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Short Communication

Interleukin-13 enhanced Ca^{2+} oscillations in airway smooth muscle cells

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ABSTRACT

Physiological mechanisms associated with interleukin-13 (IL-13), a key cytokine in asthma, in intracellular Ca^{2+} signaling in airway smooth muscle cells (ASMCs) remain unclear. The aim of this study was to assess effects of IL-13 on Ca^{2+} oscillations in response to leukotriene D4 (LTD4) in human cultured ASMCs.

LTD4-induced Ca^{2+} oscillations in ASMCs pretreated with IL-13 were imaged by confocal microscopy. mRNA expressions of cysteinyl leukotriene 1 receptors (CysLT1R), CD38, involved with the ryanodine receptors (RyR) system, and transient receptor potential canonical (TRPC), involved with store-operated Ca^{2+} entry (SOCE), were determined by real-time PCR. In IL-13-pretreated ASMCs, frequency of LTD4-induced Ca^{2+} oscillations and number of oscillating cells were significantly increased compared with untreated ASMCs. Both xestospongin C, a specific inhibitor of inositol 1,4,5-triphosphate receptors (IP_3R), and ryanodine or ruthenium red, inhibitors of RyR, partially blocked LTD4-induced Ca^{2+} oscillations. Ca^{2+} oscillations were almost completely inhibited by 50 μM of 2-aminoethoxydiphenyl borate (2-APB), which dominantly blocks SOCE but not IP_3R at this concentration. Pretreatment with IL-13 increased the mRNA expressions of CysLT1R and CD38, but not of TRPC1 and TRPC3.

We conclude that IL-13 enhances frequency of LTD4-induced Ca^{2+} oscillations in human ASMCs, which may be cooperatively modulated by IP_3R, RyR systems and possibly by SOCE.

1. Introduction

Airway inflammation is a fundamental feature of asthma. Among a number of inflammatory cytokines and mediators involved in asthma, interleukin (IL)-13, a pleiotropic Th2 cytokine, plays a pivotal role [1]. In addition to its well-established effects, such as stimulation of eosinophilic inflammation, induction of goblet cell hyperplasia and airway fibrosis, previous studies have revealed that IL-13 augments agonist-induced contraction of the tracheal ring and increases intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in airway smooth muscle cells (ASMCs) [2]. Cysteinyl leukotrienes (Cys-LTs; LT-C4, -D4, and -E4) are metabolites of arachidonic acid and are important agonists for ASMCs by binding the CysLT1 receptor (CysLT1R), a G protein-coupled receptor (GPCR), and activating phospholipase C, which leads to the generation of inositol 1,4,5-triphosphate (IP_3) followed by Ca^{2+} release from sacroplasmic reticulum (SR) [3,4]. Both IL-13 and Cys-LTs are produced by mast cells that infiltrate airway smooth muscle layer [5], and a close relationship between IL-13 and Cys-LTs in asthma pathogenesis has been suggested recently [6].

Although intracellular Ca^{2+} signaling is usually assessed by $[Ca^{2+}]_i$, previous studies using mouse lung slices [7,8] have highlighted the importance of Ca^{2+} oscillations by showing that an increase in oscillation frequency was associated with augmentation of airway contraction. Ca^{2+} oscillation is an efficient system for the Ca^{2+} signaling pathways by reducing possible deleterious effects due to sustained increases in $[Ca^{2+}]_i$ [9]. Currently, repetitive Ca^{2+}-release systems, such as inositol 1,4,5-triphosphate receptors (IP_3R) and/or ryanodine receptors (RyR) on the SR membrane, are thought to be key systems in agonist-induced Ca^{2+} oscillations. Furthermore, an interrelationship has been

Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; ASMCs, airway smooth muscle cells; CICR, calcium induced calcium release; Cys-LTs, cysteinyl leukotrienes; CysLT1R, cysteinyl leukotriene 1 receptor; GPCR, G protein-coupled receptor; IICR, IP_3-induced Ca^{2+} release; IL, interleukin; IP_3R, inositol 1,4,5-triphosphate receptors; RyR, ryanodine receptors; SOCE, store-operated Ca^{2+} entry; SR, sacroplasmic reticulum; TRPC, transient receptor potential canonical.

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recently proposed between sustained Ca^{2+} oscillations and plasma membrane Ca^{2+} influx, particularly store-operated Ca^{2+} entry (SOCE) triggered by depletion of Ca^{2+} in SR [10–12].

Thus far, however, leukotriene D4 (LTD4)-induced Ca^{2+} oscillations in ASMCs have not been demonstrated, and the effects of IL-13 on this pathway remain unknown.

In the present study, we aimed to assess the effects of IL-13 on Ca^{2+} oscillations in response to LTD4 in human cultured ASMCs, and elucidate the mechanism(s) underlying a putative augmentation of LTD4-induced Ca^{2+} oscillations with IL-13 pretreatment.

2. Methods

2.1.1. Study population and cell preparation

Human ASMCs were obtained from the lungs of 15 patients (10 males; average 68.0 years old; lung cancer in 14 patients and granulomatous inflammation in 1) who underwent surgical resection in accordance with procedures approved by the ethics committee of Kyoto University. Airway smooth muscle bundles were dissected out and cultured as described previously [13,14]. When cells grew to confluence, they were seeded at a density of $1 \times 10^4/cm^2$ into collagen-coated (5 μg/cm^2) glass-bottom dishes (Matsunami Glass Ind. Ltd., Osaka, Japan) for measurements of Ca^{2+} oscillations or into 6-well culture plates for measurements of mRNA. In each experiment, cells were cultured in fresh medium containing IL-13 (10 ng/ml) (Sigma Aldrich, Osaka, Japan) or diluent (PBS with 0.1% BSA) for 24 h when they reached subconfluence. The effects of IL-13 on mRNA expression were also examined at earlier time points, i.e., after 8 h and 12 h of incubation with IL-13.

2.1.2. Measurements of Ca^{2+} oscillations

Pretreated ASMCs were washed and loaded with a Ca^{2+}-sensitive dye, Fluo-4 AM (5 μM) (Invitrogen, Molecular probes, CA, USA) [15] for 30 min at 37 °C. After rinsing twice, the dishes were mounted on a Zeiss LSM510 confocal microscope (Axiovert 200 M, Carl Zeiss, Jena, Germany). In each experiment, 10 min perfusion with modified Krebs solution for equilibration was followed by perfusion with modified Krebs solution containing LTD4 (100 nM) (Cayman Chemical, Michigan, USA) in the presence or absence of inhibitors: 2-aminoethoxydiphenyl borate (2-APB) (Sigma Aldrich), xestospongin C (Wako, Osaka, Japan), ruthenium red (Wako), or ryanodine (Calbiochem, Dalmstadt, Germany). The acquired images were transferred to a BV analyzer (BrainVision, Tokyo, Japan), and the frequency of Ca^{2+} oscillations was analyzed. Fluorescence magnitude was expressed as the ratio of fluorescence relative to the fluorescence level immediately prior to the addition of an agonist. The temperature of the chamber in which glass-bottom dishes were placed to measure Ca^{2+} oscillations was set at 37 °C using a temperature controller (Zeiss Model CZI-3, Carl Zeiss). The room temperature was set at 26 °C to keep the focus plane constant. All measurements were done in duplicate using at least three cell lines each obtained from a different donor.

2.1.3. Reverse transcription and real-time PCR analysis

Total RNA was extracted from ASMCs using the RNeasy Mini Kit (Qiagen, Osaka, Japan). cDNA was synthesized and real-time PCR was performed using the ABI Prism 7300 sequence detection system (Applied Biosystems, Tokyo, Japan) with SYBR green (Roche Diagnostics, Tokyo, Japan). Specific primer sets used are shown in Table 1. The relative quantity of mRNA expression level of a target molecule was normalized to the mRNA expression levels of β-actin and β_2 microglobulin in the same sample.

2.1.4. Western blotting

Protein expression of CysLT1R and CD38 was analyzed by Western blotting and enhanced chemiluminescence. ASM cells were lysed in a buffer containing 50 mM Tris, 1% proteinase inhibitor cocktail (Sigma Aldrich), 1% Triton X-100. Protein concentration was determined measured by the Bio-Rad DC protein assay (Bio-Rad, Osaka, Japan). 10 μg of protein/lane was electrophoresed through 10% SDS–PAGE and transferred to PVDF membranes (GE Healthcare, Tokyo, Japan). Membranes were blocked with 5% skimmed-milk in Tris-buffered saline and 0.05% Tween 20 and then incubated overnight at 4 °C with the primary antibodies, rabbit anti-CysLT1R antibody (1:200) (Cayman Chemical) or with mouse anti-CD38 antibody (Santa Cruz Biotechnology, Inc. CA, USA) (1:200) in blocking solution overnight at 4 °C. Secondary anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase for use with ECL plus (1:5000) (GE Healthcare) was incubated for 1 h at room temperature and visualized using ECL plus Western Blotting Detection Reagents (GE Healthcare), and quantified by densitometry using ChemiDoc XRS (Bio-Rad). Data were normalized to the values for GAPDH in the same lane.

2.2. Statistics

Each result for a single treatment is given as the mean ± SE. Statistical analysis used paired *t* tests to compare untreated and treated cells. For the comparison of mRNA expression at different time points, repeated measures one-way ANOVA with Fisher's protected least significant difference correction was employed. p-value < 0.05 indicated statistical significance.

3. Results

3.1. LTD4 induces Ca^{2+} oscillations in ASMCs; pretreatment with IL-13 increases the number of oscillating cells and oscillation frequency

We first found that 100 nM LTD4 induced an increase in $[Ca^{2+}]_i$ followed by Ca^{2+} oscillations in human cultured ASMCs (Fig. 1a). In untreated cells, Ca^{2+} oscillations were observed in a small percentage of cells (13.0 ± 4.8% of total cells in a visual field) with relatively low frequencies (0.446 ± 0.140 min^{-1}). However, pretreatment with IL-13 (10 ng/ml) for 24 h significantly increased the number of oscillating ASMCs to 46.5 ± 5.2% and augmented the

Table 1
Sequences of primer sets for real-time PCR.

CysLT1R	Forward primer, 5′-GCACCTATGCTTTGTATGTCAACC-3′
	Reverse primer, 5′-ATACCTACACACACAAACCTGGC-3′
CD38	Forward primer, 5′-TGGCCAACTGCGAGTTCAG-3′
	Reverse primer, 5′-GACGAGGATCAGGACCAGGAT-3′
IP_3R type1	Forward primer, 5′-TCAATTCGGGAGAGGATGTC-3′
	Reverse primer, 5′-TCGACCAAGTGGATGTGGTA-3′
IP_3R type2	Forward primer, 5′-CAACCCTCCCAAGAAGTTCA-3′
	Reverse primer, 5′-GTTTGGCTTGCTTTGCTTTC-3′
IP_3R type3	Forward primer, 5′-GCCTTCGACTCTACCACTGC-3′
	Reverse primer, 5′-TTGTCTTCCCCACTCCAAAC-3′
RyR type3	Forward primer, 5′-CGGATGACGTGGTAAGCTG-3′
	Reverse primer, 5′-AGCCCGTCTGTGTTGAAGTTC-3′
TRPC1	Forward primer, 5′-GCCCGGAATTCTCGTGA-3′
	Reverse primer, 5′-AGGTGGGCTTGCGTCGGT-3′
TRPC3	Forward primer, 5′-CAGGCCTAAGGGAGCAGACCATAG-3′
	Reverse primer, 5′-ACTGTGATATTGGGCAGCGTGGTG-3′
β-actin	Forward primer, 5′-AAGAGAGGCATCCTCACCCT-3′
	Reverse primer, 5′-TACATGGCTGGGGTGTTGAA-3′
β_2-MG	Forward primer, 5′-TGTCTTTCAGCAAGGACTGGTC-3′
	Reverse primer, 5′-CA AACCTCCATGATGCTGC -3′

CysLT1R: cysteinyl leukotriene 1 receptor, IP_3R: inositol 1,4,5-triphosphate receptors, RyR: ryanodine receptor, β_2-MG: β_2-microglobulin, TRPC: transient receptor potential canonical.