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Polyunsaturated fatty acids inhibit stimulated coupling between the ER Ca²⁺ sensor STIM1 and the Ca²⁺ channel protein Orai1 in a process that correlates with inhibition of stimulated STIM1 oligomerization

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Abstract

Polyunsaturated fatty acids (PUFAs) have been found to be effective inhibitors of cell signaling in numerous contexts, and we find that acute addition of micromolar PUFAs such as linoleic acid are effective inhibitors of Ca²⁺ responses in mast cells stimulated by antigen-mediated crosslinking of FcεRI or by the SERCA pump inhibitor, thapsigargin. In contrast, the saturated fatty acid, stearic acid, with the same carbon chain length as linoleic acid does not inhibit these responses. Consistent with this inhibition of store-operated Ca²⁺ entry (SOCE), linoleic acid inhibits antigen-stimulated granule exocytosis to a similar extent. Using the fluorescently labeled plasma membrane Ca²⁺ channel protein, AcGFP-Orai1, together with the labeled ER Ca²⁺ sensor protein, STIM1-mRFP, we monitor stimulated coupling of these proteins that is essential for SOCE with a novel spectrofluorimetric resonance energy transfer method. We find effective inhibition of this stimulated coupling by linoleic acid that accounts for the inhibition of SOCE. Moreover, we find that linoleic acid induces some STIM1-STIM1 association, while inhibiting stimulated STIM1 oligomerization that precedes STIM1-Orai1 coupling. We hypothesize that linoleic acid and related PUFAs inhibit STIM1-Orai1 coupling by a mechanism that involves perturbation of ER membrane structure, possibly by disrupting electrostatic interactions important in STIM1 oligomerization.

Keywords

Store-operated calcium entry (SOCE); IgE receptors (FcεRI); linoleic acid; fluorescence resonance energy transfer (FRET)

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1.1. INTRODUCTION

Polyunsaturated fatty acids (PUFAs) have been found to modulate cell signaling processes in multiple contexts [1, 2]. Among other receptor-stimulated functions, they have been shown to be effective inhibitors of immunoreceptor-stimulated, Ca^{2+} -dependent signaling under conditions of acute addition [3], as well as when added to cell culture over longer periods of time [4]. This latter study presented evidence that culturing T cells with 50 μM eicosapentaenoic acid (20:5(n-3)) for several days in serum-free medium reduced T cell receptor signaling by inhibiting stimulated tyrosine phosphorylation of the adaptor protein LAT and phospholipase $\text{C}\gamma$ in a process that interfered with LAT association with detergent-resistant, ordered lipid membrane domains. In a different context, PUFAs added to cell culture resulted in enhancement of stimulated EGF receptor phosphorylation by inhibition of EGF receptor coupling to the Ras signaling cascade [5].

As for many other receptors that activate Ca^{2+} mobilization to mediate functional responses, the high affinity receptor for IgE on mast cells, $\text{Fc}\epsilon\text{RI}$, activates the coupling of the endoplasmic reticulum (ER) Ca^{2+} sensor, STIM1, and the plasma membrane (PM) Ca^{2+} channel, Orai1, in a process known as store-operated Ca^{2+} entry (SOCE; [6]). In this process, stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) produces inositol 1,4,5-trisphosphate (IP_3) to initiate depletion of ER stores followed by SOCE, which leads to sustained Ca^{2+} oscillations and consequent granule exocytosis. A genetic knockout study showed that SOCE responses and granule exocytosis in mast cells require Orai channels [27]. We have previously characterized a role for ordered regions of the plasma membrane (PM) in segregating activated receptors from inactivating tyrosine phosphatases [7], and, although we first considered the possibility that PUFAs interferes with this signaling cascade by disrupting ordered PM domains, our investigation led us to a different conclusion.

In experiments described in this study, we find that acute addition of micromolar concentrations of the PUFA linoleic acid (C18:2 (n-6)) rapidly and strongly inhibits $\text{Fc}\epsilon\text{RI}$ -activated Ca^{2+} mobilization by inhibiting antigen-stimulated release of Ca^{2+} from ER stores, as well as by inhibiting SOCE stimulated by either antigen or the SERCA pump inhibitor, thapsigargin. The saturated fatty acid with the same carbon chain length, stearic acid, does not inhibit these responses. We determined that linoleic acid does not inhibit early signaling events that depend on ordered PM structure, but rather, more directly inhibits coupling between STIM1 and Orai1 monitored by fluorescence resonance energy transfer (FRET) between these labeled proteins. These and other results point to perturbation by linoleic acid of ER membrane structure in the mechanism of inhibition of SOCE.

2.1 MATERIALS AND METHODS

2.2 Chemicals and Reagents

FITC-dextran, thapsigargin, 2-aminoethyl diphenylborinate (2-APB), ATP, and stearic acid were purchased from Sigma-Aldrich. Linoleic acid (C18:2 (n-6)) was from Nu-Chek Prep., Inc. Unless otherwise noted, all cell culture reagents were purchased from Invitrogen. Anti-

DNP IgE was purified as described previously [8]. Multivalent antigen, DNP-BSA, was prepared as described previously [9].

2.3 Cells and Expression Plasmids

RBL-2H3 mast cells were maintained in monolayer culture through weekly passage as described previously [10]. For stimulation, cells were sensitized with 1 μ g/ml anti-DNP IgE for 4-24 hours. COS-7 cells were maintained in culture as previously described [11].

The genetically encoded Ca^{2+} indicators GCaMP3 [12] and R-geco1 [13] were purchased from Addgene (plasmid #22692 and plasmid #32444 respectively). Plasmids containing AcGFP-Orai1, STIM1-mRFP [14], YFP-STIM1, and mRFP-STIM1 or their untagged versions [15] were previously described. For transfection, cells were sparsely plated ($1-3 \times 10^5$ /ml) in six well plates for fluorimetry experiments, or on # 1.5 coverslips or in 35 mm glass bottom dishes (MatTek Corp.) for confocal imaging. After overnight culture, cells were transfected using 1-1.5 μ g DNA and 2 μ l Lipofectamine 2000 in 1 ml OptiMEM per well for 3-4 hr for COS-7 cells, or 2-2.5 μ g DNA and 10 μ l FuGENE HD (Promega) in 1 ml OptiMEM per well for 3-4 hr in the presence of 1 ng/ml phorbol 12,13-dibutyrate to enhance DNA uptake for RBL-2H3 cells [10]. Samples were then washed into full media and cultured for 16-24 hours to allow for protein expression.

2.4 Fluorescence Measurements

Cytoplasmic Ca^{2+} levels were measured using an SLM 8100C steady-state fluorimeter (SLM Instruments, Urbana, IL). RBL cells previously transfected with R-geco1 or COS-7 cells previously transfected with GCaMP3 together with untagged Orai1 and STIM1 plasmid DNA were harvested using PBS/EDTA and resuspended in buffered salt solution (BSS: 135 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 5.6 mM glucose, 20 mM HEPES, pH 7.4 in the presence or absence of 1.8 mM CaCl_2). Cells were stirred in an acrylic cuvette at 37°C, and the time course of either GCaMP3 fluorescence (ex 490 nm, em 520 nm) or R-geco1 fluorescence (ex 560, em 580 nm LP) was monitored. Linoleic acid and stearic acid were added from 5 mM stock solutions in absolute ethanol, stored under N_2 at -80°C . No effects on Ca^{2+} responses or FRET measurements were detected by addition of the solvent alone (data not shown). Inhibition of SOCE was determined as % decrease in the sustained phase of the Ca^{2+} response following addition of linoleic acid, and inhibition of the stimulated release from ER stores was determined by comparing the integrated transient responses in the presence and absence of linoleic acid.

FRET measurements were carried out in COS-7 cells transfected with either AcGFP-Orai1 (cytoplasmic donor) and STIM1-mRFP (cytoplasmic acceptor) or YFP-STIM1 (luminal donor) and mRFP-STIM1 (luminal acceptor). Transfected cells were harvested, and fluorescence changes were monitored as above with ex 490 nm and em both at 515 nm (donor emission) and at >580 nm (sensitized acceptor emission). Stimulation of SOCE and STIM1-Orai1 coupling monitored by FRET between the cytoplasmic labeled proteins was initiated by 50 μ M ATP (to synchronize responses), together with 200 nM thapsigargin. A control experiment was carried out to monitor nonspecific fluorescence changes under these conditions by using Orai1 and Stim1 labeled donors and acceptors on cytoplasmic and

luminal sides of the ER, respectively, a distance too large for FRET. These experiments showed no detectable changes fluorescence parameters after stimulation (Supplementary Figure S1).

Degranulation experiments were carried out as described previously [16]. Briefly, RBL-2H3 cells were plated overnight in the presence of FITC-dextran (1 mg/ml) and anti-DNP IgE (1 µg/ml), then harvested with PBS/EDTA and monitored by steady-state fluorimetry (ex 490, em 520) before and after stimulation by DNP-BSA (0.2 µg/ml) in BSS containing Ca^{2+} and 0.5 µM cytochalasin D to enhance signaling.

2.5 Immunoblot Analysis

IgE-sensitized cells were harvested, suspended in BSS, pre-treated for 5 min as indicated, then stimulated with DNP-BSA for 0-10 min, and whole cell lysates (WCLs) were prepared as previously described [17]. The WCL were resolved by SDS/PAGE, and the proteins were transferred to PVDF membranes. The filters were blocked in 100 mg/ml BSA diluted in 20 mM Tris, 135 mM NaCl, and 0.02% Tween 20 and then incubated with 4G10 anti-phosphotyrosine diluted in the same buffer. The primary antibody was detected with HRP-conjugated secondary antibody followed by exposure to ECL reagent (Invitrogen).

2.6 Confocal microscopy

COS-7 cells were transfected with AcGFP-Orai1 and STIM1-mRFP as described above and plated overnight at a subconfluent density of 0.5×10^6 cells/mL in 35 mm coverslip dishes (MatTek Corp.), then fixed in 4% para-formaldehyde and 0.1% glutaraldehyde and quenched with 10 mg/ml BSA in PBS with 0.01% sodium azide. Confocal imaging was performed using a Zeiss LSM 710 inverted confocal microscope with a 63× Oil Plan-Apochromat lens. A DF 488/561 filter set was used to perform sequential color imaging of the samples.

2.7 Statistical analysis

Uncertainties are expressed as standard deviations (SD) for $n = 3$ and as standard error (SE) for $n > 3$. Data were analyzed by a paired Students t-test, and significance was accepted at $p < 0.05$.

3.1 RESULTS

Linoleic acid (C18:2 (n-6)) represents a structurally minimal PUFA that has been found to inhibit signaling in cytotoxic T cells as potently as the more complex “omega 3 fatty acid,” linolenic acid (C18:3 (n-3)) [3]. To investigate the capacity of linoleic acid to modulate FcεRI signaling in mast cells, we first evaluated its effects on antigen-stimulated Ca^{2+} mobilization. As shown by the representative experiment in Figure 1A, acute addition of micromolar quantities of linoleic acid following stimulation by antigen results in rapid decreases in the elevated levels of cytoplasmic Ca^{2+} , with inhibition of $70.5 \pm 4.9\%$ (SE, $n=4$ independent experiments, $p = 0.0088$) after addition of 2.5 µM linoleic acid. We observe >90% inhibition after a total of 5 µM linoleic acid is added as represented in this experiment. Under these conditions, the cells are >90% viable. Larger concentrations of

linoleic acid ($\geq 10 \mu\text{M}$) resulted in some cell lysis, as evidenced by leakage of Ca^{2+} indicators and uptake of trypan blue in some cells (data not shown). A more complex PUFA, docosahexaenoic acid (C22:6), also strongly inhibits Ca^{2+} mobilization by antigen at low μM concentrations (data not shown), but the cells are more sensitive to lysis by this PUFA, such that we did not evaluate its effects further.

To evaluate the effects of linoleic acid on both antigen-stimulated release of Ca^{2+} from ER stores, as well as on SOCE, RBL cells were stimulated in the absence of extracellular Ca^{2+} , followed by the addition of 1.8 mM Ca^{2+} to initiate SOCE. As shown by representative experiments in Figure 1B and C, addition of $2.5 \mu\text{M}$ linoleic acid prior to antigen inhibits stimulated Ca^{2+} release from ER stores by $37 \pm 5.8\%$ (SD; $p = 0.036$), and inhibits SOCE by $65 \pm 5.9\%$ (SE, $n = 4$; $p = 0.012$). Addition of the CRAC channel inhibitor 2-APB following Ca^{2+} addition provides an indicator of the SOCE that remains. In other experiments with RBL cells, we found that stimulation of SOCE by thapsigargin, which bypasses $\text{Fc}\epsilon\text{RI}$ signaling to activate Ca^{2+} entry via irreversible inhibition of the ER Ca^{2+} pump, is similarly inhibited by linoleic acid (data not shown; see results below with COS-7 cells). In contrast, stearic acid, the saturated fatty acid with the same carbon chain length as linoleic acid, fails to inhibit Ca^{2+} responses to antigen or thapsigargin at similar concentrations as the unsaturated fatty acid, indicating a role for this unsaturation in the mechanism of inhibition (data not shown; see results below with COS-7 cells). We showed previously that the sustained phase of Ca^{2+} influx in response to thapsigargin is completely inhibited by $1 \mu\text{M}$ Gd^{3+} in these cells, consistent with its dependence on Orai1 in the absence of voltage-gated Ca^{2+} channels [22]. These results, taken together, demonstrate potent inhibition of Ca^{2+} responses in these cells, particularly SOCE, by linoleic acid.

In mast cells, sustained Ca^{2+} mobilization is necessary for secretory granule exocytosis, and we previously characterized a method to monitor degranulation in these cells that utilizes selective fluid phase uptake of FITC-dextran into secretory lysosomes [16]. Using this method, granule exocytosis can be observed as individual bursts of fluorescence due to release of low pH-quenched FITC-dextran in confocal imaging experiments, or as a time-dependent increase in stimulated fluorescence in a steady-state fluorimeter. The representative experiment in Figure 2 shows that pre-addition of $2.5 \mu\text{M}$ linoleic acid causes $48 \pm 11\%$ (SD; $p = 0.012$) inhibition of antigen-stimulated degranulation, consistent with a similar amount of inhibition of SOCE at this dose (Figure 1). These results show that an important functional response in mast cells that is downstream of Ca^{2+} mobilization is effectively inhibited by linoleic acid.

Previous studies in RBL mast cells demonstrated a role for cholesterol-dependent ordered lipids in plasma membrane structure for the initial steps of $\text{Fc}\epsilon\text{RI}$ signaling [17, 18]. To investigate whether linoleic acid inhibits antigen-stimulated Ca^{2+} mobilization by inhibiting the initial steps in $\text{Fc}\epsilon\text{RI}$ signaling, we evaluated the effects of linoleic acid on the stimulation of tyrosine phosphorylation in these cells, including stimulated phosphorylation of $\text{Fc}\epsilon\text{RI}$ β and LAT, a key adaptor protein for the activation of phospholipase $\text{C}\gamma$ ($\text{PLC}\gamma$) [19]. As shown in a representative experiment in Figure 3, pre-addition of $5 \mu\text{M}$ linoleic acid or $10 \mu\text{M}$ stearic acid failed to inhibit antigen-stimulated tyrosine phosphorylation of $\text{Fc}\epsilon\text{RI}$ β LAT, or the $\sim 70 \text{ kDa}$ substrates of the tyrosine kinase Syk, which is activated as a

consequence of FcεRI tyrosine phosphorylation and is necessary for the activation of PLCγ [20]. In three different experiments of this design, no inhibition of stimulated tyrosine phosphorylation was detected under conditions in which linoleic acid inhibits >90% of stimulated Ca²⁺ mobilization. These results suggest that inhibition of Ca²⁺ mobilization occurs at a step or steps that are more downstream of FcεRI activation than stimulated tyrosine phosphorylation.

Because the inhibition by linoleic acid of SOCE stimulated by either antigen or thapsigargin was found to be more substantial than inhibition of antigen-stimulated release of Ca²⁺ from ER stores (Figure 1B and C, and data not shown), we focused on understanding the mechanism of inhibition of SOCE by this PUFA. For these experiments, we initially utilized COS-7 cells, which exhibit more effective transfection of plasmids than RBL cells. As shown by the representative experiment in Figure 4A, stimulation of SOCE by thapsigargin + ATP in COS-7 cells co-expressing STIM1 and Orai1, together with the Ca²⁺ indicator, GCaMP3, resulted in a robust response that was nearly completely inhibited by 5 μM linoleic acid. In three experiments, 5 μM linoleic acid inhibited this response by 88 ± 11% (SD; p = 0.036). In contrast, addition of sequential aliquots of stearic acid in the same dose range did not inhibit this response (Figure 4B), consistent with its failure to inhibit Ca²⁺ mobilization in RBL cells (data not shown).

To investigate whether linoleic acid inhibits SOCE by interfering with the coupling of STIM1 and Orai1, we co-expressed the FRET donor AcGFP-Orai1 together with the FRET acceptor STIM1-mRFP in COS-7 cells, and we used steady-state fluorimetry to monitor donor emission (D) simultaneously with the ratio of sensitized acceptor emission to donor emission (A/D) for maximal sensitivity to FRET changes. As shown by the representative experiment in Figure 5, stimulation of SOCE with ATP + thapsigargin results in a time-dependent decrease in D, together with a corresponding time-dependent increase in A/D, consistent with stimulated coupling between STIM1 and Orai1 [14]. After several hundred seconds, two sequential additions of 2.5 μM linoleic acid resulted in nearly complete reversal of this FRET response, consistent with the nearly complete inhibition of SOCE shown in Figure 4A. In four separate experiments, we observed that addition of 5 μM linoleic acid causes 82 ± 8% (SE, n=4, p = 0.017) reversal of the stimulated decrease in D. In other experiments, pre-addition of 5 μM linoleic acid prevented FRET stimulated by ATP + thapsigargin (data not shown). Similar results were also observed for RBL cells co-expressing AcGFP-Orai1 and STIM1-mRFP: Stimulation with either thapsigargin or with antigen resulted in FRET responses that could be largely reversed by 5 μM linoleic acid (data not shown). These results provide strong evidence that linoleic acid inhibits stimulated SOCE in both COS-7 and RBL cells by inhibiting the stimulated association of these key proteins.

In response to ER store depletion, STIM1 coupling to Orai1 is subsequent to STIM1 oligomerization [21]. To evaluate whether linoleic acid inhibits STIM1-Orai1-coupling by altering stimulated oligomerization of STIM1, we examined the effect of linoleic acid on stimulated STIM1/STIM1 oligomerization in COS-7 cells by monitoring stimulated FRET between YFPSTIM1 and mRFP-STIM1 with steady-state fluorimetry. As shown by the representative experiment in Figure 6A, addition of ATP + thapsigargin results in stimulated

FRET between YFP-STIM1 and mRFP-STIM1 co-expressed in COS-7 cells, consistent with previous observations [21]. Addition of 5 μ M linoleic acid subsequent to ATP + thapsigargin results in a further increase in FRET that declines after several hundred seconds. Importantly, addition of linoleic acid prior to stimulation by ATP + thapsigargin results in an increase in FRET, but subsequent stimulation of FRET by ATP + thapsigargin is substantially inhibited by this PUFA (Figure 6B). In four experiments, we observed that addition of 5 μ M linoleic acid causes $75 \pm 5\%$ (SE, $n = 4$, $p = 0.0004$) inhibition of the ATP + thapsigargin-stimulated increase in the A/D fluorescence ratio. Because Orai1 is not co-expressed in these COS-7 cells under these conditions, STIM1/STIM1 oligomerization is independent of its coupling to Orai1, and the inhibition of this process by linoleic acid is likely to reflect a direct effect of this PUFA on STIM1 and/or ER membrane structure.

Consistent with this, confocal imaging shows that addition of 5 μ M linoleic acid to COS-7 cells co-expressing STIM1-mRFP with AcGFP-Orai1 results in a substantial alteration in the distribution of STIM1-mRFP in the ER, with the appearance of micron-scale clusters of the protein in unstimulated COS-7 cells (compare Figure 7A and C), and its localization in large sheets of ER membrane instead of discrete puncta that are observed in thapsigargin-stimulated cells (compare Figure 7B and D). These results, taken together, provide evidence that linoleic acid at concentrations that inhibit STIM1-Orai1 coupling alter ER membrane structure but do not alter early events in Fc ϵ RI signaling.

4.1 DISCUSSION

Our results show that coupling between the ER sensor protein STIM1 and the PM Ca^{2+} channel Orai1 in the process of SOCE is sensitive to interference by acutely added PUFAs, such as linoleic acid, with as few as two double bonds in their acyl chain. Furthermore, our observations that linoleic acid can induce STIM1-STIM1 association detected by FRET, while inhibiting STIM1-STIM1 oligomerization due to store depletion (Figure 6), is consistent with a direct effect on ER membranes. The altered distribution of STIM1-mRFP that we observe in the presence of linoleic acid (Figure 7) is consistent with a perturbation of ER membrane structure by this PUFA, and the modest inhibition of antigen-stimulated Ca^{2+} release from ER stores (Figure 1B) is also consistent with a more global effect on ER membrane structure. However, we cannot rule out additional effects of linoleic acid on PM structure that could contribute to the potent inhibition of SOCE and STIM1-Orai1 coupling that we observe. Despite these uncertainties, it is clear that acute addition of micromolar linoleic acid inhibits STIM1-Orai1 coupling with similar potency as the inhibition of SOCE stimulated by either antigen or thapsigargin, making it likely that this inhibition of coupling is the principal basis for inhibition of sustained Ca^{2+} mobilization.

In a previous study we found that modest depletion of cholesterol from RBL cell membranes is very effective at inhibiting thapsigargin-stimulated SOCE, suggesting a role for an ordered membrane structure in this process [22]. Consistent with this, PM-localized PIP_2 is found in an ordered lipid, detergent-resistant membrane pool, as well as in a disordered lipid membrane pool that is solubilized by the non-ionic detergent Triton X-100, and these pools have differential effects on the regulation of SOCE. In particular, we found that the PIP_2 pool in ordered membrane domains has a positive effect on the regulation of SOCE, whereas

the PIP₂ pool in disordered membrane domains has a negative effect, as evidenced by selective alteration of each of these pools by different PIP 5-kinases and inositol 5-phosphatases. These differential effects on SOCE were paralleled by differential effects on STIM1-Orai1 coupling detected by FRET, such that increasing the pool of PIP₂ in ordered PM domains enhanced this coupling whereas increasing the pool of PIP₂ in the disordered PM domains inhibited this coupling. These results indicate that PM structure can also influence SOCE mediated by STIM1-Orai1 coupling.

The lack of inhibition of antigen-stimulated tyrosine phosphorylation by concentrations of linoleic acid that effectively inhibit STIM1-Orai1 coupling and SOCE was unexpected based on our previous studies and pointed to the particular sensitivity of SOCE to membrane perturbations. Linoleic acid has the capacity to alter plasma membrane structure, as evidenced by its inhibition of ordered-disordered phase separation in giant plasma membrane vesicles (data not shown; [23]). However, this inhibition is only observed at higher concentrations of linoleic acid that reduce cell viability and are thus less meaningful for studies on live cells. Previous studies by Stulnig and colleagues in T cells that demonstrated the capacity of PUFAs to inhibit Ca²⁺ mobilization and other downstream functional responses suggested that this inhibition most closely correlates with reduction of stimulated tyrosine phosphorylation of LAT and PLC γ [4]. These previous studies utilized biosynthetic incorporation of PUFAs into a variety of phospholipids, so that the specific lipid modifications that may be important for the function consequences observed are difficult to unravel. In our studies, the rapid effects of acute addition of linoleic acid makes it likely that this PUFA is exerting a direct effect on membrane structure to inhibit SOCE and STIM1/Orai1 coupling.

The mechanism by which PUFAs so effectively inhibit SOCE is not yet clear. Although our results are not consistent with perturbation of lipid order in the PM as a principal cause, they do not distinguish between more large-scale perturbations of ER membrane organization from more local effects that could be selective for STIM1-STIM1 and STIM1-Orai1 interactions. Our finding that linoleic acid causes some inhibition of antigen-stimulated release of Ca²⁺ from ER stores (Fig. 1B,C) indicates an effect on ER function independent of STIM1-Orai1 coupling. Our previous studies provided evidence that electrostatic interactions between an acidic sequence in the C-terminal sequence of Orai1 and a polybasic sequence in the “CRAC-activating domain” (CAD) of STIM1 are critical for functional coupling of these proteins [14, 22]. A more recent crystallographic study highlights the position of these basic residues in STIM1 at the “top” of a paired dimer of an R-shaped structure of CAD that implies the capacity of this region for association with Orai1 [24]. The functional coupling via these basic and acidic sequences can be inhibited by basic sphingosine derivatives, that have the capacity to flip to the inner leaflet of the PM in a process that correlated with their capacity to displace a polybasic peptide from this inner leaflet [14, 25].

These results highlight the importance of electrostatic interactions in SOCE, and it is possible that linoleic acid may also inhibit SOCE by interfering with electrostatic interactions between STIM1 and Orai1. If this is relevant, it is unclear why stearic acid is not effective in inhibiting SOCE (Figure 4) or STIM1/Orai1 coupling (data not shown). Like

linoleic acid, stearic acid has the potential to flip to the inner leaflet of the PM, and we have evidence that this occurs with similar efficiency (data not shown). However, a saturated fatty acid may have a greater tendency to remain in the PM, where it can pack more readily with the largely ordered membrane structure there [26]. Unlike sphingosine bases at the inner leaflet, these fatty acids would enhance the net negative charge at this leaflet, and thus would not serve to neutralize the effective concentration of negatively charged phosphoinositides that the sphingosine derivatives can achieve. In comparison to stearic acid, linoleic acid and other PUFAs are more likely to translocate from the PM to other internal membrane pools, including the ER. In this capacity, they may be able to provide more effective perturbation of these internal membranes and the finely tuned regulation of key proteins, including STIM1, within them.

In conclusion, our results highlight the utilization of fluorimetric measurements of FRET between labeled STIM1 and labeled Orai1 as an effective tool for monitoring functionally important protein-protein interactions, and they reveal the sensitivity of this association to uncoupling by long-chain PUFAs acutely added to live cells. The role of these SOCE proteins in bridging the PM to the ER to mediate this process, and its capacity to be disrupted effectively by PUFAs provides a new example of how lipids can effectively regulate a critical process in cell signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Berquin IM, Edwards IJ, Kridel SJ, Chen YQ. Polyunsaturated fatty acid metabolism in prostate cancer. *Cancer metastasis reviews*. 2011; 30:295–309. [PubMed: 22015690]
2. Shaikh SR, Edidin M. Polyunsaturated fatty acids, membrane organization, T cells, and antigen presentation. *The American journal of clinical nutrition*. 2006; 84:1277–1289. [PubMed: 17158407]
3. Richieri GV, Mescher MF, Kleinfeld AM. Short term exposure to cis unsaturated free fatty acids inhibits degranulation of cytotoxic T lymphocytes. *J Immunol*. 1990; 144:671–677. [PubMed: 2295805]
4. Zeyda M, Staffler G, Horejsi V, Waldhausl W, Stulnig TM. LAT displacement from lipid rafts as a molecular mechanism for the inhibition of T cell signaling by polyunsaturated fatty acids. *J Biol Chem*. 2002; 277:28418–28423. [PubMed: 12029091]
5. Turk HF, Barhoumi R, Chapkin RS. Alteration of EGFR Spatiotemporal Dynamics Suppresses Signal Transduction. *PloS one*. 2012; 7:e39682. [PubMed: 22761867]
6. Holowka D, Calloway N, Cohen R, Gadi D, Lee J, Smith NL, Baird B. Roles for Ca²⁺ mobilization and its regulation in mast cell functions. *Frontiers in immunology*. 2012; 3:104. [PubMed: 22586429]
7. Holowka D, Gosse JA, Hammond AT, Han X, Sengupta P, Smith NL, Wagenknecht-Wiesner A, Wu M, Young RM, Baird B. Lipid segregation and IgE receptor signaling: a decade of progress. *Biochimica et biophysica acta*. 2005; 1746:252–259. [PubMed: 16054713]

8. Posner RG, Lee B, Conrad DH, Holowka D, Baird B, Goldstein B. Aggregation of IgE-receptor complexes on rat basophilic leukemia cells does not change the intrinsic affinity but can alter the kinetics of the ligand-IgE interaction. *Biochemistry*. 1992; 31:5350–5356. [PubMed: 1534998]
9. Weetall M, Holowka D, Baird B. Heterologous desensitization of the high affinity receptor for IgE (Fc epsilon R1) on RBL cells. *J Immunol*. 1993; 150:4072–4083. [PubMed: 8473750]
10. Gosse JA, Wagenknecht-Wiesner A, Holowka D, Baird B. Transmembrane sequences are determinants of immunoreceptor signaling. *J Immunol*. 2005; 175:2123–2131. [PubMed: 16081778]
11. Korzeniowski MK, Manjarres IM, Varnai P, Balla T. Activation of STIM1-Orai1 involves an intramolecular switching mechanism. *Sci Signal*. 2010; 3:ra82. [PubMed: 21081754]
12. Tian L, Hires SA, Mao T, Huber D, Chiappe ME, Chalasani SH, Petreanu L, Akerboom J, McKinney SA, Schreiter ER, Bargmann CI, Jayaraman V, Svoboda K, Looger LL. Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. *Nature methods*. 2009; 6:875–881. [PubMed: 19898485]
13. Zhao Y, Araki S, Wu J, Teramoto T, Chang YF, Nakano M, Abdelfattah AS, Fujiwara M, Ishihara T, Nagai T, Campbell RE. An expanded palette of genetically encoded Ca(2+)(+) indicators. *Science*. 2011; 333:1888–1891. [PubMed: 21903779]
14. Calloway N, Vig M, Kinet JP, Holowka D, Baird B. Molecular clustering of STIM1 with Orai1/CRACM1 at the plasma membrane depends dynamically on depletion of Ca2+ stores and on electrostatic interactions. *Mol Biol Cell*. 2009; 20:389–399. [PubMed: 18987344]
15. Varnai P, Toth B, Toth DJ, Hunyady L, Balla T. Visualization and manipulation of plasma membrane-endoplasmic reticulum contact sites indicates the presence of additional molecular components within the STIM1-Orai1 Complex. *J Biol Chem*. 2007; 282:29678–29690. [PubMed: 17684017]
16. Cohen R, Corwith K, Holowka D, Baird B. Spatiotemporal resolution of mast cell granule exocytosis reveals correlation with Ca2+ wave initiation. *J Cell Sci*. 2012; 125:2986–2994. [PubMed: 22393234]
17. Young RM, Zheng X, Holowka D, Baird B. Reconstitution of regulated phosphorylation of FcepsilonRI by a lipid raft-excluded protein-tyrosine phosphatase. *J Biol Chem*. 2005; 280:1230–1235. [PubMed: 15537644]
18. Sheets ED, Holowka D, Baird B. Critical role for cholesterol in Lyn-mediated tyrosine phosphorylation of FcepsilonRI and their association with detergent-resistant membranes. *J Cell Biol*. 1999; 145:877–887. [PubMed: 10330413]
19. Saitoh S, Arudchandran R, Manetz TS, Zhang W, Sommers CL, Love PE, Rivera J, Samelson LE. LAT is essential for Fc(epsilon)RI-mediated mast cell activation. *Immunity*. 2000; 12:525–535. [PubMed: 10843385]
20. Zhang J, Berenstein EH, Evans RL, Siraganian RP. Transfection of Syk protein tyrosine kinase reconstitutes high affinity IgE receptor-mediated degranulation in a Syk-negative variant of rat basophilic leukemia RBL-2H3 cells. *J Exp Med*. 1996; 184:71–79. [PubMed: 8691151]
21. Liou J, Fivaz M, Inoue T, Meyer T. Live-cell imaging reveals sequential oligomerization and local plasma membrane targeting of stromal interaction molecule 1 after Ca2+ store depletion. *Proc Natl Acad Sci U S A*. 2007; 104:9301–9306. [PubMed: 17517596]
22. Calloway N, Owens T, Corwith K, Rodgers W, Holowka D, Baird B. Stimulated association of STIM1 and Orai1 is regulated by the balance of PtdIns(4,5)P(2) between distinct membrane pools. *J Cell Sci*. 2011; 124:2602–2610. [PubMed: 21750194]
23. Sengupta P, Hammond A, Holowka D, Baird B. Structural determinants for partitioning of lipids and proteins between coexisting fluid phases in giant plasma membrane vesicles. *Biochimica et biophysica acta*. 2008; 1778:20–32. [PubMed: 17936718]
24. Yang X, Jin H, Cai X, Li S, Shen Y. Structural and mechanistic insights into the activation of Stromal interaction molecule 1 (STIM1). *Proc Natl Acad Sci U S A*. 2012; 109:5657–5662. [PubMed: 22451904]
25. Smith NL, Hammond S, Gadi D, Wagenknecht-Wiesner A, Baird B, Holowka D. Sphingosine derivatives inhibit cell signaling by electrostatically neutralizing polyphosphoinositides at the plasma membrane. *Self/nonself*. 2010; 1:133–143. [PubMed: 21423874]

26. Holowka D, Baird B. Fc(epsilon)RI as a paradigm for a lipid raft-dependent receptor in hematopoietic cells. *Semin Immunol.* 2001; 13:99–105. [PubMed: 11308293]
27. Vig M, DeHaven WI, Bird GS, Billingsley JM, Wang H, Rao PE, Hutchings AB, Jouvin MH, Putney JW, Kinet JP. Defective mast cell effector functions in mice lacking the CRACM1 pore subunit of store-operated calcium release-activated calcium channels. *Nat Immunol.* 2008; 9:89–96. [PubMed: 18059270]

Highlights for Holowka et al

1. The polyunsaturated fatty acid, linoleic acid, potently inhibits FcεRI signaling.
2. Inhibition involves uncoupling of the ER sensor STIM1 from the Ca²⁺ channel Orai1.
3. Results suggest that PUFAs regulate Ca²⁺ entry by perturbing ER membrane structure.

Figure 1. Linoleic acid (LA) inhibits Ag-stimulated Ca^{2+} mobilization

A) Ca^{2+} mobilization monitored by the genetically encoded indicator R-gecol is stimulated by an optimal dose of multivalent antigen (Ag; 0.2 mg/ml DNP₁₅-BSA) for anti-dinitrophenyl (DNP) IgE-sensitized RBL mast cells, and the sustained phase of this response is rapidly inhibited by two successive additions of 2.5 μM LA. The SOCE inhibitor 2-APB (10 μM) does not further inhibit this response. B) Pre-addition of 2.5 μM LA to RBL cells in the absence of extracellular Ca^{2+} inhibits Ag-stimulated Ca^{2+} release from ER stores by 33% and inhibits SOCE upon addition of Ca^{2+} by 54% in this representative experiment. C) Control cells without LA addition. For B and C, cells were suspended in nominally Ca^{2+} -free BSS, and a small amount of EGTA (5 μM) was added in each case to chelate trace amount of extracellular Ca^{2+} that remain.

Figure 2. Ag-stimulated degranulation from RBL cells is substantially inhibited by LA
 RBL cells were labeled with FITC-dextran overnight, and Ag-stimulated degranulation was monitored as release of this secretory granule marker by steady-state fluorimetry. A) Ag-stimulated release of FITC-dextran is near maximal after 900 s at 37°C. B) Pre-addition of 2.5 μ M LA causes 59 % inhibition of degranulation after 900 s in this representative experiment.

Figure 3. LA does not inhibit Ag-stimulated tyrosine phosphorylation at concentrations that inhibit Ag-stimulated Ca^{2+} mobilization and degranulation

Representative western blot of RBL whole cell lysates with anti-phosphotyrosine mAb 4G10 shows stimulated tyrosine phosphorylation of Syk substrate ~p70, LAT, and FcεRI β. No significant decrease in stimulated phosphorylation by 5 μM LA or 10 μM stearic acid (SA) was detected in 3 separate experiments. Mol wt. markers (kDa) are labeled at left.

Figure 4. Ca^{2+} mobilization by ATP + thapsigargin in COS-7 cells is inhibited by LA (A) but not by SA (B)

COS-7 cells expressing Orai1, STIM1, and GCaMP3 were stimulated by 0.2 μM thapsigargin + 50 μM ATP to synchronize Ca^{2+} responses. Additions of LA or SA were made as indicated. 10 μM 2-APB was added as indicated.

Figure 5. Stimulated FRET between AcGFP-Orai1 and STIM1-mRFP in COS-7 cells is inhibited by LA

Addition of LA ($2 \times 2.5 \mu\text{M}$) after stimulation of coupling between AcGFP-Orai1 and STIM1-mRFP reverses this association.

Figure 6. Oligomerization of STIM1 is perturbed by 5 μM LA

A) 1.8 mM CaCl_2 is added to COS-7 cells in BSS, and store depletion by ATP + thapsigargin stimulates FRET between YFPSTIM1 and mRFP-STIM1. Addition of 5 μM LA transiently increases the FRET response. B) Addition of 5 μM LA induces a small FRET response between YFP-STIM1 and mRFP-STIM1 in BSS + Ca^{2+} , and it reduces the subsequent FRET response to ATP + thapsigargin.

Figure 7. LA causes perturbation of ER structure

COS-7 cells were transfected with AcGFP_{Orai1} and STIM1-mRFP, incubated or not with 5 μ M linoleic acid for 5 min, then stimulated or not with 400 nM thapsigargin for 5 min at 37°C and fixed as described in Materials and Methods. Bar represents 50 μ M.