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Perilla Derived Compounds Mediate Human TRPA1 Channel Activity

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Abstract

Compounds from food plants affecting the somatosensory system, like *Perilla frutescens* (L.), are well known for their flavoring, pharmacological and medical properties. Yet the exact mechanisms underlying their activity are still poorly understood. Transient Receptor Potential (TRP) channels involved in chemestetic sensations likely represent some of the primary targets for these compounds. Using a heterologous expression system and calcium imaging we show that a number of *Perilla* derived compounds (S-(-)-1,8-*p*-menthadiene-7-al (perillaldehyde, PA); 3-(4-methyl-1-oxopentyl)furan (perillaketone, PK); 1,2,4-trimethoxy-5-[(E)-prop-1-enyl]benzene (α-asarone, ASA)) and synthetic compounds derivative from *Perilla* (3-(4-methoxy-phenyl)-1-furan-2-yl-propenone (PK-16) and 3-(4-chloro-phenyl)-1-furan-2-yl-propenone (PK-18)) are capable of activating the human TRP Ankyrin family channel (h-TRPA1). The compounds tested appear to be partial agonists of the channel with the potency sequence (EC50, μM): PK-16(107.7)>PA (160.5)>ASA(210.9)>PK(350). Our findings provide important insight into the functional properties of the compounds derived from *P. frutescens* and reveal new perspectives for the design of tools for pharmaceutical, agricultural and food industry applications.

Keywords: *Perilla frutescens*; Somato-sensory compounds; Transient receptor potential; TRPA1 ion channel; Calcium imaging

Abbreviations: MO: Mustard Oil, 3-Isothiocyanato-1-Propene; PA: Perillaldehyde: S-(-)-1,8-P-Menthadiene-7-Al; PK: Perillaketone: 3-(4-Methyl-1-Oxopentyl) Furan; IK: Isoegomaketone: 3-(4-Methyl-1-Oxo-2-Pentenyl) Furan; ASAA: Asarone: 1,2,4-Trimethoxy-5-[(E)-Prop-1-Enyl]Benzene; PK-16: 3-(4-Methoxy-Phenyl)-1-Furan-2-yl-Propenone; PK-18: 3-(4-Chloro-Phenyl)-1-Furan-2-yl-Propenone.

Introduction

Transient Receptor Potential (TRP) channels are an evolutionarily ancient and diverse superfamily of cation channels [1,2]. Many TRP channels are involved in the transduction of stimuli of different sensory modalities: photo-, thermoreception, chemosensory reception [3] and perhaps, mechanoreception [4,5] and the activity of several TRP channels appear to be directly mediated by osmolarity and/or humidity [6]. Like many other species across different phyla, mammals use TRP channels to detect a variety of physical and chemical stimuli including compounds derived from food plants and spices [3,7-9]. An interesting feature of some TRP channel family members is their ability to detect and integrate qualitatively different stimuli. For example, the temperature sensitive mammalian TRPV1 (sensitive to high temperature) and TRPM8 (sensitive to low temperature) channels were also demonstrated to interact with chemical compounds associated with high or low temperature perception, such as chili pepper derived capsaicinoids [7] and menthol from peppermint [9] respectively.

Mammalian TRPA1 channels are abundantly expressed in the somatosensory system, including in trigeminal neurons. They were initially suggested to be involved in noxious cold-sensing, potentially perceived as a burning pain [8,10-13], but in parallel were identified as receptors for mustard oil (MO) derived from *Sinapis* ssp. (Brassicales: Brassicaceae) (allyl-isothiocyanates) and phytocannabinoids from *Cannabis sativa* L. (Rosales: Cannabaceae) (tetra-hydro-cannabinol) [8]. Like MO and phytocannabinoids, several additional ligands derived from food plants and spices can activate TRPA1 channels. For instance, allycil and diallyl disulfide from garlic [11] cinnamaldehyde from essential oils of cinnamon [14], and several other natural ligands, e.g. Artepillin C, ligustilide, unsaturated dialdehyde terpenes, stilbenoids and phenol derivatives [15,16] have been reported to cause TRPA1 channel mediated Ca^{++} influx.

The Asian plant *Perilla frutescens* L. (Lamiales: Lamiaceae), known as *kaennip* in Korea and as *shiso* in Japan, is commonly used in traditional Eastern cuisine. Applications of extracts of this plant also have been used in traditional Chinese medicine for the treatment of atopic dermatitis as well as other inflammatory and allergy-related symptoms [17], resulting in a renewed interest in the economic importance and cultivation of different varieties of this species [18]. There are several varieties of *P. frutescens* characterized by different chemical composition of the essential oils extracted from their leaves, they are referred to as chemotypes and are named for the primary component of the oil. Among these, some have been identified as monoterpens like S-(-)-1,8-*p*-menthadiene-7-al (perillaldehyde, PA), which is the main component in the PA-type varieties used primarily for cooking in Japan and 3-(4-methyl-1-oxopentyl) furan (perillaketone, PK) the main component in the PK-type varieties very diffused in Korea. Despite the large diffusion of *Perilla* in Asian food and the absence of epidemiological evidences seems to indicate that there is not a relevant safety problem for humans, the plant is suspected to arise some safety issues. PK resulted toxic to some animals being associated to the insorgence of pulmonary disease in equine and ovine [19,20]; PA has been long used as a natural aroma compound for food ingredients, but a very recent report by the European Food Safety Authority (EFSA) concluded that PA is genotoxic *in-vivo* and that, accordingly, there is a safety concern for its use as flavouring substance [21].

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At the molecular level, the properties of *P. frutescens* derived compounds and their mechanisms of action are still poorly understood. PA and PK were reported to interact with rat TRPA1 (r-TRPA1) and TRPM8 channels [22]. Further experiments revealed that synthetic derivatives of PK are more potent than the natural variant [23].

Here we show that the human TRPA1 (h-TRPA1) channel orthologue is sensitive to *Perilla* derived compounds. Among the varieties of *P. frutescens*, we screened essential oils derived from the PA and PK chemotypes, as well as from the PKIK chemotype which contains varying amounts of 3-(4-methyl-1-oxo-2-pentenyl)furan (isoegomaketone, IK) in combination with PK [24], for their ability to activate h-TRPA1. In our screen, we also included two PK-derivatives recently reported to activate r-TRPA1: 3-(4-methoxy-phenyl)-1-furan-2-yl-propenone (PK-16) and 3-(4-chloro-phenyl)-1-furan-2-yl-propenone (PK-18) [23]. Additional tests were conducted with the somatosensory phenylpropanoid 1,2,4-trimethoxy-5-[(E)-prop-1-enyl]benzene, identified in the essential oil content of *P. frutescens* var. *acutifolia*, a cultivar mainly used in traditional medicine [25]. The compounds tested appear to be partial agonists of the h-TRPA1 channel with the potency sequence (EC50, μM): PK-16(107.7)>PA (160.5)>ASA (210.9)>PK (350). Our findings provide important insight into the functional properties of the compounds derived from *P. frutescens.*

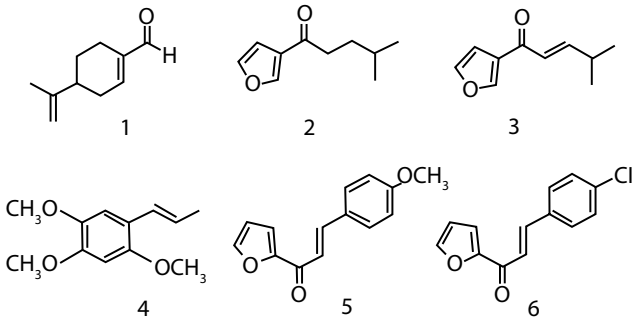

Figure 1: *Perilla* derived compounds**.** Structures of compounds derived from *P. frutescens*. **PA**, S-(-)-1,8-*p*-menthadiene-7-al (perillaldehyde); PK, 3-(4-methyl-1-oxopentyl)furan (perillaketone); IK, 3-(4-methyl-1-oxo-2-pentenyl)furan (isoegomektone); ASA, 1,2,4-trimethoxy-5-[(E)-prop-1-enyl] benzene (α-asarone); PK-16, 3-(4-methoxy-phenyl)-1-furan-2-yl-propenone (synthetic derivative); PK-18, 3-(4-chloro-phenyl)-1-furan-2-yl-propenone (synthetic derivative).

Material and Methods

Preparations of essential oils and compounds

The *P. frutescens* plants used in the present study were cultivated in an open field. The samples with the highest concentration of perillaldehyde (in PA-type plants), perillaketone (in PK-type plants) and perillaketone with isoegomaketone (in PKIK-type plants) were collected from May to October, which covers the entire period of the year during which the plants produce these compounds at the altitude and under the climatic conditions in which the plants were cultivated (Fondazione Minoprio, province of Como, Italy; 380 AMSL). Essential oils from PA- PK- and PKIK-type *P. frutescens* were prepared as previously reported [22,26]. PK derivatives 3-(4-methoxy-phenyl)-1-furan-2-yl-propenone (PK-16) and 3-(4-chloro-phenyl)-1-furan-2-yl-propenone (PK-18) were synthesized according with Bassoli et al. [23]. α-Asarone was isolated as the main component of the essential oil obtained from dry leaves of *P. frutescens var. acutifolia* (*soyeop*, Bassoli and Borgonovo, unpublished) bought at the medicinal plants market in Seoul, Korea. The essential oil has an average yield of 0.25 g/100 g dry leaves. The chemical structure of this metabolite was determined by NMR and GC-MS investigations and was consistent with those described in the literature for α-asarone [24] and with an authentic sample. For the *in-vitro* assays a commercial sample of asarone (Sigma Aldrich, St. Louis, MO, USA, >97% GC) was used. Structures of *Perilla* derived compounds are reported in Figure 1.

Heterologous expression and transient transfection

HEK293T cells (ATCC, Manassas, VA, USA) were grown in HEK293T cell media [Dulbecco's modified Eagle's medium (DMEM) enriched with 10% heat-inactivated fetal bovine serum (MP Biomedicals, Solon, OH, USA), 2 mM L-glutamine, and 100 μg/mL penicillin/streptomycin (Invitrogen, Life Technologies, Grand Island, NY, USA) at 37°C with 5% CO_2.

For transient expression, semi-confluent HEK293T cells were grown in 35-mm cell culture-treated dishes and transfected with pcDNA5-FRT carrying the entire protein-coding region for h-TRPA1 described by Doerner et al. [27] and generously provided by Dr. Gunter Gisselmann and Dr. Hanns Hatt. For parallel control of the channel expression in HEK293T cells, 0.67 μg of a separate plasmid [pEBFP2-Nuc a gift from Robert Campbell (Addgene plasmid # 14893, [28])] carrying the coding sequence of a nucleus-targeted blue fluorescent protein (BFP) under the regulation of the same CMV promoter used for expression of h-TRPA1 was co-transfected with calfectin following the manufacturer's recommended protocol (SignaGen Laboratories, Rockville, MD, USA). At 10 to 18 hours post-transfection, the transfection mix was replaced with fresh HEK293T cell media and the cells were incubated for eight additional hours. Cells were then split in 35-mm cell culture treated dishes and allowed to recover for up to 4 hours prior calcium imaging experiments. Measurements were performed within 44 to 72 hours post-transfection. To estimate transfection efficiency, a parallel transfection was conducted using the positive control vector pcDNA5/TO/LACZ (Invitrogen) and staining with 0.1% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XGal, Thermo Scientific, Wilmington, DE, USA). The predominant majority of LACZ transfected cells demonstrated a strong staining suggesting well optimized transfection protocol/transfection efficiency.

Calcium imaging

HEK293T cells co-transfected with the h-TRPA1 and pEBFP2-Nuc plasmids, were incubated for 30m-2h at room temperature in 0.5-1.0 mL HEK cell solution (mM: 140 NaCl, 5 KCl, 1.0 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES, 10 Glucose, pH 7.5) including the fluorescent calcium indicator Fluo-4AM (Invitrogen) at 5.0-15 μM prepared with 0.2-0.06% Pluronic F-127 (Invitrogen). After incubation, the buffer was removed and cells were rinsed with 4.0 mL HEK Ca^{++} solution (mM: 140 NaCl, 2.0 $CaCl_2$, 10 HEPES, pH 7.5). Cells were placed on the stage of an inverted microscope (Olympus IX-71) equipped with a cooled CCD camera (ORCA R2, Hamamatsu, Hamamatsu City, Japan). A standard FITC or Fura-2 filter set (excitation at 510 nm, emission at 530 nm and excitation at 340 nm or 380 nm, emission at 510 nm) were used to estimate Fluo-4 and BFP related fluorescence intensity. Two gravity fed perfusion contours were used. The first contour continuously washed the cells with HEK Ca^{++} solution (~250 μL/min). The second was used for stimulation and/or application of the compounds tested. Switches between the perfusion channels and regulation of pulse duration were controlled by a multi-channel rapid solution changer (RSC-160, Bio-Logic) and Clampex 9 software (Molecular Devices, Sunnyvale, CA,

USA). Stimulus duration was 10 seconds when cells were stimulated with MO and 20 seconds when stimulated with other compounds. More complex stimulation protocols are specified in the Results.

Calcium imaging experiments were carried out under the control of Imaging Workbench 6 software (INDEC BioSystems, Santa Clara, CA, USA). Stored time series image stacks were analyzed off-line using Imaging Workbench 6, Clampfit 10.5, SigmaPlot 11 or exported as TIFF files into ImageJ 1.42 (available from public domain at http://rsbweb.nih.gov/ij/index.html). Continuous traces of multiple responses were compensated for slow drift of the baseline fluorescence when necessary. All recordings were performed at room temperature (22°C to 25°C).

Stimulus

Aliquots of stimulus solutions were prepared in different volumes of HEK Ca^{++} solution depending on experimental needs, diluting stock solutions of our samples previously dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich).

MO (Sigma Aldrich), PK, PA, ASA, PK-16 and PK-18 samples were dissolved in DMSO at millimolar (mM) concentrations and diluted in HEK Ca^{++} solution to prepare stimuli at micromolar (μM) concentrations. PKIK was dissolved in DMSO at milligram/mL (mg/mL) to prepare stimulus in HEK Ca^{++} solution at microgram/mL (μg/mL). For the latter, concentrations of PK and IK compounds were calculated according with their respective percentage content in the essential oil sample. Concentrations of stimulus solutions and their sequences of application varied between different experiments.

According to HPLC based estimations, PKIK-type essential oil from *P. frutescens* contains a mixture of PK as the major component and IK as the secondary metabolite; the relative content of the two compounds changes during growing of the plant (Bassoli and Borgonovo, unpublished). For experiments reported here, we used a mixture of samples collected throughout the entire harvesting season. The amount of PK and IK in the sample determined by HPLC is 88% and 11% respectively. In our experiments, we used *P. frutescens* samples diluted to a final PK dose of ~535 μM (saturating PK concentration). Thus, the PK dose in the PKIK-type essential oil used in this study contained ~67 μM IK.

Generation of dose/response relationships

Dose/response relationships were generated for pure compounds (ASA, PK-16, PK-18) and *P. frutescens* essential oils predominantly containing only one compound, as estimated by HPLC [PK-type (PK > 98%) and PA-type (PA > 98%)]. Different concentration ranges were chosen for different compounds/samples, depending on compound concentration eliciting the minimal detectable response and the stimulus saturating concentration (Table 1). To estimate and if necessary correct the system sensitivity to excitation light intensity and to the mechanical disturbance of the cells caused by perfusion system we performed control tests before each experiment using HEK Ca^{++} solution without stimulus. MO (200 μM) was used as a positive control and as a reference for normalization. To minimize rundown of the calcium responses especially to saturating stimuli the consecutive stimuli were applied every 45-60 min and multiple 35-mm dishes were used for different doses. To estimate EC50s, the normalized dose/response data were approximated by a modified Hill equation.

Sample	Human TRPA1 (μM)	Rat TRPA1 (μM)
MO	85.25 ± 14.99	2.5 ± 0.7
PK-16	107.66 ± 10.71	20.9 ± 2.27
PA	160.47 ± 9.12	40.7 ± 7.63
ASA	210.92 ± 36.43	Inactive
PK	349.92 ± 53.01	21.9 ± 1.93

Table 1: Comparison of the potencies (EC50) (Potencies were calculated for *P. frutescens* samples comparing human and rat TRPA1, according with our findings and Bassoli et al. in 2013. MO: Mustard oil, as a reference).

Results

To confirm functional expression of h-TRPA1, we began by applying MO, a known agonist. Many of the HEK293T cells transfected with h-TRPA1 generated transient calcium signal upon application of MO. The amplitude of the responses depended on MO concentration, and the overall sensitivity of the cells to the agonist correlated with BFP expression. The parameters of concentration dependence for the MO were estimated to be EC50~ 85.3 ± 15 μM with Hill coefficient h~ 2.9 ± 1.3 μM, consistent with previous estimates [14,29]. Untransfected cells were not responsive to MO when tested under the same conditions. The responses to MO at close to saturating concentration (200 μM) were used for normalization to allow comparison among the agonists tested in further experiments.

We then screened a panel of *P. frutescens*-derived compounds, including those previously found to be r-TRPA1 channel modulators [22,23]. The main components of *P. frutescens* essential oils (PA, PK, and ASA) and the PK synthetic derivative PK-16 were able to activate h-TRPA1 channel mediated calcium responses. However, all of the compounds tested evoked substantially lower calcium responses in comparison to MO (saturating concentrations of agonists, Figure 2). The dose response relationship of the h-TRPA1 channel response to PA yielded an EC50 of ~160.5 ± 9.1 μM (n = 76-164) with a normalized peak amplitude of 0.33 ± 0.04. Similarly, PK activated a much less robust h-TRPA1 channel dependent calcium response in comparison to MO, with normalized peak amplitude of 0.44 ± 0.03. The concentration-dependence yielded an EC50 of ~349.9 ± 53 μM (n = 78-151). The concentration dependences for ASA and PK-16 yielded comparable EC50s of ~210.9 ± 36.4 μM and ~107.66 ± 10.71 μM and normalized amplitudes of 0.28 ± 0.04 (n = 52-139) and 0.47 ± 0.05 (n = 16-114), respectively. HEK293T cells not expressing h-TRPA1 were not appreciably responsive to any of the compounds tested. Overall, the potency sequence of the compounds was PK-16>PA>ASA>PK, with the synthetic derivative PK-16 and its natural analog PK acting as the most and the least potent ligands, respectively.

Unlike the other agonists tested, synthetic PK-derivative, PK-18, resulted in relatively slow, incoherent and mostly irreversible responses in h-TRPA1 transfected cells (Figure 3). These properties as well as low solubility of PK-18 [1.2E-4 mol/L, Log P=3.48, Scifinder, 2015; Chemical Abstracts Service: Columbus, OH, 2015; CAS Registry Number 111042-59-2 (accessed Nov 12, 2015); calculated using ACD/Labs software, version 11.02; ACD/Labs 1994-2015] made the compound impractical in terms of obtaining experimental data and interpretation.

The relatively low efficacy of the compounds tested in comparison to MO suggests that they are partial agonists of the h-TRPA1 channel. To probe whether they may promote the h-TRPA1 channel desensitisation [23] and/or inhibit channel activity by competing with MO, we used following experimental paradigm: the h-TRPA1 channel-mediated calcium signal was initially activated by exposure of the cells to MO (40 μM, 20 seconds), followed by application of a close to saturating concentration of an agonist (PA or PK) and then by a combined application of MO and an agonist (PA or PK). Each

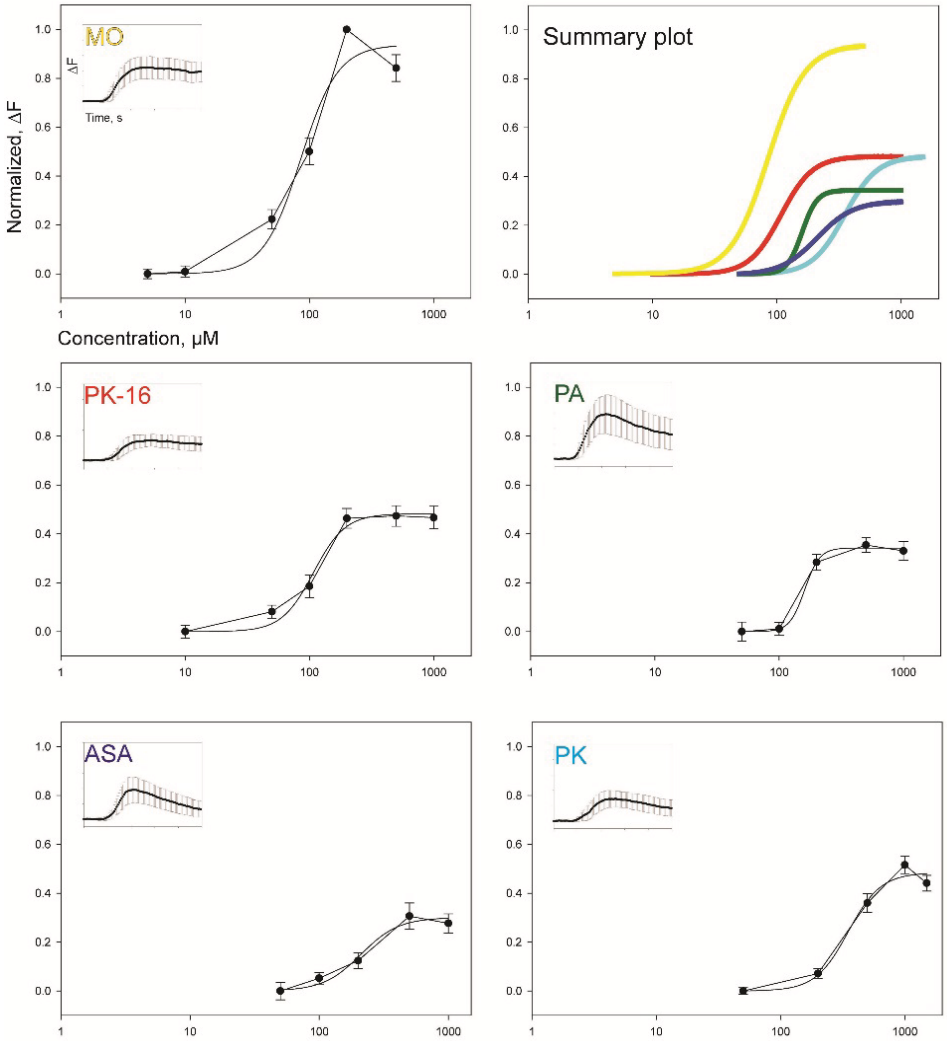

Figure 2: Effects of *Perilla* derived compounds on human TRPA1 channel activity. The HEK293T cells expressing h-TRPA1 channels generate calcium signals in response to application of the compounds (insets). The effects were concentration dependent and reversible. Graphs show the concentration dependences of the compounds expressed as a function of normalized mean peak fluorescence intensity change (ΔF) vs drug concentration. In all cases, the responses were individually normalized to the saturating concentration of MO (200 μM). Error bars represent standard error of mean. Data were fit with the Hill equation (solid lines). Insets represent average calcium response elicited by a drug application obtained in a single experiment. Grey color depicts standard deviation. Top right panel, summary plot of the concentration dependences.

compound was tested in an individual series of experiments. The amplitudes of the calcium responses were (mean ± SEM): MO, 40 μM 19.48 ± 8.30; PA, 300 μM 13.73 ± 9.10; PA, 300 μM + MO, 40 μM 28.33 ± 15.22; MO, 40 μM 32.73 ± 14.82; PK, 400 μM 32.08 ± 12.12; PK, 400 μM + MO, 40 μM 54.82 ± 16.41 (Figure 4). In each of the series, the combined application of the ligands (MO + PA or PK) generated the strongest responses.

Discussion

Here we found that a number of compounds from the Asian food plant *P. frutescens* can activate the h-TRPA1 channel. The potency sequence of h-TRPA1 activation derived for the compounds, PK-16>PA>ASA>PK, differs from that previously derived for r-TRPA1 activation, PK-16>PK>PA>ASA (Table 1). Notably, r-TRPA1 is almost equally responsive to PK-16 and PK, while h-TRPA1 is more sensitive to PK-16 than PK. Furthermore, tests conducted on r-TRPA1 verified that it does not respond to ASA (Dr. Luciano De Petrocellis, personal communication; Table 1), which is a compound that we have found to activate h-TRPA1 (EC50~210.9 ± 36.4 μM). Consistent with previous data for r-TRPA1, the synthetic derivative of PK, PK-16 (EC50~107.7 ± 10.7 μM), is the most potent of the non-MO h-TRPA1 agonists tested. The previous findings [23] also suggested that some *Perilla*-derived compounds promoted r-TRPA1 channel desensitization and/or inhibited the channel activity, possibly by competing with MO for receptor binding. In direct contrast, we found that the strongest of the antagonists reported for r-TRPA1, PA and PK did not reduce the h-TRPA1 calcium signal activated by MO. Instead, the h-TRPA1 channel mediated calcium signal was proportionally augmented in the presence of close to saturating concentrations of these compounds. Further structure-function relationship analysis is necessary to understand what structural features of these compounds and the TRPA1 orthologues account for the different potencies and the different kinetic parameters of the responses.

The significant differences in overall channel properties of r-TRPA1

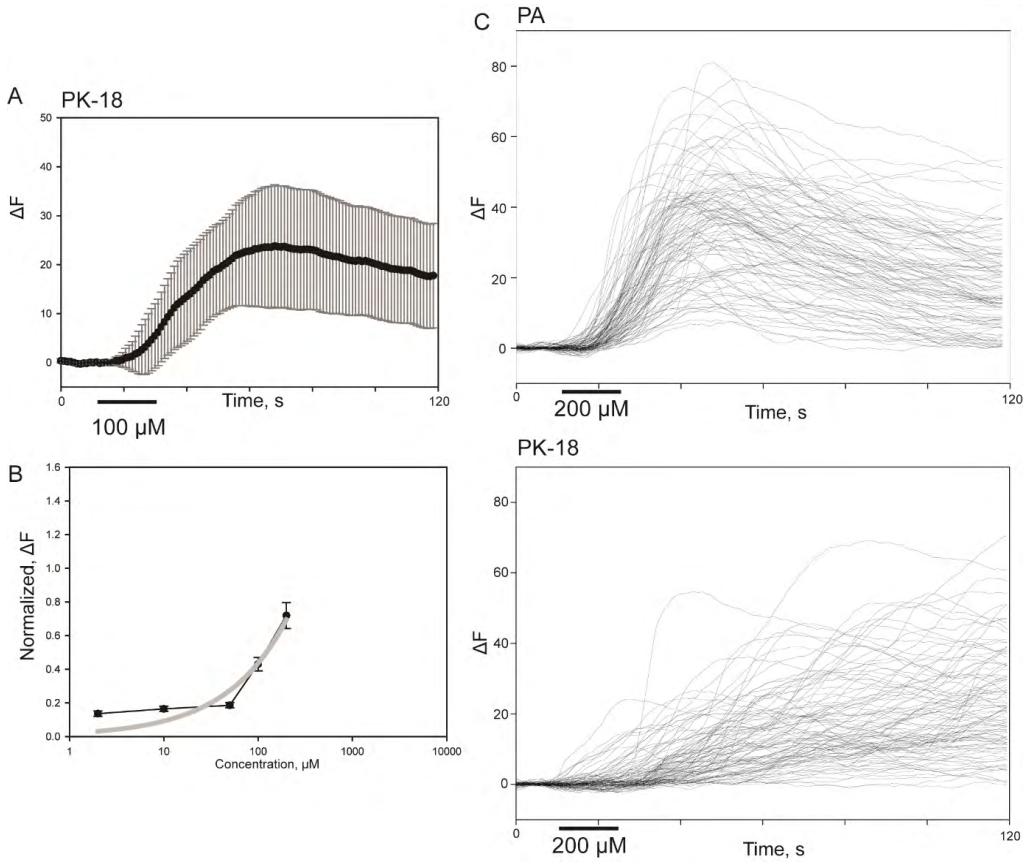

Figure 3: Effects of PK-18 on the calcium signal mediated by the h-TRPA1 channel expressed in HEK293T cells. A, Typical average response generated by PK-18 (100 μM, n=83). Grey bars depict standard deviation. B, PK-18 concentration dependence limited by the solubility threshold of the drug (1.2E-4 mol/L (Scifinder, 2015; Chemical Abstracts Service: Columbus, OH, 2015; CAS Registry Number 111042-59-2 (accessed Nov 12, 2015); calculated using ACD/Labs software, version 11.02; ACD/Labs 1994-2015)). Data represent normalized mean ± standard error of mean. C, Comparison of the calcium responses of the same set of cells (n=104) to two different *Perilla* compounds: PA (top panel) and PK-18 (bottom panel). Note: responses to PK-18 are relatively slow and incoherent. Horizontal black bars below traces mark timing of the drug pulse application (20 s).

and h-TRPA1 could be explained by either the different experimental approaches used in the two studies or, most likely, by species specific heterogeneity in their functional properties. The full length (untrimmed) protein sequences of the two orthologues differ in amino acid identity by approximately 20%, with differences throughout including within transmembrane domains 5 and 6 which have been found to determine the specificity of proton sensitivity of TRPA1. More targeted structure - function correlation analyses of these TRPA1 channel orthologues, similar to those of previous studies [30-32], will be required to identify the differences in the critical functional domains/residues that result in the biophysical and pharmacological heterogeneity of the responses to the compounds tested here.

The agonists of TRPA1 channels are usually divided into two main categories based on their mechanism of activation: electrophiles and non-electrophiles. Electrophiles such as allyl-isothiocyanates [8] and α-β unsaturated aldehydes [33,34], activate TRPA1 channels through covalent modifications, condensing α-β-unsaturated bonds with nucleophilic mercapto-groups of cysteine residues of the channels in a Michael addition. Non-electrophiles, such as carvacrol from oregano [35], activate TRPA1 channels without causing covalent modifications. According to the molecular features of the ligands tested here (Figure 1), ASA lacks of α-β-unsaturated bonds, indicate that this compound may be involved in non-covalent interactions with the channel. In contrast, the electrophilic carbonyl of PA as well as those of ketones PK and its synthetic analogs PK-16 and PK-18 may serve as electrophilic targets in the reversible attack of nucleophilic residues of N-terminal cysteines in a Michael addition.

One of the compounds that we identified as a partial agonist of h-TRPA1 is ASA (EC50~210.9 ± 36.4 μM), a phenylpropainoid normally present in essential oils from ginger species within *Asarum* (Piperales: Aristolochioaceae), but also found in *P. frutescens* var. *acutifolia* (Bassoli and Borgonuovo, unpublished data). Contrary to our findings, tests conducted on r-TRPA1 found no response to ASA (Table 1), which is consistent with significant differences in the overall channel properties between r-TRPA1 and h-TRPA1. ASA and other phenylpropanoids (allyltetramethoxybenzene, caffeic acid, dillapiole, elemicin, myristicin, nothoapiole, rosmarinic acid and methyl ester) contained in *P. frutescens* extracts, were recently found to inhibit proinflammatory cytokine generation in lipopolysaccharide-treated murine lung cells [36]. Conversely, monoterpens from *P. frutescens* such as PK have long been known to induce pulmonary toxicity in mammals such as horses and sheep [19,20]. While the molecular targets for these compounds in these processes were not identified, there is evidence that implicates TRP channels, including TRPA1, in the physiological and pathophysiological reactions associated with respiratory depression and neurogenic lung inflammation [37,38].

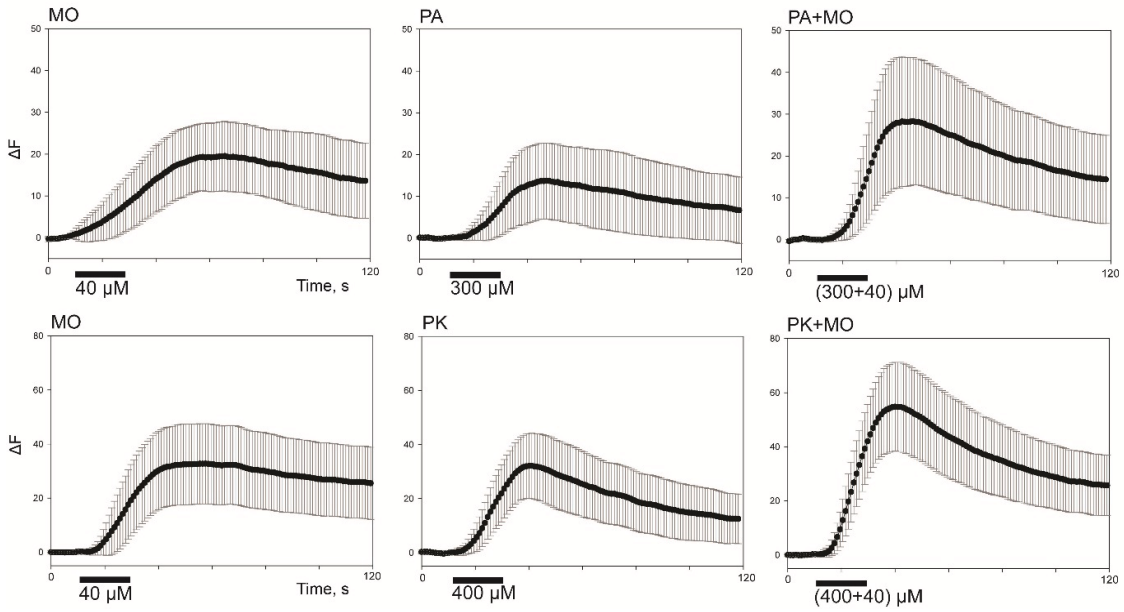

Figure 4: Effects of PA and PK on the MO activated h-TRPA1 channel. The h-TRPA1 channel mediated calcium signal was initially activated by MO (40 μM, left panels), then by high responsive doses of an agonist (PA: 300 μM, n=111, or PK: 400 μM, n=86, middle panels) followed by combined application of MO and an agonist (PA or PK, right panels). Amplitudes of the calcium responses were (mean ± standard error of mean): MO, 40 μM 19.48 ± 8.30; PA, 300 μM 13.73 ± 9.10; PA, 300 μM+MO, 40 μM 28.33 ± 15.22; MO, 40 μM 32.73 ± 14.82; PK, 400 μM 32.08 ± 12.12; PK, 400 μM+MO, 40 μM 54.82 ± 16.41. Note, in both experimental sets the combination of ligands (MO+PA or PK) generated the highest responses. Horizontal black bars below traces mark timing of the drug pulse application (20 s).

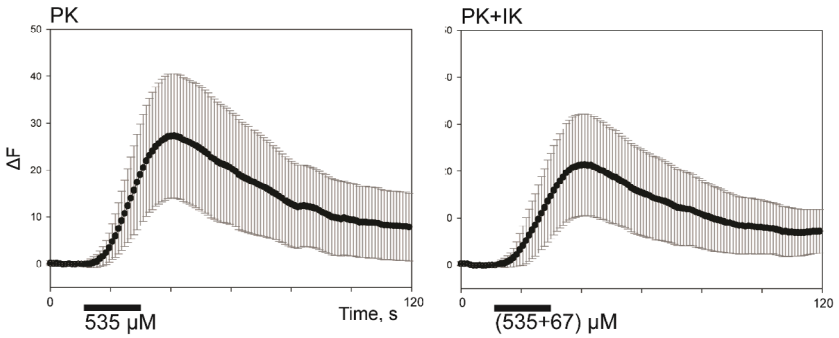

Figure 5: Effects of PK and PKIK on the h-TRPA1 channel. The h-TRPA1 channel mediated calcium signal was initially activated by PK (535 μM, n=84, left panel) and then by a PKIK dose containing the same concentration of PK (535 μM PK+67 μM IK, right panel). The amplitudes of the calcium responses were (mean ± standard error of mean) PK, 535 μM 27.27 ± 13.30; PK, 535 μM+IK, 67 μM 21.36 ± 10.86. Horizontal black bars below traces mark timing of the drug pulse application (20 s).

Our findings indicate that these compounds can activate h-TRPA1, suggesting a potential molecular mechanism for their role in human inflammatory processes in general, and in pulmonary inflammation in particular.

The major components of the essential oil derived from the PKIK chemotype of *P. frutescens* [24] are PK and IK [3-(4-methyl-1-oxo-2-pentenyl)furan]. IK is an unsaturated form of PK and a *P. frutescens* monoterpen known for antitumor properties [39]. Although its molecular targets have not been identified *in-vivo*, IK was recently reported to activate r-TRPA1 channels (EC50 = 7.6 ± 0.2 μM), even more potently than *P. frutescens* derived natural ligands (PA: 40.7 ± 7.63; PK: 21.9 ± 1.93; Table 1) [23]. Although it has not been tested with r-TRPA1, the h-TRPA1-based calcium signal activated by the essential oil derived from the PKIK chemotype was lower (~78%) than that activated by a similar concentration of PK alone (Figure 5). While speculative in the context of the current study given the molecular complexity of essential oils, the lower response to PKIK suggests that PK and IK may be competitive agonists of h-TRPA1 channel.

Conclusion

In conclusion, the chemotypic variations of *P. frutescens* and resulting differing physiological effects underlie the distinct uses of specific varieties of this plant species in food [18] and medicine [17]. Here we report that two natural terpenoids, PA and PK, found in *P. frutescens* essential oils and PK-16, a synthetic derivative of PK, can activate the h-TRPA1 channel. We use dose response experiments to demonstrate that the channel is more sensitive to PK-16 and PA and find that the ability of these compounds to activate TRPA1 differs between the human and rat orthologues. Notably, we also report that h-TRPA1 but not r-TRPA1 responds to a phenylpropanoid ASA

which has a possible inhibitory role of inflammatory responses and is a compound known to be synthesized by *Asarum* plants that was recently identified in the essential oil content of a novel chemotype of *P. frutescens* (*var. acutifolia*). Contrary to previous findings with r-TRPA1, we find no detectable inhibitory activity of PK and PA towards the well-known TRPA1 agonist MO. We rather demonstrate a potential inhibitory interaction between two monoterpens, PK and IK, emitted by the PKIK chemotype of this plant. The varying levels of TRPA1 activation in response to the compounds derived from the essential oils of different *P. frutescens* chemotypes, likely resulting from differences in their modes of interaction with the channels, highlight the importance of the chemotype mapping of this food and medicinal plant [24]. Further, they draw attention to the potential importance of this plant as a source for naturally derived drugs that may be used to target the TRPA1 channel.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interests.

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