The NADPH oxidase inhibitor diphenyleneiodonium activates the human TRPA1 nociceptor

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The NADPH oxidase inhibitor diphenyleneiodonium activates the human TRPA1 nociceptor. Am J Physiol Cell Physiol 307: C384–C394, 2014. First published June 18, 2014; doi:10.1152/ajpcell.00182.2013.—Transient receptor potential ankyrin 1 (TRPA1) is a Ca2⁺-permeable nonselective cation channel expressed in neuronal and nonneuronal cells and plays an important role in acute and inflammatory pain. Here, we show that an NADPH oxidase (NOX) inhibitor, diphenyleneiodonium (DPI), functions as a TRPA1 activator in human embryonic kidney cells expressing human TRPA1 (HEK-TRPA1) and in human fibroblast-like synoviocytes. Application of DPI at 0.03–10 μM induced a Ca2⁺ response in HEK-TRPA1 cells in a concentration-dependent manner. The Ca2⁺ response was effectively blocked by a selective TRPA1 antagonist, HC-030031 (HC). In contrast, DPI had no effect on HEK cells expressing TRPV1-V4 or TRPM8. Four other NOX inhibitors, apocynin (APO), VAS2870 (VAS), plumbagin, and 2-acetylphenothiazine, also induced a Ca2⁺ response in HEK-TRPA1 cells, which was inhibited by pretreatment with HC. In the presence of 5 mM glutathione, the Ca2⁺ response to DPI was effectively reduced. Moreover, mutation of cysteine 621 in TRPA1 substantially inhibited the DPI-induced Ca2⁺ response, while it did not inhibit the APO- and VAS-induced responses. The channel activity was induced by DPI in excised membrane patches with both outside-out and inside-out configurations. Moreover, internal application of neomycin significantly inhibited excised membrane patches with both outside-out and inside-out configurations. Additionally, internal application of neomycin inhibited DPI-induced inward currents. The channel activity was induced by DPI in excised membrane patches with both outside-out and inside-out configurations. Moreover, internal application of neomycin inhibited DPI-induced inward currents. Because numerous structurally unrelated compounds stimulate TRPA1, greater attention should be given to whether common clinical drugs and experimental pharmacological agents activate the channel.

The NADPH oxidases (NOXs), a family of transmembrane proteins comprising seven members (NOX1–NOX5, DUOX1, and DUOX2), function as transmembrane electron transporters that use cytosolic NADPH as electron donor and oxygen as electron acceptor. All redox centers are conserved among the NOX family members, and all of these proteins share a similar catalytic mechanism and generate reactive oxygen species (ROS), mainly superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$). Because oxidative stress has been suggested to play a major role in the pathogenesis of numerous diseases, NOX inhibitors, which reduce production of O$_2^-$ and H$_2$O$_2$, are potential drug candidates for diseases associated with increased levels of ROS. Several compounds, including natural substances, have been reported to inhibit NOX activity. One of these compounds, diphenyleneiodonium (DPI), is a classical nonspecific potent flavoenzyme inhibitor, which reduces the activity of NOX, nitric oxide synthase, and xanthine oxidase (25). APO is one of the most frequently used NOX inhibitors and may also act as an antioxidant (13). However, because of the lack of specificity of DPI and APO, novel and more selective NOX inhibitors, such as VAS, VAS3947, and 2-acetylphenothiazine (2APT), have been developed (9, 35, 40). Because these NOX inhibitors have been used in numerous physiological, cell biological, and biochemical studies, knowing the effects of these compounds on targets unrelated to NOX can be quite helpful and informative.

In the present study, we show that DPI functions as an activator of human TRPA1, without affecting TRPV1-V4 or TRPM8. Four other NOX inhibitors tested [APO, VAS, plumbagin (PLU), and 2APT] also effectively activated TRPA1. A comparison of the response of mutant TRPA1 to DPI with those to APO, VAS, and allyl isothiocyanate (MO) revealed that cysteine 621 of the channel is critical for the DPI-induced response. On the other hand, the channel activity was induced by DPI in excised membrane patches with both outside-out and inside-out configurations. Moreover, internal application of neomycin inhibited DPI-induced inward currents. DPI also effectively activated TRPA1 in inflammatory...
human synoviocytes and caused a pain-related response in mice. Our findings indicate that DPI and other commonly used NOX inhibitors may directly activate human TRPA1 without mediating NOX.

MATERIALS AND METHODS

The present study was approved by the Animal Care Committee at Aichi-Gakuin University and performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

Cell culture. Human embryonic kidney (HEK) cells were obtained from Health Science Research Resources Bank (HSRBB). HEK cells were maintained in Dulbecco’s modified minimum essential medium (D-MEM; Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS; JRS Biosciences), penicillin G (100 U/ml), and streptomycin (100 μg/ml). Human synoviocytes were purchased from Cell Applications and cultured as described previously in synoviocyte growth medium containing 10% growth supplement, 100 U/ml penicillin G, and 100 μg/ml streptomycin (11).

Recombinant expression of TRP channels in HEK cells. Partially confluent HEK cells (40–60% confluency) were transfected with the pcDNA3.1(neo+) plasmid containing human TRPA1, TRPV1, TRPV2, TRPV3, TRPV4, or TRPM8 using Lipofectamine 2000 (Invitrogen). The TRPA1 mutations were constructed by PCR using mutant oligonucleotide primers in which the cysteine residues at amino acid positions 414, 421, 621, 633, or 641 were changed to serine (Aigilent Technologies). All constructs were sequenced. All experiments were performed within 48 h of transfection.

Western blotting. HEK cells were lysed in 50 μl lysis buffer (in mM: 50 Tris-HCl (pH 8.0), 150 NaCl, 5 EDTA) including 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors (11). The cell lysates were incubated on ice for 30 min with vortexing every 5 min and then centrifuged at 20,000 × g for 30 min at 4°C. Each lysate (10 μg protein) was separated on an 8% polyacrylamide gel, and proteins were then transferred to a PVDF membrane. Nonspecific binding of antibodies was blocked by incubation for 2 h in Tris-buffered saline (TBS) containing 5% skim milk and 0.1% Tween-20. The PVDF membrane was subsequently incubated with the first antibody (goat anti-TRPA1; Santa Cruz Biotechnology; 1:1,000 dilution) and then washed three times with buffered saline (TBS) containing 0.1% Tween-20, and secondary antibody (IgG-HRP; 1:10,000 dilution) was added to the PVDF membrane. Blots were washed again, and detection reagents (Millipore) were added to generate a chemiluminescence signal. To determine the relative quantity of TRPA1 to β-actin in each sample, the PVDF membrane was also exposed to mouse anti-β-actin monoclonal antibody (1:2,000 dilution). Finally, gels were scanned with an LAS-3000 mini apparatus (Fujifilm, Tokyo, Japan).

Patch-clamp experiments. Patch-clamp experiments were performed as described previously (16). The resistance of electrodes was 3–5 MΩ when filled with the pipette solution in whole cell and outside-out patch configurations. In inside-out patch configuration, a pipette solution containing 140 CsCl, 1 MgCl2, 10 HEPES, 1 EGTA (adjusted to pH 7.4 with NaOH) was used in whole cell and outside-out patch configurations. In inside-out patch configuration, a bath solution containing (in mM) 140 CsCl, 1 MgCl2, 1 EGTA, 10 HEPES, 2.89 triplyphosphate (TPP) (adjusted to pH 7.2 with CsOH). All experiments were performed at 25 ± 1°C.

Measurement of the Ca2+ fluorescence ratio. HEK cells and human synoviocytes were loaded with 10 μM fura 2-AM in the HEPS solution for 30 min at room temperature. After cells were superfused with the HEPS solution for 10 min, fura-2 fluorescence signals were measured using the Argus/HisCa imaging system driven (Hamamatsu Photonics, Hamamatsu, Japan) by Imagework Bench 6.0 (Indec). The frequency of image acquisition was set at 0.2 Hz. The efficacy of gene transfection was similar, but not identical, from cell to cell. To reduce variation, we collected 30–70 single cells in one coverslip for analysis and repeated the same experiment with the other coverslips. In each analysis, a whole cell area was chosen as a region of interest to average the fluorescence ratio of the area. Since protein expression level of mutant TRPA1 is different from that of wild TRPA1 (see Fig. 5A), we normalized Ca response to NOX inhibitors with that to 30 μM MO for each comparison in Fig. 5.

Pain-related behavioral test. The ddY mice (6–7 wk of age) were individually placed in transparent cages (30 × 20 × 12 cm) for 30 min before experiments. An intraplantar injection of 10 μl DPI (2 mM, solvent: Kolliphor EL with 20% DMSO) was then injected into the right hindpaw with or without intraperitoneal administration with HC030031 (300 mg/kg at 0.5 h prior to injection of DPI; solvent: saline with 0.5% methyl cellulose). The time spent licking or biting the injected paw was recorded for 45 min after injection.

Data analysis. Data are expressed as means ± SE. Statistical significance between two groups and among multiple groups was examined using Student’s t-test and Tukey’s multiple comparison test, respectively.

Chemicals and reagents. The following drugs were used: diphenylhydrizidium chloride (DPI; Enzo Life Sciences), allyl isothiocyanate (MO; Kanto Chemical), HC030031 (HC; Enzo Life Sciences), apocynin (APO; Sigma), VAS2870 (VAS; Calbiochem), plumbagin (PLU; Wako), 2-acetylphenothiazine (2APT; Sigma), auranofin (AUR; Enzo Life Sciences), capsacin (CAP; Sigma), cannabidiol (CAB; Sigma), 2-aminoethoxydiphenyl borate (2APB; Sigma), GSK1016790A (GSK; Sigma), menthol (MEN; Wako), glutathione (GSH; Sigma), ascorbic acid (Wako), uric acid (Wako), catalase (Sigma), Nα,Nβ-diaminocaproyl methyl ester hydrochloride (NAME; Sigma), neomycin sulfate (Wako), triplyphosphate sodium (TPP; Wako), Kolliphor EL (Sigma), and methyl cellulose (Sigma). Each drug was dissolved in the vehicle recommended by the manufacturer.

RESULTS

DPI activates human TRPA1. TRPA1 expression in HEK cells transfected with TRPA1 plasmid (HEK-TRPA1 cells) was confirmed using the following three assays, as previously described (11, 12): TRPA1 mRNA transcript expression, TRPA1 protein expression (see Fig. 5A) and TRPA1 channel function (see Fig. 5B). Using these HEK-TRPA1 cells, we examined the effects of DPI, a NOX inhibitor, on TRPA1. As shown in Fig. 1A, application of DPI to HEK-TRPA1 cells at a concentration range of 0.03–10 μM effectively induced a Ca2+ response; the EC50 of DPI needed to activate TRPA1 was 1–3 μM. In contrast, DPI failed to evoke a Ca2+ response in control HEK cells, even at a relatively high dose of 10 μM (control, Fig. 1A). To confirm that DPI activates Ca2+-permeable TRPA1, we applied 3 μM DPI to HEK-TRPA1 cells with or without 30 μM HC, a TRPA1 antagonist. The treatment significantly inhibited the Ca2+ response induced by 3 μM DPI.
Because DPI has been shown to elevate intracellular Ca$^{2+}$ concentration by inhibiting uptake of the cation into stores (39), we sought to clarify the effect of DPI in our cell line. DPI was applied to HEK-TRPA1 cells without extracellular Ca$^{2+}$. As summarized in Fig. 1B, DPI failed to evoke a Ca$^{2+}$ response under 0 Ca$^{2+}$ conditions, demonstrating that DPI induces Ca$^{2+}$ entry into the cells. In the whole cell recording mode, application of 3 μM DPI to a HEK-TRPA1 cell induced inward and outward currents at −100 and +40 mV, respectively (Fig. 1, C and D). The evoked currents had a similar I-V relationship to that induced by MO, a typical TRPA1 agonist (11). After withdrawal of DPI, these evoked currents were abolished. Taken together, our findings indicate that DPI activates human TRPA1.

For comparison, we also examined the effects of DPI on other types of human TRP channels expressed in HEK cells. Here, we chose TRPV1–4 and TRPM8, which are functionally expressed in sensory neurons, similar to TRPA1. To demonstrate the presence of these channels, we used their respective agonists—CAP (10 μM) for TRPV1, CAB (30 μM) for TRPV2, 2APB (100 μM) for TRPV3, GSK (3 nM) for TRPV4, and MEN (100 μM) for TRPM8—to evoke activity in Ca$^{2+}$ measurement assays (Fig. 2). In HEK cells transfected successfully with TRPV1, TRPV2, TRPV3, TRPV4, or DPI at 0.3 and 3 μM failed to evoke a Ca$^{2+}$ response, showing that DPI selectively activates only TRPA1 among the six TRP channels.

**Other NOX inhibitors also activate human TRPA1.** Several structurally unrelated compounds, including natural substances, have been shown to inhibit NOX activity (4). We therefore examined whether four additional NOX inhibitors, APO, VAS, PLU, and 2APT, could also activate human TRPA1. Application of these NOX inhibitors effectively induced a Ca$^{2+}$ response in HEK-TRPA1 cells in a concentration-dependent manner (Fig. 3A). Interestingly, the potency of each NOX inhibitor in activating TRPA1 parallels its ability to inhibit NOX activity (6, 9, 34, 37). Moreover, the Ca$^{2+}$ response to these NOX inhibitors was substantially inhibited in the presence of 30 μM HC (Fig. 3B), suggesting that the five NOX inhibitors tested, including DPI, are potential TRPA1 agonists.

**Mechanism of action of DPI.** The conservative cysteine-to-serine mutations (C414S, C421S, C621S, C633S, and C641S) in the predicted NH2-terminal ankyrin repeats of TRPA1 have revealed that these cysteines play a critical role in the activation of the channel by electrophilic agonists, which are able to interact with the sulfide moiety of these residues. To test whether DPI also interacts with these sites, we first applied DPI to HEK-TRPA1 cells in the presence of the nucleophile glutathione (GSH), which binds to electrophilic compounds. As
shown in Fig. 4, 5 mM GSH effectively inhibited the DPI-induced Ca\textsuperscript{2+} response. Moreover, GSH significantly inhibited the Ca\textsuperscript{2+} response to MO and auranofin (AUR), which are both electrophilic compounds that activate TRPA1 (Fig. 4; Ref. 12).

We further examined the interaction of DPI with the cysteine residues in TRPA1 using HEK cells transfected with mutant TRPA1 (HEK-muTRPA1) containing either the C414S, C421S, C621S, C633S, or C641S substitution. The expression and channel function of these TRPA1 mutants in HEK cells was verified by Western blotting (Fig. 5A) and by electrophysiological experiments (Fig. 5B). The reversal potentials of 30 \mu M MO-induced membrane currents in HEK-muTRPA1 were not significantly different from wild-type TRPA1 (HEK-wTRPA1) (WT: \(-4.6 \pm 2.1\) mV, \(n = 6\); C421S: \(-6.8 \pm 0.8\) mV, \(n = 4\); C621S: \(-2.8 \pm 3.2\) mV, \(n = 6\); C633S: \(-5.2 \pm 1.6\) mV, \(n = 6\); C641S: \(-0.3 \pm 4.3\) mV, \(n = 4\)), suggesting that mutations except C414S (4 independent cells) had little effect on channel function. DPI at a concentration range of 0.01–3 \mu M was applied to HEK-wTRPA1 and HEK-muTRPA1 cells (Fig. 5D). Some of these cells were also exposed to APO or VAS to evaluate their effect on the Ca\textsuperscript{2+} response (Fig. 5, E and F). Although Ca\textsuperscript{2+} response of each mutant to 30 \mu M MO was not significantly different from that of wild TRPA1 (Fig. 5C), we normalized the Ca\textsuperscript{2+} response to NOX inhibitors with that to 30 \mu M MO for their comparison. The Ca\textsuperscript{2+} responses to DPI were markedly inhibited in HEK-muTRPA1 cells expressing TRPA1 with the C414S, C421S, and C621S mutations (Fig. 5D). In particular, as shown by the concentration-response relationship, the DPI-induced Ca\textsuperscript{2+} response in HEK-muTRPA1 cells with the C414S mutation was abolished (Fig. 5D), suggesting that this cysteine is essential for activation of TRPA1 by DPI, similar to electrophilic compounds, such as MO and cinnamonaldehyde (CA) (12). On the other hand, when normalized with the response to 30 \mu M MO, among DPI at 10 \mu M, APO at 3 mM, and VAS at 10 \mu M (all of which fully activate wild-type TRPA1), the Ca\textsuperscript{2+} response induced by APO was substantially smaller than that in HEK-wTRPA1 cells expressing wild-type TRPA1 (Fig. 5E). We then examined the effects of 10 \mu M DPI, 3 mM APO, and 10 \mu M VAS on the Ca\textsuperscript{2+} response in HEK-wTRPA1 cells and in HEK-muTRPA1 cells expressing TRPA1 with the C621S mutation. The response of the mutant TRPA1 to DPI was much smaller than that of the wild type, whereas responses to APO and VAS were comparable to the wild type (Fig. 5F), suggesting that C621 in TRPA1 is critical for the DPI-induced Ca\textsuperscript{2+} response.
To exclude the possibility of involvement of ROS in DPI-induced TRPA1 activation, HEK-TRPA1 cells were exposed to DPI in the presence of ROS scavengers, ascorbic acid (300 μM), uric acid (300 μM), and catalase (500 U/ml), and a nitric oxide synthase (NOS) inhibitor, L-NAME (500 μM). As shown in Fig. 6, ascorbic acid, uric acid, and L-NAME had little effects on TRPA1 response to 3 μM DPI, while catalase significantly inhibited the response. Nevertheless, more than 60% DPI response sustained in the presence of catalase, suggesting that ROS including H2O2 should have minor roles in the activation of TRPA1 by DPI.

In intact cells, ion channel proteins might be modulated by factors which are intracellularly produced. To reduce the possibility that such factors might influence the channel activity of TRPA1, we next examined whether DPI directly activates TRPA1 channel in patches excised from HEK-TRPA1 cells in outside-out (Fig. 7) and inside-out (Fig. 8) configurations. As shown in Fig. 7A, application of 10 μM DPI and 30 μM MO induced the channel activity in an excised outside-out patch. These unitary current amplitudes were similar between DPI-induced (2.18 ± 0.12 pA, n = 9) and MO-evoked (2.20 ± 0.05 pA, n = 8) currents at −50 mV (Fig. 7B). Moreover, the single-channel conductance in the presence of 10 μM DPI (69.1 ± 6.4 pS, n = 4), which was calculated between −150 and 0 mV, was not significantly different from that in the presence of 30 μM MO (77.4 ± 4.8 pS, n = 4) (Fig. 7C).

When the cytosolic side of the excised patches was exposed to an intracellular solution containing TPP (19, 21), 10 μM DPI and 30 μM MO also induced the channel activity in the inside-out configuration (Fig. 8, A and B). These unitary current amplitudes at −50 mV were not significantly different (Fig. 8C; DPI: 2.86 ± 0.2 pA, n = 8; MO: 2.64 ± 0.28 pA, n = 5). These results strongly suggest that human TRPA1 is activated by DPI in a membrane-delimited manner.

MO-induced TRPA1 currents were effectively inhibited by inclusion of neomycin in the pipette solution, due to a shielding effect of the negative charges of phosphatidylinositol 4,5-bisphosphate (PIP2) by neomycin (19). Therefore, we next examined effects of intracellularly applied neomycin on DPI-induced currents. As shown in Fig. 9, the current response to 10 μM DPI was substantially inhibited in cells dialyzed with 5 mM neomycin.

DPI activates endogenous TRPA1 expressed in human synoviocytes. To examine whether DPI can activate TRPA1 expressed in human tissue, we used inflammatory human synoviocytes where TRPA1 is functionally expressed (11). We confirmed the functional expression of TRPA1 by measuring the MO-induced Ca2+ response of synoviocytes treated with 100 U interleukin-1α (IL1α) for 24 h (Fig. 10A). Exposure of these cells to 3 μM DPI evoked a Ca2+ response when cells were treated with IL1α (Fig. 10B). Moreover, the DPI-induced response was effectively inhibited by pretreatment with 30 μM HC (Fig. 10C), suggesting that DPI activates endogenous TRPA1 expressed in human synoviocytes.

DPI-induced pain response in mice. There is no information regarding TRPA1-dependent irritation by NOX inhibitors in animals. Therefore, we examined whether DPI causes pain-related response in mice. As shown in Fig. 11A, intraplantar injection of 2 mM DPI to the hindpaw caused licking or biting behavior. Moreover, the response was significantly inhibited in mice preadministered 300 mg/kg HC intraperitoneally (Fig. 11B), suggesting that DPI may be a potential irritant mediating activation of TRPA1.

DISCUSSION

In the present study, we show that five structurally unrelated NOX inhibitors activate TRPA1 with potencies similar to that for NOX inhibition. In particular, the activation of TRPA1 by DPI depends on the cysteine residues at 414 and 621 in the NH2 terminus of the channel. On the other hand, the channel activity of TRPA1 was induced by DPI in excised membrane patches with both outside-out and inside-out configurations. Moreover, intracellular dialysis of neomycin inhibited DPI-induced inward currents. In mice, intraplantar injection of DPI caused a pain-related response which was inhibited by predadministration with HC. To our knowledge, this is the first study to demonstrate that TRPA1 is activated by frequently used NOX inhibitors.

Some reactive species are proposed to be potential TRPA1 agonists. In particular, extracellular H2O2 is involved in activation of TRPA1 induced by inflammation in human synoviocytes (11). However, generation of OH·radicals can also explain a part of activation of TRPA1 by H2O2 (2). NOX inhibitors generally reduce ROS production, hence ruling out the possibility that the decrease in ROS production by NOX inhibitors results in the activation of TRPA1. Nevertheless, to exclude the involvement of ROS in the activation of TRPA1 by DPI, we tested the effects of ROS scavengers and L-NAME on DPI-induced response. Although H2O2 is in part responsible for the activation, a major component of the response is independent of ROS. A further study is, however, required for the explanation of inhibition of DPI response by catalase.

TRPA1 is stimulated by electrophilic compounds such as MO and CA, and by endogenous substances such as 15-deoxy-
\(\Delta(12,14)\) prostaglandin J2 and H2O2, all of which activate the channel by covalent modification of cysteines and/or lysines in the NH2 terminus (2, 14, 26, 31). Although it is not yet known whether other nucleophilic sinks are involved in channel activation, mutagenesis experiments have identified the importance of C414, C421, and C621 in the activation of human

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Fig. 4. Inhibition of the DPI-induced Ca2+ response by glutathione. DPI at 1 μM was applied to HEK-TRPA1 cells with (n = 142, 5 independent experiments) or without (n = 261, 5 independent experiments) 5 mM glutathione (GSH), and the peak evoked Ca2+ response was determined. For comparison, 1 μM allyl isothiocyanate (MO) and 1 μM auranofin (AUR) were also applied with (GSH: MO, n = 81, 3 independent experiments; AUR, n = 96, 3 independent experiments) or without (control: MO, n = 158, 3 independent experiments; AUR, n = 142, 3 independent experiments) 5 mM GSH. **P < 0.01 vs. control. Bars: SE.
TRPA1 by electrophilic compounds. C621 is particularly crucial for channel activation by DPI; even a high dose of DPI failed to evoke a substantial Ca\(^{2+}\)/H\(_{11001}\) response from cells expressing TRPA1 with the C621S mutation. Similarly, DPI evoked a negligible response in cells expressing TRPA1 with the C414S mutation, similar to MO, CA, and AUR in previous studies (12, 14). Moreover, the importance of C421 for channel activation by APO is clear in this study. As an electrophilic compound, DPI likely affects the cysteine residues within TRPA1, including C621, which induces activation of the channel. Consistently, the DPI-induced response in HEK cells expressing wild-type TRPA1 was effectively inhibited by GSH, which also attenuated the response to the other electrophilic compounds, MO and AUR.

In the present study, we demonstrated that DPI directly activated channels in excised patches, which were similar to those by MO, hence implying that DPI may activate MO-sensitive TRPA1 channel in a membrane-delimited manner. However, even in the presence of TPP, the channel activity disappeared in some inside-out patches and addition of DPI

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**Fig. 5. Contribution of NH\(_2\)-terminal cysteines in TRPA1 to the DPI-induced Ca\(^{2+}\) response.**

A: wild-type (WT) and mutant TRPA1 proteins (C414S, C421S, C621S, C633S, and C641S) were assayed by Western blotting. As a control, levels of β-actin protein were also measured. B: channel function of TRPA1 with mutations. Current-voltage relationships of membrane currents during application of 30 μM MO in a representative HEK cell with wild-type TRPA1 cell (WT), with C414S mutant TRPA1 (C414S), with C421S (C421S), with C621S (C621S), with C633S (C633S), and with C641S (C641S). C: Ca\(^{2+}\) response to 30 μM MO of wild and mutant TRPA1 (WT, 4 independent experiments, C414S, 4 independent experiments; C421S, 6 independent experiments; C621S, 4 independent experiments; C633S, 4 independent experiments; C641S, 4 independent experiments). D: concentration-response relationships of the DPI-induced Ca\(^{2+}\) response in HEK cells transfected with wild-type (WT, n = 159, 4 independent experiments) or mutant (C414S, n = 210, 4 independent experiments; C421S, n = 326, 6 independent experiments; C621S, n = 204, 4 independent experiments; C633S, n = 232, 4 independent experiments; C641S, n = 224, 4 independent experiments) TRPA1. DPI at a concentration range of 0.01–3 μM was applied to each cell type. Ca\(^{2+}\) response to DPI was normalized with that to 30 μM MO in each cell. Bars: SE.

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TRPA1 by electrophilic compounds. C621 is particularly crucial for channel activation by DPI; even a high dose of DPI failed to evoke a substantial Ca\(^{2+}\) response from cells expressing TRPA1 with the C621S mutation. Similarly, DPI evoked a negligible response in cells expressing TRPA1 with the C414S mutation, similar to MO, CA, and AUR in previous studies (12, 14). Moreover, the importance of C421 for channel activation by APO is clear in this study. As an electrophilic compound, DPI likely affects the cysteine residues within TRPA1, including C621, which induces activation of the channel. Consistently, the DPI-induced response in HEK cells expressing wild-type TRPA1 was effectively inhibited by GSH, which also attenuated the response to the other electrophilic compounds, MO and AUR.

In the present study, we demonstrated that DPI directly activated channels in excised patches, which were similar to those by MO, hence implying that DPI may activate MO-sensitive TRPA1 channel in a membrane-delimited manner. However, even in the presence of TPP, the channel activity disappeared in some inside-out patches and addition of DPI
and MO thereafter did not induce the activity (DPI; 3 of 12 patches, MO; 5 of 11 patches). These suggest that certain factors essential for activation of TRPA1 might disappear, and the channel run down is irreversible. Indeed, although inorganic polyphosphate (PPPi) reconstituted channel activity of TRPA1 in excised patches, the channel reactivation was not observed even in the presence of PPPi after the long cessation (19, 21). On the other hand, in whole cell recordings, we found that the PIP2 scavenger neomycin diminished DPI-induced TRPA1 currents. Consistently, MO-induced TRPA1 currents were also inhibited by intracellular dialysis of neomycin (19). Moreover, PIP2 supplied via the patch pipette delayed the decay of the MO-induced TRPA1 currents (19). Therefore, PIP2 can modulate TRPA1 and the activation by DPI as well as MO: DPI may directly activate TRPA1 in the presence of enough content of the plasma membrane PIP2. However, a direct block of DPI-induced TRPA1 currents by neomycin cannot be excluded. In addition, further studies are required for full understanding of activation mechanisms of TRPA1 by NOX inhibitors.

DPI’s interaction with the cysteine residues within NOX may also underlie its ability to inhibit the enzyme. Indeed, similar to MO and AUR, DPI inhibits thioredoxin reductase (24). Nevertheless, other mechanisms have been reported for the inhibition of NOX by DPI: the inhibition of reduction of iron-sulfur clusters in NADPH (27) and covalent binding to flavin (32). Therefore, we cannot exclude the possibility that DPI activates TRPA1 in a non-electrophilic manner.

The cysteine residues in p47phox, a component of NOX2, are targets for the trimer hydroxylated quinone derivative of APO, which disrupts the subunit interaction in NOX2, which blocks the activation of the enzyme (29). Both MO and AUR easily form conjugates with the cysteine residues of thioredoxin, an enzyme related to NOX (10, 15, 41). APO may also be an electrophilic activator, although it has lower efficacy than DPI. Indeed, APO evokes a TRPA1 response 1,000-fold weaker than DPI (Fig. 3), and APO inhibits NOX4 only weakly [IC50 of APO: ~215 μM; IC50 of DPI: ~0.2 μM, (34)]. Moreover, the Ca2+ response elicited by APO in cells expressing TRPA1 with the C421S mutation was significantly suppressed, suggesting that APO is an electrophilic compound that selectively targets specific cysteine residues within TRPA1.

In the present study, we show that VAS, PLU, and 2APT are also effective TRPA1 activators. The triazolo pyrimidine derivatives, VAS and VAS3947, which both have similar pharmacological properties (in addition to solubility in water), are more selective NOX inhibitors than APO and DPI (40). However, VAS alkylates cysteine 3635 within the ryanodine receptor type I (RyR1), which abrogates regulation of RyR1 by nitric oxide (37). Therefore, this ability of VAS to modify cysteine thiols possibly underlies its ability to inhibit NOX, as shown in previous studies, and its ability to activate TRPA1, as shown in the present study. However,
it should be mentioned that a plant-derived naphthoquinone, PLU, and a phenothiazine derivative, 2APT, effectively reduce NOX activity, although the mechanisms involved have not been identified (6, 9). Because our preliminary experiments revealed that both compounds induce smaller Ca\textsuperscript{2+}/H\textsuperscript{1+} responses in HEK cells expressing TRPA1 with the C421S mutation (peak Ca\textsuperscript{2+}/H\textsuperscript{1+} response ratio change in WT and C421S: 1.2 \pm 0.04 and 0.25 \pm 0.02, respectively, for 3 \muM PLU in 4 or 5 independent experiments; 0.5 \pm 0.03 and 0.03 \pm 0.01, respectively, for 30 \muM 2APT in 4 independent experiments), it is likely that the cysteine residues in TRPA1 are targets of PLU and 2ATP during activation. Taken together, this study raises the possibility that NOX inhibitors and TRPA1 agonists share targets with a similar moiety between NOX and TRPA1 protein.

The pain-related response caused by DPI was significantly inhibited in mice preadministered HC, hence being clear that DPI is a potential irritant in vivo, mediating activation of...
TRPA1. However, the inhibition by HC was ~50% of the vehicle (Fig. 11B). Since the vehicle itself (the control group in Fig. 11A: Kolliphor EL with 20% DMSO) caused the basal response, the component resistant to HC of DPI response may be smaller. Nevertheless, it is obvious that DPI causes HC-resistant pain-related response. Concentration of HC and/or the exposure time (30 min) might not be enough to abolish the TRPA1 response by DPI in our in vivo study. Alternatively, DPI has nonspecific actions which cause pain-related responses in vivo. In fact, MO causes TRPA1-dependent and -independent licking in mice (22).

In summary, we demonstrate that TRPA1 can be activated by five different NOX inhibitors. In particular, we show that DPI is a TRPA1 activator which has a high potency and selectivity. Although previous studies have shown that electrophilic compounds can stimulate TRPA1, this is the first study to identify specific cysteine residues in TRPA1 as critical components of the molecular machinery mediating the effects of DPI and other NOX inhibitors on the channel. Moreover DPI can directly activate TRPA1 possibly with dependence on plasma membrane PIP2 content. Since DPI causes a pain-related response in mice, DPI is a potential irritant. Our results therefore should assist in the development of NOX inhibitors to remove their agonistic activity for TRPA1 because the activation of TRPA1 could cause allergic response and/or inflammatory pain.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


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