reagents bind to TRPA1 through noncovalent hydrogen bonds with C621 and Y658 residues and a unique halogen bond between the fluorine of DNFB and T684 residue (Fig. 5), consistent with the observation that a noncovalent ligand binding confers a biased agonism of TRPA1 channels (47). The noncovalent binding agonists DNFB and DNCB that activate TRPA1 without causing the channel desensitization are widely used for the model establishment of persistent dermatitis (29), which is unlike agonist AITC that covalently binds to and induces TRPA1 channel desensitization (47, 48). Other noncovalent TRPA1 agonists such as peptide scorpion toxin (WaTx) or small-molecule GNE551 can activate TRPA1 in a slow kinetics fashion without inducing channel desensitization and cause persistent pain (47, 49). Noncovalent agents are nonreactive to cytosolic abundant nucleophiles (such as glutathione) and are expected to sustain their concentration for longer time, thus leading to more persistent activation of TRPA1 (47). In contrast, covalent agents are highly reactive and are not stable in a cytosolic environment containing high concentrations of nucleophiles (47). Covalent TRPA1 agonists such as benzoquinone, JT010, and AITC, covalently binding to the cysteine residues of the electrophilic

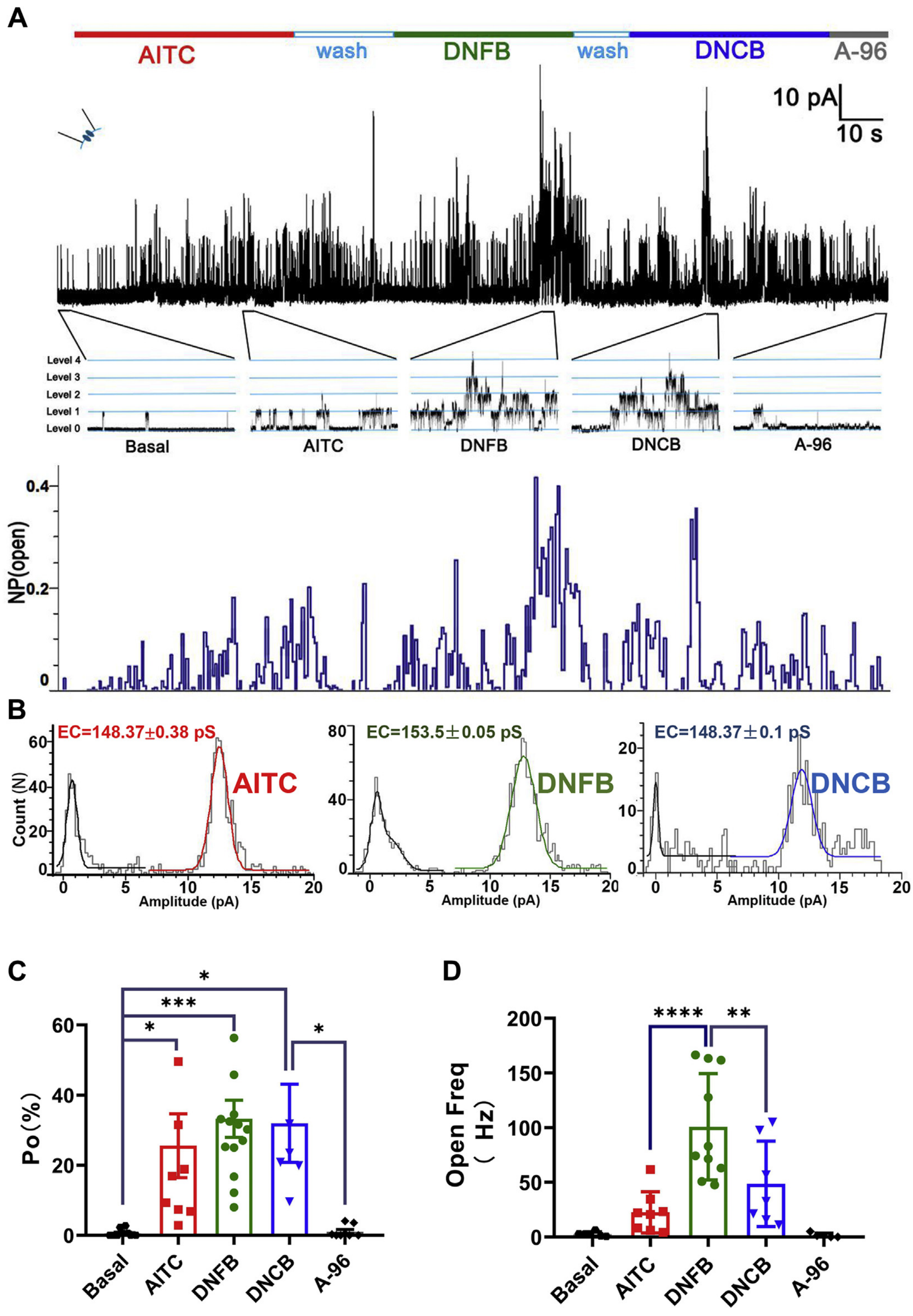

Figure 4. Increase of single-channel open probability of TRPA1 by DNFB, DNCB, and AITC. *A*, single-channel recordings in inside-out patches excised from HEK293-TRPA1 cells held at +80 mV. Different compounds of 300 μM AITC (*red bar*), 300 μM DNFB (*green bar*), 300 μM DNCB (*blue bar*), and 10 μM A-96 (*gray bar*) were added and indicated in their color bars above the raw current traces (*upper panel*), an expanded timescale (*middle panel*), and NPo in 500-ms bins (*bottom panel*). *B*, conductance and amplitude histograms of single-channel openings recorded at +80 mV were fitted with Gaussians functions (basal-*black line*, AITC-*red line*, DNFB-*green line*, DNCB-*blue*). *C* and *D*, summary for calculated mean of P_O (open probability) values and open frequency in the presence of 300 μM AITC, 300 μM DNFB, 300 μM DNCB, and 10 μM A-96 (n = 6–14). Data are shown as the mean ± SD. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001, by one-way ANOVA with post-hoc Sidak's multiple comparison test. AITC, allyl isothiocyanate; DNCB, 2, 4-dinitrochlorobenzene; DNFB, 2, 4-dinitrofluorobenzene.

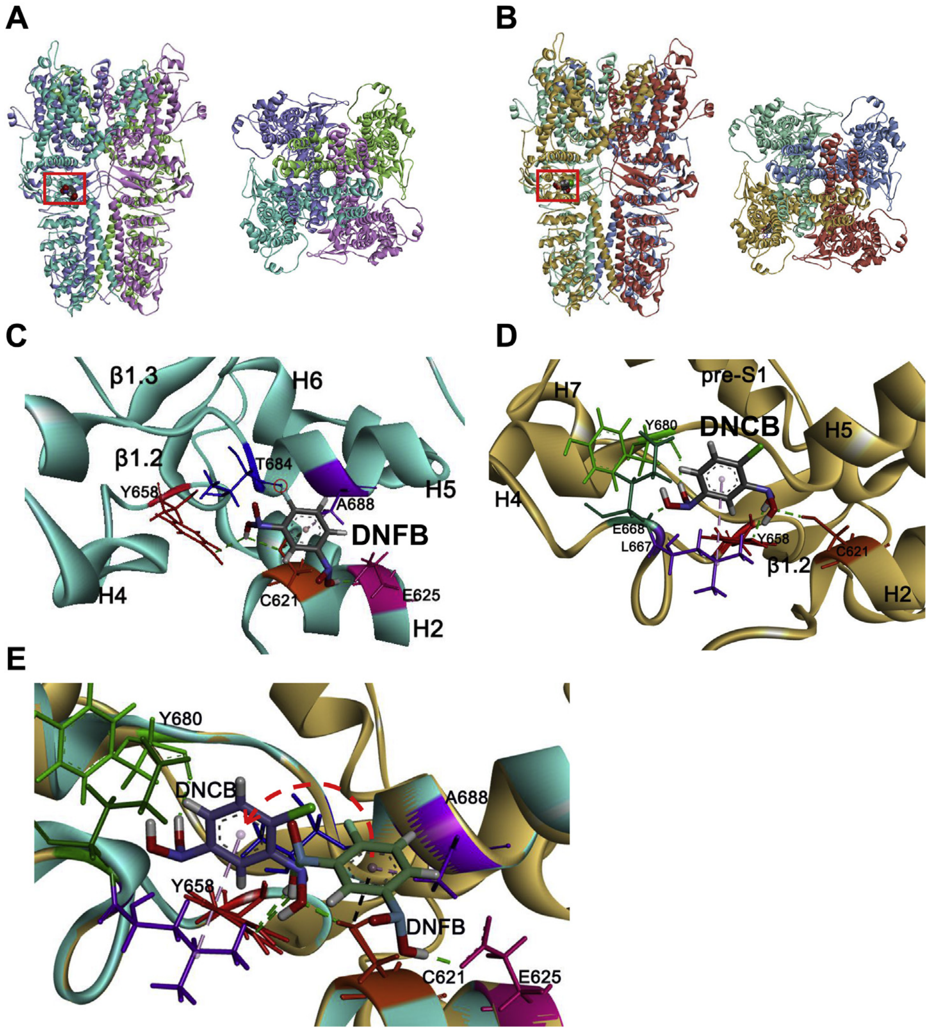

Figure 5. The residues Cys621, Glu625, and Tyr 658 in the channel coupling domain critical for DNFB and DNCB binding. *A* and *B*, representative bound conformations of DNFB (*A*) and DNCB (*B*) confined to the pocket consisting of three short helixes (H2, H4, and H5) in the coupling domain in the side view (*left*) and the top-down view (*right*). *C* and *D*, local view of DNFB or DNCB interacting with a TRPA1 subunit from hydrogen bonds (*green*), π-Alkyl (*pink*), and π-sulfur (*black*) are shown as *dotted lines*. Halogen bond is *circled* in *red*. *E*, comparison of DNFB (*right*) and DNCB (*left*) binding sites. DNCB, 2, 4-dinitrochlorobenzene; DNFB, 2, 4-dinitrofluorobenzene.

sensing domain of TRPA1, can produce TRPA1 desensitization and deactivation and only cause acute pain (17, 50, 51). We envision that higher open frequency may cause more stable and longer channel openings than short openings caused by AITC, which is consistent with the observation for the channel rapid desensitization caused by AITC. We also made an effort in docking DNCB, the derivative of DNFB, into the same electrophilic sensing pocket, revealing a similar noncovalent binding through hydrogen bonds without the halogen bond to T684. DNCB, however, is about 16 times less potent that DNFB, thus explaining that DNCB-mediated skin sensitization is much milder than DNFB (1, 4).

In summary, the nitrobenzene skin sensitizers DNFB and DNCB were found to selectively activate TRPA1 channels through binding to the channel irritant sensing domain. DNFB can serve as a molecular tool for better understanding of TRPA1 pharmacology and pathology. In addition, pharmacological inhibition of TRPA1 channel may hold a promise for therapy of dermatitis.

Experimental procedures

Reagents and compounds

Compounds DNFB (MW: 186.1), DNCB (MW: 202.55), 2-aminoethoxydiphenyl borate (2-APB), capsaicin (Cap), ruthenium red (RR), GSK1016790A (GSK101), A-967079 (A-96), and allyl isothiocyanate (AITC) were purchased from Sigma-Aldrich. RR was made as stock solutions in before use internal solution. Other compounds were made as stock solutions in DMSO before use. Compounds used for the measurement of intracellular fluorescent calcium were diluted in the Hanks' balanced salt solution (HBSS). Compounds were diluted in perfusion solution for patch-clamp recordings.

Cell culture and transfection

The human embryonic kidney cells (HEK293) were cultured in Dulbecco's minimal essential medium (DMEM) with supplement of 10% of fetal bovine serum (FBS) at 37 °C with 5%

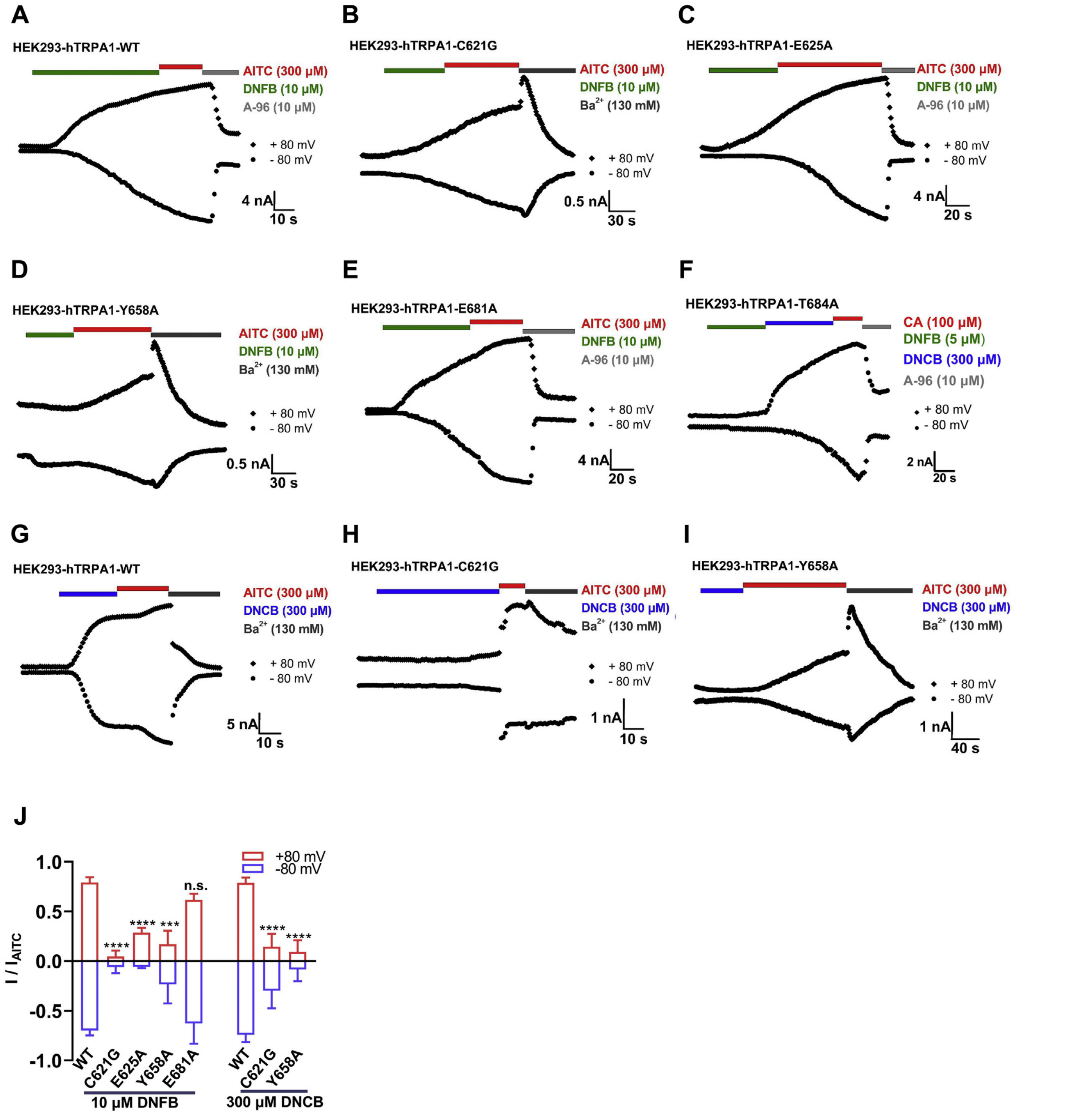

Figure 6. Three residues Cys621, Glu625, and Tyr658 critical for TRPA1 activation by nitrobenzenes. Representative current traces of wild-type human TRPA1 (*A*), C621G (*B*), E625A (*C*), Y658A (*D*) and E681A (*E*) mutants expressed in HEK293 cells in response to DNFB or AITC. *F*, the current traces of T684A mutant expressed in HEK293 cells in responses to DNFB, DNCB, and cinnamaldehyde (CA). *G–I*, representative current traces of wild type human TRPA1 (*G*), C621G (*H*) and C658A (*I*) mutants expressed in HEK293 cells in response to DNCB and AITC. *J*, summary for normalized WT TRPA1 or mutant channel currents activation by 10 μM DNFB, 300 μM DNCB, and 300 μM AITC, showing the ratio of DNFB current to AITC current (n = 5) (the current in 60-s application of DNFB or DNCB and the strongest current activated by AITC were recorded). Data are shown as the mean ± SD. ***$p < 0.001$, ****$p < 0.0001$, n.s., not significant, by one-way ANOVA with post-hoc Sidak's multiple comparison test. DNCB, 2, 4-dinitrochlorobenzene; DNFB, 2, 4-dinitrofluorobenzene.

CO_2. HEK293 cells were seeded in a 6-well plate for intracellular calcium measurement. For whole-cell patch-clamp recordings, cells were cultured on glass coverslips. The HEK293 cells were transiently transfected in a 350-mm Petri dish or a 350-mm well on a 6-well plate with 1600 ng of cDNA plasmid encoding wild-type or mutant human TRPA1 and other TRP channels, using Lipofectamine 2000 (Invitrogen). The cells were used after 24 to 48 h transfection. Human TRPA1 plasmids (Gene ID: 8989) including wild-type or mutants were verified by DNA sequencing.

Measurement of intracellular calcium in fluorescent imaging plate reader FlexStation 3 assay

The Cal-520TM PBX Calcium Assay Kit (AAT Bioquest) was used to detect the change of intracellular calcium in a population of cells with Multi-Mode Microplate Reader in FlexStation 3 assay (Molecular Devices). HEK293 cells were transiently transfected with cDNAs of TRP channels before being seeded at a density of ∼40,000 cells per well in a 96-well black-walled plate (Thermo Fisher Scientific) for an overnight culture at 37 °C with 5% CO_2. Cells were incubated with the calcium fluorescent dye provided in the Cal-520TM PBX Calcium Assay Kit at 37 °C for 1.5 h, and the values of relative fluorescence unit (RFU) were measured by FlexStation3 under the wavelength at 485 nm (excitation) and 515 nm (emission) with an interval of 1.6 s (52).

Patch-clamp recordings

All patch-clamp recordings in two different configurations of macroscopic whole-cell and microscopic inside-out patch clamp recordings were conducted after 12 to 24 h transfection using an EPC10 amplifier powered by PatchMaster software (HEKA) at room temperature (23–25 °C). The borosilicate glass pipettes were pulled using a DMZ universal electrode puller (Zeitz-Instruments GmbH) with the resistances of 3 to 5 MΩ (whole-cell) or 5 to 7 MΩ (inside-out) when filled with a solution containing (in mM) (1): 150 NaCl, 2 EGTA, 10 HEPES, 1 InsP6 (pH 7.2, adjusted with NaOH) for human-TRPA1 (inside-out) (2); 130 NaCl, 0.2 EGTA, 3 HEPES (pH 7.2, adjusted with NaOH) for others. External solution was prepared ditto to internal solution. Currents were recorded at a potential ramp from −100 mV to + 100 mV for 1000 ms with 1000-ms intervals (whole-cell) or a persistent potential of + 80 mV (inside-out).

Molecular docking

The cryo-EM structures of hTRPA1 (Protein Data Bank (PDB) code: 3J9P) were selected as the docking model. The binding pockets were determined according to the reported TRPA1 agonist binding site (17) and the Glide method. The conformation was conducted according to the result score using Rosetta software with default parameters. Binding poses of DNFB and DNCB with top ten scores were considered as possible binding modes with hTRPA1.

Site-directed mutagenesis

The Mut Express II rapid mutagenization kit was used to generate site-directed mutations in TRPA1 (Gene ID: 8989). All mutants were verified by autosequencing to ensure the correct generation of mutations.

Data analysis

All data are expressed with the mean ± SD. Statistical significance was calculated by one-way ANOVA with post-hoc Sidak's multiple comparison test using GraphPad Prism 8.0 and Origin 9 software. A value of $p < 0.05$ is considered to be statistically significant.

Data availability

All data are contained within the manuscript.

Acknowledgments—We are grateful to Drs Fan Yang, Xiaowen Tang and Mr Heng Zhang for assistance and discussion in the molecular docking. This work was supported by National Natural Science Foundation of China (81903734 and 81973299) and the Ministry of Science and Technology of China (2018ZX09711001-004-006).

Author contributions—X. S. and K. W. W. conceptualization; H. W., Y. Q., and X. S. formal analysis; K. W. W. funding acquisition; H. W. and X. S. methodology; K. W. W. project administration; X. S. and K. W. W. resources; K. W. W. supervision; H. W. validation; H. W. visualization; H. W., Y. Q., X. S., and K. W. W. writing—original draft; K. W. W. writing—review and editing.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: 2-APB, 2-aminoethoxydiphenyl borate; A-96, A-967079; ACD, allergic contact dermatitis; AD, atopic dermatitis; AITC, allyl isothiocyanate; ANOVA, one-way analysis of variance; CA, cinnamaldehyde; Cap, capsaicin; cryo-EM, cryoelectron microscopy; DMEM, Dulbecco's modified Eagle's medium; DNCB, 2, 4-dinitrochlorobenzene; DNFB, 2, 4-dinitrofluorobenzene; FBS, fetal bovine serum; GFP, Green fluorescent protein; GSK101, GSK1016790A; HBSS, Hanks' balanced salt solution; HEK293, human embryonic kidney 293; IL-1, interleukin-1; MO, mustard oil; PDB, Protein Data Bank; PGE2, Prostaglandin E2; RFU, relative fluorescence unit; RR, ruthenium red; thermo-TRPs, thermal transient receptor potentials; TRP, transient receptor potential; TRPA1, TRP ankyrin 1; TRPV, TRP vanilloid.

References

1. Gouin, O., L'Herondelle, K., Lebonvallet, N., Le Gall-Ianotto, C., Sakka, M., Buhé, V., Plée-Gautier, E., Carré, J.-L., Lefeuvre, L., Misery, L., and Le Garrec, R. (2017) TRPV1 and TRPA1 in cutaneous neurogenic and chronic inflammation: Pro-inflammatory response induced by their activation and their sensitization. *Protein Cell* **8**, 644–661
2. Bourane, S., Duan, B., Koch, S. C., Dalet, A., Britz, O., Garcia-Campmany, L., Kim, E., Cheng, L. Z., Ghosh, A., Ma, Q. F., and Goulding, M. (2015) Gate control of mechanical itch by a subpopulation of spinal cord interneurons. *Science* **350**, 550–554
3. Barry, D. M., Munanairi, A., and Chen, Z.-F. (2017) Spinal mechanisms of itch transmission. *Neurosci. Bull.* **34**, 156–164
4. Hsieh, G. C., Kolano, R. M., Andrews, W., Fey, T. A., Collins, K. A., Gauvin, D., Kawai, M., Mollison, K. W., and Luly, J. R. (1996) Immunosuppressant effects on improved Guinea pig contact hypersensitivity (CH) model: Induction with DNFB and elicitation with DNCB. *FASEB J.* **10**, 1284
5. Garcia-Perez, A. (1978) Occupational dermatitis from DNFB with cross sensitivity to DNCB. *Contact Dermatitis* **4**, 125–127
6. Rijnierse, A., van Zijl, K. M. F., Koster, A. S., Nijkamp, F. P., and Kraneveld, A. D. (2006) Beneficial effect of tachykinin NK1 receptor antagonism in the development of hapten-induced colitis in mice. *Eur. J. Pharmacol.* **548**, 150–157

7. Rijnierse, A., Koster, A. S., Nijkamp, F. P., and Kraneveld, A. D. (2006) TNF-alpha is crucial for the development of mast cell-dependent colitis in mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* **291**, G969–G976
8. Parenti, A., De Logu, F., Geppetti, P., and Benemei, S. (2016) What is the evidence for the role of TRP channels in inflammatory and immune cells? *Br. J. Pharmacol.* **173**, 953–969
9. Tingle, M. D., Clarke, J. B., Kitteringham, N. R., and Park, B. K. (1990) Influence of glutathione conjugation on the immunogenicity of dinitrophenyl derivatives in the rat. *Int. Arch. Allergy Appl. Immunol.* **91**, 160–165
10. Landsteiner, K., and Chase, M. W. (1941) Studies on the sensitization of animals with simple chemical compounds: IX. Skin sensitization induced by injection of conjugates. *J. Exp. Med.* **73**, 431–438
11. Caterina, M. J. (2014) TRP channel cannabinoid receptors in skin sensation, homeostasis, and inflammation. *ACS Chem. Neurosci.* **5**, 1107–1116
12. Rodriguez, A. L., Grier, M. D., Jones, C. K., Herman, E. J., Kane, A. S., Smith, R. L., Williams, R., Zhou, Y., Marlo, J. E., Days, E. L., Blatt, T. N., Jadhav, S., Menon, U. N., Vinson, P. N., Rook, J. M., *et al.* (2010) Discovery of novel allosteric modulators of metabotropic glutamate receptor subtype 5 reveals chemical and functional diversity and *in vivo* activity in rat behavioral models of anxiolytic and antipsychotic activity. *Mol. Pharmacol.* **78**, 1105–1123
13. Lin, J., Xu, R., Hu, L., You, J., Jiang, N., Li, C., Che, C., Wang, Q., Xu, Q., Li, J., and Zhao, G. (2018) Interleukin-32 induced thymic stromal lymphopoietin plays a critical role in the inflammatory response in human corneal epithelium. *Cell Signal.* **49**, 39–45
14. Gao, Z.-R., Chen, W.-Z., Liu, M.-Z., Chen, X.-J., Wan, L., Zhang, X.-Y., Yuan, L., Lin, J.-K., Wang, M., Zhou, L., Xu, X.-H., and Sun, Y.-G. (2019) Tac1-expressing neurons in the periaqueductal gray facilitate the itch-scratching cycle *via* descending regulation. *Neuron* **101**, 45–59.e9
15. Ishibashi, T., Takumida, M., Akagi, N., Hirakawa, K., and Anniko, M. (2008) Expression of transient receptor potential vanilloid (TRPV) 1, 2, 3, and 4 in mouse inner ear. *Acta Otolaryngol.* **128**, 1286–1293
16. Sandor, Z., Dekany, A., Kelemen, D., Bencsik, T., Papp, R., and Bartho, L. (2016) The TRPA1 activator allyl isothiocyanate (AITC) contracts human jejunal muscle: Pharmacological analysis. *Basic Clin. Pharmacol. Toxicol.* **119**, 341–342
17. Sun, Y. G., and Chen, Z. F. (2007) A gastrin-releasing peptide receptor mediates the itch sensation in the spinal cord. *Nature* **448**, 700–703
18. Engel, M. A., Leffler, A., Niedermirtl, F., Babes, A., Zimmermann, K., Filipovic, M. R., Izydorczyk, I., Eberhardt, M., Kichko, T. I., Mueller-Tribbensee, S. M., Khalil, M., Siklosi, N., Nau, C., Ivanovic-Burmazovic, I., Neuhuber, W. L., *et al.* (2011) TRPA1 and substance P mediate colitis in mice. *Gastroenterology* **141**, 1346–1358
19. Tóth, B. I., Oláh, A., Szöllősi, A. G., and Bíró, T. (2014) TRP channels in the skin. *Br. J. Pharmacol.* **171**, 2568–2581
20. Mandadi, S., and Roufogalis, B. D. (2008) ThermoTRP channels in nociceptors: Taking a lead from capsaicin receptor TRPV1. *Curr. Neuropharmacol.* **6**, 21–38
21. Atoyan, R., Shander, D., and Botchkareva, N. V. (2009) Non-neuronal expression of transient receptor potential type A1 (TRPA1) in human skin. *J. Invest. Dermatol.* **129**, 2312–2315
22. Bertin, S., Aoki-Nonaka, Y., Lee, J., de Jong, P. R., Kim, P., Han, T., Yu, T., To, K., Takahashi, N., Boland, B. S., Chang, J. T., Ho, S. B., Herdman, S., Corr, M., Franco, A., *et al.* (2017) The TRPA1 ion channel is expressed in CD4+T cells and restrains T-cell-mediated colitis through inhibition of TRPV1. *Gut* **66**, 1584–1596
23. Li, M. W., Fan, X. S., Yue, Q. F., Hu, F. Y., Zhang, Y. M., and Zhu, C. (2020) The neuro-immune interaction in airway inflammation through TRPA1 expression in CD4+T cells of asthmatic mice. *Int. Immunopharm.* **86**, 106696
24. Oh, M. H., Oh, S. Y., Lu, J., Lou, H., Myers, A. C., Zhu, Z., and Zheng, T. (2013) TRPA1-dependent pruritus in IL-13-induced chronic atopic dermatitis. *J. Immunol.* **191**, 5371–5382
25. Zeng, D., Chen, C., Zhou, W., Ma, X., Pu, X., Zeng, Y., Zhou, W., and Lv, F. (2021) TRPA1 deficiency alleviates inflammation of atopic dermatitis by reducing macrophage infiltration. *Life Sci.* **266**, 118906
26. Grandclement, C., Pick, H., Vogel, H., and Held, W. (2016) NK cells respond to haptens by the activation of calcium permeable plasma membrane channels. *PLoS One* **11**, e0151031
27. Saarnilehto, M., Chapman, H., Savinko, T., Lindstedt, K., Lauerma, A. I., and Koivisto, A. (2014) Contact sensitizer 2,4-dinitrochlorobenzene is a highly potent human TRPA1 agonist. *Allergy* **69**, 1424–1427
28. Takaoka, A., Arai, I., Sugimoto, M., Honma, Y., Futaki, N., Nakamura, A., and Nakaike, S. (2006) Involvement of IL-31 on scratching behavior in NC/Nga mice with atopic-like dermatitis. *Exp. Dermatol.* **15**, 161–167
29. Kaplan, D. H., Igyarto, B. Z., and Gaspari, A. A. (2012) Early immune events in the induction of allergic contact dermatitis. *Nat. Rev. Immunol.* **12**, 114–124
30. Bailon, E., Cueto-Sola, M., Utrilla, P., Nieto, A., Garrido-Mesa, N., Celada, A., Zarzuelo, A., Xaus, J., Galvez, J., and Comalada, M. (2011) DNFB-DNS hapten-induced colitis in mice should not be considered a model of inflammatory bowel disease. *Inflamm. Bowel Dis.* **17**, 2087–2101
31. Rijnierse, A., Koster, A. S., Nijkamp, F. P., and Kraneveld, A. D. (2006) Critical role for mast cells in the pathogenesis of 2,4-dinitrobenzene-induced murine colonic hypersensitivity reaction. *J. Immunol.* **176**, 4375–4384
32. Wilson, S. R., Nelson, A. M., Batia, L., Morita, T., Estandian, D., Owens, D. M., Lumpkin, E. A., and Bautista, D. M. (2013) The ion channel TRPA1 is required for chronic itch. *J. Neurosci.* **33**, 9283–9294
33. Bautista, D. M., Pellegrino, M., and Tsunozaki, M. (2013) TRPA1: A gatekeeper for inflammation. *Annu. Rev. Physiol.* **75**, 181–200
34. Facchinetti, F., Amadei, F., Geppetti, P., Tarantini, F., Serio, C. D., Dragotto, A., Gigli, P. M., Catinella, S., Civelli, M., and Patacchini, R. (2007) Alpha,beta-unsaturated aldehydes in cigarette smoke release inflammatory mediators from human macrophages. *Am. J. Respir. Cell Mol. Biol.* **37**, 617–623
35. Andre, E., Campi, B., Materazzi, S., Trevisani, M., Amadesi, S., Massi, D., Creminon, C., Vaksman, N., Nassini, R., Civelli, M., Baraldi, P. G., Poole, D. P., Bunnett, N. W., Geppetti, P., and Patacchini, R. (2008) Cigarette smoke-induced neurogenic inflammation is mediated by ±,-unsaturated aldehydes and the TRPA1 receptor in rodents. *J. Clin. Invest.* **118**, 2574–2582
36. Nilius, B., Owsianik, G., Voets, T., and Peters, J. A. (2007) Transient receptor potential cation channels in disease. *Physiol. Rev.* **87**, 165–217
37. Vay, L., Gu, C., and McNaughton, P. A. (2012) The thermo-TRP ion channel family: Properties and therapeutic implications. *Br. J. Pharmacol.* **165**, 787–801
38. Luo, J., Feng, J., Yu, G., Yang, P., Mack, M. R., Du, J., Yu, W., Qian, A., Zhang, Y., Liu, S., Yin, S., Xu, A., Cheng, J., Liu, Q., O'Neil, R. G., *et al.* (2018) Transient receptor potential vanilloid 4-expressing macrophages and keratinocytes contribute differentially to allergic and nonallergic chronic itch. *J. Allergy Clin. Immunol.* **141**, 608–619.e7
39. Southall, M. D., Li, T., Gharibova, L. S., Pei, Y., Nicol, G. D., and Travers, J. B. (2003) Activation of epidermal vanilloid receptor-1 induces release of proinflammatory mediators in human keratinocytes. *J. Pharmacol. Exp. Ther.* **304**, 217–222
40. Yoshioka, T., Imura, K., Asakawa, M., Suzuki, M., Oshima, I., Hirasawa, T., Sakata, T., Horikawa, T., and Arimura, A. (2009) Impact of the Gly573Ser substitution in TRPV3 on the development of allergic and pruritic dermatitis in mice. *J. Invest. Dermatol.* **129**, 714–722
41. Hinman, A., Chuang, H. H., Bautista, D. M., and Julius, D. (2006) TRP channel activation by reversible covalent modification. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 19564–19568
42. Kim, S., Barry, D. M., Liu, X. Y., Yin, S. J., Munanairi, A., Meng, Q. T., Cheng, W., Mo, P., Wan, L., Liu, S. B., Ratnayake, K., Zhao, Z. Q., Gautam, N., Zheng, J., Karunarathne, W. K., *et al.* (2016) Facilitation of TRPV4 by TRPV1 is required for itch transmission in some sensory neuron populations. *Sci. Signal.* **9**, ra71
43. Kang, S. M., Han, S., Oh, J. H., Lee, Y. M., Park, C. H., Shin, C. Y., Lee, D. H., and Chung, J. H. (2017) A synthetic peptide blocking TRPV1

activation inhibits UV-induced skin responses. *J. Dermatol. Sci.* **88**, 126–133
44. Yogianti, F., Kunisada, M., Nakano, E., Ono, R., Sakumi, K., Oka, S., Nakabeppu, Y., and Nishigori, C. (2014) Inhibitory effects of dietary Spirulina platensis on UVB-induced skin inflammatory responses and carcinogenesis. *J. Invest. Dermatol.* **134**, 2610–2619
45. Li, Y. X., Yu, H. Y., Jin, Y., Li, M., and Qu, C. J. (2018) Verbascoside alleviates atopic dermatitis-like symptoms in mice via its potent anti-inflammatory effect. *Int. Arch. Allergy Immunol.* **175**, 220–230
46. Deng, Z. Q., Paknejad, N., Maksaev, G., Sala-Rabanal, M., Nichols, C. G., Hite, R. K., and Yuan, P. (2018) Cryo-EM and X-ray structures of TRPV4 reveal insight into ion permeation and gating mechanisms. *Nat. Struct. Mol. Biol.* **25**, 252–260
47. Mohania, D., Chandel, S., Kumar, P., Verma, V., Digvijay, K., Tripathi, D., Choudhury, K., Mitten, S. K., and Shah, D. (2017) Ultraviolet radiations: Skin defense-damage mechanism. *Adv. Exp. Med. Biol.* **996**, 71–87
48. Dien, P. H., Nhan, N. T., Le Thuy, H. T., and Quang, D. N. (2012) Main constituents from the seeds of Vietnamese Cnidium monnieri and cytotoxic activity. *Nat. Prod. Res.* **26**, 2107–2111
49. Danso-Abeam, D., Zhang, J. G., Dooley, J., Staats, K. A., Van Eyck, L., Van Brussel, T., Zaman, S., Hauben, E., Van de Velde, M., Morren, M. A., Renard, M., Van Geet, C., Schaballie, H., Lambrechts, D., Tao, J. S., *et al.* (2013) Olmsted syndrome: Exploration of the immunological phenotype. *Orphanet J. Rare Dis.* **8**, 79
50. Heber, S., Gold-Binder, M., Ciotu, C. I., Witek, M., Ninidze, N., Kress, H. G., and Fischer, M. J. M. (2019) A human TRPA1-specific pain model. *J. Neurosci.* **39**, 3845–3855
51. Ibarra, Y., and Blair, N. T. (2013) Benzoquinone reveals a cysteine-dependent desensitization mechanism of TRPA1. *Mol. Pharmacol.* **83**, 1120–1132
52. Sun, X., Wei, N., Sun, X., and Wang, K. (2017) Development of a cell-based HTS adaptable calcium fluorescent assay in FlexStation3 for the screening of TRPV3 inhibitors. *J. Chin. Pharm. Sci.* **26**, 675–683