Molecular and cellular mechanisms of the pungent and tingling impression of black pepper (*Piper nigrum* L.)

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Abstract

Sensory-directed fractionation of an ethanol extract prepared from black pepper corns (*Piper nigrum* L.) followed by LC-TOF-MS, LC-MS/MS, and 1D/2D-NMR experiments as well as synthesis revealed in sum 25 key tingling and/or pungent stimuli, which belong to two chemical classes of non-volatiles: the piperine-type analoges and unsaturated, long-chain fatty acid amides. While sensory evaluation of human recognition threshold concentrations by means of a modified half tongue test demonstrated the structural features causing the tingling and/or pungent impression of these pepper amides, the data obtained did not correlate with those reported for heterologously expressed TRP channels TRPV1 and TRPA1 in literature. Screening experiments with two-pore domain (KCNK, K_{2P}) K^+ channels suggest that pungent/tingling chemosensates from pepper exhibit a marked effect on three KCNK channels, namely TASK-1 (KCNK3, K_{2P} 3.1), TASK-3 (KCNK9, K_{2P} 9.1) and TRESK (KCNK18, K_{2P} 18.1), respectively, which are likely to play a complementary role to TRP channels in the complex orosensory impression elicited by black pepper corns.

Introduction

Due to the fact that nearly every cuisine all around the world appreciates black pepper (*Piper nigrum* L.) for its characteristic pungent and tingling orosensory impression, its corns remain the world's most important spice today. Although multiple research groups isolated next to piperine (**1a**), several other piperine-type analoges, like piperylin (**1b**), piperettine (**2b**) or retrofractamide (**3c**), all of which share a piperonal moiety, and identified them as the key players imparting the pungent impression of pepper [1], reliable data published on their human taste threshold concentrations are still lacking (Figure 1). In addition, next to its pungency black pepper corns exhibit a long-lasting tingling impression, which has never been characterized before. Therefore, the first objective of the present investigation was to target the sensory active key molecules in black pepper corns by application of a Sensomics approach. Thereby, we identified 25 key pungent and tingling chemosensates, among which, interestingly, exclusively piperine analogues exhibited a clear pungent orosensory impression while a group of 2,4-dienoic acid amides with an additional *cis*-configurated double bond exhibited both, a pungent and a long-lasting tingling sensation at higher concentrations [2]. However, the data obtained did not correlate with those from heterologously expressed TRP channels, like TRPV1 and TRPA1, which are known to be activated by several pungent sensing amides from black pepper [2-4]. For decades, the pungency perception has rested almost exclusively on polymodal TRP channels. However, there are publications suggesting additional targets for pungent substances [3, 5-9] and we found that even in the presence

B. Siegmund & E. Leitner (Eds): Flavour Sci., 2018, Verlag der Technischen Universität Graz
DOI: 10.3217/978-3-85125-593-5-49, CC BY-NC-ND 4.0

of TRP-antagonists, piperine (**1a**) was still able to activate a fraction of trigeminal neurones. Two-pore domain K^+ channels (KCNK) channels are among the most plausible candidates for a complementary role in the chemoperception of pungent stimuli [8,9]. Three of those channels have been previously shown in mice to be molecular targets of the tingling active hydroxy-α-sanshool [10].

Therefore, the aim of the present investigation was, on the one hand to target the sensory active key molecules in black pepper corns by means of a Sensomics approach and on the other hand to investigate whether two-pore domain K^+ channels (KCNK) channels could play a physiologically relevant role in their perception.

Experimental

Materials

Black peppercorn samples were purchased from the German retail market. Prior to the cell-culture assays and the psychophysical experiments, spectroscopic data and the purity (>98%) of each individual pepper amides **1a**−**11c** were checked by means of HPLC-UV, $^{1}H/^{13}C$ NMR, LC-MS/MS, and LC-TOF-MS experiments. Thereby, spectroscopic data were in good agreement with those published in the literature [2]. All experimental procedures including isolation, identification and psychophysical experiments of pepper amides as well as all cell experiments have been described in detail previously [2,8,9].

Results and discussion

Aimed at characterizing the pungent and tingling orosensory impression of black pepper corns data from human psychophysical experiments, collected by means of a Sensomics approach, were combined with their effect on background K^+ currents.

Molecular definition of black pepper taste by means of a Sensomics approach

A Sensomics approach, including taste dilution analysis, followed by UHPLC-TOF-MS, LC-MS/MS and 1D/2D NMR experiments as well as synthesis, led to the structure identification of 12 piperine analogues (**1a-6c**) and 13 2,4-dienoic acid amides (**7a-11c**) [2] (Figure 1). Depending on the chemical structure of the amides, sensory studies by means of a modified half-tongue test revealed human orosensory recognition thresholds of these phytochemicals to range from 3.0 to 1150.2 nmol/cm^2 for pungency and from 520.6 to 2162.1 nmol/cm^2 for tingling [2]. Interestingly, while the piperine analogues **1a-6c** exclusively exhibited a clear pungent orosensory impression, 2,4-dienoic acid amides with an additional *cis*-configurated double bond in the fatty acid chain (**7a-8c**,**10a-11c**) were found to exhibit both a pungent (at lower concentrations) and a tingling impression (at higher concentrations) [2].

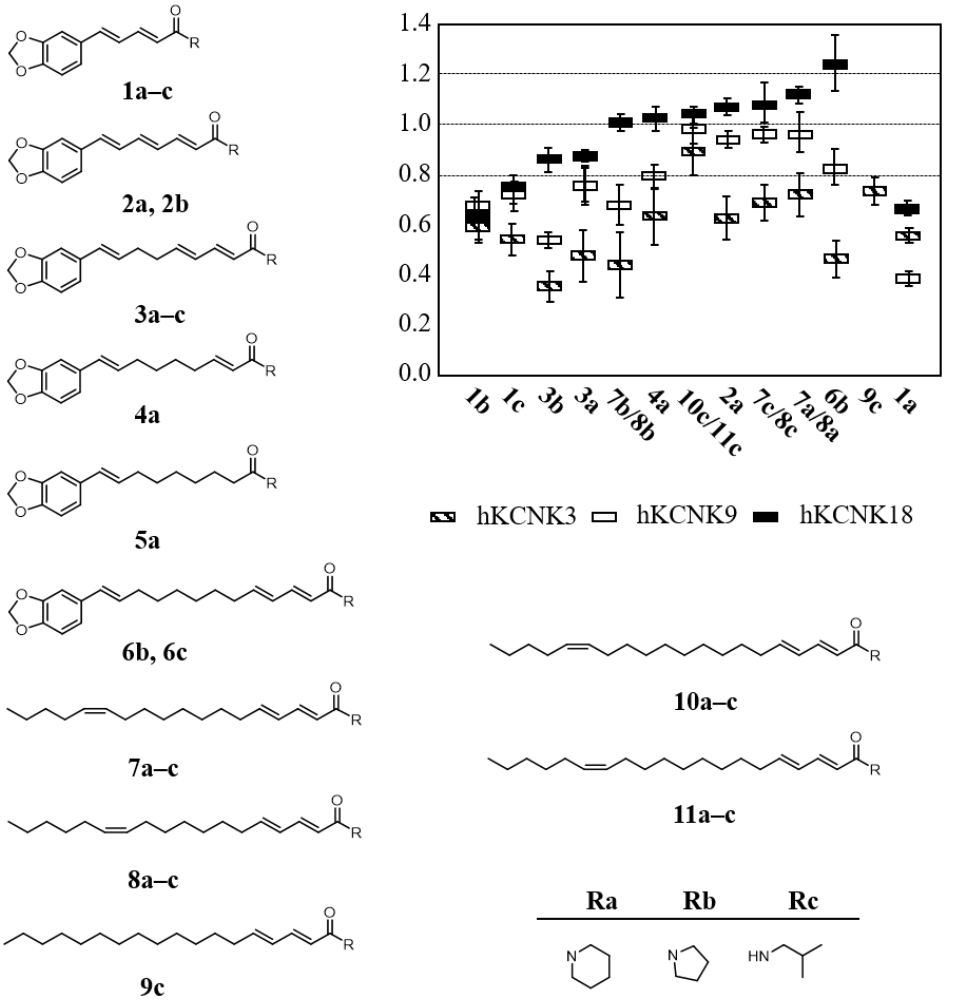

Figure 1: The chemical structures of pungent and tingling compounds **1a**-**11c** in black pepper corns and its pungent[p] and tingling[t] recognition threshold concentrations in nmol/ cm^2 as reported ealier [8]: piperine (**1a**) [3.0[p]], piperyline (**1b**) [5.1[p]], piperlonguminine (**1c**) [10.4[p]], piperettine (**2a**) [5.2[p]], piperoleine (**2b**) [10.3[p]], dehydropipernonaline (**3a**) [152.1 [p]], 1-[1-oxo-9(3,4-methylenedioxyphenyl)-2E,4E,8E-nonatrienyl]-pyrrolidine (**3b**), retrofractamide A (**3c**) [25.3[p]], pipernonaline (**4a**), piperroleine B (**5a**) [1150.2[p]], brachyamide A (**6b**), guineensine (**6c**) [810.1[p]], 1-(octadeca-2E,4E,13Z-trienyl)piperidine (**7a**), 1-(octadeca-2E,4E,13Z-trienyl)pyrrolidine (**7b**), (2E,4E,13Z)-N-isobutyl-octadeca-2,4,13-trienamide (**7c**) [540.5[p], 2162.1[t]], 1-(octadeca-2E,4E,12Z-trienyl)piperidine (**8a**), 1-(octadeca-2E,4E,12Z-trienyl)pyrrolidine (**8b**), (2E,4E,12Z)-N-isobutyl-octadeca-2,4,12-trienamide (**8c**), (2E,4E)-N-isobutyl-octadeca-2,4-dienamide (**9c**) [763.0[p]], 1-(eicosa-2E,4E,15Z-trienyl) piperidine (**10a**), 1-(eicosa-2E,4E,15Z-trienyl)pyrrolidine (**10b**), isobutyl-eicosa-2,4,15-trienamide (**10c**), 1-(eicosa-2E,4E,14Z-trienyl) piperidine (**11a**), 1-(eicosa-2E,4E,14Z-trienyl)pyrrolidine (**11b**), (2E,4E,14Z)-N-isobutyl-eicosa-2,4,14-trienamide (**11c**) [741.2[p], 1482.3[t]]. The compounds **7c**/**8c** [540.5[p], 2162.1[t]], **10a**/**11a** [260.2[p], 520.6[t]], **10b**/**11b** [405.8[p], 811.6[t]] and **10c**/**11c** [741.2[p], 1482.3[t]] were tasted as binary mixtures. Graphic top right: Normalized currents showing the effect of 12 chemosensates at 1 mM on *Xenopus laevis* oocytes expressing hKCNK3, hKCNK9 and hKCNK18. The currents were normalized to the current measured prior to the application of each tastant (middle line). If a pepper amide induces a change greater than 20% of the basal activity (upper and lower lines), a relevant effect is suggested. Figure adapted by [9].

Pungent and tingling substances inhibit the human two-pore domain potassium channels TASK-1, TASK-3 and TRESK

For a long time, the focus of pungent trigeminal chemoperception has rested almost exclusively on two members of the TRP family, TRPV1 and TRPA1 [3,4,8,9]. However, we found that the human recognition threshold concentrations for many pepper amides did not correlate with the data obtained from heterologously expressed TRP channels [2,3,9]. In addition to this, we observed that even in the presence of TRP-antagonists, piperine (**1a**) was still able to activate a large fraction of trigeminal neurones [8]. Therefore, we assumed that additional receptors, like two-pore domain (K_{2P}) potassium channels, which have been shown by Noël et al. [11] to "fine-tune" the cellular response to stimuli that activate TRP channels [8, 9], possibly interact with our taste stimuli.

Next to piperine, 6-gingerol and capsaicin 12 other pungent/tingling amides from black pepper corns, which were additionally screened, exhibited a marked effect on two-pore domain (KCNK, K_{2P}) K^+ channels, namely TASK-1 (K_{2P} 3.1), TRESK (K_{2P} 18.1) and especially TASK-3 (K_{2P} 9.1) (Figure 1) [8,9]. Although tingling compounds from Szechuan pepper have been shown to induce neuronal excitation by inhibiting KCNK channels before [10], our results demonstrate, for the first time, that next to tingling and pungent stimuli, exclusively pungent tasting compounds from *Piper nigrum*, like **1a-c**, **2a**, **3a** or **6c**, possess an inhibitory effect on two-pore domain K^+ channels. This inhibitory effect was dose-dependent and fully, although slowly, reversible. Thereby, 1-(octadeca-2*E*,4*E*,13/12*Z*-trienoyl)-pyrrolidine (**7b/8b**) was found to be the most potent naturally occurring inhibitor of *h*KCNK3. In addition, we observed when His98, the amino acid which is thought to be the main proton sensor in TASK-1 and TASK-3 [12,13], is mutated to Glu, the piperine-induced inhibition is significantly reduced [8].

In conclusion, a Sensomics approach led to the structure determination of 25 key phytochemicals, which elicit the typical pungent/tingling flavour of black pepper corns. In addition, our results suggest that pungent/tingling tasting pepper compounds, possesses a marked effect on KCNK channels, especially on KCNK3, which are likely to play a complementary role to TRP channels in the complex orosensory impression elicited by black pepper corns.

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