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Polyunsaturated fatty acids inhibit stimulated coupling between the ER Ca^{2+} sensor STIM1 and the Ca^{2+} channel protein Orai1 in a process that correlates with inhibition of stimulated STIM1 oligomerization ☆

David Holowka *, Marek K. Korzeniowski, Kirsten L. Bryant, Barbara Baird

Department of Chemistry and Chemical Biology, Baker Laboratory, Cornell University, Ithaca, NY 14853, USA

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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 effectively inhibit of Ca^{2+} 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 Ca^{2+} entry (SOCE), linoleic acid inhibits antigen-stimulated granule exocytosis to a similar extent. Using the fluorescently labeled plasma membrane Ca^{2+} channel protein, AcGFP–Orai1, together with the labeled ER Ca^{2+} 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. This article is part of a Special Issue entitled Tools to study lipid functions.

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 Cγ in a process that interfered with LATassociation 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, FcεRI, 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 interfere 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 FcεRI-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

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* Corresponding author. Tel.: +1 607 255 6140; fax: +1 607 255 4139.
E-mail address: dah24@cornell.edu (D. Holowka).

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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. Materials and methods

2.1. 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].

2.2. 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 μg/ml anti-DNP IgE for 4–24 h. 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 μg DNA and 2 μl Lipofectamine 2000 in 1 ml OptiMEM per well for 3–4 h for COS-7 cells, or 2–2.5 μg DNA and 10 μl FuGENE HD (Promega) in 1 ml OptiMEM per well for 3–4 h 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 h to allow for protein expression.

2.3. 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 μM 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 in fluorescence parameters after stimulation (Supplementary Fig. 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.

2.4. 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).

2.5. 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.

2.6. 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. 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 Fig. 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 ± 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 (>10 μM) 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-stimulatedrelease 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