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Aspirin and salicylates modulate IgE-mediated leukotriene secretion in mast cells through a dihydropyridine receptor-mediated Ca^{2+} influx

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Abstract Aspirin is a well-known nonsteroidal anti-inflammatory drug (NSAID) that may potentiate some acute allergies and causes adverse immunological reactions collectively referred to as aspirin intolerance. Aspirin intolerance is accompanied by increased leukotriene (LT) synthesis, and high levels of serum IgE are a risk factor for NSAID sensitivity. Here we demonstrate that aspirin modulates LTC_4 secretion in mast cells. Therapeutic levels of aspirin and salicylates (≤ 0.3 mM, i.e., the concentrations observed in vivo in the use of antipyretic analgesic) increased IgE-mediated LTC_4 secretion. Aspirin-induced stimulation was accompanied by increased Ser-505 phosphorylation of cytosolic phospholipase A_2 , which occurred independently of extracellular signal-regulated protein kinase-1/2 and p38 mitogen-activated protein kinase pathways. Aspirin also increased IgE-mediated Ca^{2+} influx, whereas aspirin at concentrations of ≥ 0.3 mM dose-dependently reduced Ca^{2+} store emptying and Ca^{2+} release-activated Ca^{2+} channel activation. Instead, aspirin facilitated a dihydropyridine receptor-mediated Ca^{2+} influx, resulting in increased LTC_4 secretion. This novel action of aspirin may play roles in exacerbation of immediate allergy and aspirin intolerance.

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Introduction

Aspirin (acetylsalicylic acid) and its analogs are among the most widely used nonsteroidal anti-inflammatory drugs (NSAIDs) worldwide but can cause adverse immunological reactions known as aspirin intolerance. Aspirin intolerance is a disorder that induces urticaria, asthma and anaphylaxis in response to oral administration of the drug [1,2]. The incidence of patients showing aspirin intolerance in general

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population ranges from 0.6% to 2.5%. Aspirin also potentiates some acute allergies such as food-dependent exercise-induced anaphylaxis (FDEIA), a food allergy induced by physical exercise. Recently, aspirin was shown to be a powerful trigger of anaphylaxis in FDEIA patients [3]. The anti-inflammatory actions of aspirin have been attributed primarily to inhibition of prostaglandin synthesis [4]. Slepian and colleagues have reported [5] that aspirin intolerance is particularly common in asthmatic patients who additionally have chronic rhinitis and/or nasal polyps. These individuals differ in several respects from patients who experience urticaria and/or angioedema after aspirin administration. Aspirin-sensitive asthma is most likely to be related to in some manner to the capacity of aspirin to inhibit cyclooxygenases. Although data regarding the latter are indirect and incomplete, different mechanisms may be involved in aspirin-sensitive urticaria and/or angioedema. Aspirin acetylates Ser-530 of cyclooxygenase (COX) I and II, thereby blocking prostaglandin (PG) and thromboxane A₂ synthesis, while therapeutic concentrations of aspirin and salicylates inhibit COX II protein expression [6]. However, several lines of evidence indicate that additional mechanisms, independent of COX inhibition, are involved in the anti-inflammatory actions of aspirin and salicylates. These mechanisms include inhibiting the activity of the transcription factors nuclear factor κ B (NF- κ B) [7] and nuclear factor of activated T cells (NF-AT) [8], as well as inhibiting degranulation of basophils and mast cells [9].

One key feature of aspirin intolerance is the overproduction of cysteinyl leukotrienes (cys-LTs) such as LTC₄/D₄/E₄, which are arachidonic acid (AA) metabolites. The cys-LTs are potent proinflammatory mediators and cause smooth muscle contraction and increased vascular permeability. Patients with aspirin intolerance have significantly higher levels of cys-LTs in their bronchoalveolar lavage fluid and urine before and after oral aspirin challenge [10].

Mast cells are important effector cells in allergic and inflammatory reactions and are the major producers of cys-LTs. Mast cells express the high-affinity IgE receptor (Fc ϵ RI) on their cell surface, and antigen cross-linking of Fc ϵ RI triggers biochemical cascades which lead to degranulation, secretion of cys-LTs and PGs, and production of cytokines. Moreover, mast cells are one of the major cell types responsible for cys-LT overproduction in aspirin intolerance [11–13]. In addition, several studies have shown that an atopic background (high levels of serum IgE) is a risk factor for NSAID sensitivity [14]. These observations led us to examine the effects of aspirin on IgE-mediated LT secretion and on the signaling pathways involved in secretion in cultured mast cells. Our results demonstrate that therapeutic concentrations of aspirin, i.e., the concentrations observed in vivo in the use of antipyretic analgesic, increase IgE-mediated LTC₄ secretion via dihydropyridine receptor (DHPR)-mediated Ca²⁺ channels, which previously reported by us [15] but not Ca²⁺ release-activated Ca²⁺ (CRAC) channels.

Materials and methods

Reagents

Aspirin (Acetylsalicylic acid, ASA) and salicylate (SA) were obtained from Wako Pure Chemicals (Osaka, Japan). ASA and SA (1 M stock solutions) were prepared in dimethylsulfoxide

(DMSO). The stock solutions were stored in the dark at –20 °C. ASA and SA were diluted in Hank's balanced salt solution (HBSS) immediately prior to use. The monoclonal anti-trinitrophenyl (TNP) IgE antibody (clone IgE-3) was purchased from BD PharMingen Japan (Tokyo, Japan). TNP-conjugated bovine serum albumin (TNP-BSA) (conjugation ratio of 25 TNP:1 BSA) was purchased from Cosmo Bio (Tokyo, Japan). Thapsigargin (Tg), 2-aminoethoxydiphenyl borate (2-APB), (S)-BayK8644, *p*-trifluoromethoxyphenylhydrazine (FCCP), antimycin A, oligomycin, and rotenone were obtained from Sigma (St. Louis, MO, USA). Nifedipine was obtained from Calbiochem (San Diego, CA, USA). Fluo3-acetoxymethyl ester (Fluo3/AM) was purchased from Dojindo (Kumamoto, Japan). 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbo-cyanine iodide (JC-1) was obtained from Invitrogen Corporation (Carlsbad, CA, USA). The JRK clone producing the monoclonal anti-rat β -chain antibody was kindly provided by Dr. J. Rivera (NIAMS, NIH, USA). All other chemicals were of analytical grade.

RBL-2H3 cells

Rat basophilic leukemia (RBL-2H3), an experimental model of mucosal-type mast cells, were obtained from the National Institute of Health Sciences (Japanese Collection of Research Biosources, [JCRB]; Cell No. JCRB0023) and grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS, SAFC Biosciences, Tokyo, Japan) in an atmosphere containing 5% CO₂. Cells were harvested by incubating them in Hank's balanced salt solution (HBSS) (pH 7.4) containing 1 mM ethylenediamine tetraacetate and 0.25% trypsin for 5 min at 37 °C. For IgE sensitization, cells suspended in complete DMEM were plated on 100-mm culture dishes (1 × 10⁶ cells/ml) or in 24-well plates (2 × 10⁵/well) and incubated with anti-TNP IgE (1 μ g/dish or 0.1 μ g/well) at 37 °C overnight. IgE-sensitized cells were washed with phosphate-buffered saline (PBS) and suspended in HBSS and then stimulated with TNP-BSA at the indicated concentrations at 37 °C.

BMMCs

All animal experiments were performed according to Nihon University guidelines. Bone marrow-derived mast cells (BMMCs) were prepared from femurs of 4- to 8-week-old C3HeBFeJ mice as previously described [16]. Cells were cultured in RPMI-1640 medium (Sigma) supplemented with 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen, Carlsbad, CA, USA), 5 × 10^{–5} M 2-mercaptoethanol (Wako, Tokyo, Japan), 100 μ g/ml sodium pyruvate (Invitrogen), 1% MEM nonessential amino acid solution (Invitrogen), and 5 ng/ml of recombinant IL-3 (Pepro Tech; Rocky Hill, NJ) in an atmosphere containing 5% CO₂ at 37 °C. After 4 to 6 weeks in culture, cells were stained for cell surface expression of Fc ϵ RI, and BMMCs were used for experiments after 4 to 8 weeks in culture (>95% mast cells).

LTC₄ secretion assay

LTC₄ secretion was determined as described previously [17]. Briefly, cells were activated, as described above, and LTC₄ content in the supernatant was determined using an ELISA kit

(Cayman Chemical, Ann Arbor, MI) according to the manufacturer's protocols.

Measurement of $[Ca^{2+}]_i$

Measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was performed using the Ca^{2+} -reactive fluorescence probe Fluo3/AM, as previously described [17]. Briefly, a cell suspension (1×10^6 cells/ml) was incubated with $4 \mu M$ Fluo3/AM for 30 min at $37^\circ C$ and then washed with HBSS and resuspended in HBSS supplemented with $1 mM$ $CaCl_2$. To study Ca^{2+} store release and Ca^{2+} entry separately, aliquots of the Fluo3-loaded cells were resuspended in HBSS supplemented with $1 mM$ EGTA instead of $1 mM$ $CaCl_2$. Fluorescence was monitored at 5-s intervals for 3 min using a microplate fluorometer (Fluoroskan Ascent CF, Labsystems, Helsinki, Finland; excitation and emission at 485 and 527 nm, respectively). $[Ca^{2+}]_i$ was calculated using the equation $[Ca^{2+}]_i = K_d [(F - F_{min}) / (F_{max} - F)]$, where K_d is the dissociation constant of the Ca^{2+} -Fluo3 complex ($450 nM$). F_{max} represents the maximum fluorescence (obtained by treating cells with $5 \mu M$ A23187), and F_{min} represents the minimum fluorescence (obtained by treating cells with A23187 in the presence of $10 mM$ EGTA). F is the actual sample fluorescence. The data are expressed as calculated $[Ca^{2+}]_i$ after background subtraction ($[Ca^{2+}]_i$ observed in unstimulated cells).

Measurement of mitochondrial transmembrane potential ($\Delta\Psi_m$)

Changes in the $\Delta\Psi_m$ were measured using the lipophilic cation JC-1, as previously described [16]. Briefly, cells (5×10^5 cells/

$500 \mu l$) were loaded with $2 \mu M$ JC-1 for 15 min in a CO_2 incubator, washed, and resuspended in HBSS. After cell stimulation, the green fluorescence (the monomeric JC-1) and red fluorescence (J-aggregates) were measured using the FL-1 and FL-2 channels, respectively, with a FACScalibur instrument (Becton Dickinson, San Jose, CA). Oligomycin, an inhibitor of the mitochondrial F_0F_1 adenosine 5'-triphosphate (ATP) synthase, was added in experiments using antimycin A to prevent the mitochondrial consumption of cellular ATP. Oligomycin alone had no significant effect, as previously described [16].

Western blotting

After stimulation, cell lysates were prepared and proteins were separated by SDS-PAGE using a 10% separating gel under reducing conditions and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The PVDF membranes were incubated with 3% BlockAce™ (Snow Brand Milk Products, Tokyo, Japan) at $4^\circ C$ overnight or 1 h at room temperature. For analyses of the dually phosphorylated (active) forms of extracellular signal-regulated protein kinase-1/2 (ERK 1/2) and p38 mitogen-activated protein kinase (p38MAPK), the membranes were incubated with a 1:4000 dilution of a rabbit polyclonal antibody raised against phosphorylated Thr²⁰² and Tyr²⁰⁴ of ERK or a 1:2000 dilution of a rabbit polyclonal antibody raised against phosphorylated Thr¹⁸⁰ and Tyr¹⁸² of p38MAPK (Cell Signaling, Beverly, MA), respectively overnight at $4^\circ C$. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Cell Signaling) for 1 h at room temperature. For analysis of the phosphorylated (active) form of cytosolic phospholipase A_2 (cPLA₂), membranes were incubated

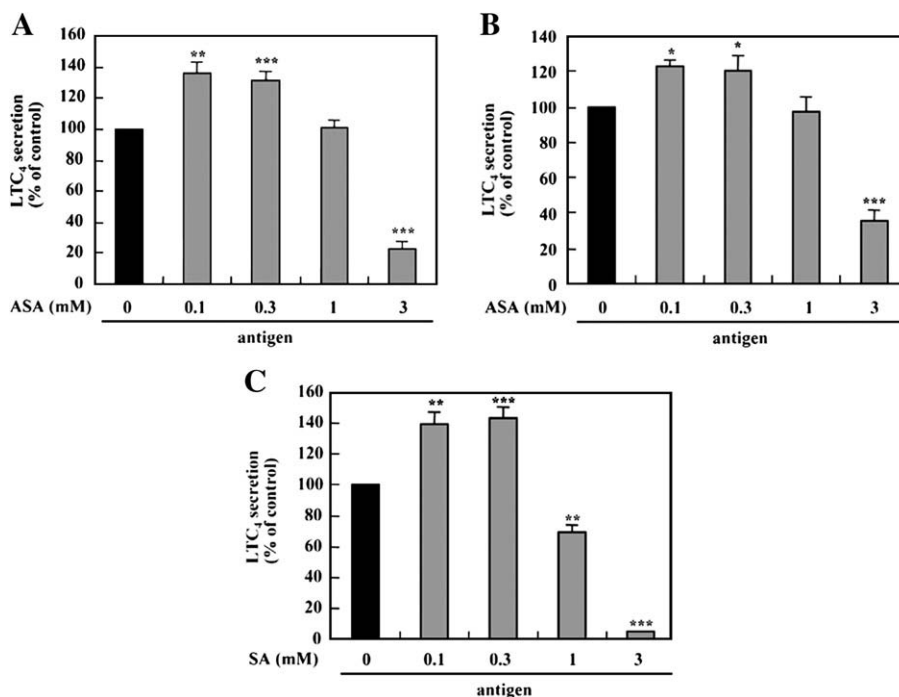


Figure 1 Effects of aspirin and salicylate on IgE-mediated LTC₄ secretion in mast cells. RBL-2H3 cells (A, C) or BMMCs (B) were treated with aspirin (ASA) or salicylate (SA) at the indicated concentrations and then immediately stimulated with 30 ng/ml TNP-BSA (antigen) for 30 min at $37^\circ C$. The LTC₄ content of the supernatant was determined by ELISA, as described in Materials and methods. The results are presented as a percent of control, in which the LTC₄ secretion in cells stimulated with antigen in the absence of drug is 100%. The data are the mean $\pm$ S.E.M. of three to five independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

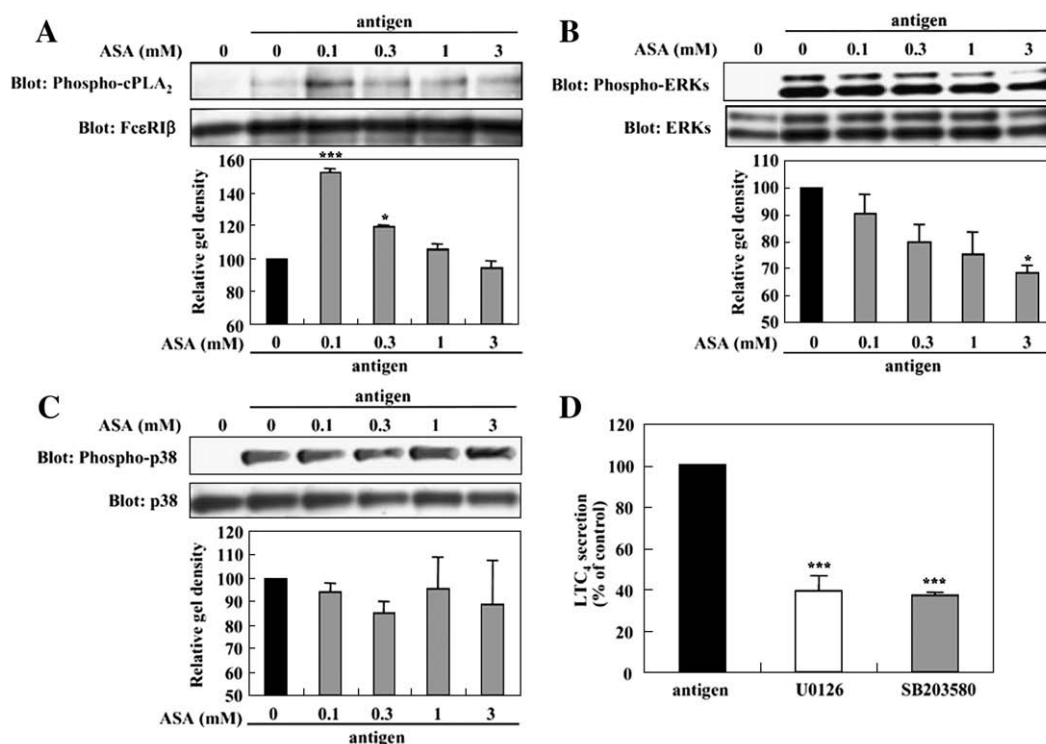


Figure 2 Role for cPLA₂, ERK1/2, and p38MAPK in IgE-mediated LTC₄ secretion and regulation by aspirin. (A–C) RBL-2H3 cells were treated with ASA at the indicated concentrations and then immediately stimulated with 30 ng/ml antigen for 5 min at 37 °C. The stimulated cells were lysed with SDS sample buffer. The proteins were separated by SDS-PAGE using a 10% separating gel under reducing conditions, and transferred to PVDF membranes. The presence of phospho-cPLA₂ (A), phospho-p44/42 ERK1/2 (B), and phospho-p38MAPK (C) was analyzed by Western blotting using specific antibodies, as described in Materials and methods. To verify equal loading, the blots were reprobed with FcεRIβ (A) or antibodies to each kinase (B, C). The data are representative of three independent experiments. (A–C, lower panels) Quantitative analyses data from three separate gels. **P* < 0.05; ****P* < 0.001 (D) RBL-2H3 cells were treated with 20 μM U0126 or 30 μM SB203580 and then immediately stimulated with 30 ng/ml antigen for 30 min at 37 °C. The LTC₄ content of the supernatant was determined by ELISA. Results are expressed as percent of control, in which LTC₄ secretion from cells stimulated with antigen in the absence of drug is 100%. The data represent the mean ± S.E.M. of four independent experiments. ****P* < 0.001.

with a 1:1000 dilution of a rabbit polyclonal antibody raised against phosphorylated Ser⁵⁰⁵ of cPLA₂ (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C and then with HRP-conjugated species-specific anti-rabbit Ig (GE Healthcare, Little Chalfont, UK) for 1 h at room temperature. After extensive washing of the membranes, the immunoreactive proteins were visualized using an ECL Plus kit (GE Healthcare) according to the recommendations of the manufacturer. The PVDF membranes were exposed to Fuji RX film (Fuji Film, Tokyo, Japan). Immunoreactive bands were scanned to produce digital images that were quantified using the NIH Image software.

Statistical analysis

Data were analyzed with a one-way ANOVA followed by a post-Tukey test. Values of *p* < 0.05 were considered significant.

Results

Aspirin and salicylate have a dual effect on IgE-mediated LTC₄ secretion in mast cells

To determine whether ASA affects IgE-mediated LT secretion in mast cells, we initially examined the effect of ASA on the LTC₄

secretion in RBL-2H3 cells. Cells that had been sensitized with anti-TNP IgE were treated with ASA at concentrations ranging from 0.1 mM to 3 mM, and then immediately stimulated with the antigen TNP-BSA (the basal level, 48 ± 11 pg/ml *n* = 5; antigenic stimulation, 3601 ± 218 pg/ml, *n* = 5). ASA alone had no effect on LTC₄ secretion in the cells. However, ASA had a dual effect on IgE-mediated LTC₄ secretion: at low concentrations (≤ 0.3 mM), ASA significantly increased LTC₄ secretion, while at high concentrations (> 1 mM) ASA suppressed secretion (Fig. 1A). The optimal concentration of ASA was 0.1 or 0.3 mM in different experiments (~1.5-fold greater secretion compared with antigenic stimulation alone). To exclude the possibility that this behavior is an aberration of tumor cells such as RBL-2H3, we also examined the effect of ASA on LTC₄ secretion in BMMCs and observed a similar trend in these mast cell primary cultures, although the magnitude of the increased secretion varied considerably in different experiments (Fig. 1B). Similar results were obtained when cells were exposed to the aspirin metabolite SA: at low concentrations (≤ 0.3 mM), SA significantly increased IgE-mediated LTC₄ secretion, while at high concentrations (> 1 mM), SA suppressed secretion (Fig. 1C). SA was approximately 3 times more toxic than ASA. Treatment with ASA up to 3 mM for 4 h had a minimal effect on cell viability (control, 92%; ASA 0.1 mM, 92%; ASA 0.3 mM, 93%; ASA 1 mM, 92%; ASA 3 mM, 91%), while ASA (10 mM) and SA (1 mM) caused

substantial cell death (ASA 10 mM, 94%; SA 1 mM, 40%), as measured by annexin V and propidium iodide double staining or trypan blue dye exclusion assay (data not shown). Moreover, DMSO concentration (0.3%) in 3 mM ASA had minimal effects of

cytotoxicity (97%). These results demonstrate that ASA and SA exhibit stimulatory effects on IgE-mediated LTC₄ secretion at non-toxic concentrations. Because ASA suppresses COX-1 and COX-2 activities and SA does not [18], these results suggest that

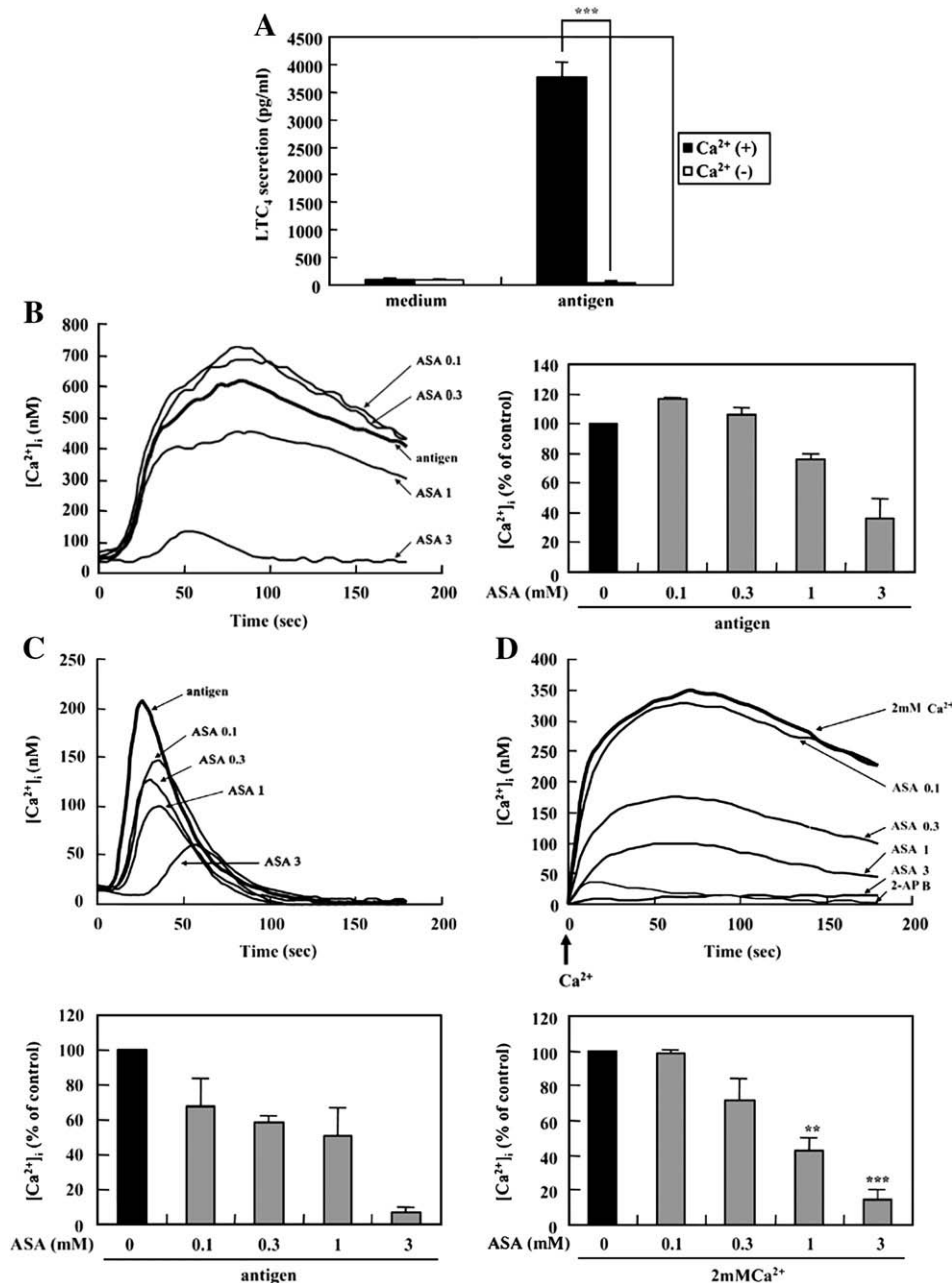


Figure 3 Ca²⁺ entry is essential for IgE-mediated LTC₄ secretion and is aspirin regulated. (A) RBL-2H3 cells were suspended in Ca²⁺-containing medium (HBSS supplemented with 1 mM CaCl₂) or nominally Ca²⁺-free medium (HBSS supplemented with 1 mM EGTA), stimulated with 30 ng/ml antigen for 30 min at 37 °C, and then the LTC₄ content of the supernatant was determined by ELISA. The data represent the mean ± S.E.M. of four independent experiments. ****P* < 0.001. (B, C) RBL-2H3 cells were loaded with Fluo3/AM, washed twice in HBSS, and resuspended in Ca²⁺-containing medium (HBSS supplemented with 1 mM CaCl₂) (B) or nominally Ca²⁺-free medium (HBSS supplemented with 1 mM EGTA) (C). The cells were incubated with ASA at the indicated concentrations and then immediately stimulated with 30 ng/ml antigen. The data are expressed as calculated [Ca²⁺]_i and are representative of two independent experiments. (D) RBL-2H3 cells loaded with Fluo3/AM were resuspended in nominally Ca²⁺-free medium and treated with thapsigargin for 10 min at 37 °C in order to deplete intracellular Ca²⁺ stores and activate SOCE. The cells were then incubated with ASA at the indicated concentrations or 10 μM 2-APB prior to the addition of 2 mM CaCl₂ (indicated by the arrow). The data are representative of five independent experiments. (B, right panel C, D, lower panels) Statistical analysis data of the maximal [Ca²⁺]_i. ***P* < 0.01; ****P* < 0.001.

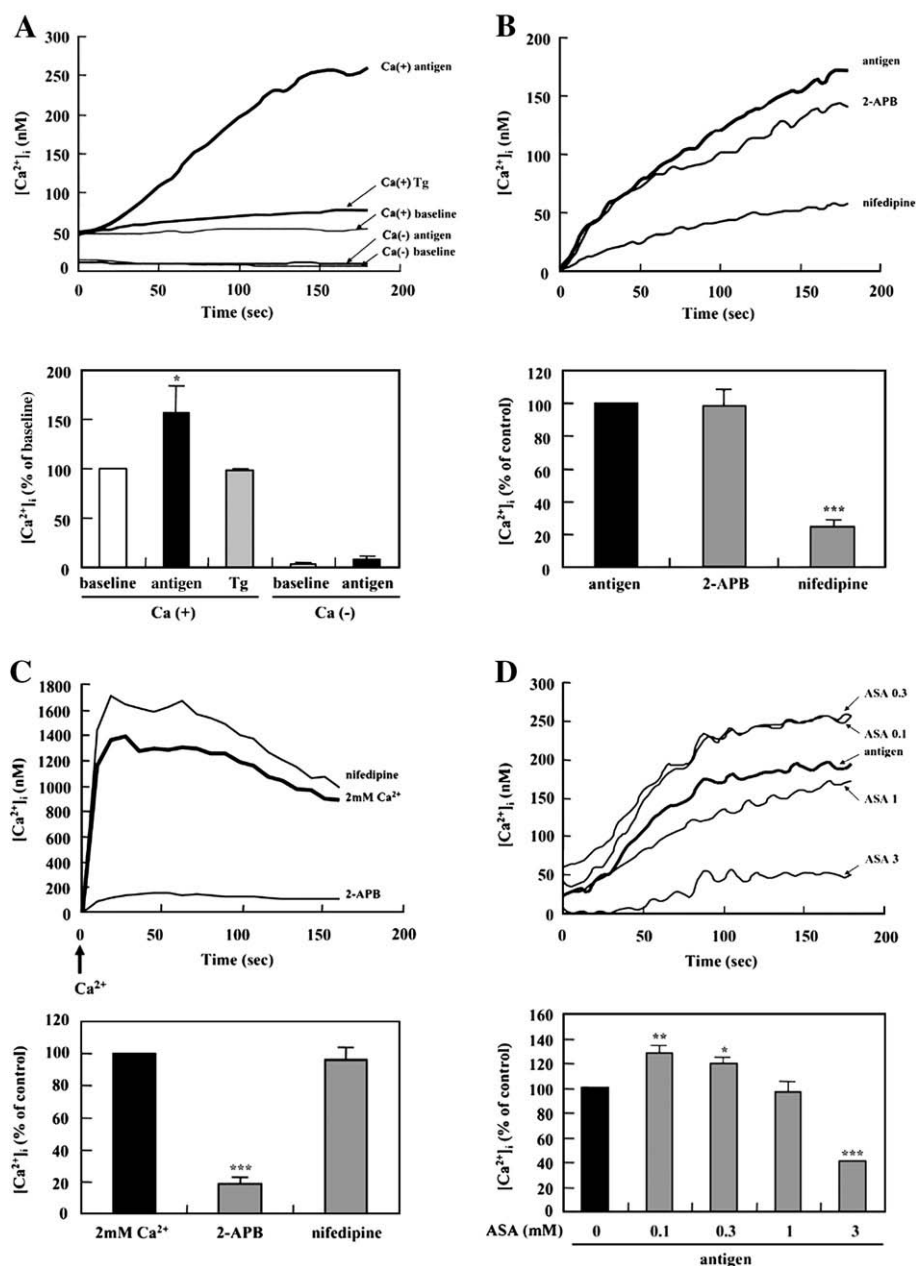


Figure 4 Antigenic stimulation evokes a DHP-sensitive Ca^{2+} entry that is distinct from SOCE and is aspirin regulated. (A) RBL-2H3 cells loaded with Fluo3/AM were resuspended in nominally Ca^{2+} -free medium and treated with Tg for 10 min at 37 °C. After extensive washing, the store-depleted cells were resuspended in Ca^{2+} -containing medium or nominally Ca^{2+} -free medium and then stimulated with 30 ng/ml antigen or with 1 μ M Tg. (B, D) The store-depleted cells resuspended in Ca^{2+} -containing medium were incubated with 10 μ M 2-APB or 1 μ M nifedipine (B) or with ASA at the indicated concentrations (D) and then immediately stimulated with 30 ng/ml antigen. (C) SOCE was induced in RBL-2H3 cells by pharmacologically depleting intracellular Ca^{2+} stores as described in the legend of Fig. 3D. The cells were treated with 10 μ M 2-APB or 1 μ M nifedipine before Ca^{2+} replenishment (indicated by the arrow). The data are representative of five independent experiments. (A–D, lower panels) Statistical analysis data of the maximal $[Ca^{2+}]_i$. * P <0.05; ** P <0.01; *** P <0.001.

the enhancement of LTC₄ secretion is not via suppression of COX activity. We investigated the effect of aspirin further in detail using RBL-2H3 cells as a model.

Aspirin increases activation of cPLA₂ independently of ERK and p38MAPK signaling pathways

To gain insight into the mechanisms by which ASA modulates LTC₄ secretion in mast cells, we examined the effect of ASA

on intracellular signaling pathways involved in LTC₄ synthesis, including activation of cPLA₂, which mediates agonist-induced AA release in most cell types [19]. The catalytic activity of cPLA₂ is phosphorylation dependent. Phosphorylation of cPLA₂ on Ser-505 by ERK1/2 is necessary for cPLA₂-mediated AA release following stimulation of various cell types by many different agonists [20]. Using a polyclonal antibody that specifically recognizes the Ser-505 phosphorylation of cPLA₂, we found that antigenic stimulation

substantially induced cPLA₂ phosphorylation, and that low concentrations (≤ 0.3 mM) of ASA substantially (1.5-fold) increased the effect (Fig. 2A). Thus, ASA both increased IgE-mediated LTC₄ secretion and Ser-505 phosphorylation of cPLA₂. We next examined whether ASA stimulated activation of ERK1/2. ERK1/2 is activated following dual phosphorylation of a threonine and a tyrosine residue by the upstream kinase MEK1/2. RBL-2H3 cells were treated with various concentrations (0.1–3 mM) of ASA prior to antigenic stimulation, and activation of ERK1/2 was then assessed using a polyclonal antibody that specifically recognizes the dually phosphorylated (active) ERK1/2 (Thr²⁰²/Tyr²⁰⁴) [21]. The phosphorylation of p42/44 ERK1/2 clearly increased following antigenic stimulation (Fig. 2B). However, ASA treatment dose-dependently reduced the activation of ERK1/2 (Fig. 2B). The cPLA₂ Ser-727 residue is another

site important for activation of the enzyme [22], and is phosphorylated by MNK1-related protein kinases, which are activated by p38MAPK [23]. We therefore examined the effect of ASA on p38MAPK activation. Western blot analysis, using a polyclonal antibody recognizing the active enzyme phospho-p38MAPK (Thr180/Tyr182), showed that antigenic stimulation induced p38MAPK activation, but ASA at concentrations ranging from 0.1 mM to 3 mM did not substantially affect the phosphorylation level (Fig. 2C). To determine the involvement of these MAPKs in LTC₄ secretion in our cell system, the effect of pharmacological inhibitors of selective MAPK pathways on IgE-mediated LTC₄ secretion was examined. As shown in Fig. 2D, 20 μ M U0126 and 30 μ M SB203580, which are selective inhibitors of ERK1/2 and p38MAPK, respectively, substantially ($\sim 50\%$ inhibition) inhibited antigen-induced LTC₄ secretion in RBL-2H3 cells,

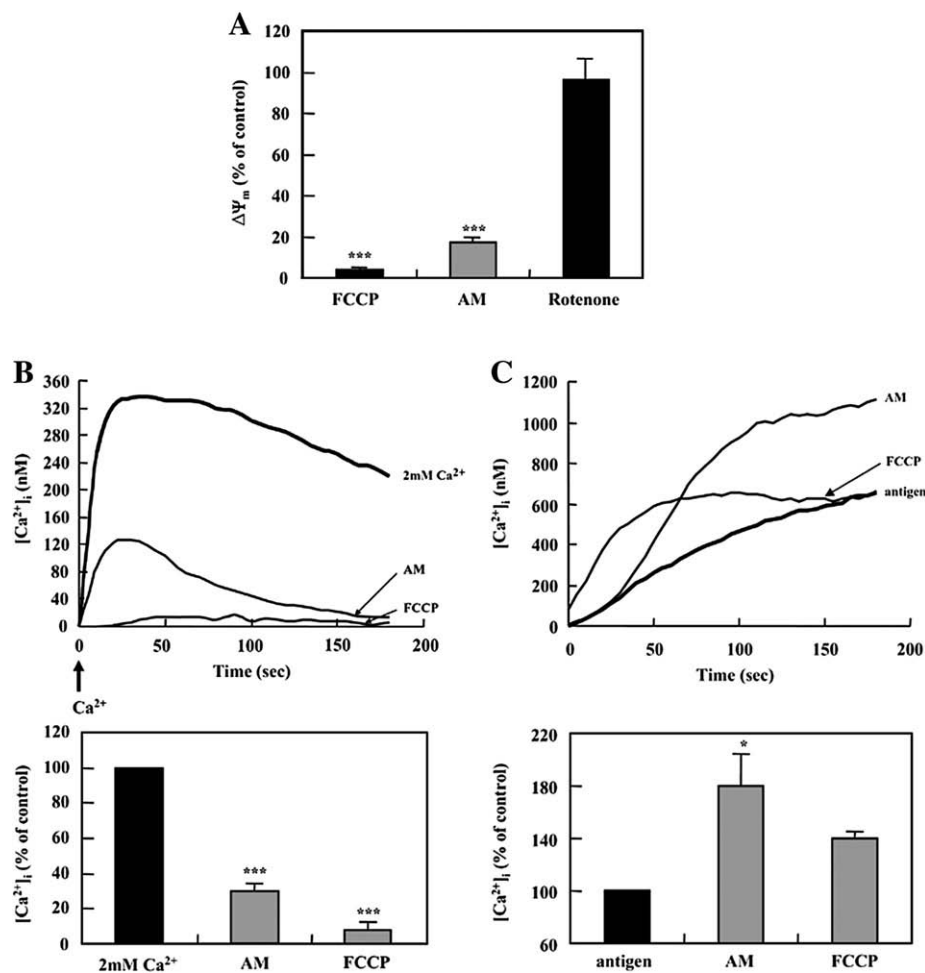


Figure 5 Differential effects of mitochondrial depolarization on SOCE and DHP-sensitive Ca²⁺ entry. (A) RBL-2H3 cells loaded with JC-1 were incubated with 5 μ M FCCP, 5 μ g/ml antimycin A plus 0.5 μ g/ml oligomycin (AM), or 5 μ M rotenone for 5 min. Green and red fluorescence were then measured by flow cytometry. The results are expressed as percent of control where $\Delta\Psi_m$ in the untreated control cells is 100%. The results represent the mean $\pm$ S.E.M. of three independent experiments. *** $P < 0.001$. (B) SOCE was induced in RBL-2H3 cells as described in the legend of Fig. 3D. The cells were treated with 5 μ M FCCP, or 5 μ g/ml antimycin A plus 0.5 μ g/ml oligomycin (AM) before Ca²⁺ replenishment (indicated by the arrow). (C) DHP-sensitive Ca²⁺ entry was induced in RBL-2H3 cells as described in the legend of Fig. 4A. The cells were treated with 5 μ M FCCP, or 5 μ g/ml antimycin A plus 0.5 μ g/ml oligomycin (AM) prior to stimulation with 30 ng/ml antigen. The results are representative of three to five independent experiments. (B, C, lower panels) Statistical analysis data of the maximal $[Ca^{2+}]_i$. * $P < 0.05$; *** $P < 0.001$.

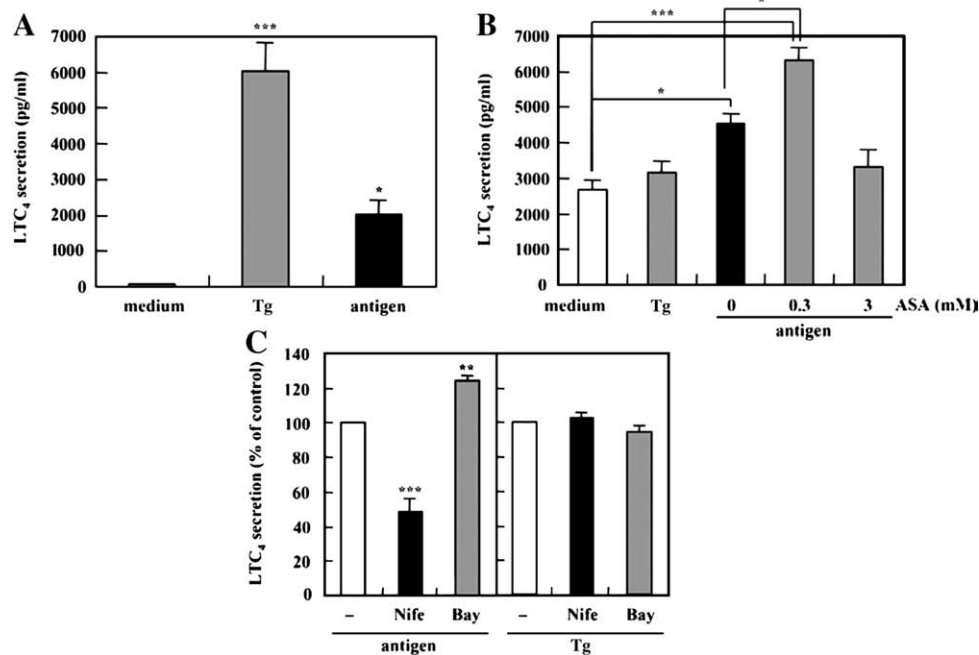


Figure 6 Role for DHP-sensitive Ca^{2+} entry in IgE-mediated LTC_4 secretion. (A) RBL-2H3 cells were suspended in Ca^{2+} -containing medium, stimulated with 30 ng/ml antigen or 1 μM Tg for 30 min at 37 °C, and then the LTC_4 content of the supernatant was determined by ELISA. The data represent the mean $\pm$ S.E.M. of four independent experiments. $*P < 0.05$; $***P < 0.001$. (B) RBL-2H3 cells suspended in nominally Ca^{2+} -free medium were depleted of intracellular Ca^{2+} stores as described in the legend of Fig. 4, and then resuspended in Ca^{2+} -containing medium. The cells were then stimulated with 30 ng/ml antigen or 1 μM Tg for 30 min at 37 °C in the absence or presence of ASA at the indicated concentrations, and the LTC_4 content of the supernatant was determined by ELISA. The data represent the mean $\pm$ S.E.M. of four independent experiments. $*P < 0.05$; $***P < 0.001$. (C) RBL-2H3 cells suspended in Ca^{2+} -containing medium were treated with 1 μM nifedipine or 10 μM (S)-BayK8644 and then immediately stimulated with 30 ng/ml antigen or 1 μM Tg for 30 min at 37 °C, and the LTC_4 content of the supernatant was determined by ELISA. The data represent the mean $\pm$ S.E.M. of three independent experiments. $**P < 0.01$; $***P < 0.001$.

indicating that both MAPK pathways are involved in LTC_4 synthesis in our cell system.

IgE-mediated LTC_4 secretion is dependent on Ca^{2+} entry and aspirin has a dual effect on Ca^{2+} responses

Another key physiological regulator of LTC_4 synthesis is Ca^{2+} . cPLA₂ is regulated by an increase in intracellular Ca^{2+} , which binds to the amino-terminal C2 domain and induces cPLA₂ translocation to the nuclear envelope and the endoplasmic reticulum [24]. Ca^{2+} is an important regulator of 5-lipoxygenase (5-LOX) as well, which catalyzes the addition of molecular oxygen to AA. Because Ca^{2+} entry from the extracellular space is a major source of increased $[\text{Ca}^{2+}]_i$, we determined whether LTC_4 secretion in mast cells was dependent on Ca^{2+} influx. As shown in Fig. 3A, EGTA chelation of extracellular Ca^{2+} completely abolished IgE-mediated LTC_4 secretion, indicating that this response is strictly dependent on Ca^{2+} influx. We then examined the effect of ASA on Ca^{2+} mobilization. ASA alone at concentrations ranging from 0.1 mM to 3 mM, caused a minimal increase in $[\text{Ca}^{2+}]_i$ (data not shown). However, ASA had a dual effect on IgE-mediated $[\text{Ca}^{2+}]_i$ increase: at low concentrations (≤ 0.3 mM), ASA increased the response, while at high

concentrations (> 1 mM), ASA suppressed the $[\text{Ca}^{2+}]_i$ increase (Fig. 3B). Next, the effect of ASA on Ca^{2+} mobilization from intracellular stores was examined in cells placed in nominally Ca^{2+} -free medium. As shown in Fig. 3C, ASA dose-dependently reduced the effect of antigenic stimulation. ASA reduced the maximal level of $[\text{Ca}^{2+}]_i$ with a minimum effective dose of 0.1 mM, and caused an increase in the time to reach peak $[\text{Ca}^{2+}]_i$ levels (for aspirin concentrations of 0, 0.3, 1, and 3 mM the times to peak $[\text{Ca}^{2+}]_i$ levels were 30, 35, 50, and 60 s, respectively).

In nonexcitable cells including mast cells, store-operated Ca^{2+} entry (SOCE) is a main pathway of Ca^{2+} influx [25]. In RBL cells, the best known store-operated Ca^{2+} channels (SOCs), the CRAC channels are activated by a pharmacological emptying of intracellular stores with thapsigargin (Tg) or by antigenic stimulation [26,27]. We therefore examined whether ASA affected Ca^{2+} influx through SOCs. Cells were treated with Tg for 10 min in nominally Ca^{2+} -free medium in order to deplete intracellular Ca^{2+} stores and activate SOCs. A substantial $[\text{Ca}^{2+}]_i$ rise was seen following readmission of external Ca^{2+} to the Tg-treated cells (Fig. 3D). This Ca^{2+} influx was almost completely abolished by 2-APB, a SOCE antagonist, indicating that the influx is SOC-mediated. ASA (≥ 0.3 mM) suppressed Ca^{2+} influx in a dose-dependent manner. These results suggest that ASA may stimulate some other Ca^{2+} entry pathway.

Aspirin facilitates DHP-sensitive Ca^{2+} response, which is pharmacologically distinct from SOCE

Recent work has demonstrated that in various immune cells including T and B cells, in addition to SOCE, antigenic stimulation can activate Ca^{2+} entry independently of Ca^{2+} store emptying. Moreover, we previously showed that antigenic stimulation induces substantial Ca^{2+} entry even when SOCE is inactive. In addition, this Ca^{2+} entry was blocked by DHP receptor (DHPR) antagonists but minimally affected by SOCE antagonists [15], indicating the occurrence of DHP-sensitive Ca^{2+} response. We therefore examined whether ASA might target this response. Consistent with our previous study, antigenic stimulation but not Tg increased $[\text{Ca}^{2+}]_i$ in Ca^{2+} store-depleted cells (Figs. 4A and B), although the amplitude of the increase varied considerably in different experiments. This effect was absent in Ca^{2+} -free conditions, indicating that the response was due to entry of extracellular Ca^{2+} . This Ca^{2+} entry was substantially reduced by the DHPR antagonist nifedipine but minimally affected by $10\ \mu\text{M}$ 2-APB (Fig. 4B). On the other hand, 2-APB completely abolished Ca^{2+} influx through SOCs, while nifedipine increased it (Fig. 4C). ASA had a dual effect on this DHP-sensitive Ca^{2+} response: at low concentrations ($\leq 0.3\ \text{mM}$), ASA increased this effect, while at high concentrations ($> 1\ \text{mM}$), ASA suppressed it (Fig. 4D). These results demonstrate that ASA increases DHP-sensitive Ca^{2+} response, which is pharmacologically distinct from SOCE.

Mitochondrial depolarization has distinct effects on SOCE and DHP-sensitive Ca^{2+} entry

Mitochondria play a facilitating role in SOC opening [28–30]. Mitochondrial depolarization evoked by the protonophore FCCP or by respiratory chain inhibitors such as the complex I inhibitor rotenone and the complex III inhibitor antimycin A impairs CRAC channel activity and the subsequent $[\text{Ca}^{2+}]_i$ rise [26–30]. To characterize DHP-sensitive Ca^{2+} entry further, we examined the effect of mitochondrial metabolic inhibitors. FCCP and antimycin A caused an acute collapse of $\Delta\Psi_m$, while rotenone had no effect on $\Delta\Psi_m$, as measured with the lipophilic cation JC-1 (Fig. 5A). Consistent with a number of previous reports [26–30], both FCCP and antimycin A profoundly reduced SOCE (Fig. 5B). In contrast, both drugs increased DHP-sensitive Ca^{2+} entry (Fig. 5C). These data indicate that mitochondrial depolarization has distinct effects on activation of SOCE and on DHP-sensitive Ca^{2+} entry.

Role of DHP-sensitive Ca^{2+} entry in LTC_4 secretion

The data presented so far demonstrated that low concentrations of ASA selectively enhance DHP-sensitive Ca^{2+} response. Next, we determined whether DHP-sensitive Ca^{2+} entry was involved in LTC_4 secretion. Tg could induce substantial LTC_4 secretion under normal conditions: $1\ \mu\text{M}$ Tg was more effective than Fc ϵ RI stimulation by optimal concentration of antigen (Fig. 6A). Depletion of intracellular Ca^{2+} stores by Tg resulted in increased LTC_4 secretion, which may result from activation of SOCE ($2689 \pm 261\ \text{pg/ml}$, $n=4$). In accordance with its inability to evoke Ca^{2+}

response, Tg no longer induced any significant increase in LTC_4 secretion in the cells ($3142 \pm 318\ \text{pg/ml}$, Fig. 6B). In contrast, antigenic stimulation still significantly increased LTC_4 secretion ($4520 \pm 289\ \text{pg/ml}$), in parallel with substantial Ca^{2+} entry. Moreover, ASA had a dual effect on the LTC_4 secretion: at low concentrations ($0.3\ \text{mM}$), ASA significantly increased secretion ($6311 \pm 350\ \text{pg/ml}$, $n=4$), while at a high concentration ($3\ \text{mM}$), ASA suppressed secretion ($3303 \pm 484\ \text{pg/ml}$, $n=4$, Fig. 6B). As shown above, nifedipine blocks DHP-sensitive Ca^{2+} entry with minimal effect on SOCE. On the other hand, the DHPR agonist (S)-BayK 8644 was found to increase DHP-sensitive Ca^{2+} response but not SOCE (data not shown). Thus, these drugs serve as powerful tools in studying the role of DHP-sensitive Ca^{2+} entry. To elucidate the role of DHP-sensitive Ca^{2+} entry in LTC_4 secretion under the conditions in which SOCs are active, we examined whether the two drugs affected LTC_4 secretion. As shown in Fig. 6C, nifedipine significantly reduced IgE-mediated LTC_4 secretion, while (S)-BayK 8644 increased secretion. In contrast, both drugs displayed minimal effects on Tg-induced LTC_4 secretion. These results demonstrate that DHP-sensitive Ca^{2+} entry is involved in LTC_4 secretion under physiological conditions and that low concentrations of ASA selectively increase this pathway.

Discussion

The current study demonstrates that ASA has a dual effect on IgE-mediated LTC_4 secretion in mast cells. At therapeutic concentrations ($0.1\text{--}0.3\ \text{mM}$) ASA can significantly increase LTC_4 secretion, while at high concentrations ($> 1\ \text{mM}$) ASA reduces LTC_4 secretion. We observed essentially similar effects with the aspirin metabolite SA, which lacks the acetyl group necessary for inhibiting COX activity, suggesting the enhancement of LTC_4 secretion is not via suppression of COX activity. Consistent with the view that cPLA₂ mediates agonist-induced AA release in most cell types [19], we demonstrated that antigenic stimulation induced Ser-505 phosphorylation of cPLA₂, which is required to activate the enzyme. Furthermore, ASA increased the phosphorylation of cPLA₂ and LTC_4 secretion in parallel, suggesting that the stimulatory effect of ASA is at least in part, attributable to activation of cPLA₂. Although the protein kinases responsible for activation of cPLA₂ are not fully understood, ERK1/2 may play a role [20,31]. In addition, in various cell types, the p38MAPK pathway has been implicated in cPLA₂ activation [32–34]. Our data indicate that antigenic stimulation activates both the ERK pathway and the p38MAPK pathway. Moreover, U0126 and SB203580, selective inhibitors of ERK1/2 and p38MAPK, respectively, both significantly reduced antigen-induced LTC_4 secretion, indicating that both kinases may be necessary for maximal response. Nevertheless, ASA dose-dependently inhibited IgE-mediated activation of ERK1/2 and had minimal effects on that of p38MAPK. Thus, inhibition of the ERK1/2 pathway may be involved in inhibition of LTC_4 secretion by high concentrations of ASA, but cannot explain the stimulatory effect of low concentrations of ASA.

Ca^{2+} is another key physiological regulator of LTC_4 synthesis. Ca^{2+} binds to the amino-terminal C2 domain of cPLA₂, thereby inducing its translocation to the nuclear

envelope and the endoplasmic reticulum [24]. Depletion of external Ca^{2+} almost completely abolishes IgE-mediated LTC_4 secretion, indicating the critical role of extracellular Ca^{2+} entry. In nonexcitable cells including mast cells, SOCE, which is mediated by SOCs such as CRAC channels and activated by depletion of intracellular Ca^{2+} stores, has been considered the main pathway of Ca^{2+} influx [25]. Recently, CRAC channels in RBL cells were shown to play key roles in Tg or IgE-mediated AA release, cPLA₂ activation, and LTC_4 secretion [26,27]. The current study demonstrates that low concentrations of ASA can increase IgE-mediated Ca^{2+} influx, while dose-dependently reducing Ca^{2+} store emptying and CRAC channels. Thus, inactivation of CRAC channels may be the mechanism by which high concentrations of ASA inhibit LTC_4 secretion. However, the stimulatory effect of low concentrations of ASA cannot be explained by CRAC channels, suggesting that ASA may target an additional Ca^{2+} entry mechanism.

Increasing evidence demonstrates that in addition to SOCE, a variety of agonists can activate store-independent Ca^{2+} entry in nonexcitable cells. We previously demonstrated that in Ca^{2+} store-depleted cells Fc ϵ RI stimulation but not Tg can evoke substantial Ca^{2+} entry, which is regulated by DHP derivatives. The data presented in the current study show that this Ca^{2+} response is a likely target of ASA. Low concentrations of ASA increased the DHP-sensitive pathway, while high concentrations of ASA inhibited it. Thus, this novel Ca^{2+} entry pathway in mast cells appears to be responsible for the stimulatory effect of ASA on Ca^{2+} influx. Mitochondrial depolarization had opposing effects on activation of CRAC channels and the DHP-sensitive Ca^{2+} entry. Increasing evidence indicates that mitochondria are key regulators of SOC opening [28–30]. Mitochondrial depolarization impairs CRAC channel activity and Ca^{2+} responses [26–30]. Moreover, mitochondrial depolarization inhibits LTC_4 secretion through inactivation of CRAC channels [26,27]. In the current study, we confirmed that mitochondrial depolarization impairs CRAC channel activity in mast cells. However, our data demonstrate that mitochondrial depolarization strongly activates DHP-sensitive Ca^{2+} entry, providing further evidence that this pathway differs from SOCE.

Our data suggest a potential role for DHP-sensitive Ca^{2+} channels in Ca^{2+} entry into mast cells. These Ca^{2+} channels, such as the α_1 subunit of voltage-gated L-type Ca^{2+} channel, are well characterized in excitable cells. Interestingly, in immune cells such as B and T cells, antigen receptor stimulation evokes a Ca^{2+} influx via DHP-sensitive Ca^{2+} channels; this Ca^{2+} influx plays roles in B and T cell functions especially the production of cytokines such as IL-4 [35–37]. Recently, we have found that DHP-sensitive Ca^{2+} response is involved in IgE-mediated cytokine production in mast cells. These observations suggest that DHP-sensitive channels with similar pharmacological and functional properties exist in these cells, although further investigations will be necessary to establish this view. Moreover, the current study demonstrates that like CRAC channels, DHP-sensitive Ca^{2+} channels are involved in immunological LTC_4 secretion in mast cells. Our data clearly demonstrate that Fc ϵ RI stimulation can evoke LTC_4 secretion even under the conditions where SOCs are inactivated and that low concentrations of ASA increases the secretion. In addition, our data indicate that the DHPR antagonist nifedipine inhibits, and the DHPR agonist (S)-BayK8644 augments, antigen-induced LTC_4 secretion, while

these drugs have minimal effects on Tg-induced LTC_4 secretion. Given that Tg can evoke SOCE but not DHP-sensitive Ca^{2+} entry, these data suggest that antigen and Tg stimulate LTC_4 secretion in mast cells by two pharmacologically distinct Ca^{2+} entry pathways or channels, i.e., CRAC channels and DHP-sensitive Ca^{2+} channels. Additionally, ASA facilitates the latter mechanism selectively and mitochondrial depolarization appears to regulate the two pathways oppositely. The findings suggest that these two pathways may be regulated coordinately by mitochondria and that ASA might affect this mitochondrial regulation. Further studies testing this hypothesis are underway.

Mortaz et al. have reported that 10 mM ASA alone increased LT release, whereas ASA inhibited IgE-mediated β -hexosaminidase release, although ASA alone had no effect [9,38]. In our cell system, however, 10 mM ASA exhibited substantial cytotoxicity, and had no effect on LT release. Selective occurrence of aspirin (NSAID) intolerance in some but not all human population does not seem to be explainable by the COX inhibition mechanism alone, because COX I and II enzyme are not specific for aspirin (NSAID) intolerance patients. The data presented in the current study demonstrate that ASA can modulate IgE-mediated mast cell activation. They are also consistent with various clinical observations regarding ASA. In ASA intolerance, several studies have shown that an atopic background (high levels of serum IgE) is a risk factor for NSAID sensitivity [14]. Moreover, our data demonstrating that low concentrations of ASA increase IgE-mediated LTC_4 secretion are consistent with the clinical observation that ASA intolerance is induced by low doses of ASA [39]. Additionally, patients with ASA intolerance can be desensitized to ASA by oral challenges with high doses of ASA, which results in reduced LT synthesis [39–42]. This phenomenon is similar to the suppressive effect of high concentrations of ASA on LTC_4 secretion observed in the current study.

In conclusion, the current study demonstrates that therapeutic concentrations of ASA facilitates DHP-sensitive Ca^{2+} entry, a novel pathway of Ca^{2+} influx, thereby modulating IgE-mediated LTC_4 secretion in mast cells. These actions of ASA may be relevant to ASA-induced adverse immunological reactions including aspirin intolerance.

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References

- [1] C.E. Grattan, Aspirin sensitivity and urticaria, *Clin. Exp. Dermatol.* 28 (2003) 123–127.
- [2] S. Ying, C.J. Corrigan, T.H. Lee, Mechanisms of aspirin-sensitive asthma, *Allergol. Int.* 53 (2004) 111–119.

- [3] E. Morita, K. Kunie, H. Matsuo, Food-dependent exercise-induced anaphylaxis, *J. Dermatol. Sci.* 47 (2007) 109–117.
- [4] J.R. Vane, Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs, *Nat. New Biol.* 231 (1971) 232–235.
- [5] I.K. Slepian, K.P. Mathews, J.A. McLean, Aspirin-sensitive asthma, *Chest* 87 (1985) 386–391.
- [6] K.K. Wu, Aspirin and other cyclooxygenase inhibitors: new therapeutic insights, *Semin. Vasc. Med.* 3 (2003) 107–112.
- [7] E. Kopp, S. Ghosh, Inhibition of NF- κ B by sodium salicylate and aspirin, *Science* 265 (1994) 956–959.
- [8] J. Román, A.F. de Arriba, S. Barrón, P. Michelena, M. Giral, M. Merlos, E. Bailón, M. Comalada, J. Gálvez, A. Zarzuelo, I. Ramis, UR-1505, a new salicylate, blocks T cell activation through nuclear factor of activated T cells, *Mol. Pharmacol.* 72 (2007) 269–279.
- [9] E. Mortaz, F.A. Redegeld, F.P. Nijkamp, F. Engels, Dual effects of acetylsalicylic acid on mast cell degranulation, expression of cyclooxygenase-2 and release of pro-inflammatory cytokines, *Biochem. Pharmacol.* 69 (2005) 1049–1057.
- [10] R. Rubinsztajn, J. Wrońska, R. Chazan, Urinary leukotriene E₄ concentration in patients with bronchial asthma and intolerance of non-steroids anti-inflammatory drugs before and after oral aspirin challenge, *Pol. Arch. Med. Wewn.* 110 (2003) 849–854.
- [11] S. O' Sullivan, B. Dahlén, S.E. Dahlén, M. Kumlin, Increased urinary excretion of the prostaglandin D₂ metabolite 9 α , 11 β -prostaglandin F₂ after aspirin challenge supports mast cell activation in aspirin-induced airway obstruction, *J. Allergy Clin. Immunol.* 98 (1996) 421–432.
- [12] H. Mita, S. Endoh, M. Kudoh, Y. Kawagishi, M. Kobayashi, M. Taniguchi, K. Akiyama, Possible involvement of mast-cell activation in aspirin provocation of aspirin-induced asthma, *Allergy* 56 (2001) 1061–1067.
- [13] X.S. Wang, A.Y. Wu, P.S. Leung, H.Y. Lau, PGE₂ suppresses excessive anti-IgE induced cysteinyl leukotrienes production in mast cells of patients with aspirin exacerbated respiratory disease, *Allergy* 62 (2007) 620–627.
- [14] J.S. Bae, S.H. Kim, Y.M. Ye, H.J. Yoon, C.H. Suh, D.H. Nahm, H.S. Park, Significant association of Fc ϵ RI α promoter polymorphisms with aspirin-intolerant chronic urticaria, *J. Allergy Clin. Immunol.* 119 (2007) 449–456.
- [15] Y. Suzuki, T. Yoshimaru, T. Inoue, C. Ra, The high-affinity immunoglobulin E receptor (Fc ϵ RI) regulates mitochondrial calcium uptake and a dihydropyridine receptor-mediated calcium influx in mast cells: role of the Fc ϵ RI β chain immunoreceptor tyrosine-based activation motif, *Biochem. Pharmacol.* 75 (2008) 1492–1503.
- [16] Y. Suzuki, T. Yoshimaru, T. Inoue, C. Ra, Mitochondrial Ca²⁺ flux is a critical determinant of the Ca²⁺ dependence of mast cell degranulation, *J. Leukoc. Biol.* 79 (2006) 508–518.
- [17] Y. Suzuki, T. Yoshimaru, T. Matsui, T. Inoue, O. Niide, S. Nunomura, C. Ra, Fc epsilon RI signaling of mast cells activates intracellular production of hydrogen peroxide: role in the regulation of calcium signals, *J. Immunol.* 171 (2003) 6119–6127.
- [18] D.D. Stevenson, M.A. Hankammer, D.A. Mathison, S.C. Christiansen, R.A. Simon, Aspirin desensitization treatment of aspirin-sensitive patients with rhinosinusitis-asthma: long-term outcomes, *J. Allergy Clin. Immunol.* 98 (1996) 751–758.
- [19] C.C. Leslie, Properties and regulation of cytosolic phospholipase A₂, *J. Biol. Chem.* 272 (1997) 16709–16712.
- [20] R.A. Nemenoff, S. Winitz, N.X. Qian, V. Van Putten, G.L. Johnson, L.E. Heasley, Phosphorylation and activation of a high molecular weight form of phospholipase A₂ by p42 microtubule-associated protein 2 kinase and protein kinase C, *J. Biol. Chem.* 268 (1993) 1960–1964.
- [21] J.A. Rosado, S.O. Sage, Role of the ERK pathway in the activation of store-mediated calcium entry in human platelets, *J. Biol. Chem.* 276 (2001) 15659–15665.
- [22] A.G. Börsch-Haubold, F. Bartoli, J. Asselin, T. Dudler, R.M. Kramer, R. Apitz-Castro, S.P. Watson, M.H. Gelb, Identification of the phosphorylation sites of cytosolic phospholipase A₂ in agonist-stimulated human platelets and HeLa cells, *J. Biol. Chem.* 273 (1998) 4449–4458.
- [23] Y. Hefner, A.G. Börsch-Haubold, M. Murakami, J.I. Wilde, S. Pasquet, D. Schieltz, F. Ghomashchi, J.R. Yates, C.G. Armstrong, A. Paterson, P. Cohen, R. Fukunaga, T. Hunter, I. Kudo, S.P. Watson, M.H. Gelb, Serine 727 phosphorylation and activation of cytosolic phospholipase A₂ by MNK1-related protein kinases, *J. Biol. Chem.* 275 (2000) 37542–37551.
- [24] M.A. Gijón, C.C. Leslie, Regulation of arachidonic acid release and cytosolic phospholipase A₂ activation, *J. Leukoc. Biol.* 65 (1999) 330–336.
- [25] A. Zweifach, R.S. Lewis, Calcium-dependent potentiation of store-operated calcium channels in T lymphocytes, *J. Gen. Physiol.* 107 (1996) 597–610.
- [26] W.C. Chang, A.B. Parekh, Close functional coupling between Ca²⁺ release-activated Ca²⁺ channels, arachidonic acid release, and leukotriene C₄ secretion, *J. Biol. Chem.* 279 (2004) 29994–29999.
- [27] W.C. Chang, C. Nelson, A.B. Parekh, Ca²⁺ influx through CRAC channels activates cytosolic phospholipase A₂, leukotriene C₄ secretion, and expression of c-fos through ERK-dependent and -independent pathways in mast cells, *FASEB J.* 20 (2006) 2381–2383.
- [28] J.A. Gilibert, A.B. Parekh, Respiring mitochondria determine the pattern of activation and inactivation of the store-operated Ca²⁺ current I (CRAC), *EMBO J.* 19 (2000) 6401–6407.
- [29] A. Makowska, K. Zablocki, J. Duszyński, The role of mitochondria in the regulation of calcium influx into Jurkat cells, *Eur. J. Biochem.* 267 (2000) 877–884.
- [30] M.D. Glitsch, D. Bakowski, A.B. Parekh, Store-operated Ca²⁺ entry depends on mitochondrial Ca²⁺ uptake, *EMBO J.* 21 (2002) 6744–6754.
- [31] L.L. Lin, M. Wartmann, A.Y. Lin, J.L. Knopf, A. Seth, R.J. Davis, cPLA₂ is phosphorylated and activated by MAP kinase, *Cell* 72 (1993) 269–278.
- [32] X. Zhu, H. Sano, K.P. Kim, A. Sano, E. Boetticher, N.M. Muñoz, W. Cho, A.R. Leff, Role of mitogen-activated protein kinase-mediated cytosolic phospholipase A₂ activation in arachidonic acid metabolism in human eosinophils, *J. Immunol.* 167 (2001) 461–468.
- [33] R.M. Kramer, E.F. Roberts, S.L. Um, A.G. Börsch-Haubold, S.P. Watson, M.J. Fisher, J.A. Jakubowski, p38 mitogen-activated protein kinase phosphorylates cytosolic phospholipase A₂ (cPLA₂) in thrombin-stimulated platelets, *J. Biol. Chem.* 271 (1996) 27723–27729.
- [34] W.H. Waterman, R.I. Sha'afi, A mitogen-activated protein kinase independent pathway involved in the phosphorylation and activation of cytosolic phospholipase A₂ in human neutrophils stimulated with tumor necrosis factor- α , *Biochem. Biophys. Res. Commun.* 209 (1995) 271–278.
- [35] A. Badou, M. Savignac, M. Moreau, C. Leclerc, R. Pasquier, P. Druet, L. Pelletier, HgCl₂-induced interleukin-4 gene expression in T cells involves a protein kinase C-dependent calcium influx through L-type calcium channels, *J. Biol. Chem.* 272 (1997) 32411–32418.
- [36] M. Savignac, A. Badou, M. Moreau, C. Leclerc, J.C. Guéry, P. Paulet, P. Druet, J. Ragab-Thomas, L. Pelletier, Protein kinase C-mediated calcium entry dependent upon dihydropyridine-sensitive channels: a T cell receptor-coupled signaling pathway involved in IL-4 synthesis, *FASEB J.* 15 (2001) 1577–1579.
- [37] M. Savignac, B. Gomes, A. Gallard, S. Narbonne, M. Moreau, C. Leclerc, P. Paulet, B. Mariamé, P. Druet, A. Saoudi, G.J. Fournié, J.C. Guéry, L. Pelletier, Dihydropyridine receptors are selective markers of Th₂ cells and can be targeted to prevent

- Th₂-dependent immunopathological disorders, *J. Immunol.* 172 (2004) 5206–5212.
- [38] E. Mortaz, F.A. Redegeld, K. Dunsmore, K. Odoms, H.R. Wong, F.P. Nijkamp, F. Engels, Stimulation of cysteinyl leukotriene production in mast cells by heat shock and acetylsalicylic acid, *Eur. J. Pharmacol.* 561 (2007) 214–219.
- [39] A. Szczeklik, D.D. Stevenson, Aspirin-induced asthma: advances in pathogenesis, diagnosis, and management, *J. Allergy Clin. Immunol.* 111 (2003) 913–921.
- [40] J.P. Arm, K.F. Austen, Leukotriene receptors and aspirin sensitivity, *N. Engl. J. Med.* 347 (2002) 1524–1526.
- [41] U.R. Juergens, S.C. Christiansen, D.D. Stevenson, B.L. Zuraw, Inhibition of monocyte leukotriene B₄ production after aspirin desensitization, *J. Allergy Clin. Immunol.* 96 (1995) 148–156.
- [42] S.M. Nasser, M. Patel, G.S. Bell, T.H. Lee, The effect of aspirin desensitization on urinary leukotriene E₄ concentrations in aspirin-sensitive asthma, *Am. J. Respir. Crit. Care Med.* 151 (1995) 1326–1330.