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RESEARCH PAPER

The ryanodine receptor agonist 4-chloro-3-ethylphenol blocks ORAI store-operated channels

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BACKGROUND

Depletion of the Ca^{2+} store by ryanodine receptor (RyR) agonists induces store-operated Ca^{2+} entry (SOCE). 4-Chloro-3-ethylphenol (4-CEP) and 4-chloro-*m*-cresol (4-C*m*C) are RyR agonists commonly used as research tools and diagnostic reagents for malignant hyperthermia. Here, we investigated the effects of 4-CEP and its analogues on SOCE.

EXPERIMENTAL APPROACH

SOCE and ORAI1-3 currents were recorded by Ca^{2+} imaging and whole-cell patch recordings in rat L6 myoblasts and in HEK293 cells overexpressing STIM1/ORAI1-3.

KEY RESULTS

4-CEP induced a significant release of Ca^{2+} in rat L6 myoblasts, but inhibited SOCE. The inhibitory effect was concentration-dependent and more potent than its analogues 4-C*m*C and 4-chlorophenol (4-ClP). In the HEK293 T-REx cells overexpressing STIM1/ORAI1-3, 4-CEP inhibited the ORAI1, ORAI2 and ORAI3 currents evoked by thapsigargin. The 2-APB-induced ORAI3 current was also blocked by 4-CEP. This inhibitory effect was reversible and independent of the Ca^{2+} release. The two analogues, 4-C*m*C and 4-ClP, also inhibited the ORAI1-3 channels. Excised patch and intracellular application of 4-CEP demonstrated that the action site was located extracellularly. Moreover, 4-CEP evoked STIM1 translocation and subplasmalemmal clustering through its Ca^{2+} store-depleting effect via the activation of RyR, but no effect on STIM1 redistribution was observed in cells co-expressing STIM1/ORAI1-3.

CONCLUSION AND IMPLICATIONS

4-CEP not only acts as a RyR agonist to deplete the Ca^{2+} store and trigger STIM1 subplasmalemmal translocation and clustering, but also directly inhibits ORAI1-3 channels. These findings demonstrate a novel pharmacological property for the chlorophenol derivatives that act as RyR agonists.

Abbreviations

2-APB, 2-aminoethoxydiphenyl borate; 4-CEP, 4-chloro-3-ethylphenol; 4-ClP, 4-chlorophenol; 4-C*m*C, 4-chloro-*m*-cresol; EYFP, enhanced yellow fluorescent protein; mCFP, monomeric cyan fluorescent protein; MH, malignant hyperthermia; RyRs, ryanodine receptors; SOCE, store-operated Ca^{2+} entry; SR/ER, sarco/endoplasmic reticulum; TG, thapsigargin

Introduction

Ryanodine receptors (RyRs) are important intracellular Ca^{2+} release channels in the sarcoplasmic/endoplasmic reticulum (SR/ER) (Fill and Copello, 2002). They are ubiquitously expressed in different cell types including both excitable and non-excitable cells (Lanner *et al.*, 2010). RyR1 is the primary isoform in skeletal muscle, while RyR2 is the cardiac isoform, and RyR3, previously referred to as the brain isoform, exists in many tissues (Fill and Copello, 2002; Endo, 2009; Lanner *et al.*, 2010). RyRs are considered particularly important in excitable cells, such as cardiac and skeletal muscles and neurons. Membrane depolarization in cardiomyocytes or neurons activates voltage-gated L-type Ca^{2+} channels, which results in Ca^{2+} influx and subsequently triggers the Ca^{2+}-induced Ca^{2+} release from SR/ER through RyRs, and thus causes muscle contraction or alter the functionality of neurons (Lanner *et al.*, 2010; Rose and Konnerth, 2001). In skeletal muscle, physiological Ca^{2+} release is induced by t-tubule membrane depolarization, which transmits the signal to RyRs through a mechanism involving a protein-protein interaction (Endo, 2009). Dysfunctional RyRs cause diseases, for example, RyR1 mutations are involved in central core disease and malignant hyperthermia (MH) (Robinson *et al.*, 2006; Hopkins, 2011), and RyR2 mutations are associated with catecholaminergic polymorphic ventricular tachycardia and arrhythmogenic right ventricular cardiomyopathy (Lanner *et al.*, 2010; Priori and Chen, 2011). RyR3 is the least studied isoform, but it has been suggested to play a role in Alzheimer's disease (Supnet *et al.*, 2010).

Caffeine and chlorophenol derivatives, structurally different RyR agonists, are commonly used as pharmacological research tools and diagnostic drugs for MH (Figure 1A) (DiJulio *et al.*, 1997; Westerblad *et al.*, 1998; Baur *et al.*, 2000; Varadi and Rutter, 2002; Gerbershagen *et al.*, 2005). These two classes of structure may have different binding sites to RyRs (Tian *et al.*, 2013). The chlorophenol derivatives 4-chloro-3-ethylphenol (4-CEP) and 4-chloro-*m*-cresol (4-C*m*C) can induce a robust release of intracellular Ca^{2+} by stimulating RyRs at concentrations about 10-fold lower than caffeine (Islam *et al.*, 1998; Jacobson *et al.*, 2006), and have advantages over caffeine and ryanodine as diagnostic drugs for MH (Gerbershagen *et al.*, 2005). It has been demonstrated that activation of RyRs by ryanodine or repetitive depolarization can induce store-operated Ca^{2+} entry (SOCE) and a Ca^{2+} release-activated current in smooth muscle and skeletal muscle cells (Fellner and Arendshorst, 2000; Yarotskyy and Dirksen, 2012). ORAI and STIM proteins are recently identified molecules that act as store-operated channels (Zhang *et al.*, 2005; Feske *et al.*, 2006; McNally *et al.*, 2012). The nomenclature and pharmacological characteristics of ORAI store-operated channels are still not known (Vig *et al.*, 2008; Alexander *et al.*, 2013), although they have been shown to be blocked by certain compounds, such as 2-aminoethoxydiphenyl borate (2-APB) (Peinelt *et al.*, 2008), lanthanides and BTP2 (Zitt *et al.*, 2004; Shuttleworth, 2012). Recently, using HEK293 cells transfected with STIM1 tagged with enhance yellow fluorescent protein (EYFP), we showed that the depletion of Ca^{2+} stores by the RyR agonists caffeine and 4-CEP evoked the release of Ca^{2+} accompanied by STIM1 translocation and subplasmalemmal clustering (Zeng *et al.*, 2012), and the RyR antagonist tetracaine prevented this intracellular movement of STIM1 (Zeng *et al.*, 2012). This provides direct evidence that RyRs induce the activation of store-operated channels (SOCs) (Zhang *et al.*, 2005). Interestingly, upon re-introduction of external Ca^{2+} into the bath solution, caffeine induced the release of Ca^{2+} followed by a significant SOCE, but 4-CEP only induced a robust release of Ca^{2+} with little Ca^{2+} influx (Zeng *et al.*, 2012). Furthermore, caffeine evokes intracellular Ca^{2+} oscillations in bullfrog sympathetic ganglion cells, but the 4-CEP analogue cresol does not (Higure *et al.*, 2006). These observations strongly suggest the existence of functional coupling between RyRs and SOCs via intracellular STIM1 movement, and that caffeine and 4-CEP probably have different effects on the dynamics of intracellular Ca^{2+}. Therefore, we hypothesized that the unexpected reduction in Ca^{2+} influx induced by 4-CEP could be due to the blockage of store-operated ORAI channels.

To test this hypothesis, we investigated the effects of 4-CEP on the SOCE and ORAI1-3 channels using rat L6 myoblasts derived from skeletal muscle and HEK293 cells transfected with ORAI1-3 and STIM1 genes. We also compared the effect of 4-CEP with those of 4-C*m*C and 4-chlorophenol (4-ClP) to determine any potential structure-activity relationship for their antagonism of ORAI channels.

Methods

Chemicals and reagents

Generally used salts and other chemicals including thapsigargin (TG), 2-APB, caffeine, 4-CEP, 4-C*m*C, 4-ClP and Fura-PE3/AM were purchased from Sigma-Aldrich (Poole, UK).

Cell culture and transfection

The HEK293 T-REx cells were cultured in DMEM/F-12 medium (Invitrogen, Paisley, UK) supplemented with 10% fetal calf serum, 100 units·mL^{-1} penicillin and 100 mg·mL^{-1} streptomycin, and maintained at 37°C under 95% air and 5% CO_2. Rat L6 myoblasts were purchased from ATCC (Manassas, VA, USA) and cultured in the same conditions. Plasmids encoding mCherry-ORAI1, mCherry-ORAI2 and monomeric cyan fluorescent protein (mCFP)-ORAI1, mCFP-ORAI2 and mCFP-ORAI3 in the pcDNA4/TO vector were transfected into stable STIM1-EYFP cells and the expression of these genes was induced with 1 μg·mL^{-1} tetracycline in the culture medium. Details of the procedures for gene cloning and plasmid constructions are as described previously (Zeng *et al.*, 2012). The stable cells were maintained in the cell culture medium containing G418 (200 μg·mL^{-1}).

Electrophysiology

Whole-cell patch-clamp recording was performed at room temperature (23–26°C). Briefly, the signal was amplified with an Axopatch 200B amplifier and controlled by the software pClamp 10. A 1-s ramp voltage protocol from –100 mV to +100 mV was applied at a frequency of 0.2 Hz from a holding potential of 0 mV. Signals were sampled at 10 kHz and filtered at 1 kHz. Glass microelectrodes with resistances of 3–5 MΩ were used. The pipette solution contained (in mM) 145 Cs-methanesulfonate, 10 BAPTA, 8 $MgCl_2$ and 10 HEPES (pH 7.2 adjusted with CsOH). The standard bath solution

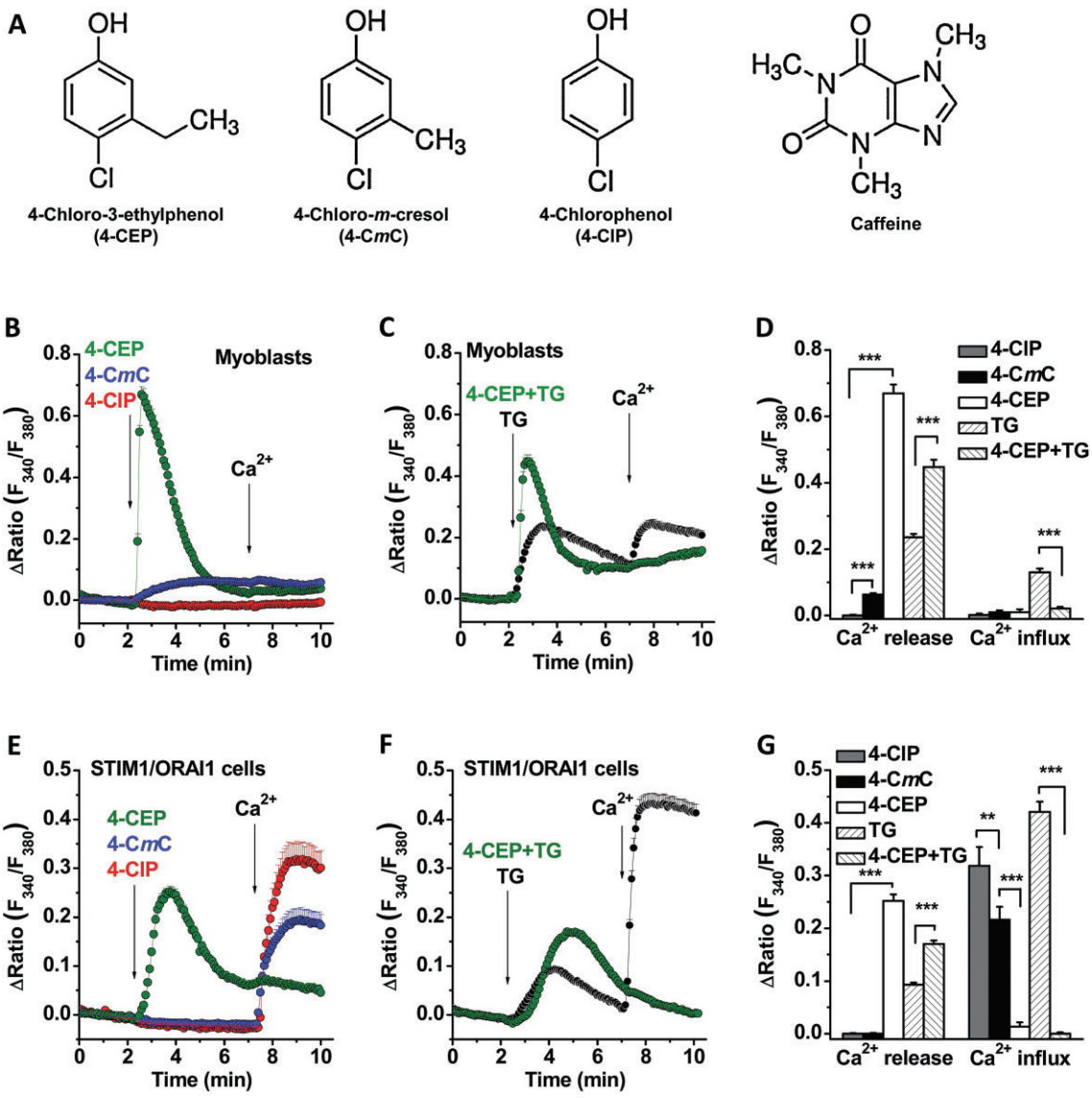

Figure 1

4-CEP induced Ca^{2+} release but abolished store-operated Ca^{2+} entry in rat L6 myoblasts and HEK293 cells co-expressing STIM1/ORAI1. (A) Structures for 4-CEP, 4-C*m*C, 4-ClP and caffeine. (B) The effects of 4-CEP, 4-C*m*C and 4-ClP on the cytosolic Ca^{2+} level in rat L6 myoblasts. The cells were maintained in Ca^{2+} free solution first and then perfused with different compounds (500 μM for each compound) in Ca^{2+} free and 1.5 mM Ca^{2+} solution as indicated by the arrows. (C) 4-CEP (500 μM) nearly abolished the TG (1 μM)-induced Ca^{2+} influx in the myoblasts. (D) Mean ± SEM data for the comparison of Ca^{2+} release and influx induced by 4-ClP, 4-C*m*C, 4-CEP and TG in the myoblasts (n = 19–40 cells in each group). (E) The effects of 4-CEP, 4-C*m*C and 4-ClP (500 μM) in STIM1/ORAI1 cells. (F) 4-CEP (500 μM) abolished the TG (1 μM)-induced Ca^{2+} influx in STIM1/ORAI1 cells. (G) Mean ± SEM data for Ca^{2+} release and influx induced by 4-ClP, 4-C*m*C, 4-CEP and TG in the STIM1/ORAI1 cells (n = 18–26 cells in each group). Ca^{2+} release was measured as the peak of Ca^{2+} release signal; and Ca^{2+} influx was measured at 1 min after Ca^{2+} re-addition. One-way ANOVA with Bonferroni test was used for (D) and (G). ** $P < 0.01$, *** $P < 0.001$.

contained (mM) 130 NaCl, 5 KCl, 8 D-glucose, 10 HEPES, 1.2 $MgCl_2$ and 1.5 $CaCl_2$. The pH was adjusted to 7.4 with NaOH.

Live cell imaging and Ca^{2+} measurement

The stably transfected STIM1/ORAI1-3 cells were seeded on 13-mm glass cover slips and cultured for 24–48 h. Live cell images for EYFP/mCherry/mCFP fluorescence were captured using a microscope equipped with a Nikon Plan Fluor 100 × /1.30 oil objective by using similar procedures to those reported previously (Zeng *et al.*, 2012). The images were analysed using NIS-Elements software (Version 3.2, Nikon, Tokyo, Japan).

For intracellular Ca^{2+} measurements, cells were loaded with 2 μM Fura-PE3/AM in standard bath solution for 30 min at 37°C, followed by 5 min wash in standard bath solution at room temperature. Cells were excited alternately by 340 and 380 nm light and emission was collected via a 510-nm filter.

Images were sampled every 5 s in pairs for the two excitation wavelengths by a CCD camera (ORCA-R2, Hamamatsu, Japan). The ratio of 340/380 nm fluorescence represented the intracellular Ca^{2+} level. The standard bath solution was the same as that used in the patch-clamp experiment. The Ca^{2+}-free solution contained (mM): NaCl 130, KCl 5, $MgCl_2$ 1.2, HEPES 10, Glucose 8, and EGTA 0.4. The pH was adjusted to 7.4 with NaOH. All experiments were performed at room temperature. The *n* values given are the numbers of cells from at least three independent Ca^{2+} imaging experiments.

Statistics

All values are expressed as mean ± SEM. Student's *t*-test was used to assess the statistical difference between two groups. One-way ANOVA with Bonferroni *post hoc* test was used for data sets with more than two groups. A *P* value less than 0.05 was considered significant.

Results

Effects of 4-CEP, 4-CmC and 4-ClP on Ca^{2+} release and SOCE

The effects of 4-CEP and its analogues on Ca^{2+} release and SOCE were observed. In rat L6 myoblasts, 4-CEP significantly evoked a Ca^{2+} release signal in Ca^{2+} free solution, but the same concentrations of 4-C*m*C showed only a small increase and 4-ClP showed no effect (Figure 1B). Re-introduction of 1.5 mM Ca^{2+} did not cause significant cytosolic Ca^{2+} increase in cells in the absence of TG treatment; however, store-operated Ca^{2+} entry (SOCE) was evident after store depletion with 1 μM TG. 4-CEP significantly inhibited the influx of store-operated Ca^{2+} (Figure 1C–D). To further confirm the effects on SOCE, the HEK293 T-REx cells overexpressing STIM1-EYFP and mCFP-ORAI1 were used. The SOCE in STIM1/ORAI1 cells was significantly higher than that in non-transfected cells (Supporting Information Figure S1). Perfusion with 4-CEP (500 μM) induced a robust Ca^{2+} release signal, as seen in the myoblasts, but 4-C*m*C (500 μM) and 4-ClP (500 μM) did not cause Ca^{2+} release (Figure 1E). The different effects on Ca^{2+} release for the three compounds in the two cell types could be due to the differential expression of RyR isoforms in these cells and the different affinities of the compounds for RyRs; indeed, RyR1 is a dominant isoform in the skeletal muscle derived myoblasts, while RyR3 is dominant in HEK293 cells (Van Petegem, 2012) and RyR3 is sensitive to 4-CEP, but almost insensitive to 4-C*m*C (Fessenden *et al.*, 2003; Hopkins, 2011). In addition, the Ca^{2+} influx in the 4-CEP-treated cells was nearly abolished after the addition of 1.5 mM Ca^{2+} in the bath solution (Figure 1E), which accords with our previous observations in the HEK293 cells transfected with STIM1-EYFP alone (Zeng *et al.*, 2012). The Ca^{2+} influx in the 4-C*m*C-treated cells was much higher than that in the 4-CEP group, but lower than that in the cells treated with 4-ClP (Figure 1E). The inhibition of the store-operated Ca^{2+} influx induced by 4-CEP was also significant in the TG-treated, store-depleted cells (Figure 1F). The IC_{50} values for 4-CEP, 4-C*m*C and 4-ClP on the TG-induced SOCE in STIM1/ORAI1 cells were 203.6, 830.9 and 1437.1 μM respectively (Supporting Information Figure S2). These results suggest that 4-CEP can evoke Ca^{2+} release via activation of RyRs, but significantly inhibits SOCs.

STIM1 translocation evoked by 4-CEP through RyR-mediated Ca^{2+} store depletion

To elucidate the mechanism of action of 4-CEP on SOCs, we examined the effects of 4-CEP on STIM1 translocation and subplasmalemmal clustering, which are critical steps for store-operated channel activation (Zhang *et al.*, 2005). We recently demonstrated, in HEK293 T-REx cells overexpressing STIM1-EYFP, that 4-CEP can induce STIM1 translocation to the plasma membrane followed by the gradual formation of cytosolic STIM1 clusters (Zeng *et al.*, 2012). However, such cytosolic STIM1 clustering did not occur in our cells co-expressing STIM1-EYFP and ORAIs, although the STIM1 puncta formation at the plasma membrane was reproducible in the STIM1-EYFP/mCFP-ORAI1 cells after the exposure to 500 μM 4-CEP in the Ca^{2+}-containing solution (Figure 2). Importantly, the puncta near the plasma membrane did not disappear or redistribute in the STIM1/ORAI1 cells and almost no STIM1-EYFP clusters were formed in the cytosol after addition of 4-CEP for 5–10 min (Figure 2A, Supporting Information Videos S1 and S2). A similar phenomenon was observed in the cells co-expressing STIM1-EYFP/mCFP-ORAI2 or STIM1-EYFP/mCFP-ORAI3 (Figure 2A). These results suggest that the STIM1/ORAIs complexes are more stable than STIM1 oligomers, and may not disassociate easily and redistribute into the cytosol. This finding is consistent with our previous observation using 2-APB; 2-APB disassembled the subplasmalemmal STIM1 clusters after store depletion in the STIM1–EYFP cells, but lost the ability to disassemble the subplasmalemmal STIM1 clusters in the STIM1–EYFP cells coexpressing mCherry-ORAI1, mCherry-ORAI2 or mCFP-ORAI3 (Zeng *et al.*, 2012). We also observed the effects of 4-C*m*C and 4-ClP, both compounds at 500 μM failed to induce STIM1 translocation in all of the three STIM1/ORAI co-expressing cell lines, whereas the subsequent perfusion with 500 μM 4-CEP clearly potentiated the formation of STIM1 puncta at the plasma membrane (Figure 2B). These results could be explained by differences in their ability to induce Ca^{2+} release through RyRs (Figure 1E), suggesting that the Ca^{2+} store depletion, such as that induced by the RyR agonist 4-CEP, is necessary for triggering STIM1 translocation and subplasmalemmal clustering.

Inhibition of ORAI channels by 4-CEP, 4-CmC and 4-ClP

The subplasmalemmal translocation of STIM1 induced by 4-CEP is apparently not the mechanism of SOC inhibition, because the persistence of STIM1/ORAI complexes at the plasma membrane induced by 4-CEP implies that STIM1 proteins may keep the active conformation to maintain the ORAI channel opening, therefore, the inhibitory effect of 4-CEP on SOCE or store-operated current seen in the STIM1/ORAI co-expressing cells and myoblasts could be a direct effect on ORAI channels *per se*. To test this, we determined the effects of 4-CEP on ORAI1-3 store-operated channels in the HEK293 T-REx cells co-expressing STIM1/ORAIs. The ORAI store-operated currents evoked by TG in the ORAI1/STIM1, ORAI2/STIM1 and ORAI3/STIM1 cells were abolished by 500 μM

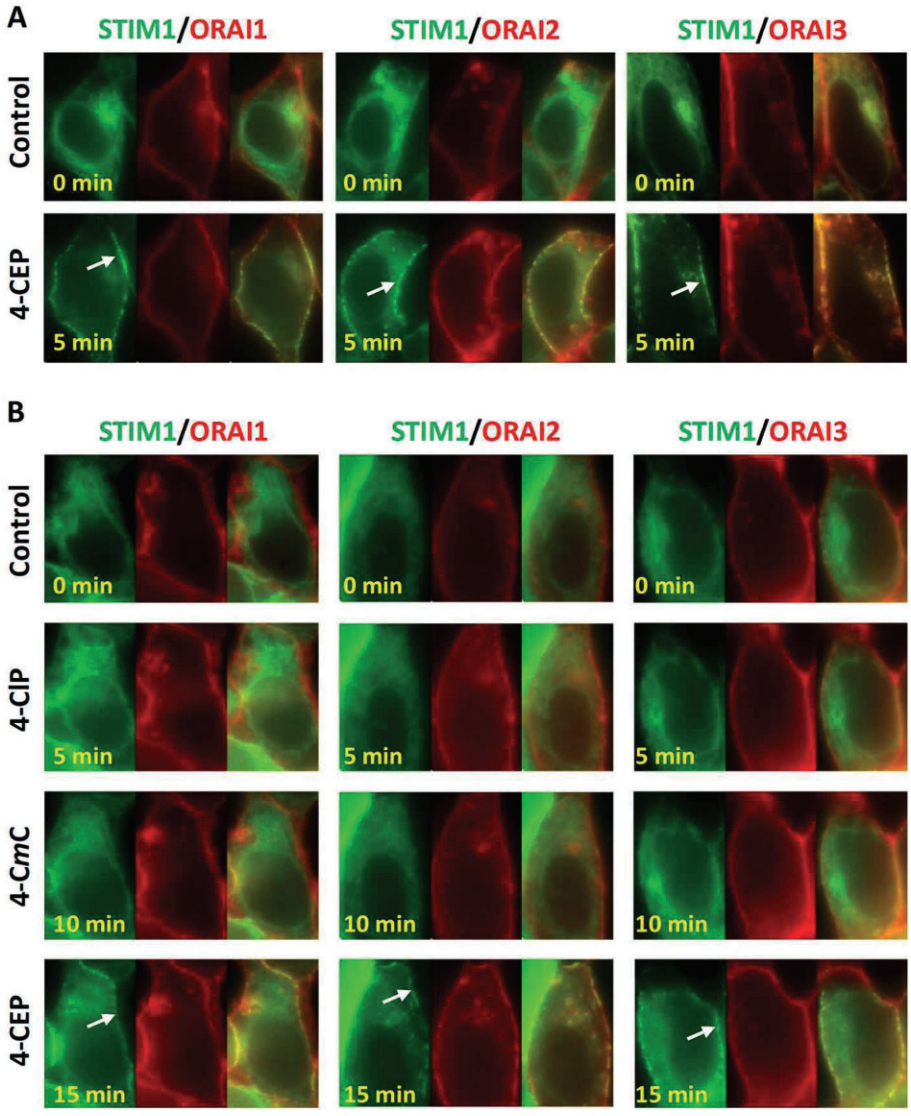

Figure 2

STIM1 translocation and clustering induced by 4-CEP, but not by 4-C*m*C and 4-ClP in the STIM1/ORAI1-3 cells. (A) Application of 500 μM 4-CEP for 5 min induced persistant STIM1-EYFP puncta (green, indicated by arrow) at the plasma membrane in STIM1-EYFP/mCFP-ORAI1-3 cells, while cytosolic clustering of STIM1 was not evident. The mCFP-ORAI fluorescence was converted into red pseudocolour in the pictures. The merged images are shown on the right of each group. The example cell for each group is a representative of 15 to 26 cells. (B) The cells were successively challenged with 500 μM 4-ClP, 500 μM 4-C*m*C and 500 μM 4-CEP in 1.5 mM Ca^{2+} solution with 5 min interval for each drug. STIM1 puncta at the plasma membrane (arrow) seen in 4-CEP-treated cells, but no STIM1 clustering was induced by 4-C*m*C and 4-ClP.

4-CEP (Figure 3A–C). The ORAI currents were also partially inhibited by 500 μM 4-C*m*C and 4-ClP (Figure 3D–I), suggesting that 4-CEP inhibits all three store-operated ORAI currents. However, the TG-induced endogenous store-operated current in the non-transfected HEK293 T-REx cells was not significant (Figure 3J). We further tested whether the 2-APB-induced ORAI3 current could be inhibited by the three drugs in the cells overexpressing ORAI3 alone. As shown in Figure 3K, 2-APB at 50 μM potentiated a large outward current and a relatively small inward current in ORAI3 cells, which is independent of STIM1 and Ca^{2+} store depletion (Shuttleworth, 2012). The ORAI3 current was significantly inhibited by 500 μM of 4-ClP, 4-C*m*C and 4-CEP successively with potencies in the order of 4-CEP > 4-C*m*C > 4-ClP (Figure 3K–L, also see Supporting Information Figure S3). These results suggest that both the TG-induced store-operated currents and 2-APB-activated ORAI3 currents are inhibited by the three drugs.

Extracellular inhibition of ORAI3 by 4-CEP

To further characterize the pharmacological actions of 4-CEP, two strategies were used to examine the action site of 4-CEP on ORAI3. Similar to our previous findings, the current-voltage curve for the 2-APB-induced ORAI3 current displayed double (inward and outward) rectifications (DeHaven *et al.*, 2008; Peinelt *et al.*, 2008). Firstly, the outside-out patch-clamp recordings were used and the characteristic ORAI3

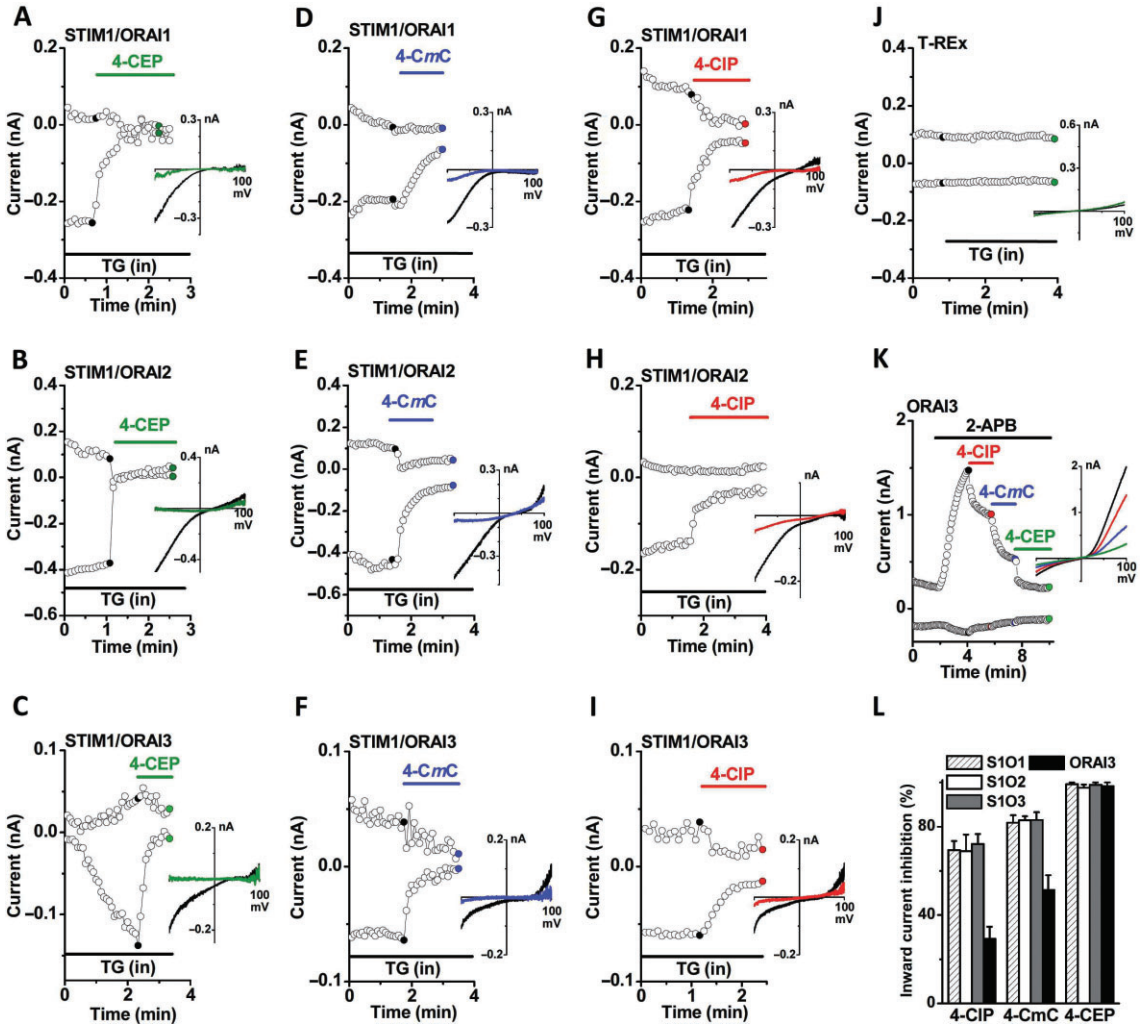

Figure 3

ORAI1-3 currents inhibited by 4-CEP, 4-C*m*C and 4-ClP in STIM1/ORAIs cells. Whole-cell currents were recorded in the HEK293 T-REx cells co-expressing STIM1-EYFP and ORAI1-3, or expressing ORAI3 alone. The store-operated currents in STIM1/ ORAI1-3 cells were evoked by TG (1 μM) in the pipette solution, and the current in ORAI3 cells was induced by 2-APB (50 μM). The time course for the amplitude of whole-cell currents was given at ±80 mV with corresponding leak-subtracted *I/V* relationships before and after treatment (inset). (A–C) Effect of 4-CEP (500 μM) on ORAI1-3 currents evoked by TG (1 μM). (D–F) Effect of 4-C*m*C (500 μM) on ORAI1-3 currents. (G–I) Effect of 4-ClP (500 μM). (J) Effect of 4-CEP on the non-transfected HEK293 T-REx cells. (K) ORAI3 current induced by 2-APB and the effects of 4-ClP, 4-C*m*C and 4-CEP (500 μM). (L) % inhibition by 4-ClP, 4-C*m*C and 4-CEP of the whole-cell currents measured at –80 mV in the STIM1/ORAI1 (S1O1), STIM1/ORAI2 (S1O2), and STIM1/ORAI3 (S1O3) (with TG); and ORAI3 (with 2-APB) cells (n = 5–10 in each group).

current evoked by 50 μM 2-APB was inhibited by 500 μM 4-CEP in the external solution (Figure 4A–C). Secondly, the intracellular application of 500 μM 4-CEP in the pipette solution failed to block the activation of ORAI3 by 50 μM 2-APB in the whole-cell configuration, whereas the subsequent addition of 500 μM 4-CEP into the extracellular solution rapidly abolished the currents (Figure 4D–E). These results demonstrate that 4-CEP inhibited ORAI3 activities from the extracellular side of the channels.

Concentration-dependence and reversibility of effects of 4-CEP

The concentration-dependence of the blocking effect of 4-CEP was examined using ORAI3 cells by patch clamp. The whole-cell current of ORAI3 was induced by 50 μM 2-APB. The ORAI3 current was significantly inhibited by 4-CEP and the channel blocking effect was reversible after washout (Figure 5A–B). The inhibition of the ORAI3 channels induced by 4-CEP was concentration-dependent with an IC_{50} of 71.0 ± 4.2 μM and a slope factor of 0.99 ± 0.04 (Figure 5C).

Discussion

In this study, we have shown that the RyR agonist 4-CEP inhibits the SOCE via extracellular sites of ORAI channels. The inhibitory effect was concentration-dependent, reversible and more potent than its analogues 4-C*m*C and 4-ClP.

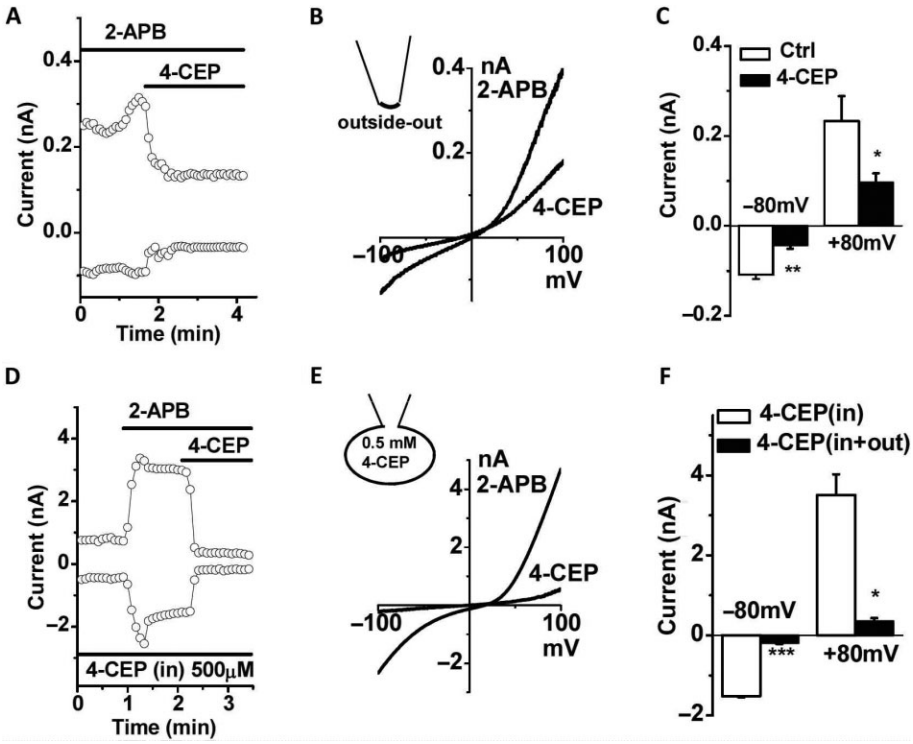

Figure 4

Extracellular effect of 4-CEP on ORAI3 channels. (A) Example of ORAI3 currents inhibited by 4-CEP (500 μM) in the STIM1/ORAI3 cells using outside-out patch configuration. (B) The *I/V* curves for (A). (C) Mean ± SEM for the current amplitude of outside-out patches (n = 3). (D) Intracellular application of 500 μM 4-CEP in pipette solution did not block the activation of ORAI3 by 2-APB, but extracellular application of 4-CEP (500 μM) significantly inhibited the ORAI3 current. (E) The *I/V* curves for (D). (F) Mean ± SEM for the amplitude of whole-cell current recorded with intracellular 4-CEP (500 μM) (in) and outside bath perfusion with 4-CEP (out) (n = 3). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

The inhibition was also independent of the Ca^{2+} release induced by activation of RyRs, because the two analogues, 4-C*m*C and 4-ClP, also inhibit ORAI channels without causing Ca^{2+} release in the HEK293 cells. In addition, 4-CEP evoked STIM1 translocation and subplasmalemmal clustering through the activation of RyRs and Ca^{2+} store depletion. However, the STIM1 redistribution in the cytosol was not evident in the STIM1/ORAI co-expressing cells, which could be explained by the stability of STIM1/ORAIs complexes in the plasma membrane. These findings are novel and important for understanding the pharmacology of RyR agonists.

The inhibition of ORAI channels induced by the 4-CEP analogues was structure-dependent. The potency of channel inhibition of the three compounds was positively correlated with the length of the 3-alkyl group, that is 4-CEP (with an ethyl group) was more effective than 4-C*m*C (with a methyl group) and 4-ClP (no side chain at 3′ position). Elongation of the 3-alkyl chain of 4-CEP may produce analogues with higher potency; however, this has not been investigated in this study due to the availability of the compounds. However, the length of alkyl side chain of chlorophenol derivatives is also associated with their affinity for RyR1 (Beeler and Gable, 1993; Jacobson *et al.*, 2006). Therefore, new compounds with longer alkyl chains may not only be more potent at inhibiting the store-operated channel, but will also strongly interfere with the ER/SR Ca^{2+} stores via RyR activation. Nonetheless, these structures with direct ORAI channel-blocking activity are important, which provides a new clue for future structure modification to search for more potent and selective compounds, although the potency of 4-CEP (IC_{50} 71 μM for ORAI3) is lower than that of other non-selective store-operated channel blockers, such as 2-APB (Xu *et al.*, 2005), SKF-96365 (Wang *et al.*, 2004), fenamate analogues (Jiang *et al.*, 2012) and BTP2 (Zitt *et al.*, 2004).

Intracellular Ca^{2+} release induced by 4-CEP and 4-C*m*C was mediated by the activation of intracellular RyRs, which suggests that the two compounds should be membrane-permeable. Therefore, they may exert their channel-blocking effects from the intracellular side. To determine their action site, we included 4-CEP in the pipette solution using whole-cell patch configuration and found that intracellular application of 4-CEP did not inhibit the ORAI3 channels, but extracellular perfusion with 4-CEP inhibited the current, suggesting that the site of action of 4-CEP is located extracellularly. The experiment using outside-out excised patches further confirmed the extracellular effect of 4-CEP. Furthermore, the blocking effect of 4-CEP on ORAI3 currents could be quickly washed out, which may indirectly indicate that the binding between 4-CEP and the channel proteins could be through non-covalent bonding at the cell surface and thus easily reversed like that of the extracellular blocker 2-APB (Xu *et al.*, 2005).

We also examined STIM1 translocation as an alternative mechanism for SOCE inhibition. 4-CEP may cause cytosolic

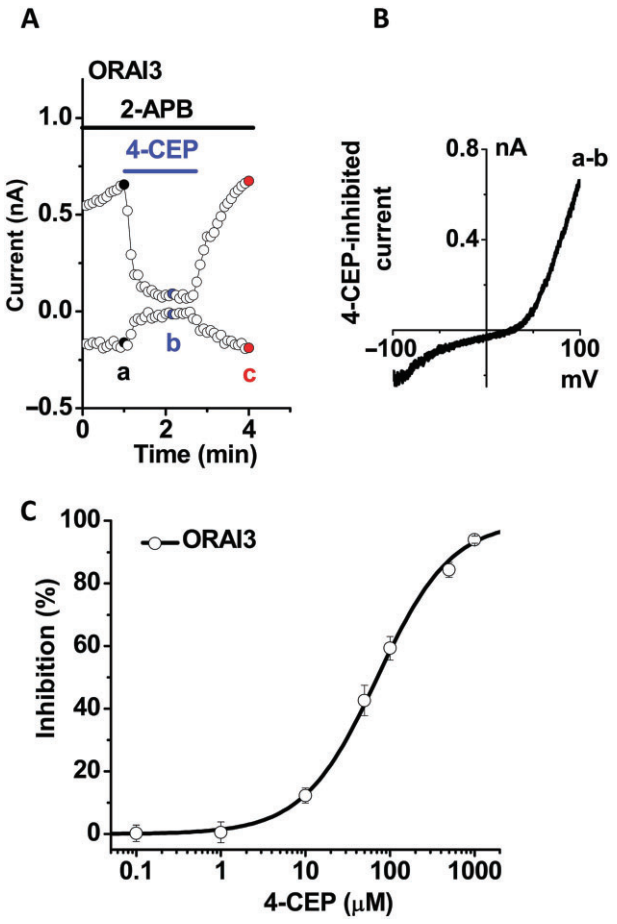

Figure 5

Concentration-dependent inhibition of ORAI3 channels by 4-CEP. The concentration-dependent inhibition by 4-CEP was examined in the HEK293 T-REx cells overexpressing mCFP-ORAI3 using whole-cell patch. (A) Example of ORAI3 current induced by 2-APB (50 μM) and the effect of 4-CEP (500 μM). The inhibition was reversible after washout. (B) The 4-CEP-inhibited *I/V* curve for (A). (C) Effect of 4-CEP at concentrations ranging from 0.1 to 1000 μM; % inhibition of whole-cell current measured at +80 mV was plotted and the curve was fitted using a logistic model with an IC_{50} for ORAI3 of 71.0 μM (n = 4–10 recordings for each point).

clustering of STIM1 and this would then lead to an impairment of SOCE, which we demonstrated previously in cells expressing STIM1 alone (Zeng *et al.*, 2012). However, the STIM1/ORAI co-expressing cells had no such cytosolic STIM1 puncta formation, instead they had stable subplasmalemmal cluster accumulation, suggesting that the ratio of STIM1 to ORAI expression in a cell is important and may affect the action of a drug. Nevertheless, the STIM1 clustering effect seems to be unique for 4-CEP, since 4-C*m*C and 4-ClP do not cause Ca^{2+} release and STIM1 translocation in the HEK293 cells.

4-CEP and 4-C*m*C are two commonly used RyR agonists for diagnosis of MH. The RyR agonist-induced skeletal muscle contraction is much stronger in MH susceptible patients with RyR1 mutations than that in the non-susceptible patients (Gerbershagen *et al.*, 2005). Recent findings have shown that SOCE exists in both normal and malignant hyperthermia-susceptible skeletal muscles and the coupling of SR Ca^{2+} release to SOCE contributes to the sustainability of hypermetabolic reactions (Kurebayashi and Ogawa, 2001; Duke *et al.*, 2010; Launikonis *et al.*, 2010; Stiber and Rosenberg, 2011). The expression of store-operated channel molecules STIM1 and ORAI1 is evident in the skeletal muscles (Lyfenko and Dirksen, 2008; Stiber *et al.*, 2008; Vig *et al.*, 2008). Therefore, the inhibition of Ca^{2+} influx or SOCE should be considered when the two compounds are used in the *in vitro* contracture test for the diagnosis of MH, particularly when the Krebs-Ringer solution containing 2.5 mM Ca^{2+} is recommended in the standard testing procedures (Ording *et al.*, 1997). In order to differentiate the contribution of Ca^{2+} release and the inhibition of Ca^{2+} influx by the two agonists, the use of Ca^{2+}-free or low Ca^{2+} solutions would be a better choice in such muscle contracture test. 4-CEP at the concentrations from 25 to 100 μM can distinguish between the MH susceptible and non-susceptible skeletal muscle samples in the muscle contracture tests, but higher concentrations of 4-CEP, such as 200 μM or higher, are indistinguishable for the diagnosis of MH (Gerbershagen *et al.*, 2005), which could be due to their direct blocking effects on SOCE and lead to changes in intracellular Ca^{2+} homeostasis.

We conclude that the RyR agonists 4-CEP, 4-C*m*C and 4-ClP are direct channel inhibitors of ORAI1–3. The use of 4-CEP and 4-C*m*C in the diagnostic test for MH should be used with caution, as at higher concentrations the two compounds significantly block SOCE, particularly in the presence of extracellular [Ca^{2+}], which may mask their effects on Ca^{2+} release via RyR activation.

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Conflicts of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Ca^{2+} influx in HEK293 T-REx cells and STIM1/ORAI1 cells. (A) The non-transfected T-REx cells were perfused with free Ca^{2+} and 1.5 mM Ca^{2+} solution as indicated by the arrow (n = 43 cells). (B) STIM1/ORAI1 cells (n = 64 cells). (C) Ca^{2+} influx in T-REx cells after store depletion with 1 μM TG (n = 27 cells). (D) Ca^{2+} influx in STIM1/ORAI1 cells after store depletion with 1 μM TG (n = 50 cells).

Figure S2 Dose-response curves for 4-CEP, 4-C*m*C and 4-ClP on the inhibition of store-operated Ca^{2+} influx. The STIM1/ORAI1 cells were pretreated with 1 μM TG for 30 min and the cytosolic Ca^{2+} were measured using Ca^{2+} imaging (n = 17–29 cells for each point). The curves were fitted by logistic equation.

Figure S3 Effects of 4-CEP, 4-C*m*C and 4-ClP on ORAI3 channels activated by 2-APB. The ORAI3 current was induced by 50 μM 2-APB in the T-REx cells expressing mCFP-ORAI3. (A) Example for the effect of 4-CEP (500 μM). (B) IV curves for (A). (C) 4-C*m*C (500 μM). (D). IV curves for (C). (E) 4-ClP (500 μM). (F) IV curves for (E).

Video S1 STIM1 cells

4-CEP induced STIM1-EYFP movement in the STIM1 cells. 4-CEP (500 μM) induced STIM1 puncta in the plasma membrane first, and then the accumulation of cytosolic STIM1-EYFP puncta. 4-CEP was added at the time scale 00:01.

Video S2 STIM1/ORAI1 coexpressed cells

4-CEP induced STIM1-EYFP translocation to the plasma membrane in the cells coexpressing STIM1/ORAI1. ORAI1 protein (red). STIM1 puncta in the plasma membrane was observed after addition of 4-CEP (500 μM), but no cytosolic STIM1-EYFP puncta were formed after perfusion with 4-CEP. 4-CEP was added at the time scale 00:03.