Bone marrow derived mast cell acquire responsiveness to Substance P with Ca^{2+} signals and release of leukotriene B_4 via mitogen-activated protein kinase

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Abbreviations: LT: leukotriene, BMMC: bone marrow derived mast cells, SP: substance P, MAP: mitogen activated protein, DNP-HSA: human serum albumin-conjugated dinitrophenol

Summary

Substance P (SP) plays a crucial role in neurogenic inflammation with granulocyte infiltration. To investigate the mechanisms of cell activation and mediator release concerned with the SP-induced inflammation, we studied the release of leukotriene (LT) B_4 and histamine from mouse bone marrow derived mast cells (BMMC) in response to SP and antigen. BMMC of three strains acquired responsiveness to SP and release of LTB_4 as well as histamine after co-culture with fibroblasts for three weeks. PD 098059 (ERK MAP kinase inhibitor) significantly inhibited the release of LTB_4 induced by either SP or antigen but not the release of histamine except for that from BMMC of BALB/c mouse induced by antigen. SB 203580 (p38 MAP kinase inhibitor) selectively inhibited the LTB_4 release from BMMC of BALB/c mouse and NC mouse induced by antigen and SP respectively. In BMMC of all strains, SP induced considerable increases of $[Ca^{2+}]_i$ after co-culture with fibroblasts. It is noteworthy that the increase of antigen-induced $[Ca^{2+}]_i$ in those cells were smaller than those in BMMC before the co-culture. These results suggest that BMMC undergoes maturation under the influence of fibroblasts, acquiring the responsiveness to SP with Ca^{2+} signals and release of LTB_4 via predominantly ERK MAP kinase.

1. Introduction

Substance P (SP) located in cutaneous sensory neurons [1] is a major mediator of neurogenic inflammation [2]. It induces the degranulation of mast cells isolated from the peritoneal cavity of rats [3] and human skin [4], releasing chemical mediators such as histamine. Intradermal injection of SP induces an immediate wheal-and-flare response [5-7] and granulocyte infiltration in both animal [8-10] and human skin [11]. Prior application of local anesthetics which prevent mast cell activation [7], and systemic administration of anti-histamines reduce wheal-and-flare responses [5-7]. It is therefore well accepted that histamine released from mast cells mediates or enhances these reactions [12].

However, the mechanism of granulocyte infiltration induced by SP has remained a matter of discussion. Using mast cell deficient mice, Matsuda et al. [8] and Yano et al. [9] have demonstrated that this reaction is dependent on mast cells. It has also been reported that this reaction was inhibited by antagonists of leukotriene (LT) B_4 [13], inhibitors of 5-lipoxygenase [10], or those of LT synthetase [14]. These studies suggest that the granulocyte infiltration induced by SP is critically mediated by mast

cell-derived LTB_4, a potent chemoattractant for neutrophils and eosinophils [15]. On the other hand, Robinson et al. [16] reported that SP induced the release of histamine but not that of LTB_4 from dispersed human skin mast cells. More recently, we have demonstrated that SP induced the release of LTB_4 as well as histamine from human skin tissues from 8 out of 22 non-allergic individuals, whereas house dust mite antigen did so from tissues of all 22 donors [17, 18]. The difference between mast cells that release LTB_4 in response to SP and that did not remains unclear.

In this study, we employed bone marrow derived mast cells (BMMC) from three different strains of mice cultured with or without 3T3 fibroblasts to investigate the functional alteration of mast cells under the influence of fibroblasts. In rodents, there are two distinct populations of mast cells, connective tissue-type mast cells (CTMC) and mucosal-type mast cells (MMC). CTMC, which reside in the serosal cavity and skin, but not MMC or BMMC are activated by nonimmunologic polycationic activators such as compound 48/80 and SP [19, 20]. BMMC, however, become capable of releasing histamine in response to SP, when co-cultured with fibroblasts [21, 22].

Here we have demonstrated that mast cells acquire a CTMC-like phenotype under

the influence of fibroblasts so that they release histamine and activate Ca^{2+} signal and the MAP kinase pathway for the release of LTB_4, in response to SP.

2. Results

2.1. Release of histamine from BMMC

SP induced histamine release from BMMC of all three different strains of mice when cultured with 3T3 fibroblasts for three weeks. The release was dependent on the dose of SP over the concentration from 4 to 100 μM (Fig. 1, A-C, panel c). On the contrary, regardless of the concentration, SP did not induce histamine release from BMMC cultured in the absence of fibroblasts (data not shown). DNP-HSA induced histamine release from BMMC in a dose dependent manner over the concentration from 1 to 1000 ng/ml after being cultured either with or without fibroblasts (Fig. 1, A-C, panel a)

2.2. Release of LTB_4 from BMMC

SP also caused the release of LTB_4 from the BMMC of all three different mouse strains cultured with 3T3 fibroblasts for three weeks in a dose dependent manner over the concentration from 4 to 100 μM (Fig. 1, A-C, panel d). On the contrary, SP could not induce the release of LTB_4 from BMMC cultured in the absence of fibroblasts, even

at the highest concentration (data not shown). DNP-HSA induced the release of LTB_4 from BMMC of all strains in a dose dependent manner over the concentration from 1 to 1000 ng/ml, regardless of the co-culture with fibroblasts (Fig. 1, A-C, panel b).

In order to ensure that the LTB_4-like immunoreactivity detected by the enzyme-immunoassay was LTB_4 itself, we fractionated the reaction mixture by reverse-phase HPLC. As shown in Fig. 4, LTB_4-like immunoreactivity was detected in the tenth fraction, in which authentic LTB_4 was eluted under the same condition (Fig. 2).

2.3. The effects of MAP kinase inhibitors on the release of histamine and LTB_4 from BMMC

In order to study the involvement of MAP kinase in the release of histamine and LTB_4, we employed two antagonists against MAP kinase; These were PD 098059, which inhibits the activation of extracellular signaling-regulated protein kinase (ERK) 2 by preventing phosphorylation of MAP kinase-ERK-kinase (MEK) 1 and SB 203580, a p38 MAP kinase inhibitor. PD 098059 did not affect the histamine release from BMMC

of any mouse strain, stimulated by 100 μM SP (Fig. 3, A-C, panel a). Similarly, it did not affect the histamine release from BMMC of NC/Kuj or C57/BL6 mice stimulated by 100 ng/ml DNP-HSA (Fig. 3A, B, panel c), whereas, it slightly, but significantly, inhibited that from BALB/c mouse BMMC (Fig. 3C, panel c). SB 203580 did not affect the histamine release from BMMC stimulated either by 100 μM SP or by 100 ng/ml DNP-HSA in all different strains (Fig. 3, A-C, panels b and d).

On the other hand, PD 098059 significantly inhibited the LTB_4 release from all mouse strains induced by SP and that by DNP-HSA in a dose dependent manner. The percent inhibition of SP-induced LTB_4 release ranged from 77 to 108% and that of antigen-induced release ranged from 53 to 78%, respectively (Fig. 3, A-C, panels a and c). SB 203580 also significantly inhibited the release of LTB_4 from BMMC of the NC mouse strain when stimulated by SP (Fig. 3A, panel b) and that by DNP-HSA from BMMC of the BALB/c mouse strain (Fig. 3C, panel d). Otherwise, SB 203580 did not show apparent effect on the release of LTB_4 induced by DNP-HSA or SP.

2.4. Calcium signal in BMMC co-cultured with fibroblasts

To investigate the mechanisms of BMMC maturation toward the CTMC like-phenotype, we investigated the increase of $[Ca^{2+}]_i$ in the BMMC stimulated with various agents. Fura-3-loaded BMMC were stimulated with 100 μM SP and $[Ca^{2+}]_i$ levels in the BMMC were measured using a fluorescence microscope with a ratio-imaging system. In BMMC of all mouse strains, SP induced only small, or even no increase of $[Ca^{2+}]_i$, whereas, the antigen induced considerable increases of $[Ca^{2+}]_i$ before co-culture with fibroblasts (Fig. 4, A-C, panel a). However, those co-cultured with fibroblasts for three weeks showed substantial increases of $[Ca^{2+}]_i$ in response to SP (Fig. 4, A-C, panel b). It is noteworthy that the antigen-induced $[Ca^{2+}]_i$ increase in those cells were smaller than those in BMMC before the co-culture procedure (Fig. 4, A-C, panel b).

3. Discussion

In this study, we have demonstrated that SP induces the release of histamine and LTB_4 from BMMC of three different mouse strains following co-culture with 3T3 fibroblasts for three weeks.

LTB_4 is synthesized from arachidonic acid (AA) via the 5-lipoxigenage-LTA_4-pathway. It induces the aggregation, chemokinetics, and chemotaxis of granulocytes and their adhesion to endothelial cells at low concentrations [15, 23]. It also has a potential to activate granulocytes to release lysosomal enzymes [24] and generate superoxide anioins [25] and nitric oxide [26]. In 1997, Yokomizo et al. reported the cloning of the cDNA for the human LTB_4 receptor in the HL-60 leukemia cell-line [24], followed by Huang et al. for the cloning of the mouse LTB_4 receptor [27]. A quantitative study of mRNA for the LTB_4 receptor revealed that it is up regulated in IL-5-transgenic mice, suggesting the involvement of LTB_4 in the accumulation of eosinophils by IL-5 [27]. Recently, Morita et al. have reported that an antagonist against the LTB_4 receptor inhibited the proliferation and cytokine production of T cells, including IL-2, interferon-γ, and IL-4, suggesting that LTB_4 is intrinsically involved in

T cell activation by various stimuli [25]. Other studies with LTB_4 receptor antagonists have suggested that LTB_4 is involved in a wide range of immune response, host defense reactions, such as bronchial asthma [26], nephrotoxic serum nephritis [28], and allograft rejection [29].

Cross-linking of the high-affinity IgE receptor (FcεRI) expressed on the surface of mast cells initiates the secretion of pre-stored inflammatory mediators such as histamine, 5-hydroxy-tryptamine, and heparin, whereas lipid mediators such as prostaglandins (PGs), LTs and platelet activating factor are synthesized *de novo* [30, 31]. In this *de novo* process, the regulation of LT synthesis depends upon initial hydrolysis of membrane phospholipid into AA by phospholipase (PL) A_2, especially cytosolic PLA_2 ($cPLA_2$) [29]. It is well accepted that the enzymatic activity of $cPLA_2$ is regulated by mitogen activated protein (MAP) kinase and intracellular Ca^{2+} signals, whereas the release of histamine is regulated primarily by intracellular Ca^{2+} signal and protein kinase C (PKC) [33]. However, in the SP-mediated response of mast cells, the mechanisms of LT synthesis and histamine release and their regulation have remained poorly understood. Moreover, the role of neurokinin $(NK)_1$ receptor, thought to be concerned

with SP-induced activation of mast cells, has not been clearly defined yet. Some reports strongly indicate that SP stimulates mast cells via direct activation of G protein [34-35], but others suggest that this occurs via NK_1 receptors [37-39]. Indeed, the expression of mRNA for the NK_1 receptor was detected in mast cells of one mouse strain but not detected in others strains even under the same experimental conditions [22, 36, 37, 39]. In the present study, we demonstrated that the release of LTB_4 from BMMC induced by DNP-HSA and that by SP were both significantly inhibited by PD 098059, an ERK MAP kinase inhibitor, in all three mouse strains (Fig. 3, A-C, panels a and c). This result suggests that both the pathway for the release of LTB_4 from mast cells stimulated by SP and that by antigen are commonly regulated by $cPLA_2$ via the activation of ERK MAP kinase. On the contrary, the histamine release induced by SP was not affected by either PD 098059 or SB 203580 in three different mouse strains (Fig. 3, A-C, panels a and b). The same results were obtained when cells were activated by DNP-HSA in either NC/Kuj or C57/BL6 mice (Fig. 3, A and B, panels c and d). We have recently demonstrated that PD 098059 did not affect the histamine release from human skin tissue stimulated by SP or that by house dust mite antigen [40]. On the other hand, PD

098059 significantly inhibited the release of histamine induced by DNP-HSA in the BALB/c mouse strain (Fig. 3C, panel c). Recently, Ishizuka, et al. have also reported that PD 098059 significantly inhibited serotonin release from antigen-stimulated BMMC of the BALB/c mouse strain [41]. These results indicate that there is an alternative pathway via MAP kinase for the degranulation of mast cells in BALB/c mice. It is noteworthy that the release of LTB_4 from BMMC of BALB/c mice in response to DNP-HSA was also inhibited significantly by SB 203580, the p38 MAP kinase inhibitor (Fig. 3C, panel d). Moreover, SP-induced release of LTB_4 was slightly but significantly inhibited by SB 203580 in NC/Kuj mice (Fig. 3A, panel a). The significance of such functional variability of MAP kinase, summarized in Fig. 5, is unclear, but may account for symptomatic heterogeneities among individuals in skin disease concerned with mast cells.

We have also demonstrated that all BMMCs from the three mouse strains had acquired the responsiveness of $[Ca^{2+}]_i$ to SP, following co-culture with fibroblasts for three weeks. On the other hand, the extent of $[Ca^{2+}]_i$ increase in BMMC stimulated with DNP-HSA was significantly reduced following co-culture with 3T3 fibroblasts for three

weeks in all mouse strains. It is well-established that antigen-induced release of histamine and that of LTB_4 are dependent on the increase of $[Ca^{2+}]_i$ [33, 42]. Taken together, the increase of $[Ca^{2+}]_i$ is necessary for both histamine release and the synthesis of LTB_4, but does not necessarily correlated with degrees of mediator release. Fibroblasts may decrease the threshold of $[Ca^{2+}]_i$, required to activate its down-stream intracellular cascades.

Further studies of the precise mechanisms by which fibroblasts convert functions of BMMC may allow us to develop new therapies for neurogenic inflammation as well as to understand the mechanisms of tissue heterogeneities of mast cells.

4 Materials and methods

4.1. Animals

Six-week-old C57BL/6 and BALB/c male mice were purchased from Nihon SLC (Hamamatsu, Japan). NC/Kuj mice were kindly donated by Dr J Hayakawa, Institute for Experimental Animals, School of Medicine, Kanazawa University, Japan.

4.2. Chemicals

Mouse recombinant IL-3, IL-4, and SCF were purchased from R&D Systems Inc. (Minneapolis, MN, USA). Substance P and human serum albumin (HSA)-conjugated dinitrophenol (DNP) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Rat DNP-specific IgE monoclonal antibody was purchased from Bio-Source International (Camarillo, CA, USA). PD 098059 (2'-amino-3'-methoxyflavone) was purchased from Research Biochemicals International (Natick, MA, USA). SB 203580 ([4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole]) was kindly donated by SmithKline Beecham (Essex, UK). All other chemicals used in the

present study were of the highest grade and were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

4.3. Cell culture

The NIH/3T3 fibroblast cell line was obtained from Dainippon Pharmaceutical Co. Ltd (Osaka, Japan) and maintained in α-minimal essential medium (αMEM) supplemented with 5% bovine-serum, 2mM glutamine, 100 IU/ml penicillin G and 100 μg/ml streptomycin. BMMC from 6-week-old male mice were obtained as previously described [44]. Briefly, bone marrow cells were suspended at a density of 1×10^6 cells/ml in αMEM supplemented with 10% fetal calf-serum (FCS), 50 μM 2-mercaptoetanol, 2 mM glutamine, 100 IU/ml penicillin G, 100 mg /ml streptomycin, 5 ng/ml IL-3, and 2 ng/ml IL-4. The cells were cultured for 4 weeks, replacing half of the existing medium with fresh medium once a week. After 4 weeks, more than 98% of non-adherent cells were stained positively by alcian blue.

4.4. Co-culture of BMMC and 3T3 fibroblasts

The NIH/3T3 fibroblasts were suspended in αMEM supplemented with 5% FCS, 2mM glutamine, 100 IU/ml penicillin G, 100mg/ml streptomycin (5%-FCS-αMEM) at a density of 6×10^4 cells/ml. Aliquots of 0.5 ml cell suspension were seeded in 24 well-multi-dish plates and cultured until confluence. The BMMC suspension (3×10^4 cells/well) in 5%-FCS-αMEM were seeded on the NIH/3T3 fibroblast monolayers and cultured in the presence of 5 ng/ml SCF for three weeks. Half of the medium was replaced every 48 hours during the culture.

4.5. Release of histamine and LTB_4

BMMC cultured in the presence or absence of NIH/3T3 fibroblasts were incubated overnight with 0.5 μg/ml DNP-specific IgE. After washing twice with α-MEM, the cells were incubated with 500 μl 5%-FCS-α-MEM containing indicated concentrations of antigen (DNP-HSA) or SP for 20 min at 37˚C. The supernatants were transferred into test tubes and centrifuged (4˚C, 1500 × *g*, 5 min) to precipitate the cell components.

4.6. Measurement of histamine

After centrifugation, 200 μl of the supernatants were mixed with equal volumes of 0.5-N perchloric acid to precipitate proteins. Both cell pellets in test tubes and residual cells in culture dishes were resolved in 1 ml of 0.25-N perchloric acid to recover all residual histamine. Histamine contents in the samples were measured fluorometrically by using an automated histamine analyzing system (Tosoh Corporation, Osaka, Japan) as described before [43]. The histamine release was expressed as percentage of the total cellular histamine.

4.7. Measurement of LTB_4

The amount of LTB_4 in the samples was measured using enzyme-immunoassay kits (Amersham International, Buckinghamshire, U.K.). The measurements were performed according to the manufacture's instructions. The minimum detectable concentration of LTB_4 was 6.0 pg/ml. Cross-reactivity for other related substances was less than 0.03%. In order to verify the measurement of LTB_4 by enzyme-immunoassay, the supernatant of the reaction mixture in one experiment was fractionated by reverse phase high

performance liquid chromatography (HPLC), as described before [43]. The amount of LTB_4 in each fraction was measured as described above.

4.8. Pretreatment of cells with MAP kinase inhibitors

After passive sensitization with DNP-specific IgE, BMMC co-cultured with NIH/3T3 fibroblasts were incubated with 5%-FCS-αMEM containing indicated concentrations of PD 098059 for 30 min, or SB203580 for 15 min and challenged with 100 ng/ml DNP-HSA or 100 μM SP as described above. The release of histamine and LTB_4 was measured as described above. The results were expressed as percent inhibition by the treatment with PD 098059 or SB 203580 for histamine release and LTB_4 release respectively.

4.9. Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in BMMC

BMMC were co-cultured with NIH/3T3 fibroblasts in 75 cm^2 flask for 3 weeks, washed twice with Ca^{2+}- and Mg^{2+}-free-phosphate-buffered-saline (PBS(-)), incubated with PBS(-) containing 0.125% trypsin and 0.01% EDTA for 3 min, and then

re-suspended in 20 ml 5%-FCS-αMEM. After 20 min incubation, most of the 3T3 fibroblasts remained attached to the bottom of the flask, and the BMMC were recovered from the supernatants. After centrifugation, the non-adherent cells were resuspended in 2 ml 5%-FCS-αMEM containing 5 ng/ml SCF and incubated overnight on a fibronectin- coated cover glass in 35-mm-diameter dishes (MatTek Co. MA, USA) with 0.5 μg/ml DNP-specific IgE. BMMC cultured with IL-3 and IL-4 in the absence of the fibroblasts were also plated on the fibronectin-coated cover glass, similarly to those cultured in the presence of fibroblasts. The cells were rinsed twice with medium that containined 119 mM NaCl, 5mM KCl, 0.4 mM KCl, 0.4mM $MgCl_2$, 2mM $CaCl_2$, 5.6 mM glucose, 1mg/ml bovine serum albumin (BSA), 25 mM 4-(2-Hydroxymethyl)-1-piperazine ethanesulfonic acid and the pH was adjusted to 7.2 with NaOH. The cells were then loaded with 2 μM fura-3/acetoxymethyl ester in the medium at 37˚C for 60 min. Fura-3-loaded cells were placed on a fluorescence-imaging microscope (Olympus, Tokyo, Japan) and perfused with the medium at 37˚C. The fura-3 fluorescence was measured with the excitation wave lengths at 340 and 380 nM, and the emission wavelength at 510 nm. The video image output was digitized by the

Merlin high performance ratio fluorescence workstation® (Olympus, Tokyo, Japan).

4.10. Statistical Analysis

The data were analyzed by one way analysis of variance (ANOVA) and Dunn's post test to test significance of the differences among groups.

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Figure legend

Fig. 1. The release of histamine and LTB_4 from bone marrow derived mast cells (BMMC) in response to substance P (SP) or antigen (DNP-HSA). BMMC from three different strains of mice (NC (1A), C57/BL6 (1B) and BALB/c (1C)) were cultured with NIH/3T3 fibroblasts for three weeks. Cells were passively sensitized with IgE against antigen and incubated in medium with various concentrations of SP or antigen at 37℃ for 20 min. The amounts of histamine and LTB_4 were measured by HPLC (a, c; histamine) or ELISA (b, d; LTB_4) as described in Materials and Methods section. Values are means ± S.D. from one representative experiment with triplicate samples. Similar results were obtained in five independent sets of experiments.

Fig. 2. Detection of LTB_4 like immunoreactivity in the fractions of HPLC. The supernatants of the reaction mixture were fractionated by reverse-phase HPLC. The data shows a representative result of one sample. The amount of LTB_4 in each fraction was measured by EIA system as described in Materials and Methods section. LTB_4 like immunoreactivity was detected with the main peak in the tenth fraction, in which authentic LTB_4 (shown by arrow) was eluted under the same condition.

Fig. 3. The effects of MAP kinase inhibitors on the release of histamine (□) and LTB_4 (◆) induced by substance P (SP) or antigen (DNP-HSA). After co-culture with NIH/3T3 fibroblasts for three weeks, sensitized BMMCs from three different mice strains (NC (3A), C57/BL6 (3B) and BALB/c (3C)) were incubated with indicated concentrations of PD 098059 for 30 min (a, c) or SB 203580 for 15 min (b, d) and stimulated with 100 μM SP (a, b) or 100 ng/ml DNP-HSA (c, d) at 37°C for 20 min. The results are expressed as inhibitory ratios of histamine and LTB_4 release, respectively under each condition vs. those in the absence of inhibitors. The data are shown as means ± S.D. from three independent experiments. * $P < 0.05$, ** $P < 0.01$ (ANOVA and Dunn's test).

Fig. 4. Calcium signal in BMMC co-cultured with fibroblasts. After co-cultured with (b) or without (a) fibroblasts for three weeks, the sensitized BMMCs from three different mice strains (NC (4A), C57/BL6 (4B) and BALB/c (4C)) were loaded with fura-3 and stimulated with 100 μM SP (▽), 100 ng/ml DNP-HSA (▼) and 5 μM ionomycin (arrow) consequently. $[Ca^{2+}]_i$ levels were calculated by the ratio of fluorescence emitted by excitation at 340 and that at 380 nm over time.

Fig. 5. The effects of PD 098059 and SB 203580 on the release of histamine and LTB_4 induced by substance P or DNP-HSA.

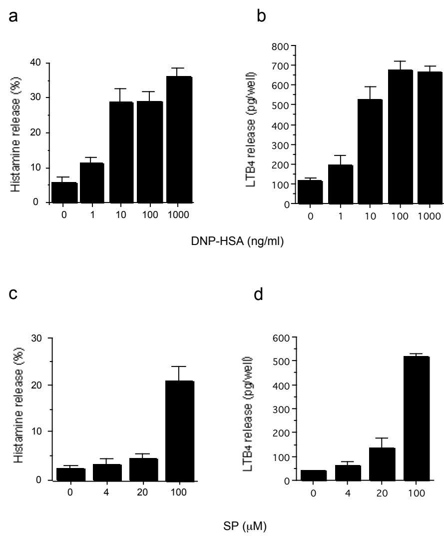


Fig. 1A.

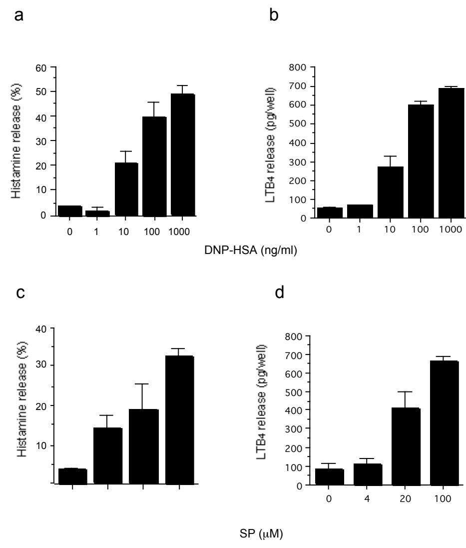


Fig. 1B.

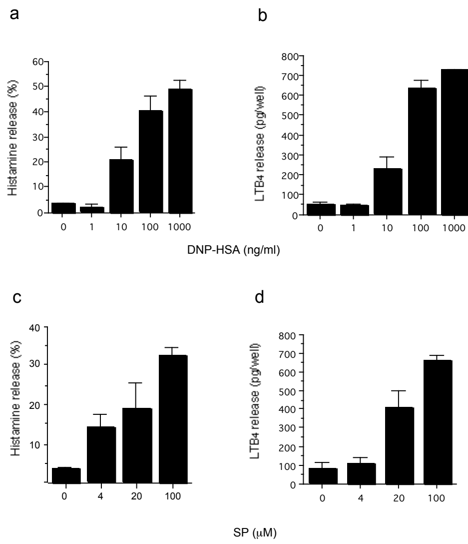


Fig. 1C.

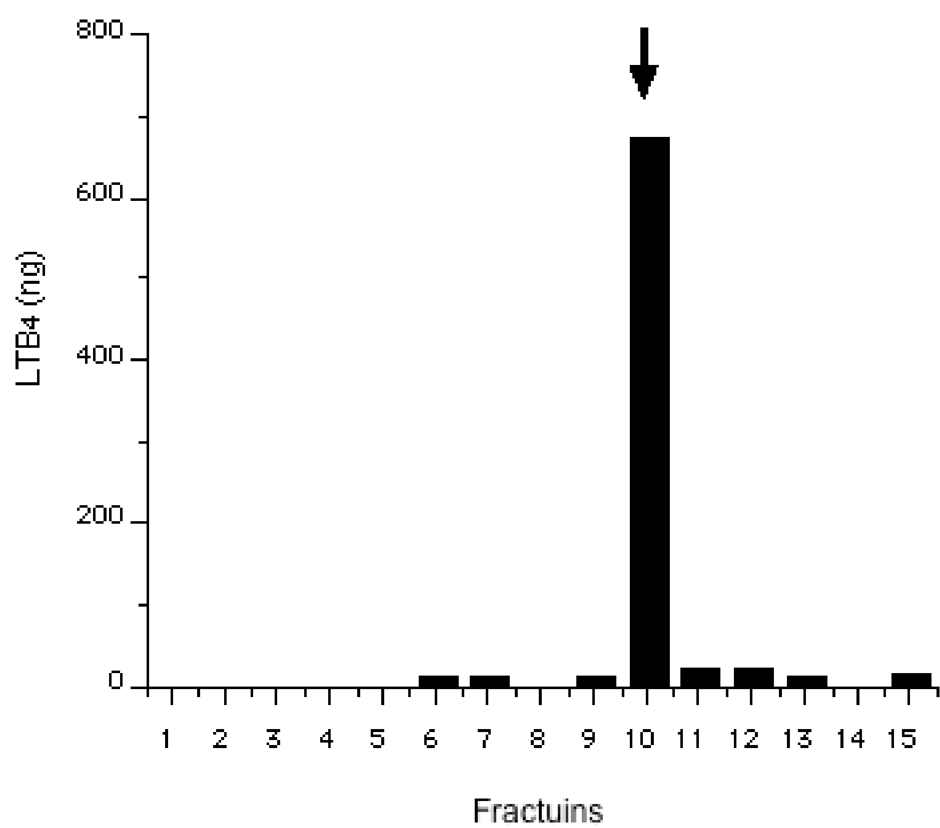


Fig. 2.

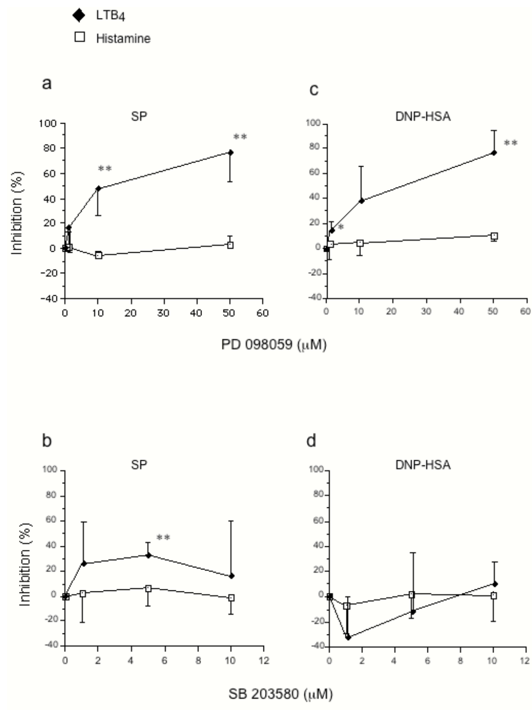


Fig. 3A.

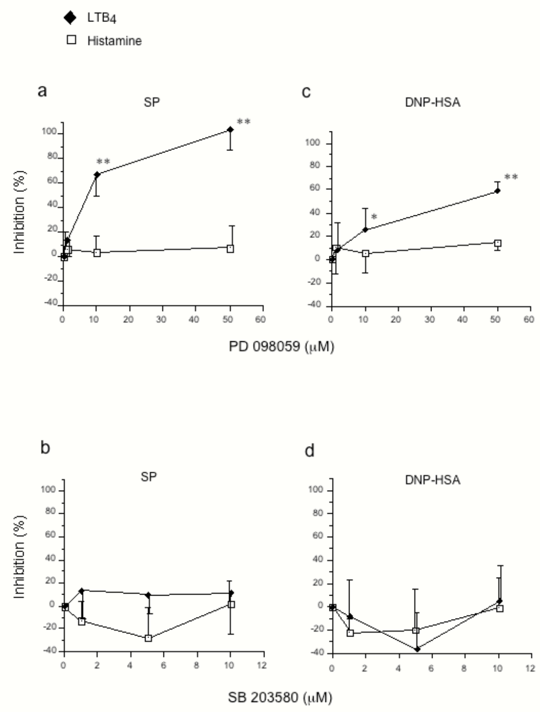


Fig. 3B.

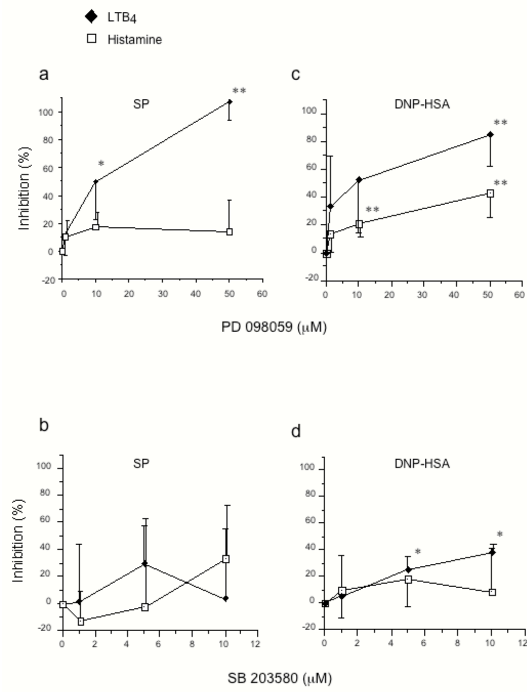
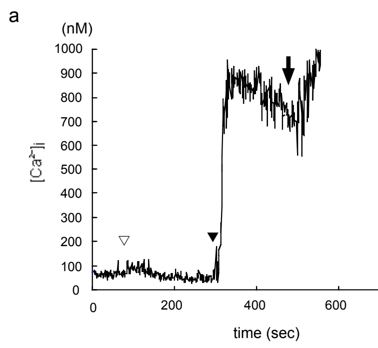


Fig. 3C.

a



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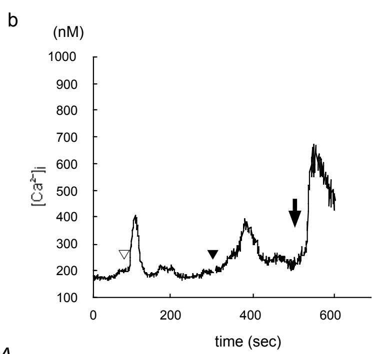
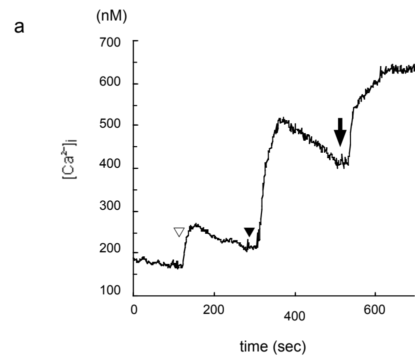


Fig. 4A.

a



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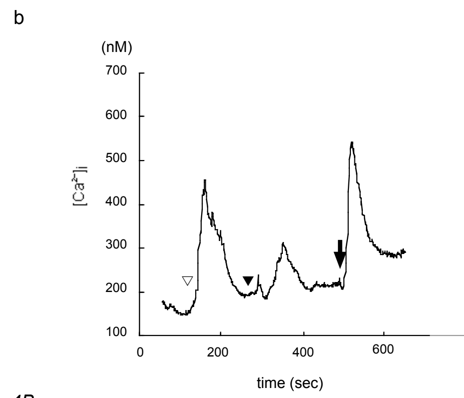
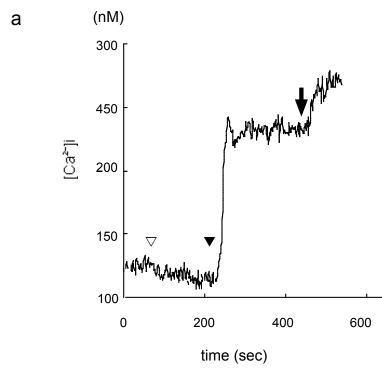


Fig. 4B.

a



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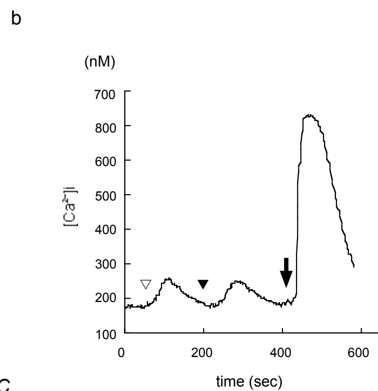

Fig. 4C.

strain		NC		C57/BL6		BALB/c	
inhibitor		PD	SB	PD	SB	PD	SB
DNP	histamine	→	→	→	→	↓	→
	LTB_4	↓	→	↓	→	↓	↓
SP	histamine	→	→	→	→	→	→
	LTB_4	↓	↘	↓	→	↓	→

no inhibition: →

strong inhibition: ↓

partial inhibition: ↘

Fig. 5.