TRPV1 activation and induction of nociceptive response by a non-pungent capsaicin-like compound, capsiate

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Abstract

Capsiate is a capsaicin-like ingredient of a non-pungent cultivar of red pepper, CH-19 sweet. To elucidate the mechanisms underlying the non-pungency of capsiate, we investigated whether capsiate activates the cloned capsaicin receptor, TRPV1 (VR1). In patch-clamp experiments, capsiate was found to activate TRPV1 expressed transiently in HEK293 cells with a similar potency as capsaicin. Capsiate induced nociceptive responses in mice when injected subcutaneously into their hindpaws with a similar dose dependency as capsaicin. These data indicate that the non-pungent capsiate is an agonist for TRPV1 and could excite peripheral nociceptors. In contrast to this, capsiate did not induce any significant responses when applied to the skin surface, eye or oral cavity of mice, suggesting that capsiate requires direct access to nerve endings to exhibit its effects. Capsiate was proved to have high lipophilicity and to be easily broken down in normal aqueous conditions, leading to less accessibility to nociceptors. Another highly lipophilic capsaicin analogue, olvanil, was similar to capsiate in that it did not produce irritant responses when applied to the skin surface, although it could activate TRPV1. Taken together, high lipophilicity and instability might be critical determinants for pungency and so help in understanding the effects of capsaicin-related compounds.

Keywords: Capsiate; Non-pungency; Capsaicin; TRPV1; Pain; Olvanil

1. Introduction

Capsiate has been extracted from a non-pungent cultivar of red pepper, CH-19 sweet, and shown to be a capsaicin analogue called capsinoid that has an ester bond instead of the amide bond between the vanillyl moiety and fatty acid chain (Fig. 1) (Kobata et al., 1998, 1999). A single intragastric administration of capsiate, like the pungent compound capsaicin, increases adrenalin secretion and oxygen consumption in mice (Ohnuki et al., 2001a; Ohnuki et al., 2001b). In addition, capsiate suppresses T cell activation by inhibiting NF-κB-dependent trancriptional activity (Sancho et al., 2002). It can, therefore, be suggested that, despite non-pungency, capsiate shares certain biological activities with capsaicin such as enhancing energy metabolism via activation of the sympathetic nervous system (Ohnuki et al., 2001a,b), and suppressing inflammation (Sancho et al., 2002). It is possible that capsiate could be a preferable medical or nutritional application over capsaicin.

The non-pungent aspect of capsiate has not yet been scrutinized. The burning sensation we feel when eating hot chili peppers is attributed to the activation of capsaicin receptors on a subset of sensory neurons that innervate the oral cavity (Szallasi and Blumberg, 1999). Previous studies of non-pungent capsaicin analogues such as olvanil (Liu et al., 1997), glyceryl nonivamide (Liu et al., 1997), ricinoleic acid (Vieira et al., 2000)
and scutigeral (Szallasi et al., 1999), have shown that these compounds either do not activate the capsaicin receptors or exhibit activation kinetics distinct from those exhibited by capsaicin. Recently, a capsaicin receptor was cloned from a rodent dorsal root ganglion cDNA library and named vanilloid receptor 1 (VR1) (Caterina et al., 1997); it is now called TRPV1 as a member of the TRP super family (Minke and Cook, 2002). TRPV1 is a non-selective cation channel expressed predominantly in unmyelinated small diameter sensory neurons (C-fiber), most of which are polymodal nociceptors (Caterina and Julius, 2001). In addition to capsaicin, acid and noxious heat activate TRPV1 receptors and depolarize neurons, suggesting that TRPV1 receptors transduce nociceptive stimuli to painful or irritant sensations (Tominaga et al., 1998). Thus, it is likely that capsiate is non-pungent either because it does not activate TRPV1 efficiently or because it cannot gain access to TRPV1-expressing sensory neurons from the mouth cavity.

To elucidate the mechanisms underlying the non-pungency of capsiate, we examined whether capsiate activates TRPV1 and excites sensory neurons. Capsiate was found to activate nonneuronal cells expressing TRPV1 heterologously as well as rat dorsal root ganglion (DRG) neurons. Capsiate injection into the hindpaw induced nociceptive behavior but failed to induce classical signs of irritation when applied to the mouth or skin. Preliminary results from this study have appeared in abstract form (Iida et al., 2002).

2. Methods

2.1. Chemicals

Capsiate was synthesized as described in Kobata et al. (2002), and capsaicin was purchased from Sigma. Olvanil was synthesized via the condensation of vanillylamine with oleoyl chloride in dry pyridine. All chemicals were dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C. Since capsiate has been shown to be unstable in aqueous solution (Sutoh et al., 2001), the DMSO stock was diluted with aqueous media just before each experiment. Capsazepine (Sigma) was dissolved in ethanol and stored at −20 °C. Morphine (Shionogi Co., Osaka, Japan) was stored according to the guidelines for the use of drugs of Mie University. Medium chain triglyceride (product name: ODO) was obtained from The Nisshin Oil Mills, Ltd (Tokyo, Japan).

2.2. Measurement of capsiate lypophilicity

To assess the lipophilicity of compounds, we measured the partition coefficient by using the octanol–water partitioning system (OECD, 1981). The log P value is an index of lipophilicity. Log P is defined as (log C_{OCT}/log C_{W}), where C_{OCT} and C_{W} are concentrations of a given compound in octanol and water, respectively. If the log P value exceeds 4, the octanol–water partitioning system cannot be applicable. In such cases, we estimated the log P value by using the retention time of the compounds in reversed-phase HPLC because there is a linear relationship between log P and log k' (Harnisch et al., 1983). The capacity factor, k', is given as (t_R/t_0), where t_R and t_0 are the retention times of a retained and an unretained solute, respectively. To get a regression line, we measured the log P and log k' of several capsaicin analogs with different acyl moieties.

2.3. Degradation of capsiate

Aliquots (1 ml) of the bath solution in the patch-clamp experiments were added to 50 µl of 1N HCl to avoid further degradation of capsiate at appropriate time intervals. Capsiate was then extracted with ethyl acetate. After evaporation of the solvent under nitrogen stream, capsiate was resolubilized by adding 50 µl of ethyl acetate. To measure the remaining capsiate, 20 µl of ethyl acetate solution was applied to the HPLC system (column, J'sphere ODS H-80, 4.6×150 mm, YMC, Kyoto, Japan; eluent, 80% methanol at 1 ml/min; detection, fluorescence Ex280 nm, Em 320 nm), using capsaicin as an internal standard.

2.4. Cell culture and electrophysiology

Human embryonic kidney-derived 293 (HEK293) cells were maintained in Dulbecco’s modified Eagle’s medium (supplemented with 10% fetal bovine serum, penicillin, streptomycin and L-glutamine) and transfected with TRPV1 cDNA as described previously (Caterina et al., 1997). Primary cultures prepared from...
male adult Wistar rat DRG neurons were incubated in a medium containing nerve growth factor (100 ng/ml). Whole-cell patch-clamp recordings were carried out one or two days after transfection of TRPV1 cDNA into HEK293 cells or one day after preparation of rat DRG neurons. Standard bath solutions contained 140 mM NaCl, 5 mM KCl, 2 mM MgCl2, 5 mM EGTA, 10 mM HEPES, 10 mM glucose, pH 7.4 (adjusted with NaOH). The pipette solutions contained 140 mM KCl, 5 mM EGTA, 10 mM HEPES, pH7.4 (adjusted with KOH). All patch-clamp experiments were performed at room temperature (22 °C).

2.5. Animals

Male ddY-strain mice (20–24 g, Japan SLC, Shizuoka, Japan) were housed in a controlled lighting environment with food and water available ad libitum. On the day of the experiments, animals were placed individually in transparent cages (19.5 cm×12.0 cm×12.0 cm), which also served as the observation chambers. After 1 h of adaptation, each mouse was removed from its cage, administered test reagent as described below, and returned to its cage for the observation period. Each animal was used for only one test. All procedures involving the care and use of mice and rats were carried out in accordance with the institutional (Mie University) guidelines and the National Institute of Health guide for the care and use of laboratory animals.

2.6. Taste aversion assay

The experiment was performed according to the methods of Spector et al. (1988), with some modification. Animals were placed in chambers bedded with chips divided from their housing cages. 20 µl of capsiate (0.5 mM or 5 mM in distilled water containing 5% DMSO), or capsaicin (0.5 mM) was applied in the mouth (0.5 mM or 5 mM in distilled water containing 5% DMSO), or olvanil (0.6 mM or 6 mM) were administered to the oral cavity or eye

2.7. Eye irritancy assay

2 µl of capsiate (0.5 mM or 5 mM in saline containing 5% DMSO), capsaicin (0.5 mM) were administered to the right eye using an Eppendorf pipette (Szolcsanyi and Jacso-Gabor., 1975). The number of wiping reactions was counted for 5 min after administration.

2.8. Evaluation of nociceptive response

20 µl of capsiate, capsaicin (0.03–0.54 mM in saline containing 5% DMSO), or olvanil (0.02–0.27 mM) were injected subcutaneously into the right hindpaw. The time spent licking and biting the infected paw was measured for 10 min after injection. In some experiments, capsazepine (10 µM) was co-administrated with capsiate or capsaicin (0.27 mM), or animals were pretreated with morphine (2.5 mg/kg) subcutaneously 15 min before the administration of capsiate or capsaicin.

2.9. Skin irritancy assay

One day before the experiment, a hair was removed from a portion of the mouse back using a depilating cream. 2 µl of capsiate (5 mM or 50 mM, in medium chain triglyceride containing 5% DMSO), capsaicin (5 mM) or olvanil (0.6 mM or 6 mM) were administered to the depilated area. The number of scratching events around the application site was counted for 5 min after administration.

3. Results

3.1. Capsiate does not exhibit irritancy-related effects on the oral cavity or eye

In order to confirm that in rodents, like humans, capsiate does not cause pungency-related responses, capsaicin and capsiate were applied to the oral cavity of mice (Fig. 2A and B). The number of chin rubbing and chip digging behaviors observed during the 5 min after capsaicin (0.5 mM) application was 13.5 ± 1.08 (mean ± S.E., n = 6). This was significantly greater than that observed upon 0.5 or 5 mM capsiate application (3.00 ± 0.87; n = 6, 2.17 ± 1.25; n = 6, p < 0.01, respectively) or in mice given control solution (2.29 ± 0.67, n = 7, p < 0.01). Another pungency-related behavior, gaping, was observed significantly more often during the 5 min period following 0.5 mM capsiate application (210 ± 15.8 s, n = 6) than after 0.5 or 5 mM capsiate application (8.00 ± 3.49 s; n = 5, 24.0 ± 2.28 s; n = 5, p < 0.01, respectively). These data indicate that capsiate does not cause pungency-related behaviors in mice. To confirm the inability of capsiate to excite sensory neurons whose cell bodies are located in trigeminal ganglion (TG), we performed an eye irritancy assay (Fig. 2C). The number of wiping reactions during the 5 min following application of 0.5 mM capsiate to the eye was 48.0 ± 3.98 (n = 6). On the other hand, the number of wipes was significantly smaller in mice treated with 0.5 or 5 mM capsiate (11.8 ± 2.20; n = 6, 7.60 ± 0.87; n = 6, p < 0.01, respectively) and 5% DMSO (7.67 ± 1.20, n = 6, p < 0.01), indicating that capsiate does not cause eye irritancy.
3.2. Capsiate activates TRPV1 expressed on cultured cells with two distinct profiles

The results described above suggest that capsiate does not have the ability to activate the capsaicin receptor in sensory neurons. To test this possibility, we decided to examine capsaicin receptor sensitivity to capsiate in both TRPV1-transfected HEK293 cells and rat DRG neurons. Surprisingly, whole-cell patch-clamp recordings demonstrated that capsiate evoked inward currents at −60 mV in the HEK293 cells expressing TRPV1 (Fig. 3). A dose–response curve for TRPV1 activation by capsiate showed an EC50 value (290 nM) and Hill coefficient (1.91) (Fig. 3B), which were similar to the values obtained in response to capsaicin (EC50 value: 99.0 nM, Hill coefficient: 1.46). Furthermore, the capsiate-evoked currents were reversibly blocked by capsazepine, a competitive inhibitor of TRPV1 (Fig. 3C). Other characteristic electrophysiological properties of TRPV1 including an outwardly rectifying current–voltage relationship and extracellular Ca2+-dependent desensitization and these were preserved in the capsiate-evoked responses (data not shown). These data indicate that capsiate does activate the cloned capsaicin receptor TRPV1 with a similar potency as capsaicin.

However, there seemed to be two differences between the capsiate-evoked current profiles and those observed in response to capsaicin. Firstly, the capsiate-evoked currents decreased with time during continuous capsiate application in the absence of extracellular calcium. Under similar conditions, little current decrease is observed with time during prolonged capsaicin application (Fig. 3A). This current decrease in the capsiate-evoked response was probably not due to the desensitization of TRPV1 because repeated capsaicin applications evoked current responses to the same extent even after current decrease by capsiate application (Fig. 3D). We attempted to examine the possibility that the decay of this response was due to capsiate breakdown, given that capsiate is easily degraded in aqueous condition (Sutoh et al., 2001). When the amount of capsiate was determined at several time points after dilution of the DMSO stock with bath solution, it became evident that the time course of capsiate degradation was consistent with that of the current reduction in the patch-clamp experiments (Fig. 4). Secondly, the capsiate-evoked currents returned to baseline after the removal of the compound more slowly than the capsaicin-evoked currents (Fig. 3D). It took about 40 s for the current to recover after cessation of 300 nM capsiate application whereas the capsaicin (100 nM)-evoked current showed a more rapid decay (about 10 s) (Fig. 3D). This slow washout could be attributed to the high lipophilicity of capsiate, which might remain for longer in the plasma membrane causing the delay in showing its effect. The lipophilicity of capsiate was evaluated by calculating the log P value. The log P value of capsiate (5.80) was greater than that of capsaicin (3.81), showing that capsiate is more lipophilic than capsaicin. Log P values for another non-pungent TRPV1 agonist, olvanil, and the ultrapotent TRPV1 agonist resiniferatoxin (RTX) were 9.12 and 6.36, respectively, indicating that they are also highly lipophilic.

To confirm the ability of capsiate to excite native sensory neurons, we examined the effects of capsiate on the membrane potentials of rat DRG neurons. Both capsaicin and capsiate could depolarize the membrane potential in responsive small diameter cells in a similar manner (from −56.3 ± 3.8 mV to −18.2 ± 4.4 mV; n = 3, from −53.6 ± 6.3 mV to −16.2 ± 9.6 mV; n = 3 in response to capsaicin and capsiate, respectively).
Fig. 3. Capsiate-evoked current responses in HEK293 cells expressing TRPV1. (A) Representative whole-cell current traces (at −60 mV) evoked by capsaicin or capsiate. Bars denote duration of the application of the compounds. (B) Concentration–response curves for capsaicin (○) and capsiate (●) evoked responses. Membrane currents were normalized to the response maximally activated by 1 µM capsaicin or 3 µM capsiate and expressed as a percent of the maximal response. Each point represents the mean values±S.E. from five independent cells. Figure shows averaged data fitted with the Hill equation. EC50 and Hill coefficient for capsaicin and capsiate concentration–response curves were 99 nM and 1.46, and 290 nM and 1.91, respectively. (C) A representative current response in the presence and absence of capsazepine (CPZ). CPZ blocks the capsiate-evoked currents with a washout phenomenon. Slash marks represent washout periods of 2 min. (D) Effects of repetitive applications of capsiate and capsaicin on the same cell. Capsiate prepared at the first application was used for the second and third applications. It is notable that it took about 40 s to return to the baseline after the first application of capsiate. Similar prolonged washout phenomena were observed in 30 cells.

3.3. Subcutaneously-injected capsiate induces nociceptive responses in mice

We explored the underlying mechanism of the ability of non-pungent capsiate to activate TRPV1. We examined the effects of subcutaneous capsiate injection into the mouse hindpaw, a route that would allow the compound to interact directly with sensory neurons. Capsiate induced typical licking and biting reactions characteristic of painful sensation. The time spent licking and biting the injected paw during the 10 min after injection was 35–80 s at 0.03–0.54 mM administration, and was maximal at 0.27–0.54 mM administration (Fig. 5A). The dose dependency of these capsiate-evoked responses was similar to that reported for the capsaicin (Sakurada et al., 1992). To make sure that these capsiate-induced behaviors indicate nociceptive responses in mice, the effect of morphine was examined. The capsiate-evoked licking and biting were significantly reduced upon pre-treatment of morphine (2.5 mg/kg) (79.2 ± 7.09 s, n =
Fig. 5. Induction of nociceptive behaviors in mice by subcutaneous injection of capsaicin or capsiate. (A) A dose–response profile of the capsiate-evoked reactions. The total time spent licking and biting the injected paw during 10 min after injection is shown. Values are the mean±S.E., n = 10 for each group, *p < 0.01 vs. control (5% DMSO). (B) and (C) Suppressive effects of morphine (B) or capsazepine (CPZ) (C) on the capsiate- and capsaicin-induced responses. Values are the mean±S.E., n = 10 for each group, *p < 0.01 vs. control, unpaired t test.

These prolonged capsiate-evoked responses are consistent with the slow decay observed in responses to capsiate in the patch-clamp experiments.

3.4. Inaccessibility to nerve endings could explain the non-pungency of capsiate

These data led us to consider the different effects of oral or eye application of capsiate versus injection into the hindpaw. Capsiate might reach sensory neurons indirectly in the former settings, but act directly on sensory neurons in the latter setting. To test this possibility, we applied capsiate to the mouse skin on the back, which would result in indirect application to the sensory neurons whose cell bodies are in DRG. Capsiate did not induce any significant scratching responses whereas capsaicin caused such responses to become more frequent during the 5 min following application (9.00 ± 2.07; n = 5 for control, 6.00 ± 1.90; n = 5 or 5.40 ± 2.54; n = 5 for 5 mM or 50 mM capsiate, respectively, vs. 30.8

10 vs. 30.7 ± 5.58 s, n = 10, p < 0.01) (Fig. 5B). Next, we investigated the effects of capsazepine to confirm that the nociceptive responses induced by capsiate occurred through the activation of the capsaicin receptor. Capsiate-evoked responses were diminished with co-administration of capsazepine (42.4 ± 9.29 s, n = 10, p < 0.01) (Fig. 5C). Similar inhibitory effects of morphine and capsazepine were observed in capsaicin-evoked responses (from 76.0 ± 3.37 s, n = 10 to 27.2 ± 5.09 s, n = 10 and 32.2 ± 4.82 s, n = 10 by morphine and capsazepine, respectively, p < 0.01) (Fig. 5B and C). These data indicate that capsiate induces nociceptive responses through the activation of the capsaicin receptor in mice, a finding in agreement with the fact that capsiate can activate the cloned capsaicin receptor TRPV1. To look at the effects of capsiate more carefully, we observed the time course of its effects. As shown in Fig. 6, capsiate-evoked licking and biting responses lasted longer than capsaicin-evoked responses although instantaneous responses were less prominent than with capsaicin.
The time spent licking and biting the injected paw was measured for 1 min up to 10 min after subcutaneous injection of the compounds into the hindpaw. Capsaicin (1.6 µg; ○), capsiate (1.6 µg; ●), olvanil (0.3 µg; ■) or 5% DMSO (×) was injected. Values are the mean±S.E., n = 10 for each group, *p < 0.05 and **p < 0.01 vs. control (5% DMSO); #p < 0.05 and ##p < 0.01 vs. capsaicin, unpaired t test.

± 2.80; n = 5 for 5 mM capsaicin, p < 0.01) (Fig. 7). These data strongly suggest that the route of application is critical for the ability of capsiate to activate sensory neurons and that capsiate is unable to act on sensory neuron capsaicin receptors when applied indirectly. We reasoned that the lipophilicity and instability of capsiate may explain this phenomenon. If so, other highly lipophilic capsaicin analogues might exhibit similar dependence on the route of application. To test this hypothesis, we chose to examine the highly lipophilic capsaicin analog olvanil, which reportedly does not cause pungency-related responses in rodents (Liu et al., 1997). First, we confirmed that olvanil, like capsiate, evoked current activation with a prolonged washout phenomenon in HEK293 cells expressing TRPV1 (Fig. 8). The EC\textsubscript{50} value of the olvanil-evoked currents was estimated to be approximately 10 nM, although it was impossible to determine the exact value because olvanil-evoked currents could not recover completely, especially at higher concentrations, which interrupted sequential application. Next, olvanil was injected into the hindpaw of mice. Olvanil induced licking and biting reactions which began slowly and lasted for longer than those induced by capsiate or capsaicin (2–10 min after injection, p < 0.05 vs. control) (Fig. 6). The dose of olvanil that evoked a maximum response was 0.03 mM, about one-eighth of that of capsaicin or capsiate, consistent with the lower EC\textsubscript{50} value estimated for the olvanil-evoked currents. However, like capsiate, olvanil caused no behavioral responses when applied to the skin surface (2.4 ± 1.05; n = 5 or 7.33 ± 3.04; n = 6 for 0.6 mM or 6 mM olvanil, respectively, p < 0.01 vs. 5 mM capsaicin) (Fig. 7). In these experiments, we continued to observe the animals until 10 min after application, but no significant response was noticed during the period from 5 to 10 min (data not shown). Taken together, it is concluded that capsiate does not excite sensory neurons sufficiently to express behavioral responses because of its lipophilicity and instability.

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4. Discussion

Although it has been of great interest that some capsaicin analogues have no irritant effects while retaining “beneficial aspects” of capsaicin, the mechanistic basis of the non-pungency has not been well elucidated. Therefore, we decided to explore the factors that explain the non-pungent nature of such compounds. Among the non-pungent capsaicin analogues, we chose capsiate for our investigation because it can be obtained from natural products and has almost the same structure as capsaicin; only one nitrogen atom and one hydrogen atom in the capsaicin molecule are replaced with one oxygen atom in the capsiate molecule (Kobata et al., 1998).

The non-pungency of capsiate was verified in a series of animal experiments. Oral application of capsiate in mice did not induce any significant aversive reactions, indicating that capsiate is not pungent in the mouth. Capsiate did not show any irritancy-related effect on the mouse eye, either. Since the nerves innervating both the mouth and eye areas branch from the TG nerve, the eye-irritancy assay has been often used for evaluating the pungency of plant ingredients and chemical compounds (Szolscanyi and Jacso-Gabor, 1975).

Contrary to our expectation, patch-clamp experiments demonstrated that capsiate does activate the cloned capsaicin receptor TRPV1 with a potency similar to that of capsaicin. However, these experiments revealed two distinct properties of capsiate, instability in aqueous conditions and high lipophilicity. The high lipophilicity was also indicated in the patch-clamp currents evoked by another non-pungent capsaicin analogue olvanil. Liu et al. had found that olvanil evokes currents with considerably slower kinetics in cultured TG neurons than does capsaicin, a phenomenon they suggested could explain the non-pungency of olvanil (Liu et al., 1997). Thus, the properties of capsiate are proposed to be relevant to its lack of pungency.

Since capsiate was shown to be an agonist of TRPV1, we examined whether direct interaction of capsiate and the capsaicin receptor would lead to painful sensation in vivo. The data obtained in the licking and biting test in mice strongly suggested that capsiate causes painful sensation through activation of the capsaicin receptor on sensory neurons. Many investigators have been interested in the ability of non-pungent capsaicin analogues to activate nociceptors. However, studies on this issue have mostly employed cultured DRG or TG neurons (Liu et al., 1997, 1998; Szallasi et al., 1999; Urban et al., 2000). The present study is significant in this regard, in that the functional activities of capsiate were detected both as an activation of the cloned capsaicin receptor TRPV1 and as nociceptive behavior, the final output of the receptor activation. Another important finding from the behavior test was that capsiate induced more prolonged responses in mice than did capsaicin, a pattern reminiscent of the lingering currents evoked by capsiate in the patch-clamp experiments. Our results demonstrate for the first time the close correlation between the time course of induction of nociceptive behavior and that of TRPV1 activation by capsaicin analogues.

Inconsistent behavioral responses observed in the animal experiments led us to focus on the accessibility of capsiate to sensory neurons. When capsiate induced nociceptive reactions in the mouse hindpaw, it was likely to reach the peripheral endings of DRG neurons directly. On the other hand, in oral and eye-irritancy assays where we did not see any significant response, the compound was likely to enter the epithelium or cornea before reaching the endings of TG cells, as suggested in the previous studies (Bryant and Moore, 1995). Therefore, we tried dermal application of capsiate on the mouse back as a
form of indirect application to the terminals of DRG neurons, a situation equivalent to that in the oral and eye-irritancy assays. The animals did not exhibit any significant responses to the indirect application, suggesting that capsiate did not reach the nerve endings in an amount sufficient to excite neurons. Thus, we conclude that the non-pungency of capsiate can be attributed to its inability to penetrate from the body surface into the space where free endings of nociceptors are located.

Based upon the capsiate-specific properties evaluated in the present study, two mechanisms are proposed to explain the poor accessibility of capsiate to sensory neurons that exist behind physiological barriers. The one possibility is that capsiate is trapped in the lipid phase of epithelium or cornea due to its high lipophilicity. This hypothesis is supported by the results obtained using another non-pungent TRPV1 agonist olvanil that was shown to be even more lipophilic than capsiate and showed no irritant response when applied to skin surface although it could activate TRPV1. A previous study has also demonstrated the low rate of penetration of olvanil beyond the epidermis (Kasting et al., 1997). Moreover, we found the ultrapotent TRPV1 agonist, RTX, to be as lipophilic as capsiate. The high lipophilicity of RTX might have relevance to the previous observation that RTX was not so pungent as it activated capsaicin-sensitive neurons in vivo (Szallasi and Blumberg, 1990). According to previous studies, one of the requirements for capsaicin analogues to be pungent is having a carbon side chain of a certain length, meaning that analogues with too low or too high lipophilicity do not possess pungency (Szolcsanyi and Jacso-Gabor, 1975; Govindarajan and Sathyarayana, 1991). The importance of lipophilicity as a determinant of pungency has also been suggested in stimulatory effects exerted by fatty acids in mouth (Bryant and Moore, 1995). These observations are in an agreement with our hypothesis.

Another possible explanation for the nonpungency of capsiate is that it is degraded before reaching the nerve endings of nociceptors. In addition to the inherent instability of capsiate in aqueous environments, there might exist esterases or lipases in vivo that can hydrolyze capsiate. Notably, many kinds of esterases and lipases have been found in the oral cavity and cornea (Nelson et al., 1977; Lee, 1983). Studies on human and rat skin have suggested that capsaicin analogues including olvanil are extensively metabolized via hydrolytic cleavage of the amide bond during passage through the skin (Kasting et al., 1997). Whether these hydrolyzing systems act on capsiate remains to be investigated.

The present results suggest that not only the efficiency of TRPV1 activation, but also the accessibility to nociceptors, is an important determinant of the pungency of capsainoids. It should be emphasized that this conclusion is drawn from the electrophysiological analyses using the cloned capsaicin receptors and the findings of the behavioral studies. The question of how capsiate activates the sympathetic nerve system might be a reasonable target for future study. One of the prominent aspects of capsiate is that it exists as a naturally occurring product. In the daily diet, one can consume a sweet pepper that has the same biological effects as a hot one. Our investigation will provide helpful information for the discovery or development of more useful capsaicin analogues.

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