Activation of TRPV1 and TRPA1 by Black Pepper Components

Yukiko Okumura,1 Masataka Narukawa,1,2,3 Yusaku Iwasaki,1,2 Aiko Ishikawa,3 Hisashi Matsuda,3 Masayuki Yoshikawa,4 and Tatsuo Watanabe1,2,3,*

1Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan
2Global COE Program, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan
3School of Food and Nutritional Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan
4Kyoto Pharmaceutical University, Misasagi, Yamashina-ku, Kyoto 607-8412, Japan

Received December 25, 2009; Accepted January 26, 2010; Online Publication, May 7, 2010

[10.1271/bbb.90964]

We searched in this study for novel agonists of transient receptor potential cation channel, subfamily V, member 1 (TRPV1) and transient receptor potential cation channel, subfamily A, member 1 (TRPA1) in pepper, focusing attention on 19 compounds contained in black pepper. Almost all the compounds in HEK cells heterogeneously expressed TRPV1 or TRPA1, increased the intracellular Ca2+ concentration ([Ca2+]i) in a concentration-dependent manner. Among these, pipericine, iso-pipericine, isochavicine, piperamine, piperonaline, dehydropiperonaline, retrofractamide C, piperolein A, and piperolein B relatively strongly activated TRPV1. The EC50 values of these compounds for TRPV1 were 0.6–128 μM. Pipericine, iso-pipericine, isochavicine, piperamine, piperolein A, piperolein B, and N-isobutyl-(2E,4E)-tetradeca-2,4-diamide also relatively strongly activated TRPA1, the EC50 values of these compounds for TRPA1 were 7.8–148 μM. The Ca2+ responses of these compounds for TRPV1 and TRPA1 were significantly suppressed by co-applying each antagonist. We identified in this study new transient receptor potential (TRP) agonists present in black pepper and found that pipericine, iso-pipericine, isochavicine, piperamine, piperolein A, and piperolein B activated both TRPV1 and TRPA1.

Key words: pepper; piperine; transient receptor potential cation channel, subfamily V, member 1 (TRPV1); transient receptor potential cation channel, subfamily A, member 1 (TRPA1); intracellular calcium concentration

Pepper, Peperineer nigrum Linne, grows on a climbing plant belonging to the Piperineerales family. The dried fruit of pepper is used as a spice throughout the world. Traditional Chinese medicine classifies pepper as a food that generates body heat. Pepper has such pharmacological effects as anti-oxidative,1) anti-protozoan,2) and prokinetic.3) Pipericine is a pungent component of pepper and has been reported as acting on the capsaicin receptor, transient receptor potential cation channel, subfamily V, member 1 (TRPV1).4,5) TRPV1 is also activated by capsaicin (CAP), a pungent component of hot pepper. It is known that TRPV1 activation enhances the energy metabolism.6) It is therefore possible that, like CAP, piperine can also enhance the energy metabolism via TRPV1, although the detailed mechanism for this is unknown. Various piperine analogs have also been identified in pepper.7,8) Since it has been reported that CAP analogs activated TRPV1 as well as CAP,9,10) it is also possible that piperine analogs could activate TRPV1.

Many thermosensitive TRP receptors have recently been cloned as TRPV1 homologs. Among these, the transient receptor potential cation channel, subfamily A, member 1 (TRPA1) is similar to TRPV1 in several respects: TRPA1 is coexpressed in TRPV1-expressing somatosensory neurons,11) and TRPA1 induces sensory stimuli such as pain and pungency like TRPV1.12) Many pungent components such as allyl isothiocyanate (AITC; wasabi),13) cinnamaldehyde (cinnamon),14) hydroxy α-sanshool (zanthoxylum fruit),15) and miogadial and miogatrial (myoga)16) have been reported to be TRPA1 agonists. We therefore hypothesized that the pungent compounds contained in pepper could also activate TRPA1. We investigated in this study the activation of TRPV1 and TRPA1 by 19 components of pepper.

Materials and Methods

Materials. CAP, piperine, and capsazepine (CPZ) were purchased from Sigma (St. Louis, MO, USA). Allyl isothiocyanate (AITC) was obtained from Wako Pure Chem. Ind. (Osaka, Japan), and HOCl0031 was purchased from ChemBridge (San Diego, CA, USA). All other chemicals were of guaranteed reagent grade.

Purification of the pepper components. We studied 19 components of black pepper: piperine, iso-piperine, isochavicine, piperamine, piperonal, methylpiperine, fragaramine, piperonaline, dehydropiperonaline, piperonluminamine, retrofractamide A (retro A), retrofractamide B (retro B), retrofractamide C (retro C), guineensine, brachystamine B, N-isobutyl-(2E,4E)-tetradeca-2,4-diamide (N-tetra), N-isobutyl-(2E,4E)-octadeca-2,4-diamide (N-octa), piperolein A, and piperolein B. The structural formulae of the black pepper components are shown in Fig. 1. With the exception of piperine, these components

1 To whom correspondence should be addressed. Fax: +81-54-264-5550; E-mail: watanbt@u-shizuoka-ken.ac.jp

Abbreviations: AITC; allyl isothiocyanate; [Ca2+]i, intracellular Ca2+ concentration; CAP, capsaicin; CPZ, capsazepine; retro A, retrofractamide A; retro B, retrofractamide B; retro C, retrofractamide C; N-octa, N-isobutyl-(2E,4E)-octadeca-2,4-diamide; N-tetra, N-isobutyl-(2E,4E)-tetradeca-2,4-diamide; TRP, transient receptor potential; TRPA1, transient receptor potential cation channel, subfamily A, member 1; TRPV1, transient receptor potential cation channel, subfamily V, member 1
were isolated and purified from black pepper (Gaban Co., Tokyo, Japan) and the fruits of *Piper chaba* Hunter purchased in Thailand. Piperolein A and B were extracted with hexane from black pepper and purified by silica gel and reversed-phase chromatography. The other compounds were obtained from *P. chaba*. The dried fruits of *P. chaba* were extracted with 80% aqueous acetone and partitioned between ethyl acetate and water, before the ethyl acetate fraction was purified by silica gel and reversed-phase chromatography. 

**Clocloning and expression of human TRPV1 and human TRPA1.** Human TRPV1 and TRPA1 cDNA were amplified by RT-PCR, using mRNA respectively obtained from human brain first-strand cDNA (Agilent Technologies, Santa Clara, CA, USA) and human WI38 cells. Human TRPV1 cDNA was subcloned into pcDNA3 (Invitrogen, Carlsbad, CA, USA) and human WI38 cells. Human TRPV1 and TRPA1 cDNA were amplified by RT-PCR, using the following primers: human TRPV1 forward primer, 5'-GCAAGGATGAAGAA-3'; human TRPA1 forward primer, 5'-TCACTTCTCCCCGGAAGGC-3'.

**Measurement of the intracellular Ca^{2+} concentration.** The intracellular Ca^{2+} concentration ([Ca^{2+}]_i) was measured by FlexStation™ II (Molecular Devices, Sunnyvale, CA, USA). The cells were seeded into 96-well plates 24 h before the assay. To obtain TRPV1-expressing HEK cells, 1 μg/ml of bisingine was added to induce the expression of the TRPA1 protein. The cells were sub-cultured every week, the highest passage number being 50. The cells were loaded with 3 μM Fluo-4-AM (Molecular Probes, Eugene, OR, USA) for 1 h at 37 °C in a loading buffer (5.37 mM KCl, 0.44 mM KH_{2}PO_{4}, 137 mM NaCl, 0.34 mM Na_{2}HPO_{4}, 7.2 mM H_{2}O, 5.56 mM D-glucose, 20 mM HEPES, 1 mM CaCl_{2}, 0.1% BSA, and 250 mM probenecid at pH 7.4). To study the inhibitory activity of the antagonists, 30 μM CPZ for TRPV1 or HC030031 for TRPA1 was added to 100 μM of each pepper compound, except for N-isobutyl-(2E,4E)-octadeca-2,4-diamide; we used 30 μM of this compound in DMSO since it did not dissolve in 100 μM. We used 0.01–100 μM piperine, isopiperine, isochavicine, pipernomaline, and piperolein A was nearly 0.1–100 μM dehydropiperonaline, retro C, piperolein A and piperolein B, and 0.1–10 μM CAP to obtain the dose-response curves for TRPV1. We used 0.03–100 μM piperine, isopiperine, isochavicine and piperonaline, 0.1–100 μM dehydropiperonaline, retro C, piperolein A and piperolein B and N-tetra, and 0.01–100 μM AITC for TRPA1. In some experiments, TRPV1 antagonist CPZ (30 μM) or TRPA1 antagonist HC030031 (30 μM) were added together with these compounds. Each test compound was prepared in DMSO and added to the loading solution, followed by loading solution to the total DMSO concentration of 0.1% to 0.2%. A 5 μM amount of ionomycin was added to each well to elicit maximum fluorescence intensity. The data values for the test compounds are expressed as the percentage response to 5 μM ionomycin. Curves were fitted and parameters estimated by using Prism 4.0a software (Graph Pad Software, San Diego, CA, USA).

**Results**

The activity of TRPV1 and TRPA1 by the 19 pepper components was compared to that by TRPV1 agonist CAP or TRPA1 agonist AITC.

**Effect of the 19 pepper components on human TRPV1**

Figure 2A shows the calcium response induced by each of the 19 pepper components in TRPV1-expressing cells. Among these components, piperine, isopiperine, isochavicine, pipernomaline, piperolein A, dehydropiperonaline, retro C, piperolein A, and piperolein B strongly increased [Ca^{2+}]_i in TRPV1-expressing HEK293T cells. A dose-response curve was drawn for these compounds (Fig. 2B). Their EC_{50} values (Table 1) were 250 to 53000 times larger than that of representative TRPV1 agonist CAP (2.4 nm). The maximum activity of piperine, isopiperine, isochavicine, pipernomaline, dehydropiperonaline, piperolein A was nearly
equal to that of CAP. The maximum response values were not calculated for piperononaline and retro C which were particularly low when compared to that of CAP (60.3%). Adding TRPV1 antagonist CPZ significantly decreased the Ca\(^{2+}\) response by these compounds (Fig. 2A). In addition, these compounds hardly increased [Ca\(^{2+}\)]\(_i\) in HEK293T cells not expressing TRPV1 (Fig. 2A). These results indicate that the black pepper components activated TRPV1.

**Effect of the 19 pepper components on human TRPA1**

Figure 3A shows the calcium response induced by each of the 19 pepper components in TRPA1-expressing cells. Among these components, piperine, isopiperine, isochavicine, piperezine, piperononaline, dehydropiperononaline, retro C, piperox A, piperox B, and N-tetra strongly increased [Ca\(^{2+}\)]\(_i\), in TRPA1-expressing HEK T-REX™ cells (Fig. 3A). The dose-response curve for these compounds is shown in Fig. 3B. A higher concentration of each of these compounds was required for action on TRPA1 than that on TRPV1. The EC\(_{50}\) values for these compounds were 16–297 times higher.

**Table 1. TRPV1 and TRPA1 Activation Potency of the Pepper Compounds**

<table>
<thead>
<tr>
<th>Component</th>
<th>TRPV1 EC(_{50}) (µM)</th>
<th>Max (%)*</th>
<th>TRPA1 EC(_{50}) (µM)</th>
<th>Max (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsaicin</td>
<td>0.0024</td>
<td>60.3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Allyl isothiocyanate</td>
<td>—</td>
<td>—</td>
<td>0.51</td>
<td>88.7</td>
</tr>
<tr>
<td>Piperine</td>
<td>0.6</td>
<td>57.7</td>
<td>29.7</td>
<td>77.0</td>
</tr>
<tr>
<td>Isopiperine</td>
<td>5.6</td>
<td>64.9</td>
<td>32.6</td>
<td>84.8</td>
</tr>
<tr>
<td>Isochavicine</td>
<td>3.4</td>
<td>61.3</td>
<td>71.1</td>
<td>NC**</td>
</tr>
<tr>
<td>Piperazine</td>
<td>6.0</td>
<td>61.3</td>
<td>148.3</td>
<td>NC**</td>
</tr>
<tr>
<td>Piperononaline</td>
<td>128.0</td>
<td>NC**</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Dehydropiperononaline</td>
<td>29.3</td>
<td>59.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Retro C</td>
<td>119.0</td>
<td>NC**</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Piperolein A</td>
<td>17.3</td>
<td>58.2</td>
<td>7.8</td>
<td>89.9</td>
</tr>
<tr>
<td>Piperolein B</td>
<td>19.1</td>
<td>39.9</td>
<td>11.1</td>
<td>70.4</td>
</tr>
<tr>
<td>N-Tetra</td>
<td>—</td>
<td>—</td>
<td>19.2</td>
<td>56.4</td>
</tr>
</tbody>
</table>

*Percent value to the response of 5 µM ionomycin
**NC, not calculated
than that of representative TRPA1 agonist AITC (0.5 mM). The relative maximum activity of piperine, isopiperine, piperolein A, piperolein B, and N-tetra to that of AITC was 0.867, 0.956, 1.01, 0.794, and 0.636, respectively (Table 1). The maximum response was not calculated for isochavicine and piperanine. The addition of TRPA1 antagonist HC-030031 (30 mM) significantly decreased the Ca\(^{2+}\) response induced by these compounds (Fig. 3B). In addition, these compounds hardly increased [Ca\(^{2+}\)]\(_i\) in HEK T-REx\(^{TM}\) cells not expressing TRPA1 (Fig. 3A). These results indicate that the black pepper components activated TRPA1.

### Discussion

This study clarified that TRP agonists other than piperine were present in pepper. Among them, piperine, isopiperine, isochavicine, piperanine, piperolein A, and piperolein B activated both TRPV1 and TRPA1. The EC\(_{50}\) values show that the piperine analogs activated TRPV1 more strongly than TRPA1. However, none of components acted more strongly than CAP and AITC. The results also clarified that piperine activated both TRPV1 and TRPA1.

Six components containing the piperidine ring (piperine, isopiperine, isochavicine, piperanine, piperolein A, and piperolein B) activated both TRPV1 and TRPA1. The piperidine ring might therefore be important for activating both of these receptors. N-tetra could strongly activate only TRPA1. Since the structure of N-tetra is similar to known TRPA1 agonist hydroxy-\(\alpha\)-sanshool, we believe that, among all the TRP agonists in pepper, only N-tetra has a structure that can selectively activate TRPA1.
TRPV1 agonist CAP is known to increase energy metabolism by stimulating the sympathetic nervous system. CAP induces adrenaline secretion through this mechanism, and this secreted adrenaline acts on the β-receptor in the liver and white adipose tissues (WAT), resulting in the decomposition of glycogen in the liver and of triglyceride in WAT to subsequently enhance the energy metabolism. On the other hand, it has been reported that the activation of TRPA1 elevated the temperature of brown adipose tissue and induced adrenaline secretion in anesthetized rats. TRPA1 activation may therefore play a role in thermogenesis.

Garlic components (diallyl sulfide, diallyl disulfide, and diallyl trisulfide), and Schihuana and Melegueta pepper components (hydroxy-α-sanshool, 6-shogaol, and 6-paradol) have also been reported to be food components that activated TRPV1 and TRPA1. Diallyl disulfide and diallyl trisulfide administrated to rats increased the UCP1 protein and blood adrenaline levels. It is therefore thought that diallyl disulfide and diallyl trisulfide would enhance the energy metabolism. It has also been reported that the secretion of adrenaline, which induces energy metabolism and temperature elevation, was promoted when piperine was administered to anesthetized rats. The thermogenesis after pepper ingestion is therefore attributed to the action of piperine. However, we found in this study that pepper contained substances other than piperine that acted on TRPV1 and additionally on TRPA1. Hence, the thermogenesis by pepper may not only be attributable to TRPV1 activation by piperine. The activation of TRPV1 and TRPA1 by pepper components, including piperine and piperine analogs, may also induce thermogenesis.

Acknowledgments

This work was supported in part by grant-aid for scientific research (C) (19580146) on the priority area of ‘Food Science’ from JSPS, Japan and by the Global Center of Excellence (COE) program from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References