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Inhibition of Ca^{2+} release-activated Ca^{2+} channel (CRAC) by curcumin and caffeic acid phenethyl ester (CAPE) via electrophilic addition to a cysteine residue of Orai1

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ABSTRACT

Ca^{2+} influx through Ca^{2+}-release activated Ca^{2+} channels (CRAC) is critical for activating immune cells. Orai and STIM proteins comprise the molecular components of CRAC. We previously observed that curcumin and caffeic acid phenethyl ester (CAPE) inhibit CRAC current in Orai1/STIM1-co-expressing HEK293 cells (Nam et al., 2009; Shin et al., 2011) [1,2]. Both compounds contain electrophilic α,β-unsaturated carbonyl groups that potentially form Michael addition with cysteine residues. We investigated the sensitivity of cysteine mutated Orai1 to curcumin and CAPE to delineate their inhibitory mechanism. Replacing the 195 cysteine residue with serine (C195S) reversed the effect of CAPE from inhibition to facilitation and significantly weakened the inhibitory effect of curcumin. Tetrahydrocurcumin, a curcumin metabolite, showed a less potent inhibitory effect on I_{CRAC}, and this effect was abolished in C195S Orai1. Additive mutation of other cysteines (C143S and C126S) had no further influence on the effects of CAPE and curcumin. These results indicate that the electrophilic addition to the Orai1 195Cys was responsible for the inhibitory effect of I_{CRAC} by curcumin and CAPE.

1. Introduction

Stimulating immune cells by various means induces Ca^{2+} release from endoplasmic reticulum (ER) and subsequent activation of sustained Ca^{2+} influx (store-operated Ca^{2+} entry, SOCE). SOCE is critical for the calcineurin-activation and subsequent dephosphorylation of NFAT [3,4]. Ca^{2+}-release activated Ca^{2+} channel (CRAC) shows a distinctive inwardly rectifying current–voltage relationship and is the electrophysiological candidate for SOCE in lymphocytes. The molecular identification of CRAC in T cells is now clearly understood; Orai1 (CRACM1) is located in the plasma membrane as the Ca^{2+} conducting pore unit, and STIM1 mediates the Ca^{2+} filling state of ER [4]. In fact, their genetic mutations result in impaired immune functions in humans [5].

Curcumin (1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione) is an orange–yellow polyphenol in the curry spice, and caffeic acid phenethylester (CAPE) is a major active component in propolis from bee hives. Both curcumin and CAPE show various pharmacological actions including anti-inflammatory and anti-cancer effects *in vitro*, some of which have been observed *in vivo* [6,7]. A large number of biomolecules have been suggested as targets of curcumin and CAPE, including the transcription factors nuclear factor-kB, nuclear factor of activated T-cells, hypoxia inducible factor-1 and signaling molecules such as hemoxygenase, cyclooxygenase, and nitric oxide synthase [8–13]. We recently reported that curcumin and CAPE dose-dependently inhibit CRAC, and thereby suppress SOCE in Jurkat T cells [1,2]. The wide spectrum of curcumin and CAPE biological effects result, at in least part, from inhibiting CRAC.

Previous studies including ours have demonstrated that curcumin inhibits not only CRAC but also other cation channels such as K^+ channels (Kv and SK4) and transient receptor potential cation channel subfamily V member 1 [2,14]. In addition, curcumin cross-links epithelial Cl^- secretory anion channels (cystic fibrosis transmembrane conductance regulator) and promotes their activity [15]. As our prior study showed that pretreatment with antioxidants right-shifted the concentration-response curve of CRAC inhibition by curcumin, redox state-sensitive residue(s) of CRAC channels might be the curcumin target.

A recent study by Niemeyer's group demonstrated that a specific cysteine residue (C195) of the Orai1 extracellular domain is responsible for the inhibitory effects of H_2O_2 on CRAC and SOCE [16]. The thiol side chain (–SH) of cysteine serves as a nucleophile.

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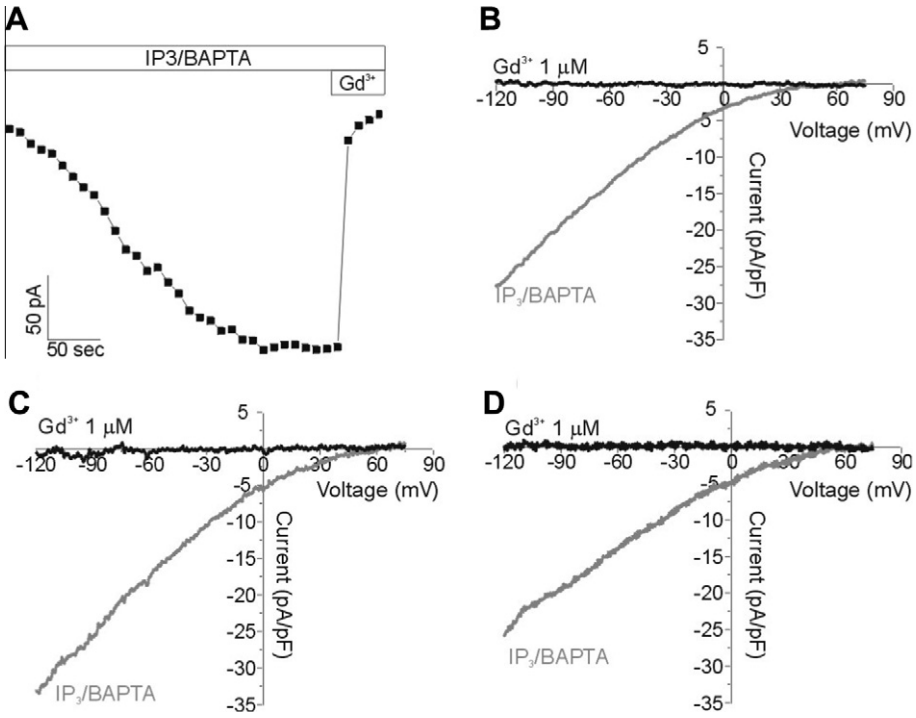

Fig. 1. Recordings of I_{CRAC} mediated by wild-type (WT) and cysteine-mutated Orai1. (A) A representative time course of I_{CRAC} development measured at −60 mV after preparing a whole-cell configuration with 10 mM BAPTA and 20 μM $InsP_3$ in the pipette solution. Applying 1 μM Gd^{3+} completely blocked the inward current after confirming steady-state I_{CRAC} activity. (B–D) Current–voltage relationships (I/V curves) obtained by ramp-depolarization showed inward rectification with a reversal potential at close to 50 mV, indicating high Ca^{2+} selectivity.

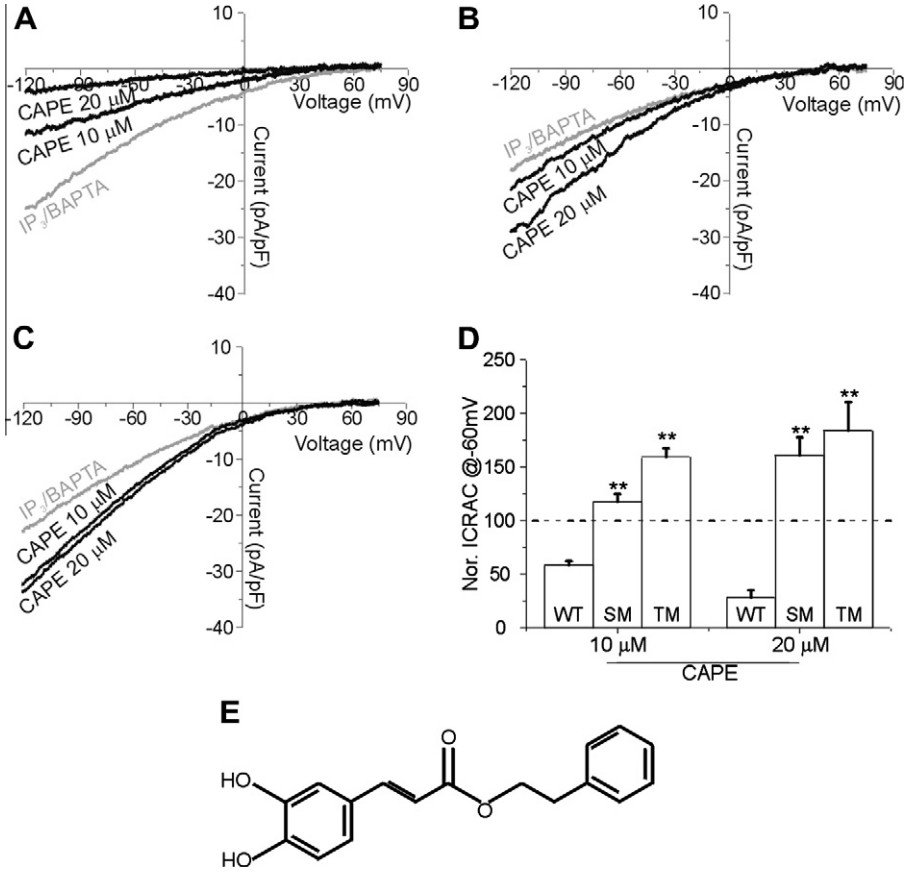

Fig. 2. C195 of Orai1-dependent inhibitory effects of curcumin and caffeic acid phenethyl ester (CAPE) on I_{CRAC}. CAPE (10 and 20 μM) was applied after confirming steady-state activation of I_{CRAC} (IP_3/BAPTA). Concentration-dependent inhibition by CAPE was confirmed in Orai1 wild-type (WT)-overexpressed in HEK293 cells (A). In contrast, CAPE increased the amplitude of I_{CRAC} in cells overexpressed with Orai1-SM and Orai1-TM (B and C, respectively). Amplitudes of I_{CRAC} at −60 mV were normalized to the steady-state level before applying CAPE and are summarized as a bar graph (D) (**$P < 0.01$). (E) CAPE chemical structure.

Both curcumin and CAPE contain electrophilic α,β-unsaturated carbonyl groups that react with channel protein nucleophilic residues. Thus, an electrophilic interaction with Orai1 C195 might be responsible for the inhibition of CRAC by curcumin and CAPE, which we investigated in the present study.

2. Materials and methods

2.1. HEK293 cells expressing Orai1/STIM1

HEK293 cells were transfected with an expression vector carrying the human Orai1 or human STIM1 genes cloned into the N-terminal portion of enhanced green fluorescent protein. Cells were grown in 6-well dishes to 60% confluence, and 0.5 μg DNA was transfected using a Fugene 6 kit (Roche, Sandhoferstrasse, Germany).

Orai1 contains three cysteines at amino acid positions 126, 143, and 195. Among them, a single mutant (C195S, Orai1-SM) and triple mutant (C126S, C143S, C195S; Orai1-TM) were overexpressed with STIM1 in HEK293 cells. The cloned genes of the hOrai1 single (C195S, Orai1-SM) and triple (C126S, C143S, C195S; Orai1-TM) mutants were kind gifts from Dr. Barbara Niemeyer (Saarland University, Saarbrücken, Germany). Transfected cells were grown in Dulbecco's Modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (Hyclone, Logan, UT, USA) at 37 °C in 5% CO_2.

2.2. Electrophysiology

Cells were transferred into a bath mounted on the stage of an inverted microscope (TE-2000S, Nikon, Tokyo, Japan). The bath (approximately 0.15 ml) was superfused at 5 ml/min, and voltage clamp experiments were performed at room temperature (23–25 °C). Patch pipettes, with a free-tip resistance of about 3 MΩ, were connected to the head stage of a patch clamp amplifier (Axopatch-1B, Axon Instruments, Foster City, CA, USA). A conventional whole-cell clamp was achieved by rupturing the patch membrane after making a giga-seal. Membrane capacitance was measured in each cell. Voltage ramps of 100 ms duration, spanning a range of −130 to +70 mV, were delivered from a holding potential of −10 mV every 5 s. pCLAMP software v.9.2 and Digidata-1322A (Axon Instruments) were used to acquire data and apply command pulses, respectively. Whole-cell currents were recorded at a rate of 10 kHz and were low-pass filtered at 1 kHz. Current traces were stored and analyzed using Clampfit v.9.2 and Origin v.7.0 (Microcal Inc., Northampton, MA, USA). The internal pipette solution used for I_{CRAC} contained (in mM) 125 Cs-glutamate, 20 CsCl, 10 BAPTA, 3 MgATP, 1 $MgCl_2$, 10 HEPES, 0.02 IP_3, and 0.002 sodium-pyruvate, and pH was titrated to 7.2 with CsOH. The normal Tyrode's (NT) bath solution contained (in mM) 145 NaCl, 3.6 KCl, 1 $MgCl_2$, 10 HEPES, 5 D-glucose, and 1.3 $CaCl_2$, and pH was titrated to 7.4 with NaOH. $CaCl_2$ was raised to 10 mM to measure I_{CRAC}. The bath ground con-

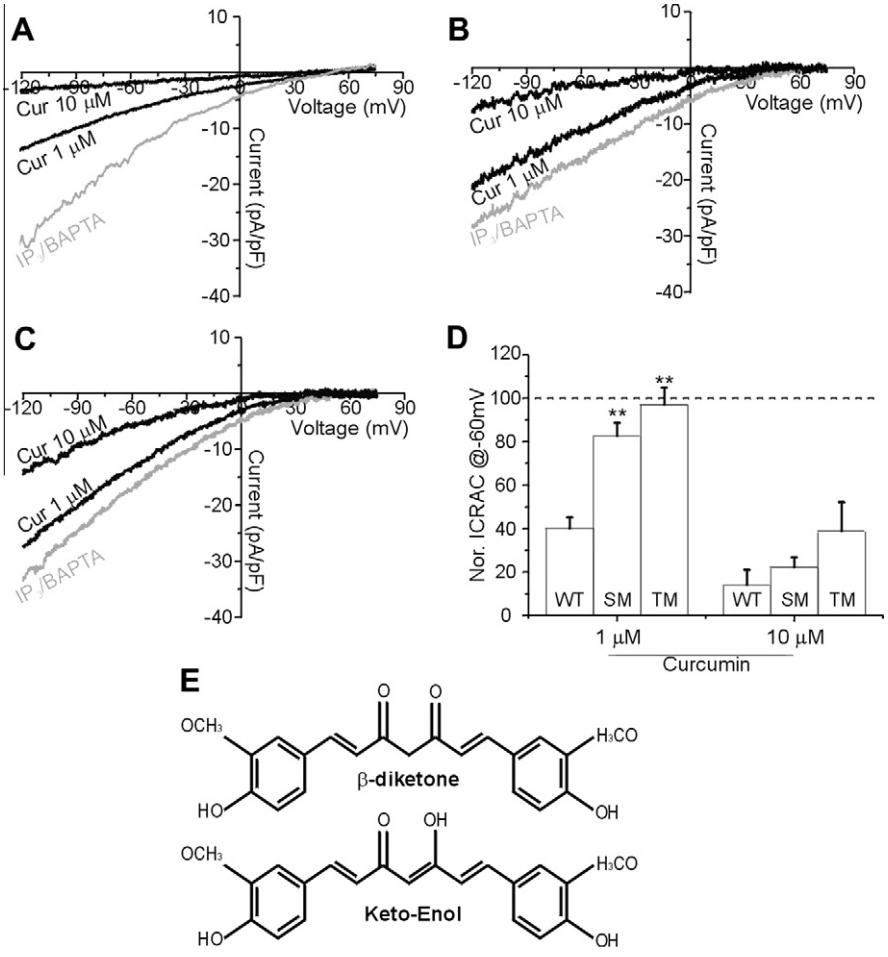

Fig. 3. Inhibition of I_{CRAC} by curcumin is partly dependent on C195 in Orai1. Curcumin (1 or 10 μM) was applied after confirming steady-state activation of I_{CRAC} mediated by wild-type (WT) Orai1 (A), Orai1-SM (B), and Orai1-TM (C). Amplitudes of I_{CRAC} at −60 mV were normalized to the steady-state level before curcumin was applied and are summarized as a bar graph (D) (**$P < 0.01$). (E) Dual chemical structures of curcumin.

sisted of Ag/AgCl wires in NT solution agar bridges. The liquid junction potential difference between the 10 mM $CaCl_2$-containing NT solution and the Cs-glutamate pipette solution was measured (approximately 1 mV) and was ignored in the I/V curves.

2.3. Chemicals and drugs

All chemicals used for experiments were purchased from Sigma-Korea (Seoul, Korea). CAPE, curcumin and tetrahydrocurcumin (THC) were dissolved in dimethyl sulfoxide as a stock solution and diluted to the final concentration in the bath perfusate and PSS. The total amount of dimethyl sulfoxide was maintained at <0.1%/vol.

2.4. Statistical analysis

Data are presented as representative original recordings and graphs of mean ± standard error. An analysis of variance followed by Tukey's post hoc test was applied in Figs. 2D, 3D. and 4D. The hypothesis of no difference was rejected if the *p*-value was <0.05.

3. Results

When the whole-cell configuration under ER Ca^{2+}-depleting conditions (10 mM BAPTA and 20 μM IP_3 in the pipette solution) was prepared, spontaneous activation of inwardly-rectifying current (I_{CRAC}) was consistently observed in HEK293 cells overexpressed with the wild type (WT) Orai1 and the cysteine mutants (Orai1-SM and Orai-TM). Current from the first ramp pulse after membrane break-in was subtracted from the subsequent responses to isolate the spontaneously developing inward I_{CRAC}. I_{CRAC} was completely blocked by 1 μM Gd^{3+} (Fig. 1). The inward current developed after the store depletion was blocked with 50 μM 2-APB, a well-known inhibitor of CRAC (data not shown).

As previously reported [1], 10 μMCAPE inhibited I_{CRAC} through WT Orai1 by about 50% (Fig. 2A, $n = 5$). Interestingly, I_{CRAC} was not decreased but was increased by CAPE in Orai1-SM (C195S) and Oria1-TM (Fig. 2B and C, $n = 10$ and 12, respectively). Current amplitudes normalized to the control (%) at −60 mV were measured in each cell and are summarized as a bar graph (Fig. 2D).

Curcumin showed more potent inhibitory effects on I_{CRAC} via WT Orai1, as previously reported [2]; about 70% inhibition at 1 μM and almost complete inhibition at 10 μM (Fig 3A, $n = 5$). However, the inhibitory effects of curcuminon I_{CRAC} were signifi-

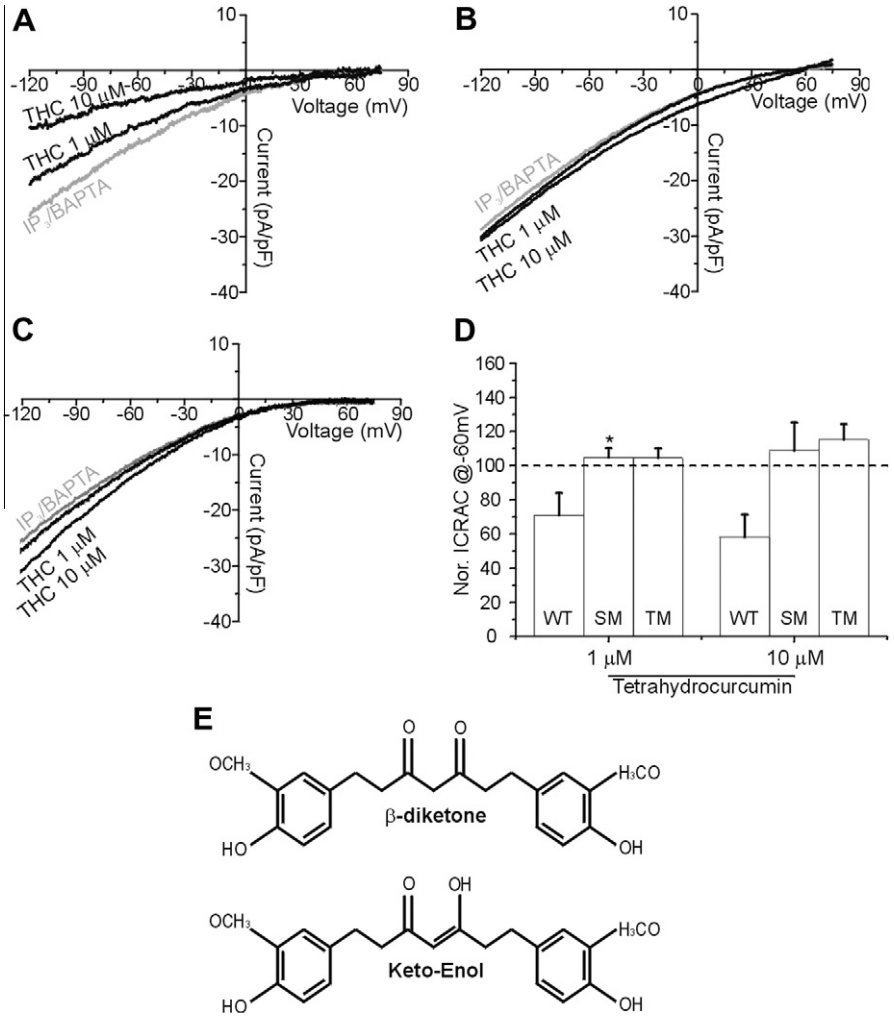

Fig. 4. C195 of Orai1-dependent inhibitory effects of tetrahydrocurcumin (THC) on I_{CRAC}. (A) Concentration-dependent inhibition by THC (1 and 10 μM) was confirmed in Orai1 wild-type (WT)-overexpressed in HEK293 cells. THC had no effect in cells overexpressed with Orai1-SM or Orai1-TM (B and C, respectively). (D) Amplitudes of I_{CRAC} at −60 mV were normalized to the steady-state level before THC was applied and are summarized as a bar graph (*$P < 0.05$). (E) Dual chemical structures of THC.

cantly attenuated in Orai1-SM and-TM overexpressed cells (Fig. 3B and C; $n = 7$ and 5, respectively). The normalized inhibitory effects of curcumin on I_{CRAC} are depicted in a bar graph (Fig 3D).

THC is a hydroxylated metabolite of curcumin that lacks a α,β-unsaturated carbonyl group (β-diketone form) or has a putative α,β-unsaturated carbonyl group (keto-enol form, Fig. 4E). Inhibition of I_{CRAC} by THC was weaker than the effect by curcumin (Fig. 4A). Interestingly, THC had no effect on I_{CRAC} via Orai1-SM or Orai1-TM (Fig. 4B and C, $n = 12$ and 7, respectively). The normalized effects of THC on I_{CRAC} at −60 mV are summarized as a bar graph (Fig. 4D).

4. Discussion

The replacement of the reactive cysteine residue with serine at a specific Orai1 (C195S) site significantly altered the sensitivity of I_{CRAC} to CAPE and curcumin, suggesting that the 195 cysteine is the target residue for the inhibitory actions of the two compounds. The importance of C195 in Orai1 was demonstrated dramatically by conversion of the effects of CAPE on I_{CRAC} from inhibition to facilitation in Orai1-SM. The opposing responses of Orai1-WT and Orai1-SM suggest that CAPE has dual effects on I_{CRAC}; removing the inhibitory effect on cysteine-mutated Orai1 may have revealed a partial positive effect of CAPE.

Both curcumin and CAPE contained two aromatic rings connected by α,β-unsaturated carbonyl group(s) (Figs. 2E and 3E), and the potential Michael adductor group may form a chemical bond with the cysteine residues. It is known that the di-ketone form of curcumin is unstable and spontaneously converts to the enol form with a single ketone group (Fig. 3E).

Different from the effects of CAPE, the sensitivity of Orai1-SM and Orai1-TM to curcumin was not largely abolished. Although significantly weakened, curcumin still decreased the I_{CRAC} via Orai1-SM and Orai1-TM (Fig. 3). These results suggest that curcumin might have an additional mechanism for inhibiting I_{CRAC} besides the nucleophilic addition to C195.

Interestingly, the inhibitory effect of THC on I_{CRAC} was totally dependent on C195 (Fig. 4). Based on the chemical structure of THC, which lacks a α,β-unsaturated carbonyl group [17–19], it was unexpected that the THC inhibitory effect was abolished in Oria1-SM and Orai1-TM. These results suggest that keto-enol derived THC with a probable electrophilic group might still interact with C195 in Orai1, resulting in less potent inhibition than that of curcumin. The lack of an inhibitory effect on Orai1-SM and Orai1-TM suggests that a conformational difference between curcumin and THC is responsible for the additional inhibitory effect of curcumin mentioned above.

Electrophilic interactions between curcumin and intracellular proteins have been proposed as a molecular mechanism explaining a number of pharmacological actions including anti-cancer effects and cellular toxicity [6,20]. The electrophile-protein interaction is a kind of post-translation modulation that occurs most frequently in free thiol groups provided by cysteine residues [21].

Transient receptor potential cation channel, subfamily A, member 1 (TRPA1) is a member of the temperature-sensitive TRP channel family, which is activated by a variety of noxious stimuli including reactive electrophilic compounds [22]. While the net effect on TRPA1 was opposite to the effect on Orai1, curcumin seems to form a Michael addition with TRPA1 cysteine and lysine residues [23,24]. Studies about the modulation of ion channels by electrophilic interaction have been limited to TRPA1. In this respect, our present report might provide the second case of ion channel modulation by Michael addition to a ion channel reactive cysteine residue.

Taken together, we suggest that the inhibition of I_{CRAC} by CAPE and curcumin is mediated by an electrophilic interaction with Orai1C195. A number of induced electrophilic substances occur both biologically (endogenous) and environmentally (exogenous). Therefore, the overlooked functional modulation of ion channels by potential electrophilic molecules might provide insights into a better understanding of their pharmacological effects *in vivo* and *in vitro*.

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