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Lipophilicity of capsaicinoids and capsinoids influences the multiple activation process of rat TRPV1

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Abstract

Analogs of capsaicin, such as capsaicinoids and capsinoids, activate a cation channel, transient receptor potential cation channel vanilloid subfamily 1 (TRPV1), and then increase the intracellular calcium concentration ($[Ca^{2+}]_i$). These compounds would be expected to activate TRPV1 via different mechanism(s), depending on their properties. We synthesized several capsaicinoids and capsinoids that have variable lengths of acyl moiety. The activities of these compounds towards TRPV1 heterologously expressed in HEK293 cells were determined by measuring $[Ca^{2+}]_i$. When an extracellular or intracellular Ca^{2+} source was removed, some agonists such as capsaicin could increase $[Ca^{2+}]_i$. However, a highly lipophilic capsaicinoid containing C18:0 and capsinoids containing C14:0, C18:0, or C18:1 (the latter was named olvanilate) could not elicit a large increase in $[Ca^{2+}]_i$ in the absence of an extracellular or intracellular Ca^{2+} source. These results suggest that highly lipophilic compounds cause only a slight Ca^{2+} influx, via TRPV1 in the plasma membrane, and are not able to activate TRPV1 in the endoplasmic reticulum.

Keywords: Capsaicinoids; Capsinoids; Intracellular calcium concentration; Lipophilicity; TRPV1

Introduction

Capsaicin (CAP; isoC10:1VA, Fig. 1), which is the major pungent principle of red peppers (*Capsicum* spp.), has diverse biological activities (Szallasi and Blumberg, 1999). There are natural and synthetic analogs, which are generally known as capsaicinoids. Their common structure is a group of acid amides of vanillylamine and fatty acids. We have also isolated a novel compound, capsiate (CST; isoC10:1VOH, Fig. 1), from 'CH-19 Sweet', a non-pungent red pepper cultivar (Kobata et al., 1998, 1999). CST and related compounds are capsaicin analogs called capsinoids that have an ester bond instead of the amide bond between the vanillyl moiety and the fatty acid chain (Kobata et al., 1998, 1999).

Several capsaicinoids and CST activate a specific receptor, transient receptor potential vanilloid subtype 1 (TRPV1) (Caterina et al., 1997; Tominaga et al., 1998; Appendino et al., 2002; Iida et al., 2003). Activation of TRPV1 causes a rapid rise in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) (Caterina et al., 1997; Tominaga et al., 1998). There are two pools of TRPV1 in cells: one in the plasma membrane ($TRPV1_{PM}$) and the other in the endoplasmic reticulum ($TRPV1_{ER}$) (Olah et al., 2001; Karai et al., 2004). Thus, TRPV1 regulates two calcium compartments: extracellular Ca^{2+} and the intracellular Ca^{2+} store (Eun et al., 2001; Marshall et al., 2003). It had been thought that all

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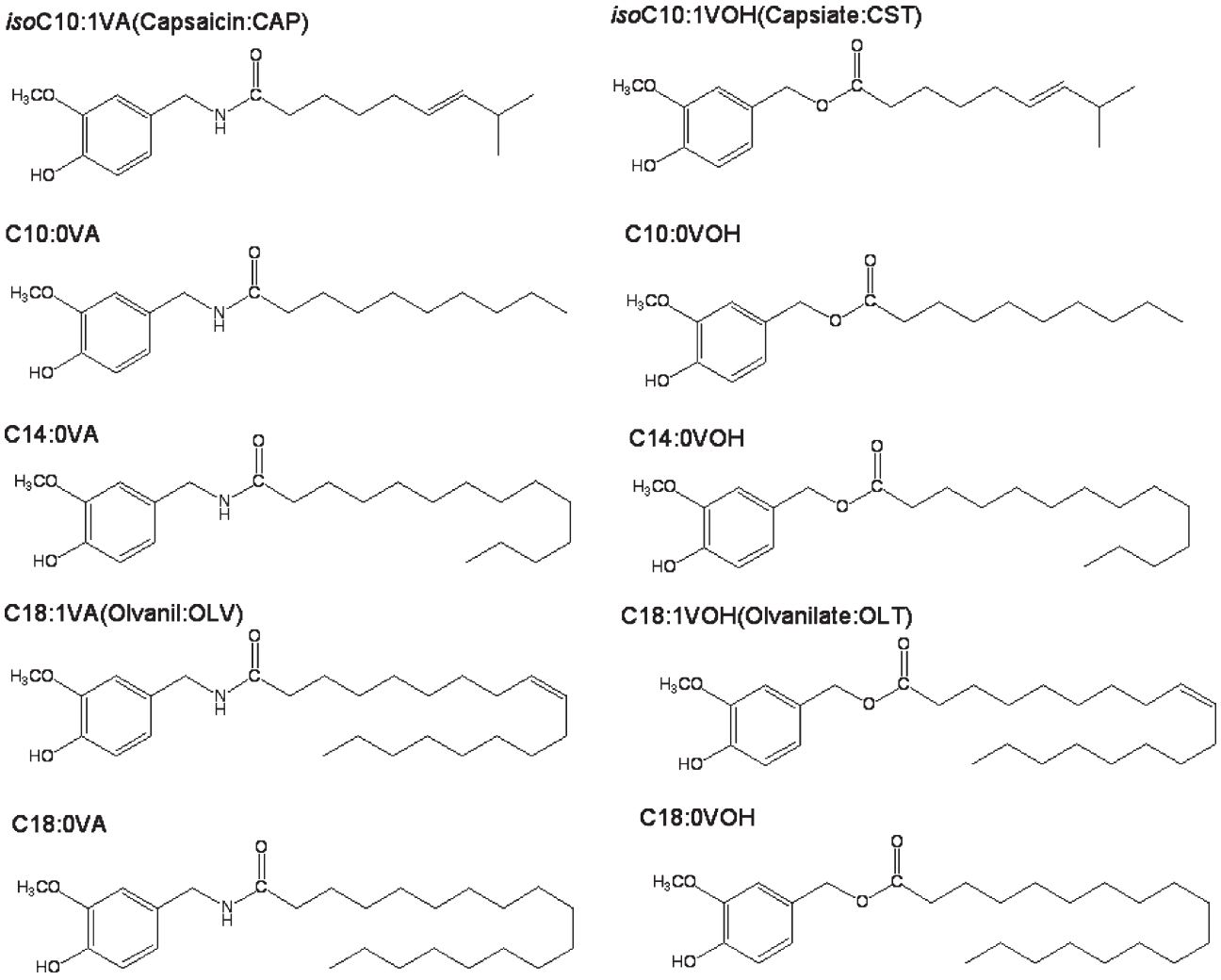

Fig. 1. Structures of capsaicinoids and capsinoids. Six capsaicinoids and six capsinoids used in this study are shown. Three compounds were previously named as capsaicin (CAP), olvanil (OLV), and capsiate (CST). One capsinoid containing oleic acid was newly synthesized and named as olvanilate (OLT).

capsaicinoids and capsinoids activate both pools of TRPV1 and mobilize both Ca^{2+} compartments. However, a recent report indicated that different vanilloid agonists cause different patterns of Ca^{2+} response in CHO cells heterologously expressing rat TRPV1 (Toth et al., 2005). The capsaicinoids and capsinoids appear to increase $[Ca^{2+}]_i$ via different mechanism(s), depending on their different properties.

For this investigation, we synthesized four capsaicinoids and five capsinoids containing varying lengths of fatty acids, as in Fig. 1. The activity of these compounds towards TRPV1 heterologously expressed in HEK293 cells was determined by measuring $[Ca^{2+}]_i$. Drugs such as *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) (Hofer et al., 1998) or tapsigargin (Bian et al., 1991; Lytton et al., 1991) were used to reduce the amount of Ca^{2+} in the intracellular Ca^{2+} store. To minimize the amount of extracellular Ca^{2+}, we used Ca^{2+}-free assay buffer containing 0.125 mM EGTA. In both cases, certain capsaicinoids and capsinoids containing long acyl moieties, such as C18:0VA, C14:0VOH, C18:1VOH (OLT), and C18:0VOH (see Fig. 1), elicited an extremely slight increase in $[Ca^{2+}]_i$. These results suggest that the high lipophilicity of the compounds influences the activation process of TRPV1.

Materials and methods

Materials

Capsaicin and capsazepine were purchased from Sigma. Capsaicinoids were synthesized by the condensation of vanillylamine hydrochloride with the corresponding acyl chlorides in dry pyridine (Rangoonwala and Seitz, 1970). Capsinoids were synthesized enzymatically (Kobata et al., 2002). The purity of all compounds was confirmed to be over 95% by reversed phase HPLC. Capsaicinoids and capsinoids were dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C. The DMSO stock was diluted with aqueous medium just before each experiment because diluted capsinoids are unstable in aqueous solution (Sutoh et al., 2001; Iida et al., 2003). All other chemicals were of guaranteed reagent grade. All cell culture media were obtained from Invitrogen. All culture dishes and plates were from Falcon.

Measurement of lipophilicity

We determined the lipophilicity of the compounds as log *P*, as described in a previous report (Iida et al., 2003). Table 1 shows that the log *P* values of the capsaicinoids and capsinoids are proportional to the acyl chain length. Compounds with unsaturated acyl chains have lower log *P* values.

Cloning and expression of rTRPV1

Total RNA of C6 glioma cells was isolated using RNeasy Mini Kit (Qiagen). The first strand cDNA was transcribed by SuperScript II (Invitrogen) using oligo dT primer. A rat TRPV1 cDNA was amplified by Expand HiFi (Roche Diagnostics) using forward and reverse primers incorporating the restriction sites shown, RVR1F1-*Hin*dIII (5′-atccaagcttgaaaggatggaacaacgggg) and RVR1R2-*Xba*I (5′-tatctctagattatttctcccctgggacc). Reaction products were cloned into the *Hin*dIII-*Xba*I sites of pcDNA3 (Invitrogen), and the sequence was confirmed using a BigDye Terminator cycle sequence kit and ABI PRISM 310 genetic analyzer (Applied Biosystems). HEK293 cells were transfected with rTRPV1-pcDNA3 construct using SuperFect Transfection Reagent (Qiagen), according to the manufacturer's instructions. Stable clones were selected in 600 μg/ml G418 (Sigma). Northern blot and Western blot analyses using antibody against rTRPV1 (Alpha Diagnostic) confirmed the rTRPV1 expression. One clone, expressing TRPV1, was named HEK293VR11.

Electrophysiology

Whole-cell patch-clamp recordings were carried out 1 or 2 days after seeding of the HEK293VR11 cells to new dishes. The standard bath solution contained 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES, and 10 mM glucose; it was adjusted to pH 7.4 with NaOH. The pipette solution contained 140 mM KCl, 5 mM EGTA, and 10 mM HEPES; it was adjusted to pH 7.4 using KOH. All patch-clamp experiments were performed at room temperature (22 °C).

Measurement of $[Ca^{2+}]_i$

HEK293VR11 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 250 ng/ml of amphotericin B under 5% CO_2/air at 37 °C. Cells were sub-cultured every week and the highest passage number used was 50.

HEK293VR11 cells were plated in 150 mm dishes at a density of 1.8×10^7 cells. After 3–4 days, cells were detached, using 10 ml of Ca^{2+}-free PBS containing 0.5 mM EDTA, and then collected by centrifugation. The collected cells were washed twice with loading buffer [5.37 mM KCl, 0.441 mM KH_2PO_4, 137 mM NaCl, 0.336 mM $Na_2HPO_4{-}7H_2O$, 5.56 mM glucose, 20 mM HEPES, 1 mM $CaCl_2$, and 0.1% bovine serum albumin (BSA), pH 7.4] and resuspended in the same buffer containing the cytoplasmic calcium indicator, Fura 2-AM (5 μM; Molecular Probes), and chromophore EL (0.15%; Sigma) at 37 °C for 30 min. After washing twice with loading buffer, the cells were stocked in the loading buffer at below 4 °C (<4 h).

A cuvette containing Fura 2-loaded cells (6×10^5 cells/2 ml) was placed in a fluorospectrophotometer (CAF-110; Jasco Inc., Tokyo, Japan). After incubation with stirring at 37 °C for at least 1 min, the test compound was added by microsyringe. Time dependent changes of the fluorescence (excitation wavelength set at 340 or 380 nm and emission wavelength at 500 nm) were recorded and analyzed using PowerLab system MacLab/4e and *Chart 4* (AD Instruments). The fluorescence ratio (340/380) was converted to $[Ca^{2+}]_i$ according to the equation published by Grynkiewicz et al. (1985), where R_{max} and R_{min} were determined using 0.1% Triton X-100 and 5 mM EGTA (pH 8.0), respectively. The effective dissociation constant for Fura 2 at 37 °C was 224 nM.

In some experiments, capsazepine (100 μM), an antagonist of TRPV1, was added at least 1 min prior to the addition of the test compounds. To analyze the effect of BSA, we treated cells with loading buffer in the presence or absence of 0.1% BSA. To exclude the contribution of the intracellular Ca^{2+} store, we used two drugs, TPEN and tapsigargin. We added 10 μM TPEN to the loading buffer during all periods. In the case of tapsigargin, we added it (50 μM) 3 min before the addition of the capsaicinoids or capsinoids. To exclude the contribution of extracellular Ca^{2+}, we suspended Fura 2-loaded cells with Ca^{2+}-free buffer, and added EGTA (0.125 mM) 1 min before the addition of the test compounds.

Data analysis

The basal level of $[Ca^{2+}]_i$ was calculated from the average value of the fluorescence ratio (340/380) during the 10 s immediately preceding the addition of test chemical. The maximum level of $[Ca^{2+}]_i$ was calculated from the maximum increase in the ratio (340/380) during the first minute after the addition of the test chemical. The change ($\Delta[Ca^{2+}]_i$) was calculated by subtraction of the former from the latter. For C18:0VA, C14:0VOH and C18:0VOH, maximum values were obtained during the first 5 min after addition. The increase in $\Delta[Ca^{2+}]_i$ induced by 10 μM CAP in the absence of TPEN was 586 nM. Using this value, we normalized the $\Delta[Ca^{2+}]_i$ values induced by the capsaicinoids or capsinoids, except for the experiments in which the extracellular Ca^{2+} was removed. Curve fitting and parameter estimation were carried out using *Prism 4* (Graph Pad Software).

Results

The rTRPV1 cDNA was cloned from rat C6 glioma cells because these cells had been shown to respond to capsaicin (Bíró et al., 1998). The nucleotide sequence of this clone was almost identical to rat TRPV1 cDNA (Caterina et al., 1997), except that it produced one amino acid substitution: Thr at position 239 was replaced by Ala. After transfecting with this clone and selecting by the presence of G418 (0.6 μg/ml), we

Table 1
Lipophilicity and pharmacokinetic parameters of capsaicinoids and capsinoids

Compounds			Log *P*	TPEN (−)		TPEN (+)	
				EC_{50} (μM)	Max (%)	EC_{50} (μM)	Max (%)
Capsaicinoids	*iso*C10:1VA	(CAP)	3.81	0.0819	99.0	4.38	62.6
	C10:0VA		4.01	0.235	98.8	11.6	63.8
	C14:0VA		7.37	0.190	100.1	7.32	63.1
	C18:1VA	(OLV)	9.12	0.00686	97.1	1.79	39.8
	C18:0VA		10.16	0.0609	82.6	0.216	10.5
Capsinoids	*iso*C10:1VOH	(CST)	5.80	0.606	91.3	16.8	58.7
	C10:0VOH		6.77	1.27	84.7	18.8	68.7
	C14:0VOH		9.86	1.44	75.5	5.46	15.9
	C18:1VOH	(OLT)	11.47	0.198	66.2	10.7	17.5
	C18:0VOH		12.93	NC	NC	4.93	10.9

The log octanol/water partition coefficient (log *P*) and pharmacokinetic parameter was calculated as described in Materials and methods. The partition coefficient of CAP was measured directly using an octanol–water partitioning system. Other log *P* values were calculated from the retention time in HPLC. The increase of $\Delta[Ca^{2+}]_i$ induced by 10 μM CAP in the absence of TPEN was 586 nM. Using this value, we normalized the $\Delta[Ca^{2+}]_i$ values induced by other capsaicinoids or capsinoids in the presence or absence of TPEN.
NC; not calculated.

obtained HEK293VR11 cells, which expressed TRPV1 mRNA and protein (data not shown).

We examined the electrophysiological properties of the cloned cells to confirm the functional expression of the rTRPV1 receptor. As shown in Fig. 2A, CAP and CST induced a concentration-dependent current in this cell line. The EC_{50} values for CAP and CST were 114 nM and 477 nM, respectively. These values were very similar to those obtained in the cells transiently expressing TRPV1 (99 nM and 290 nM for CAP and CST, respectively) (Iida et al., 2003). In addition to the electrophysiological properties, we measured the change in $[Ca^{2+}]_i$ (Fig. 2B). CAP increased $[Ca^{2+}]_i$ concentration-dependently. The EC_{50} value was 81.9 nM, which was a little smaller than that found by measuring the membrane current (Fig. 2A) and similar to the values (15 to 190 nM) previously reported by others (Jerman et al., 2000; Appendino et al., 2002). CST also increased $[Ca^{2+}]_i$, and its EC_{50} value was 606 nM, which was a little greater than that found by measuring the membrane current (Fig. 2A). These results indicate that CST opens the TRPV1 channel and then increases $[Ca^{2+}]_i$, similarly to CAP.

All five capsaicinoids (Fig. 1) increased $[Ca^{2+}]_i$ concentration-dependently (Table 1). These changes in $[Ca^{2+}]_i$ response were not observed in the parent HEK293 cells and were inhibited by capsazepine, a specific antagonist for TRPV1 (data not shown). Therefore, these effects appeared via activation of TRPV1. The potency (EC_{50} values) for four of the capsaicinoids (CAP, OLV, C10:0VA, C14:0VA) were comparable to those reported by others (Jerman et al., 2000; Appendino et al., 2002). Their efficiency (maximum response) was almost the same.

In our experiments, 0.1% BSA was added into the loading buffer to promote dissolution of the lipophilic compounds in aqueous solution. BSA binds a variety of lipophilic ligands, such as fatty acids and lysolecithin (Peters, 1996). It has been reported that BSA decreases the effect of anandamide on TRPV1 (De Petrocellis et al., 2001). However, our data (Fig. 3A) show that BSA had no effect on the action of CAP and C18:0VA.

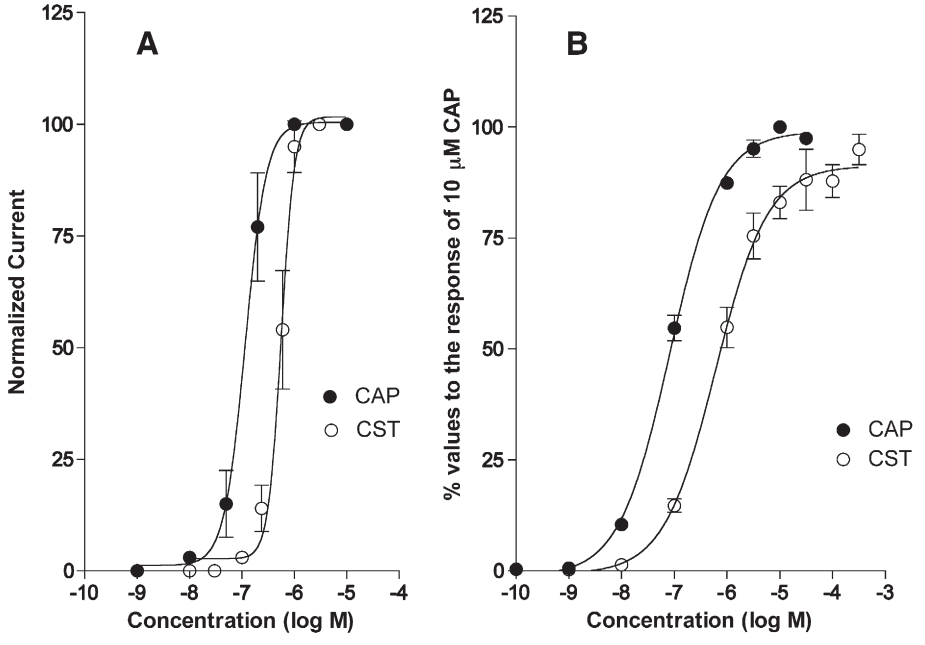

Fig. 2. CAP- and CST-induced changes in membrane potential and $[Ca^{2+}]_i$. (A) Membrane currents were normalized to the response maximally activated by 1 μM capsaicin or 3 μM capsiate and expressed as a percent of the maximal response. Each point represents a mean value±SEM from three independent cells. Averaged data were fitted with the Hill equation. EC_{50} values for capsaicin and capsiate concentration–response curves were 112 nM and 477 nM, respectively. (B) $\Delta[Ca^{2+}]_i$ values were normalized to the response maximally activated by 10 μM capsaicin and expressed as a percentage of the maximal response. Each point represents the mean values±SEM from six to seven independent experiments. Data were fitted with the Hill equation. EC_{50} values for capsaicin and capsiate concentration–response curves were 81.9 nM and 606 nM, respectively.

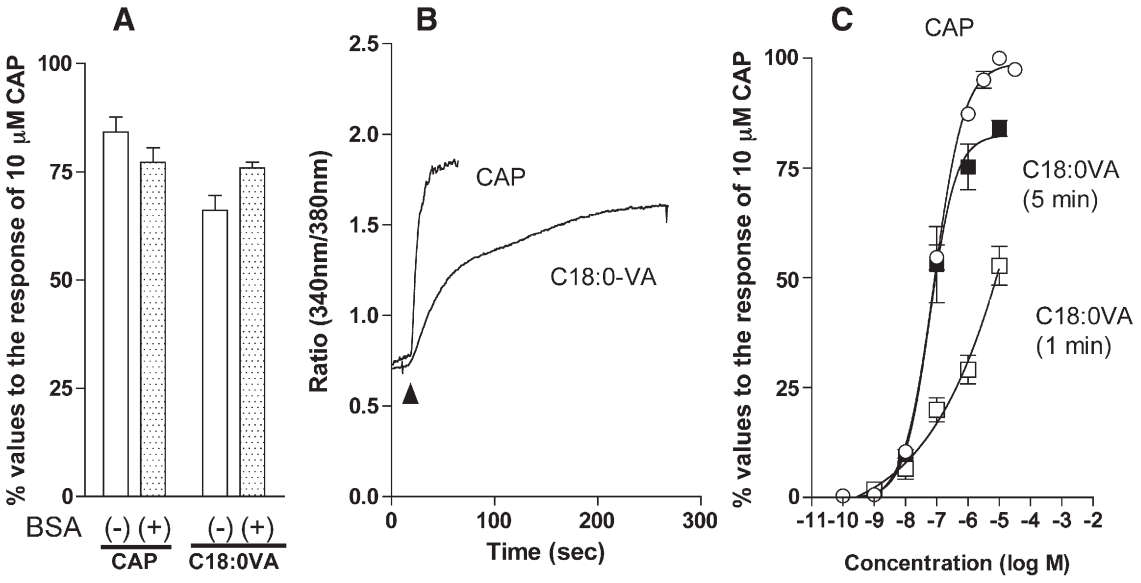

Fig. 3. The different responses to $\Delta[Ca^{2+}]_i$ induced by C18:0VA or CAP. (A) $\Delta[Ca^{2+}]_i$ is indicated after the addition of 1 μM each of CAP or C18:0VA in the absence (open bar) or presence (dotted bar) of 0.1% BSA. $\Delta[Ca^{2+}]_i$ values were normalized to the maximal response activated by 10 μM capsaicin in the presence of BSA and expressed as a percent of the maximal response. Each point represents the mean values±SEM from three independent experiments. (B) The fluorescence ratio (excitation 340 nm/excitation 380 nm) is indicated after the addition of 1 μM each of CAP or C18:0VA. Arrows indicate the time of the sample addition. (C) Concentration–response curves for CAP (circles) and C18:0VA (squares) are indicated. Open symbols indicate maximum values during 1 min after sample addition (short periods). Closed symbols indicate the maximum value during 5 min after sample addition (long periods). Each point represents the mean values±SEM from three and seven independent experiments for C18:0VA and CAP, respectively.

The potency of C18:0VA (60.9 nM) was similar to that of CAP (81.9 nM), and the efficiency of C18:0VA (maximum response) was 82.6% of that of CAP (Table 1). This result differs from that of a previous report (Appendino et al., 2002). The reason for this discrepancy remains obscure. However, we observed that C18:0VA gradually increases the fluorescence ratio (340/380) (Fig. 3B). When $[Ca^{2+}]_i$ was measured during the first minute, the EC_{50} value for C18:0VA was not measurable and its maximum response was 50% (Fig. 3C), similar to the previous results (Appendino et al., 2002). On the contrary, when we measured $[Ca^{2+}]_i$ over 5 min, C18:0VA increased the fluorescence ratio to a maximal level (Fig. 3B). The effect of C18:0VA was almost the same as that of CAP (Fig. 3C). Therefore, C18:0VA is one of the TRPV1 agonists, but it has a somewhat unique property.

CST is known to activate TRPV1 (Iida et al., 2003), but the activity of the other capsinoids has not been reported. We found that all the newly synthesized capsinoids increased $[Ca^{2+}]_i$ via

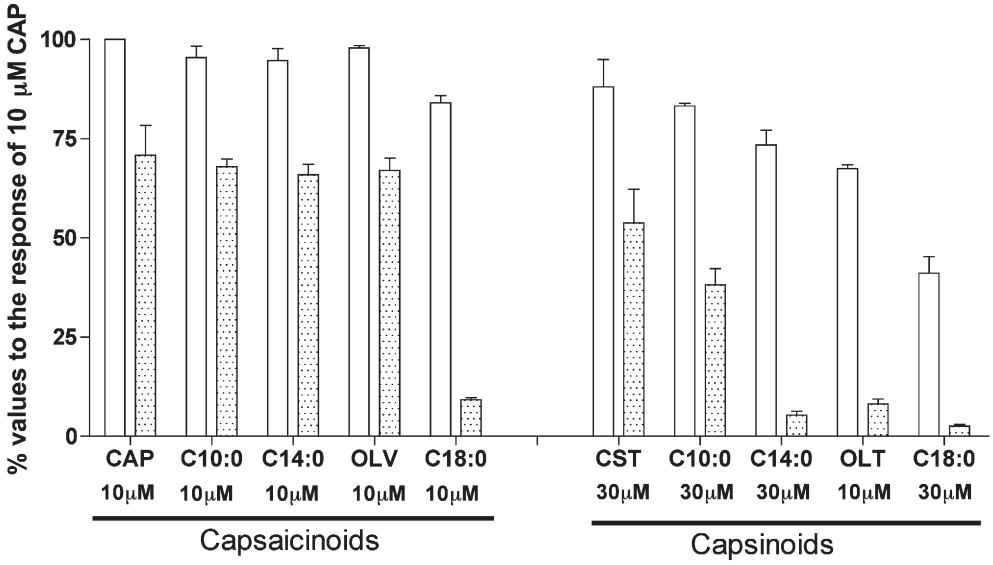

Fig. 4. Tapsigargin inhibits the increase of $\Delta[Ca^{2+}]_i$ induced by highly lipophilic agonists. $\Delta[Ca^{2+}]_i$ values were normalized to the maximal response activated by 10 μM capsaicin and expressed as a percent of the maximal response. Vehicle (open bar) or 50 μM of tapsigargin (dotted bar) were added to a loading buffer at 1 min prior to the addition of the indicated capsaicinoids (A) or capsinoids (B). Each bar represents the mean values±SEM from three experiments.

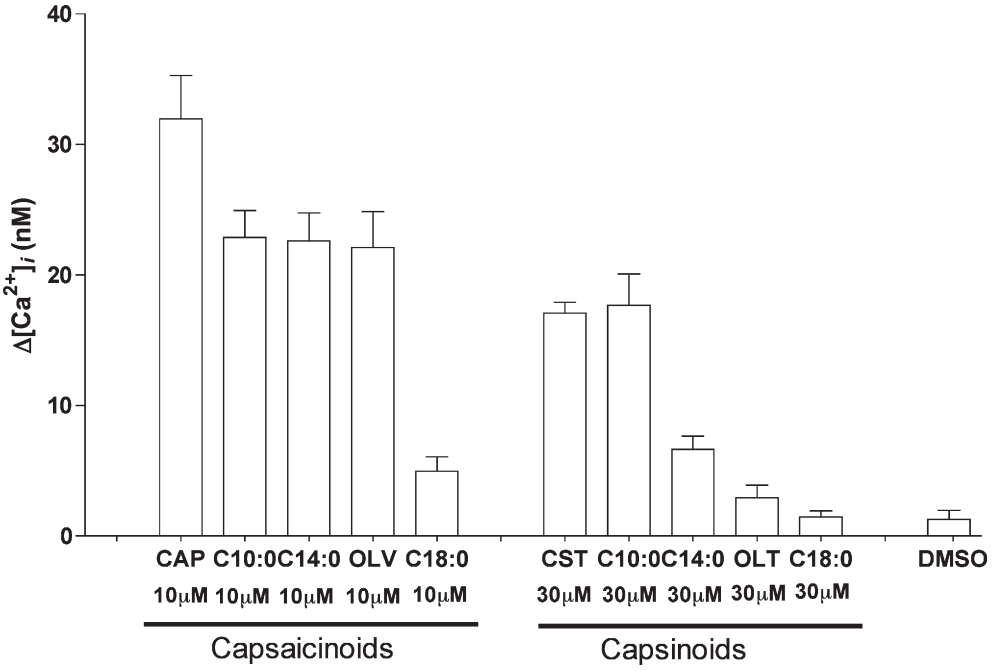

Fig. 5. The absence of extracellular Ca^{2+} inhibits the increase of $\Delta[Ca^{2+}]_i$ induced by highly lipophilic agonists. The Fura 2-loaded cells were resuspended with Ca^{2+}-free loading buffer. EGTA (0.125 mM) was added at 1 min prior to the addition of 10 μM of the capsaicinoids (A) or 30 μM of the capsinoids (B). Each bar represents the mean values±SEM from three experiments.

the activation of TRPV1, as these effects were not observed in the parent HEK293 cells and were inhibited by capsazepine (data not shown). The EC_{50} value of C10:0VOH was two-fold larger than that of CAP, and its maximum response was almost identical to that of CAP (Table 1). C14:0VOH and OLT increased $[Ca^{2+}]_i$, but the maximum responses were lower at 75.5% and 66.2%, respectively. The properties of the latter two capsinoids are similar to those of C18:0VA. The addition of more than 100 μM C18:0VOH made the assay solution turbid because of its low solubility. Therefore, we could not obtain the correct value for the fluorescence. The response of 30 μM C18:0VOH was about 60% of that of 10 μM capsaicin, but did not reach the maximum level. Thus, the EC_{50} value and the maximum response of C18:0VOH could not be calculated.

CAP regulates $[Ca^{2+}]_i$ via TRPV1 with multiple mechanisms. TRPV1 distributes in the plasma and the ER membranes (Olah et al., 2001; Karai et al., 2004). Thus, there are two Ca^{2+} sources, extracellular and intracellular (Eun et al., 2001; Marshall et al., 2003).

To eliminate contributions to $[Ca^{2+}]_i$ from the intracellular Ca^{2+} store, we treated the cells with 10 μM TPEN, a membrane-permeable multivalent cation chelator. Because TPEN has only moderate affinity for Ca^{2+} (K_d=130 mM), it should chelate Ca^{2+} in the intracellular store (Hofer et al., 1998). Four capsaicinoids (CAP, C10:0VA, C14:0VA and OLV) elicited a $[Ca^{2+}]_i$ increase even in the presence of TPEN, although the EC_{50} values increased and the efficiencies (maximum responses) decreased (Table 1). These capsaicinoids are able to increase $[Ca^{2+}]_i$ via $TRPV1_{PM}$ in the absence of an intracellular Ca^{2+} store.

Interestingly, the effect of C18:0VA was inhibited by the presence of TPEN (Table 1). The efficiency of C18:0VA was decreased from 82.6% (in the absence of TPEN) to 10.5% (in the presence of TPEN) (Table 1). This inhibition suggests that C18:0VA elicits a very slight influx of calcium via $TRPV1_{PM}$ in the absence of an intracellular Ca^{2+} store.

Capsinoids containing medium chain acyl groups (C10:0VOH and CST) increased $[Ca^{2+}]_i$ even in the presence of TPEN (Table 1). As expected from the effect of C18:0VA, capsinoids containing long fatty acids (C14:0VOH, C18:0VOH, and OLT) could not elicit a large increase in $[Ca^{2+}]_i$ (Table 1). Because the log *P* values of the latter group are higher than that of C18:0VA (Table 1), these compounds are extremely lipophilic. It is possible that these highly lipophilic capsaicinoids and capsinoids elicit only a slight calcium influx via $TRPV1_{PM}$ in the absence of an intracellular Ca^{2+} store.

In addition to TPEN, tapsigargin also depletes the intracellular Ca^{2+} store (Bian et al., 1991; Lytton et al., 1991). Fig. 4 shows the effects of tapsigargin on the increase in $[Ca^{2+}]_i$ induced by capsaicinoids and capsinoids. As expected from the results with TPEN, tapsigargin also decreased the efficiencies of the highly lipophilic capsaicinoid and capsinoids to less than 10%. Therefore, we believe that the extremely lipophilic capsaicinoid (C18:0VA) and capsinoids (C14:0VOH, C18:0VOH and OLT) elicit only a slight calcium influx via $TRPV1_{PM}$ in the absence of an intracellular Ca^{2+} store.

To eliminate the influence of extracellular Ca^{2+}, we suspended Fura 2-loaded cells with Ca^{2+}-free assay buffer, and added EGTA (0.125 mM) 1 min prior to the addition of the test compounds. CAP increased $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} (Fig. 5). This increase is due to the release of Ca^{2+} from the intracellular Ca^{2+} store via TRPV1 in the ER membrane, as previously reported by others (Olah et al., 2001; Karai et al., 2004). Three capsaicinoids (C10:0VA, C14:0VA and OLV) and two capsinoids (CST and C10:0VOH) also increased

$[Ca^{2+}]_i$, but to a lesser extent than CAP (Fig. 5). These six ligands, which are of low- or mid-level lipophilicity, can activate $TRPV1_{ER}$. However, one capsaicinoid (C18:0VA) and three capsinoids (C14:0VOH, OLT and C18:0VOH) elicited only a slight release of calcium from the intracellular Ca^{2+} store (Fig. 5). It is possible that highly lipophilic capsaicinoids and capsinoids are not able to activate $TRPV1_{ER}$.

Discussion

All capsaicinoids and capsinoids increased $[Ca^{2+}]_i$ under normal conditions. However, one capsaicinoid (C18:0VA) and three capsinoids (C14:0VOH, C18:0VOH and OLT) that have high lipophilicity (log $P > 9.5$) exhibited different effects in the absence of extracellular Ca^{2+} or intracellular Ca^{2+} sources.

When the extracellular Ca^{2+} was removed from the medium, agonists such as CAP and CST increased $[Ca^{2+}]_i$ (Fig. 5). These agonists activated the $TRPV1_{ER}$, as described by others (Olah et al., 2001; Karai et al., 2004). However, highly lipophilic agonists did not elicit large increases in $[Ca^{2+}]_i$ (Fig. 5). It has been reported that the high lipophilicity of fatty acids decreases the dissociation of these compounds from the plasma membrane (Hamilton, 1998; Zhang et al., 1996). Electrophysiological evidence indicated that the lipophilic agonists such as OLV were not washed out from the membrane but CAP was easily (Iida et al., 2003). Therefore, highly lipophilic agonists do not penetrate the plasma membrane and are not able to activate $TRPV1_{ER}$.

When the intracellular Ca^{2+} store was depleted by the addition of TPEN or tapsigargin, agonists such as CAP and CST increased $[Ca^{2+}]_i$ (Table 1 and Fig. 4). It is remarkable that the highly lipophilic agonists increased $[Ca^{2+}]_i$ only very slightly in the presence of TPEN and tapsigargin (Table 1 and Fig. 4). These results suggest that highly lipophilic agonists elicit little Ca^{2+} influx via $TRPV1_{PM}$. This suggestion is supported by evidence that C18:0VA did not increase $[^{45}Ca^{2+}]$ uptake into DRG neurons (Walpole et al., 1993).

It is necessary to consider why highly lipophilic agonists only slightly open the $TRPV1_{PM}$ channels. We postulate that highly lipophilic agonists would partially bind to $TRPV1_{PM}$. Recently, three laboratories have reported that Met 547 of TRPV1, near the extracellular side of transmembrane domain 4, was functioning as a binding site of TRPV1 to CAP (Chou et al., 2004; Gavva et al., 2004; Phillips et al., 2004). The other candidates for binding sites are located at the intracellular N-terminal cytosolic domain (Jordt and Julius, 2002) or the C-terminal cytosolic domain (Jung et al., 2002). Agonists such as CAP may have adequate lipophilicity to penetrate the plasma membrane. They can bind to both transmembrane and intracellular sites; they then elicit prolonged channel openings. On the other hand, highly lipophilic compounds do not easily dissociate from the plasma membrane (Hamilton, 1998; Zhang et al., 1996). Thus, highly lipophilic agonists would stay in the plasma membrane and act only at Met 547 in the transmembrane domain. They would bind to TRPV1 partially and open the TRPV1 channel partially, so that only small amounts of Ca^{2+} penetrate into the cells. Recent results from measuring single channel activity have also shown that TRPV1 has multiple binding sites, with at least five to seven closed conformations, and that partial binding would lead to partial channel opening (Hui et al., 2003). Further experiments are necessary to confirm this possibility.

Furthermore, we must consider several amplification processes, including intracellular Ca^{2+} stores, because highly lipophilic TRPV1 agonists can increase $[Ca^{2+}]_i$ under normal conditions. It had been postulated that a Ca^{2+} influx via TRPV1 was amplified by several mechanisms, such as CICR (calcium-induced calcium release) from the intracellular Ca^{2+} store or SOCE (store operated calcium entry) from an extracellular site (Karai et al., 2004). Recently, van der Stelt et al. (2005) demonstrated that anandamide acted as an intracellular messenger amplifying Ca^{2+} influx via TRPV1 channels. They hypothesize that anandamide may function as a store-operated messenger signaling to TRPV1 to gate extracellular Ca^{2+}. It is possible that highly lipophilic TRPV1 agonists stimulate metabolically the production of anandamide, which then activates $TRPV1_{PM}$. Therefore, the response to C18:0VA would be slower than that to CAP (Fig. 3). Further experiments are necessary to elucidate the precise mechanism(s) by which highly lipophilic agonists increase $[Ca^{2+}]_i$ under normal conditions.

Our results indicate that TRPV1 agonist potency is partially dependent on its lipophilicity, which influences its transport across the cell membrane. Indeed, some endogenous compounds, called endovanilloids (Chu et al., 2003), need to be carried inside the cell via a selective transporter to interact with TRPV1 (Huang et al., 2002). Using several agonists with different lipophilicities, we would be able to distinguish the multiple mechanisms of the activation process of TRPV1.

Conclusion

A highly lipophilic capsaicinoid containing C18:0 and capsinoids containing C14:0, C18:0, or C18:1 (the latter was named olvanilate) could not elicit a large increase in $[Ca^{2+}]_i$ in the absence of an extracellular or intracellular Ca^{2+} source. These results suggest that highly lipophilic compounds cause only a slight Ca^{2+} influx via TRPV1 in the plasma membrane and are not able to activate TRPV1 in the endoplasmic reticulum. The capsaicinoids and capsinoids increase $[Ca^{2+}]_i$ through different mechanism(s), depending on their properties.

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