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Taste-guided identification of high potency TRPA1 agonists from *Perilla frutescens*

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ABSTRACT

Perilla frutescens is a food plant widely used in Asian cuisine. This plant was investigated for its interesting taste and somatosensory properties. Perillaldehyde and perillaketone are among the components of the aromatic extracts from *P. Frutescens*. These compounds were shown here to activate the cloned TRPA1 channel when expressed in an heterologous cell system and are therefore suggested to be responsible for the chemesthetic properties of this plant.

1. Introduction

The research of novel taste compounds is very active. The interest arises from both the gastronomic and pharmaceutical sides. For what concerns the taste aspects, there is a great interest in consumers for 'unusual' tastes, which are different from those well known from 'globalized' foods. Among these those that are now known to activate Transient Receptor Potential (TRP) cation channels and referred to as 'chemesthetical' or 'trigeminal' (such as hot, cooling, tingling and similar) are of great importance, since they are found in many spices and food plants used in traditional cuisine and strongly contribute to their character. Indeed, gustative properties are not the sole reasons of interest for TRP active compounds in food; these receptors have in fact an important role in other biological mechanisms, behaving as authentic 'cellular sensors' for external different stimuli.[1] The importance of TRP channels in disease[2] and in general in biomedical and nutrition research[3] have been recently reviewed. It is very interesting from the pharmacological point of view the role of TRPs in pain perception, which is well described in the literature. TRP active compounds, such as capsaicin on TRPV1, as well as irritating (or pungent) compounds from wasabi or garlic on TRPA1 are known to produce a desensitisation effect depending on exposure: therefore, is has been suggested that a diet rich in such compounds might raise the threshold of pain perception through a smooth but systematic 'deactivation' of these pain sensors. Thus a diet rich in TRP active compounds might constitute a sort of 'pain preventing menu'.

A relatively little number of chemical classes of compounds is clearly involved in such mechanisms, including the well known vanilloids, which activate TRPV1, menthol, which activates TRPM8, and allyl isothiocyanates, such as those contained in horseradish and mustard oils, which activate the TRPA1 receptor and are likely involved in the generation of the tingling or pungent taste of these plants. Beside pungent isothiocyanates, TRPA1 is activated also by other compounds among which aldehydes, such as cinnamaldehyde and acrolein, a lacrimating and pungent compound formed during food cooking at high temperature, and by icilin, a synthetic compounds that also interacts with TRPM8. TRPA1 seems to play a role also in noxious cold perception and in mechanical bending of stereocilia in the inner ear hair cells. TRPA1 is highly conserved in evolution and has a very restricted pattern of expression suggesting a specificity of roles unique in sensory functions.

Perilla frutescens is a food plant commonly used in Asian cuisine, especially in Korea (*kaennip*) and Japan (*shiso*). Despite its wide use, this plant has not yet been exhaustively investigated; a recent review has been published showing a renewed interest for its

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cultivation and properties.[4] Application of extracts of this plant is described in traditional Chinese medicine, for the treatment of atopic dermatitis and for other anti-inflammatory and anti-allergic properties.[5] From the leaves of *P. frutescens* the perillaldehyde (PA), (which is an intermediate for synthesis of the intensive sweetener perillartine) has been extracted, although the plant is not sweet. Kaennip is instead very 'unusual' to western consumers for its olfactive, gustative and somatosensory properties. In a research project on the comparison of tastes of Italian and Korean cuisine, we started the isolation of pure taste active compounds from Perilla to be submitted to sensory analysis and in vitro assays with cloned taste receptors. In fact, a systematic study on taste active compounds from this plant has not been carried out previously, even though many volatile components of the aroma have been already identified by GCMS chromatography.

2. Results

For this study three samples of *P. frutescens* were used: (1) adult plant; the plant was grown in a garden from seeds obtained from the Korean market and harvested at the end of the flowering season; (2) young plant: the plant was grown in greenhouses in controlled conditions (Faculty of Agricoltural Sciences, Milano), and extracted at the two leaves developing stadium; (3) kaennip leaves; commercial adult kaennip leaves from a food market in Korea. Both fresh or freeze-dried leaves of *P. frutescens* were used. From the volatile fraction of Perilla leaves we initially isolated the perillaketone (PK), a furylketone derivative that has been previously identified as one of the major secondary metabolites in this plant together with perillaldehyde (PA)[6] (Fig. 1). No references to the properties of perillaldehyde and perillaketone in relationship to chemosensory perception have been previously reported.

In our samples, perillaldehyde PA was not present in significant amounts; only traces could be identified by comparison with an authentic sample. Perillaketone PK was isolated by steam distillation followed by extraction of the distillate or by direct solvent extraction of either fresh or freeze-dried leaves. From adult plants also seeds, flowers and stems were extracted, but PK was not found in these samples in appreciable amounts. PK was analysed to confirm its structure. It is a colorless oil with a characteristic and penetrating odour, with strong and herbaceous notes.

Despite kaennip is commonly used in Korean and other Asian countries' cuisine and is therefore to be considered safe for human consumption, pure PK has been previously reported to be responsible of pulmonary toxicity in mice[7] and suspected to have some role in the toxicity of the plant to cattle through ingestion. In the absence of a detailed toxicological information, we did not conduct sensory evaluations on the pure compound to determine its specific gustatory and somatosensory properties, such as pungency or refreshing activity. Instead, we submitted the isolated PK to in vitro assays with rat recombinant TRPA1 and TRPM8 receptors, in order to identify a possible activity due to cooling, pungent or tingling compounds that were suggested from the overall taste profile of the plant as described in gastronomic preparations. Also PA was tested on the two TRP receptors, after a careful control of its chemical and optical purity.

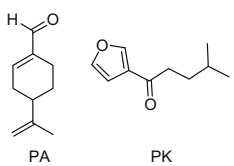

Figure 1. The structure of perillaldehyde (PA) and perillaketone (PK).

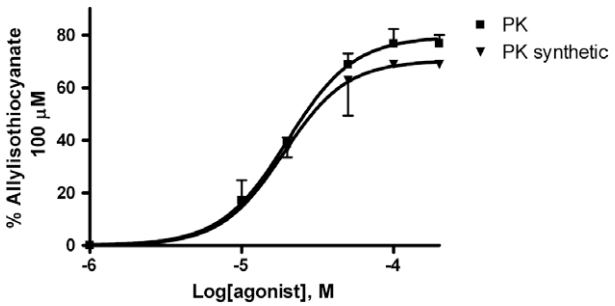

Figure 2. Dose-related effects of PK and PK synthetic on $[Ca^{2+}]_i$ in HEK-293 cells stably transfected with the rat recombinant TRPA1 channel. Data are expressed as percentage of the maximal effect observed with MO 100 μM. Data are the means ± S.E. of at least $n = 3$ separate determinations.

2.1. TRPA1 receptor is activated by PK and PA

Using a fluorometric test, we showed that rat TRPA1-HEK293 cells exhibit a sharp increase in intracellular $[Ca^{2+}]_i$ upon application of PK. The activity of the compounds was normalised to the maximum intracellular Ca^{2+} elevation generated by application of allylisothiocyanate (mustard oil, MO) 100 μM. Both natural and synthetic PK were tested and yielded similar results (Fig. 2).

In the same assay, PA were tested and resulted more than two-fold more efficacious than PK in activating TRPA1. Using this test, we determined the concentration for half-maximal activation to be 19.7 ± 1.7 μM (Hill slope 2.0 ± 0.4) for PK and 41.0 ± 7.5 μM for PA (Hill slope 1.0 ± 0.1) (Fig. 3). Ruthenium red at 10 μM, an unselective blocker of TRP channels, inhibited the effect (data not shown).

Note that 5-min preincubation of TRPA1-HEK-293 cells with PK prevented the elevation of $[Ca^{2+}]_i$ induced by MO in TRPA1-HEK-293 cells (data not shown).

Potency and efficacy of PA and PK are also shown in Table 1.

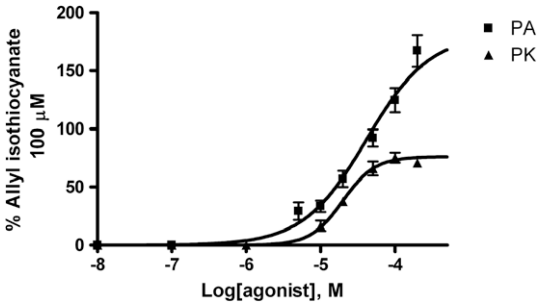

Figure 3. Dose-related effects of PA and PK on $[Ca^{2+}]_i$ in HEK-293 cells stably transfected with the rat recombinant TRPA1 channel. Data are expressed as percentage of the maximal effect observed with MO 100 μM. Data are the means ± S.E. of at least $n = 3$ separate determinations.

Table 1
The efficacy and the potency of PK and PA on $[Ca^{2+}]_i$ in HEK-293 cells stably transfected with the rat recombinant TRPA1 receptor channel

Cpd	Efficacy (%MO 100 μM ± S.E.)	Potency ($pEC_{50\%}$ ± S.E.)
PK	74.6 ± 2.3	4.71 ± 0.03
PA	181.2 ± 11.3	4.39 ± 0.09

The efficacy is the maximal response of the agonists expressed as percentage of the analogous effect observed with MO 100 μM. The potency (EC_{50}) is determined as the concentration of test substances required to produce half-maximal increases in $[Ca^{2+}]_i$. Data are means ± S.E. of at least $n = 3$ experiments.

2.2. Effect of PK and PA on TRPM8 activation by icilin

As shown previously,[8] icilin dose-dependently elevated intracellular Ca^{2+} in TRPM8-HEK-293 cells, but not in non-transfected cells, with an EC_{50} of 0.19 ± 0.03 μM. Preincubation (5 min) with different doses of PK and PA (which were inactive per se up to a 200 μM concentration), and then continued incubation with icilin (0.25 μM) of TRPM8-HEK-293 cells, caused a slight inhibition of TRPM8 response to icilin (PA 200 μM 64.3% of inhibition; PK 200 μM 39.3% of inhibition of icilin at 0.25 μM concentration). A longer (15 min) preincubation time did not dramatically modify the performances, whereas shorter preincubation times were not tested.

3. Discussion

Several plants are known to contain TRP active compounds that usually have a defensive role against herbivorous predators, achieved through the biosynthesis of chemical agents that produce irritation and inflammation.[9,10] Irritants such as capsaicin from hot pepper and isothiocyanates and thiosulfinates contained in garlic produce their psychophysical effects by targeting excitatory TRP channels on primary afferent nerve fibers of the pain pathway. The ability of these agents to produce neurogenic inflammation and vasodilatation has been attributed to the fact that their molecular sites of action (TRPV1 and TRPA1, respectively) are expressed on neurons whose activation leads to the peripheral release of CGRP, substance P, and other pro-nociceptive, inflammatory and vasodilatory neurotransmitters. Several pieces of evidence suggest that TRPA1 is the major site of isothiocyanate and garlic derivative action on nociceptive sensory neurons.[11] Beside isothiocyanates, few other chemicals are known to activate TRPA1. Among them, some unsaturated aldehydes exhibit irritating or even noxious properties at high concentrations, such as cinnamaldehyde from cinnamon or acrolein formed during thermal treatments of food.

Here we report for the first time that PA an PK from *P. frutescens* strongly activate in vitro the TRPA1 channel. Remarkably, both compounds have an unsaturated carbonyl group in their structure and this could support the hypothesis that this functional group is an important pharmacophore in generating this bioactivity. In fact, it has been suggested[12–14] that TRPA1 can be activated through an 1,4-addition of nucleophilic cystein groups of the receptor protein to appropriate acceptors such as isothiocyanates and unsaturated carbonyls. The activation of TRPA1 might explain at the molecular level the somatosensory characteristics of kaennip, the taste and flavour of which is described as 'refreshing', 'pungent', 'tingling' or 'mint-like'. Interestingly, PK, PA and Perilla extracts do not activate the TRPM8 receptor; on the other hand the physiological role of TRPA1 in the transmission of cold sensation is still a matter of discussion.[15]

The relationship between the activation of TRP channels by compounds contained in vegetable food and the mechanisms of food preference and choice are extremely interesting. In fact, the somatosensory contribution to the overall gustative impact is quite important in determining our food preferences. Differently from the so called 'basic tastes', which determine a 'raw' selection of main nutrients, the perception of chemestetic properties plays probably a more subtle role both in positive and in negative selection. It is a fact that vegetables that are rich in TRP active compounds, such as garlic, onions, rockets and other brassicaceous plants, are often associated to positive effects on health; therefore, the identification of such a property in an food plant such as *P. frutescens*, which is easily cultivated and largely diffused (at least in the Asiatic cuisine) is an interesting finding. It is interesting to note that, although the efficacy and potency of PK and, particularly, PA at eliciting elevation of intracellular Ca^{2+} via TRPA1 in HEK293 cells was similar, or even superior, to that of pungent TRPA1 agonists, such as the mustard oil isothiocyanates, or carvacrol,[16] the major ingredient of oregano, or isovelleral, the pungent product of the fungus *Lactarius vellereus*, and polygodial,[17] isolated from the leaves of water pepper, this does not seem to be sufficient to confer to *P. frutescens* a strong pungent or otherwise 'aggressive' taste similar to that experienced instead with mustard, garlic or oregano. This might be due to several reasons, including: (1) the capability of some of these compounds (e.g. isovelleral, polygodial, carvacrol) to activate other TRP channels involved in heat sensitivity (i.e., TRPV1 and TRPV3), (2) the capability, shown here, of PK and PA to immediately desensitise TRPA1, and also to antagonize the TRPM8 channel; (3) the presence in *P. frutescens* of lower amounts of PK and PA as opposed to the possible higher abundance of other TRPA1 agonists in other plants. Whatever the underlying reason, the lack of pungency of *P. frutescens* resembles that of *Cannabis sativa*, which contains several compounds capable to activate TRPA1 (and antagonize TRPM8) at concentrations 2 or 3 orders of magnitude lower than those necessary to PK and PA to exert the same effect.[18]

4. Conclusions

Starting from the 'unusual' taste and the chemesthetic properties of the food plant *P. Frutescens* we were able to identify that two of its major secondary metabolites, PA and PK, are able to activate in vitro the TRPA1 channel. This finding might explain the taste properties of the plant at the molecular level and identify it as an interesting target for its culinary and pharmaceutical applications. PA and especially PK—which has a relatively simple chemical structure—might be considered as new interesting lead compound for future structure-activity relationship studies. This could help in a near future to identify pharmacophores and mechanisms involved in TRPA1 activation and to design new potentially active compounds with applications in the pharmaceutical and food industries.

5. Experimental

5.1. Extraction of perillaketone from *P.* frutescens

Perillaldehyde PA was not detected in appreciable amounts in our samples. Commercial S-(−)-PA was purified by distillation followed by TLC preparative chromatography. Chemical purity was checked by HPLC and NMR, optical purity by $[\alpha]_D$ measurements. Perillaketone PK was isolated with steam distillation followed by extraction with ethyl acetate or DCM of the distillate or by extraction with DCM at room temperature on fresh leaves or with hexane on freeze-dried leaves. Every single sample was distillated or extracted three times, and usually, by steam distillation pure perillaketone was obtained, while solvent extraction gives an impure sample. The purity was detected by GC and HPLC analysis. From the adult plant sample also seeds, flowers and stems were extracted but PK was not identified in these samples. Extraction of 30 g of freeze-dries leaves, followed by chromatography purification on column of silica gel, gave 23 mg of pure product as a colorless oil.

Perillaketone (PK) (RN 553-84-4): 1H NMR data are consistent with those in the literature.[19,20] ^{13}C NMR ($CDCl_3$) δ: 22.2 (CH_3, two signals overlapping), 27.6, 33.0, 38.2, 108.5(C4), 127.5(C3), 144.0(C5), 146.9 (C2), 195.2 (CO). The assignments were performed by 2D cosy-dqf experiments and the data compared to those in the literature.[21] GC–MS (AT-1ms column (Alltech), He carrier, flow 0.8 mL/min, from 50 °C to 240 °C in 40 min, 240 °C for another

15 min); retention time 14.05 min, *m/z*, (%): 166 (M^+, 5), 110 (90), 95 (100). Lit[22]: MS (EI) *m/z* 166, 110, 95.

5.2. In vitro assays with TRP receptors

HEK-293 cells stably over-expressing recombinant rat TRPA1 cDNA[16] or rat TRPM8[8] were selected by G-418 (Geneticin; 600 μg/ml), grown on 100-mm diameter Petri dishes as monolayers in minimum essential medium supplemented with non-essential amino acids, 10% foetal bovine serum, and 2 mM glutamine, and maintained under O_2/CO_2 (95%/5%) at 37 °C; stable transfections were checked by quantitative real-time polymerase chain reaction (RT-PCR). The cells were loaded for 1 h at 25 °C with the cytoplasmic calcium indicator Fluo-4-methylester (4 μM; Invitrogen), a selective intracellular fluorescent probe for Ca^{2+}, containing Pluronic (0.02%; Invitrogen) in minimum essential medium without foetal bovine serum. After the loading, cells were washed twice in Tyrode's buffer (145 mM NaCl, 2.5 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM D-glucose, and 10 mM HEPES, pH 7.4), resuspended in Tyrode's buffer, and transferred to the quartz cuvette of the spectrofluorimeter (λ_{EX} 488 nm; λ_{EM} 516 nm) (Perkin–Elmer LS50B equipped with PTP-1 Fluorescence Peltier System; PerkinElmer Life and Analytical Sciences, Waltham, MA) under continuous stirring. Experiments were carried out by measuring cell fluorescence at 25 °C before and after the addition of the test compounds at various concentrations (1 nM–200 μM). Agonist activity was determined in comparison to the maximum increase of intracellular Ca^{2+} due to the application of 4 μM ionomycin (Alexis). EC_{50} values were determined as the concentration of test substances required to produce half-maximal increases in $[Ca^{2+}]_i$.

Antagonist behaviour was evaluated against 100 μM allylisothiocyanate (mustard oil, MO) by adding the compounds directly in the quartz cuvette under stirring and 5 min before starting the measurement. In experiments in TRPM8-HEK-293 cells, varying doses of test compounds were added 5 min (or in some experiments 15 min) before EC_{90} concentrations of icilin (0.25 μM). All determinations were at least performed in triplicate.

All dose response curves were fitted by a sigmoidal regression with variable slope, and 50% effective concentration (EC_{50}) values were derived by use of PRISM® Version 3.0 (GraphPad, San Diego, CA, USA). The efficacy of the agonists was first determined by normalizing their effect to the maximal effect on $[Ca^{2+}]_i$ observed with 4 μM ionomycin.

5.3. Statistical analysis

Statistical analysis of the data was performed by analysis of variance (ANOVA) at each point followed by the Bonferroni test. Differences were considered significant at $P < 0.05$.

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