



Inhibitory effects of a cationic liposome on allergic reaction mediated by mast cell activation



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ABSTRACT

Several studies have shown that cationic liposomes exert immunomodulatory effects with low immunogenicity and toxicity, and offer advantages such as easy preparation and targeting. Cationic liposomes not only transport DNA to immune cells but also enhance the function of antigen presenting cells such as dendritic cells and macrophages. Here, we investigated the effect of a particular cationic liposome on mast cell function during allergic reaction. We found that the cationic liposomes bound to the mast cell surface suppressed degranulation induced by cross-linking of high affinity immunoglobulin E receptors in a time- and dose-dependent manner. The suppression of degranulation was mediated by impairment of the sustained level of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) derived from the inhibition of store-operated Ca^{2+} entry. The decrease in sustained elevation of $[\text{Ca}^{2+}]_i$ led to the suppression of phosphorylation of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor proteins such as SNAP-23, syntaxin-4, which are necessary for membrane fusion between secretory granules and the plasma membrane during degranulation. Furthermore, the cationic liposomes suppressed vascular permeability elevation induced by mast cell activation in mice. These results showed that cationic liposomes possess the novel property of inhibiting mast cell activation, suggesting the possibility of developing cationic liposomes as anti-allergic effectors.

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1. Introduction

Cationic liposomes are used as a transfection reagent for nucleic acids, peptides, and proteins *in vitro* and *in vivo* [1–7]. Liposomes have recently attracted considerable attention as DNA vaccine carriers [8]. During these developmental studies, it was shown that cationic liposomes could enhance antigen uptake by antigen presenting cells (APC) such as dendritic cells (DC), B lymphocytes, and macrophages, and induce APC activation [8–14]. These properties have prompted further investigation of cationic liposomes as DNA vaccine carriers and APC activators. Furthermore, several reports have revealed that cationic liposomes activated specific signaling cascades, such as Toll-like receptor 4-dependent pathways [15,16], mitogen-activated protein (MAP) kinase cascades [17,18], and other signaling cascades in immunocompetent cells [19]. It is essential to investigate the effect of cationic liposomes on immunocompetent cell function for the

development of DNA vaccine therapy and as potential therapeutic tools.

Mast cells express high-affinity immunoglobulin E (IgE) receptors (FcεRI) on their surface [20]. Cross-linking of FcεRI on mast cells by a multivalent antigen induces the activation of several protein tyrosine kinases and the phosphorylation of phosphatidylinositol 3-kinase (PI3K). PI3K activates phospholipase Cγ (PLCγ), which leads to the production of inositol 1,4,5-triphosphate, thereby eliciting the elevation of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) by releasing Ca^{2+} from the endoplasmic reticulum (ER). The decrease in Ca^{2+} concentration in ER induces the binding of stromal interaction molecule 1 on the ER to Orai1 on the plasma membrane. This permits Ca^{2+} influx through the plasma membrane and is known as store-operated Ca^{2+} entry (SOCE). PLCγ also catalyzes the release of diacylglycerol, which in turn activates protein kinase C (PKC) [21]. The activation of intracellular signaling cascades by FcεRI ligation leads to the exocytotic release of granular contents (degranulation), resulting in allergic responses such as an increase in vascular permeability and stimulation of smooth muscle cell contraction [22–24]. With regard to exocytotic release, several recent reports have shown that soluble *N*-ethylmaleimide-sensitive factor attachment protein

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receptor (SNARE) proteins are involved in membrane fusion between the plasma membrane and secretory granules [25–29].

The aim of this study was to examine the effects of cationic liposomes on allergic responses mediated by mast cell activation. Although there are reports pertaining to the interaction between mast cells and nanoparticles, such as gold, polymeric nanoparticles, and fullerene [30,31], only a limited number of studies have focused on the effects of cationic liposomes on mast cell function. Cationic liposomes containing 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE) and cholesteryl-3 β -carboxyamidoethylene-*N*-hydroxyethylamine (OH-Chol) were used in this study because they were found to show high transfection efficiency of foreign genes to target cells as a result of excellent interaction with the cells [32–35]. We found that the cationic liposomes could suppress mast cell degranulation by impairing SOCE *in vitro* and diminish the increase in vascular permeability induced by mast cell activation *in vivo*. Our results reveal the potential of cationic liposomes as a useful therapeutic tool for allergic disease mediated by mast cell activation.

2. Materials and methods

2.1. Materials

The synthesis of the cationic cholesterol derivative OH-Chol was described in our previous study [32] and DOPE was purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse anti-dinitrophenyl (DNP)-IgE was provided by Prof. T. Kishimoto (Osaka University). DNP-conjugated bovine serum albumin (DNP-BSA) was prepared using previously described methods [36]. In the present experiments, six DNP groups, on average, were conjugated with a single BSA molecule. Thapsigargin, phorbol-12-myristoyl-13-acetate (PMA), and Evans blue were purchased from Wako Pure Chemical Industries (Osaka, Japan). FITC-conjugated goat anti-IgE antibody was purchased from Bethyl laboratories Inc. (Montgomery, TX, USA). MTT labeling reagent was purchased from Roche Diagnostics (Mannheim, Germany).

2.2. Preparation of liposomes

The cationic liposomes were prepared by ultrasonication methods according to our previously described procedures [33–35]. In brief, DOPE (20 nmol) and OH-Chol (30 nmol) were mixed and dried with N₂ gas under reduced pressure to remove the chloroform solvent. The lipid film was hydrated with 400 μ l of phosphate buffered saline (PBS) for 1 h. Samples were sonicated in a bath-type sonicator (Branson model B 1200) to generate small unilamellar vesicles.

2.3. Cell culture

The rat mast cell line RBL-2H3, which is the most widely used as a model for mast cells [37], was cultured in minimum essential medium (Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum (Gibco, Grand Island, NY, USA).

2.4. Cell viability assay

Cultured RBL-2H3 cells were seeded in 96-well plates at a density of 5×10^3 cells/well 24 h before introduction of the cationic liposomes. Cells were incubated with the different concentrations of cationic liposomes for 3 h and washed in PBS. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT assay is based on the cleavage of the yellow tetrazolium salt to purple formazan crystals by viable cells. After addition of the cationic liposomes, 10 μ l of MTT labeling

reagent was added to each well and incubated for 4 h at 37 °C. After addition of 100 μ l of solubilization solution, cells were incubated overnight at 37 °C. The absorbance at 570 nm was measured using a plate reader (Model 680; BIO-RAD, Tokyo, Japan).

2.5. Confocal laser scanning microscopy (CLSM)

We observed the distribution of IgE on the surface of RBL-2H3 cells using FITC-conjugated goat anti-IgE antibody. RBL-2H3 cells were incubated with 46.5 μ g/ml cationic liposomes for 3 h and washed in HEPES buffer containing 10 mM HEPES (pH 7.2), 140 mM NaCl, 5 mM KCl, 0.6 mM MgCl₂, 1 mM CaCl₂, 0.1% glucose, 0.1% BSA, and 0.01% sulfinpyrazone. After sensitizing the RBL-2H3 cells with anti-DNP IgE for 30 min at 37 °C, they were incubated with FITC-conjugated goat anti-IgE antibody for 15 min at 4 °C. The distribution of IgE was scrutinized in an observation chamber (Elecon, Chiba, Japan) by CLSM (LSM 510; Zeiss, Oberkochen, Germany). The FITC-conjugated goat anti-IgE antibody was excited by an Ar ion laser (488 nm), and FITC fluorescence was detected through a band-pass filter at 505–530 nm. Liposomes were visualized by excitation of rhodamine-conjugated phosphatidylethanolamine (3% in lipids composed of liposomes) by a He–Ne laser (543 nm), and rhodamine fluorescence was detected through a band-pass filter at 560–580 nm.

2.6. Measurement of degranulation

Mast cell degranulation (exocytotic release) was monitored by measuring the activity of the granule-stored enzyme β -hexosaminidase, which is secreted into cell supernatants [38]. RBL-2H3 cells (5×10^4 cells/well) plated in a 24-well plate and cultured overnight were sensitized with anti-DNP IgE for 30 min at 37 °C and washed twice with HEPES buffer. Aliquots of mast cell supernatant were transferred to a 96-well plate (20 μ l/well) and incubated with 20 μ l of substrate solution (2 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide (Sigma-Aldrich) in 100 mM citrate [pH 4.5]) for 1 h at 37 °C. After terminating the reaction with 160 μ l of 167 mM Na₂CO₃–NaHCO₃ buffer, the absorbance was measured at 405 nm using a microplate reader. The release activity relative to the total β -hexosaminidase content of cells was determined by disrupting the cells with 0.1% Triton X-100.

2.7. Measurement of [Ca²⁺]_i

RBL-2H3 cells (5×10^5 cells) were loaded with 1 μ M Fura 2-AM (Molecular Probes, Eugene, OR, USA.), sensitized with anti-DNP IgE for 30 min at 37 °C, and then washed twice with HEPES buffer. Fluorescence intensities at 500 nm using excitation wavelengths of 340 and 360 nm were measured at 37 °C, and the F_{340}/F_{360} ratio was calculated using a spectrofluorometer connected to a personal computer (RF-5300PC; Shimadzu, Kyoto, Japan). Ratios were converted to Ca²⁺ concentrations by a previously described procedure [39].

2.8. Phosphorylation assay of SNARE proteins

Enrichment and separation of phosphorylated SNARE proteins from cell lysates was performed using a Phosphoprotein Purification Kit (Qiagen, Hilden, Germany) according the manufacturer's instructions [40]. After pretreatment of RBL-2H3 cells with the cationic liposomes for 3 h at 37 °C in a CO₂ incubator, RBL-2H3 cells (1×10^7 cells) were washed twice with HEPES buffer, and stimulated for 15 min at 37 °C. The reaction was terminated with ice-cold TBS (137 mM NaCl, 2.68 mM KCl, 25 mM Tris–HCl) and cells were harvested. The collected cells were lysed for 30 min on ice with lysis buffer containing 0.25% CHAPS. After centrifugation

(10,000 × g, 30 min at 4 °C), cleared lysates were loaded onto equilibrated phosphoprotein purification columns supplied with the kit and washed with 6 ml of lysis buffer. The phosphorylated proteins were eluted using elution buffer containing 0.25% CHAPS. Samples were separated by SDS-PAGE and were subjected to Western blotting analysis. Western blotting was performed using primary antibodies: rabbit polyclonal anti-SNAP-23 (Göttingen, Germany) (1:1000) and rabbit polyclonal anti-syntaxin-4 (Göttingen, Germany) (1:2000). Immunoreactivities were detected with horseradish peroxidase-conjugated anti-rabbit IgG (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) using an LAS-3000 mini image analyzer (Fuji Film, Tokyo, Japan).

2.9. Effect of the cationic liposome on vascular permeability in mice

BALB/c mice were housed under standard conditions and in compliance with Aichi Gakuin University guidelines for the use of experimental animals. All animal experiments were performed after approval by the Animal Care and Use Committee of Aichi Gakuin University. Evans blue (40 mg/g body weight; Sigma-Aldrich) was intravenously administered into BALB/c mice 1 h after subcutaneous injection of 200 μ l of cationic liposomes into the abdomen. Thereafter, allergic reactions were induced by subcutaneous injection of 100 μ l of compound 48/80 (0.06 mg/ml) into the abdomen. Mice were sacrificed after 30 min and their skins were harvested with 1N KOH for 24 h at 37 °C. Evans blue was extracted in 5 ml of 0.2 M phosphoric acid and acetone mixture (5:13). After centrifugation (3000 rpm, 10 min), the absorbance of the supernatant at 620 nm was determined using a spectrophotometer (UV-1800; Shimadzu) and normalized for protein amount determined by bicinchoninic acid (BCA) assay.

2.10. Statistical analysis

Results are expressed as mean $\pm$ standard error (S.E.). A one-way analysis of variance (ANOVA) was used to compare differences between groups. A *p*-value of <0.05 was considered significant.

3. Results

3.1. Effects of the cationic liposomes on mast cell viability

Although cationic liposomes were considered to be a promising method of introducing a foreign gene or protein into target cells

with low toxicity, an excess of cationic liposomes has been shown to induce cytotoxicity and apoptosis that is dependent on cell type, liposome size, and cationic composition [11,12,41,42]. Therefore, we first determined the non-toxic concentration of cationic liposomes by liposome-pretreatment of mast cells followed by the MTT assay to evaluate cell viability. Cell viability was maintained above 80% 3 h after the addition of 11.6, 23.3, and 46.5 μ g/ml cationic liposomes to RBL-2H3 cells. However, cell viability fell below 80% when the concentration of cationic liposomes added to RBL-2H3 cells was increased to 69.8 μ g/ml (data not shown). On the basis of these results, subsequent exposure of RBL-2H3 cells to cationic liposomes was limited to concentrations of less than 46.5 μ g/ml.

Next, we observed the distribution of anti-DNP IgE on RBL-2H3 cell surface to investigate whether adhesion of cationic liposomes to the plasma membrane influenced antigen (DNP-BSA)-induced IgE aggregation, which is a trigger for mast cell activation. IgE was distributed along the plasma membrane of RBL-2H3 cells pretreated with or without cationic liposomes (46.5 μ g/ml) for

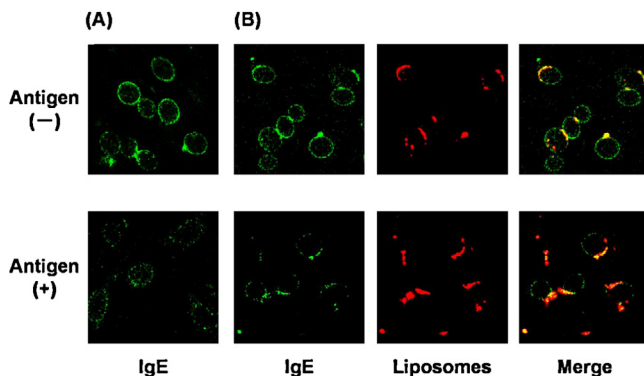


Fig. 1. The distribution of IgE on the plasma membrane of RBL-2H3 cells. (A) Typical confocal fluorescence images of IgE on the plasma membrane of cells in the absence of liposomes before and after antigen (DNP-BSA; 50 ng/ml) addition (for 5 min) (*n* = 6). (B) Typical confocal fluorescence images of IgE and cationic liposomes on the plasma membrane of cells pretreated with cationic liposomes (46.5 μ g/ml) for 3 h before and after antigen (DNP-BSA; 50 ng/ml) addition (for 5 min) (*n* = 6).

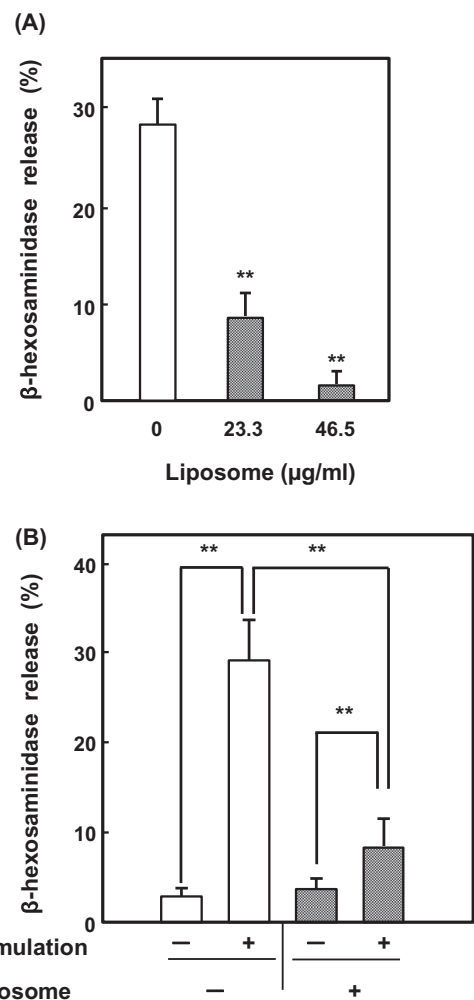


Fig. 2. Inhibition of β -hexosaminidase release from mast cells by the cationic liposomes. (A) RBL-2H3 cells were sensitized with anti-DNP IgE for 30 min at 37 °C. The quantity of β -hexosaminidase released from RBL-2H3 cells for 30 min after antigen (DNP-BSA; 50 ng/ml) addition. Cells were pretreated with the indicated concentration of cationic liposomes for 3 h. Values are expressed as the percent of total enzyme activity, which was assessed by Triton X-100 treatment (*n* = 6). ****p* < 0.01 compared with the absence of cationic liposomes (0 μ g/ml). (B) The quantity of β -hexosaminidase released from RBL-2H3 cells for 30 min after costimulation with thapsigargin (20 nM) and PMA (50 ng/ml). Cells were pretreated with cationic liposomes (46.5 μ g/ml) for 3 h. Values are expressed as the percent of total enzyme activity (*n* = 6) (***p* < 0.01).

3 h: the amount of IgE bound to FcεRI on the cells was similar in both treatments (Fig. 1 upper panels). A punctate distribution of IgE was observed in antigen-stimulated cells as shown in Fig. 1A lower panels. The change in antigen-induced IgE distribution in cells pretreated with the cationic liposomes was similar to that observed in the absence of liposomes, as shown in Fig. 1B. These data suggested that the cationic liposomes affected neither the binding of IgE to FcεRI nor the distribution of IgE on the plasma membrane induced by antigen addition.

3.2. Effects of the cationic liposomes on degranulation

The effects of the cationic liposomes on degranulation in RBL-2H3 cells were examined. When RBL-2H3 cells were pretreated with cationic liposomes at 23.3 or 46.5 μg/ml for 3 h, the amount of β-hexosaminidase released for 30 min after antigen addition was significantly decreased in a dose-dependent manner, as shown in Fig. 2A. To study the inhibitory pathway of degranulation by the cationic liposomes, we stimulated RBL-2H3 cells with thapsigargin and PMA. Costimulation with thapsigargin and PMA is often used to mimic antigen stimulation without cross-linking of FcεRI because thapsigargin and PMA induce Ca²⁺ leakage from the ER by inhibition of the sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPases and activation of PKC, respectively. The cationic liposomes significantly inhibited degranulation caused by costimulation of PMA and

thapsigargin, as shown in Fig. 2B. When RBL-2H3 cells were stimulated by thapsigargin or PMA individually, degranulation did not increase with or without cationic liposomes pretreatment (data not shown). This suggested that pretreatment with cationic liposomes suppressed degranulation of antigen-activated RBL-2H3 cells by inhibiting the intracellular signaling pathway after cross-linking of FcεRI.

3.3. Effects of the cationic liposomes on [Ca²⁺]_i

Mobilization of [Ca²⁺]_i has a marked effect on degranulation; therefore, [Ca²⁺]_i in RBL-2H3 cells was measured after antigen stimulation in order to elucidate step at which the cationic liposomes inhibited the intracellular signaling pathway. The increase in [Ca²⁺]_i in RBL-2H3 cells was maintained at a high concentration after antigen addition. When RBL-2H3 cells pretreated for 3 h with cationic liposomes were stimulated with antigen, the increase in [Ca²⁺]_i diminished in a dose-dependent manner, as shown in Fig. 3A and B. Furthermore, the reduction of the antigen-induced maintained elevation of [Ca²⁺]_i was dependent on the duration of cationic liposome-pretreatment as shown in Fig. 3C and D. Subsequently, we investigated whether the cationic liposomes suppressed the increase in [Ca²⁺]_i after costimulation with thapsigargin and PMA. While costimulation with thapsigargin and PMA led to a marked increase in [Ca²⁺]_i in untreated RBL-2H3 cells, pretreatment with

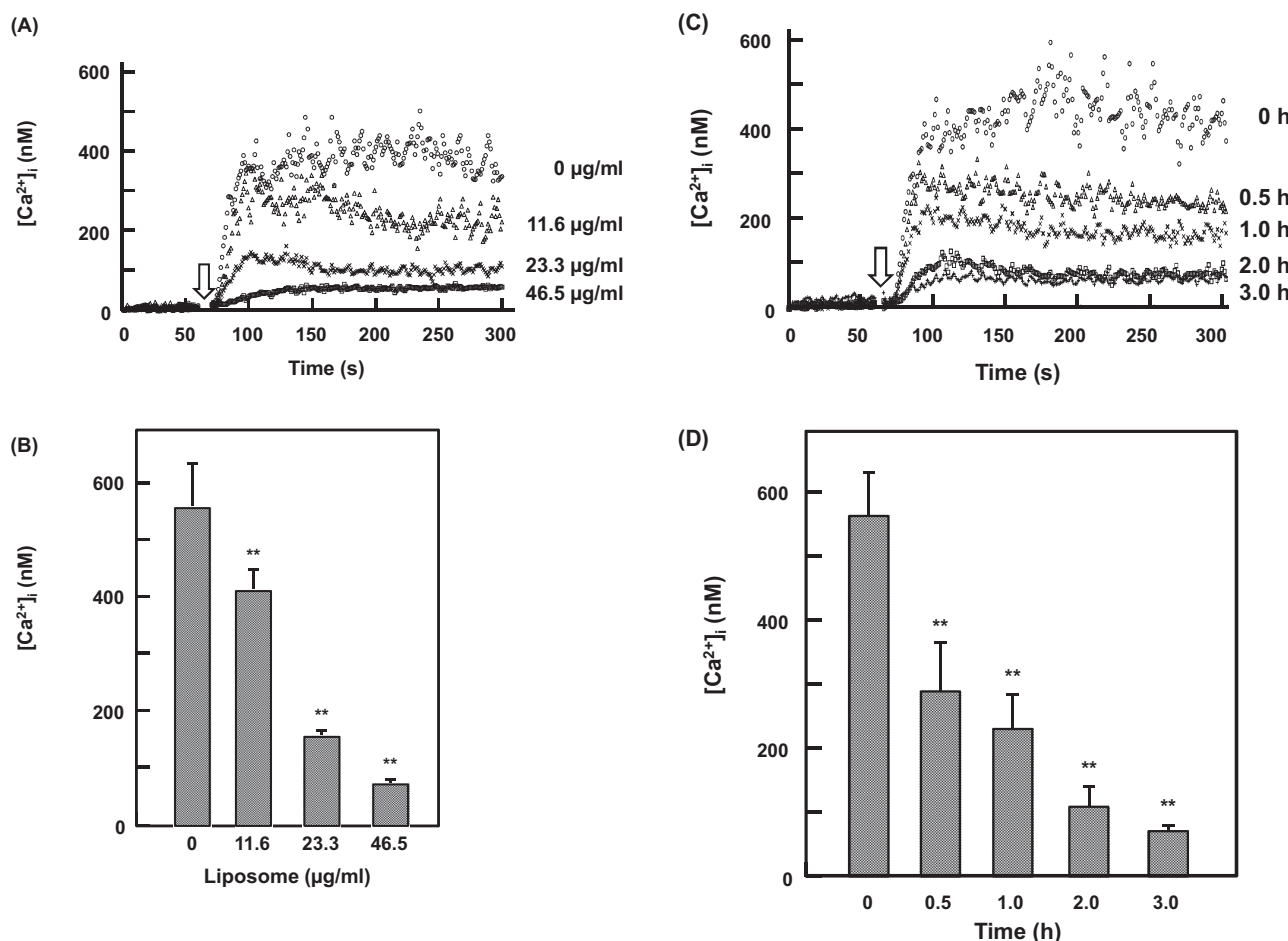


Fig. 3. The increase in [Ca²⁺]_i in RBL-2H3 cells after antigen stimulation. Cells incubated with anti-DNP IgE and Fura 2-AM for 30 min were stimulated with antigen (DNP-BSA; 50 ng/ml) at the time indicated by arrows (60 s). A typical time-course of [Ca²⁺]_i is shown from 3 independent experiments. (A) A typical time-course of [Ca²⁺]_i in RBL-2H3 cells pretreated with the indicated concentration of cationic liposomes for 3 h. (B) The average [Ca²⁺]_i in (A) after antigen stimulation. (C) The time-course of [Ca²⁺]_i in RBL-2H3 cells pretreated with cationic liposomes (46.5 μg/ml) for the indicated period. (D) The average [Ca²⁺]_i in (C) after antigen stimulation.

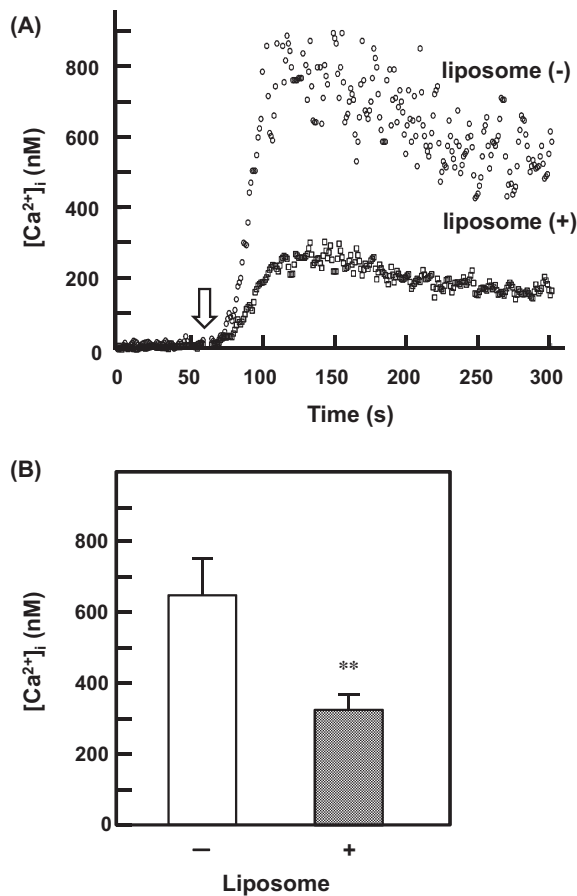


Fig. 4. Effect of the cationic liposomes on the increase in $[Ca^{2+}]_i$ after costimulation with thapsigargin and PMA. (A) A typical time-course of $[Ca^{2+}]_i$ in RBL-2H3 cells pretreated with and without cationic liposomes (46.5 $\mu\text{g}/\text{ml}$) for 3 h. Cells were stimulated with thapsigargin (20 nM) and phorbol-12-myristoyl-13-acetate (50 ng/ml) at the time indicated by an arrow. A representative result is shown from 3 independent experiments. (B) Comparison with the increased $[Ca^{2+}]_i$ in costimulated RBL-2H3 cells pretreated with and without cationic liposomes (46.5 $\mu\text{g}/\text{ml}$). ** $p < 0.01$ compared with the absence of cationic liposomes.

cationic liposomes (46.5 $\mu\text{g}/\text{ml}$) for 3 h diminished the increase in $[Ca^{2+}]_i$, as shown in Fig. 4A. Comparison of the average $[Ca^{2+}]_i$ elevations showed that pretreatment with cationic liposomes significantly suppressed the increase in $[Ca^{2+}]_i$ after costimulation with thapsigargin and PMA, as shown in Fig. 4B. These results suggested that the cationic liposomes inhibited Ca^{2+} release from the ER and/or Ca^{2+} entry through the plasma membrane in activated RBL-2H3 cells.

3.4. Inhibition of SOCE by the cationic liposomes

To address the mechanism of diminished $[Ca^{2+}]_i$ elevation by cationic liposomes, RBL-2H3 cells were stimulated with antigen in the absence of extracellular Ca^{2+} . As shown in Fig. 5A, there was no difference in $[Ca^{2+}]_i$ elevation induced by antigen in the absence of extracellular Ca^{2+} in RBL-2H3 cells pretreated with or without cationic liposomes (46.5 $\mu\text{g}/\text{ml}$) for 3 h. The same result is shown for the average $[Ca^{2+}]_i$ elevation in Fig. 5B (white bars). However, the subsequent addition of Ca^{2+} (3 mM) to the extracellular buffer, which induced SOCE through the plasma membrane, showed that pretreatment with cationic liposomes significantly inhibited SOCE (Fig. 5A and B; gray bars). This indicated that the cationic liposomes inhibited SOCE but not Ca^{2+} release from ER in activated mast cells.

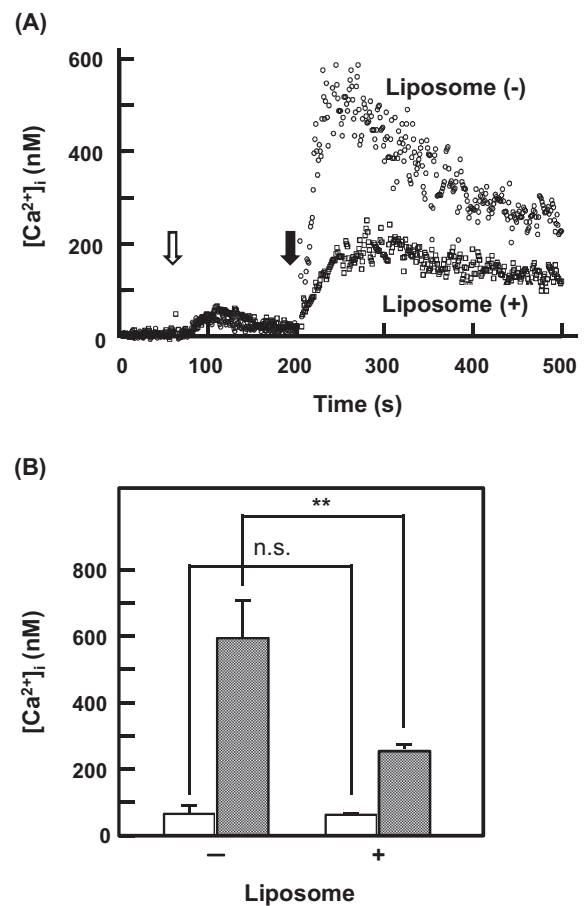


Fig. 5. Effect of the cationic liposomes on Ca^{2+} release from ER and SOCE. RBL-2H3 cells incubated with anti-DNP IgE and Fura 2-AM for 30 min were stimulated with antigen (DNP-BSA; 50 ng/ml) at the time indicated by the white arrow (60 s) in Ca^{2+} -free HEPES buffer. The addition of Ca^{2+} (3 mM) was subsequently made to the extracellular buffer at the time indicated by the black arrow (200 s), when $[Ca^{2+}]_i$ returned to a basal level. The average $[Ca^{2+}]_i$ elevation in RBL-2H3 cells pretreated with and without cationic liposomes (46.5 $\mu\text{g}/\text{ml}$) for 3 h is shown before (white bars) and after (gray bars) Ca^{2+} addition to the extracellular buffer ($n = 4$) (** $p < 0.01$; n.s., not significant).

3.5. Inhibition of SNARE phosphorylation by the cationic liposomes

SNAREs are widely accepted to play a major role in the exocytotic activity of mast cells [25–29]. Here, we detected the phosphorylation of SNAP-23 and syntaxin-4 SNARE proteins, which are reported to have major roles in the exocytic pathway in RBL-2H3 cells. Both SNAP-23 and syntaxin-4 were phosphorylated after antigen stimulation, whereas pretreatment with cationic liposomes for 3 h significantly inhibited their phosphorylation, as shown in Fig. 6A and B. This suggested that the cationic liposome-mediated decrease in sustained $[Ca^{2+}]_i$ after antigen addition led to the inhibition of SNAP-23 and syntaxin-4 phosphorylation.

3.6. Inhibition of vascular permeability by cationic liposomes in mice

The effect of cationic liposomes was investigated on immediate-type allergic reaction in mice because the cationic liposomes were found to suppress degranulation in mast cells by inhibiting SOCE and SNARE phosphorylation *in vitro*. The specific mast cell activator compound 48/80 was subcutaneously injected into the abdomen of mice that had received an intravenous injection of Evans blue. Leakage of the dye from blood vessels to skin tissue was observed. The amount of leakage was lesser in mice that had been subcutaneously injected with the cationic liposomes than in

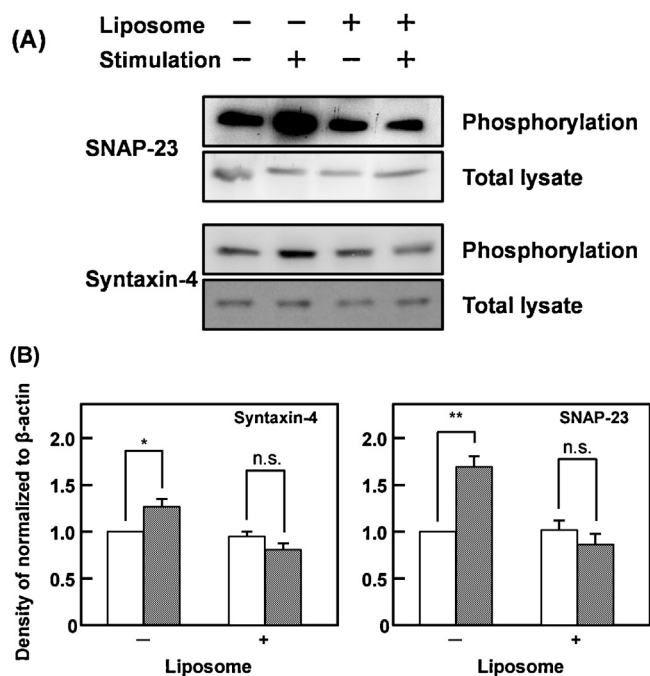


Fig. 6. Inhibition of SNARE phosphorylation by the cationic liposomes. RBL-2H3 cells pretreated with and without cationic liposomes (46.5 μ g/ml) for 3 h were stimulated with DNP-BSA for 30 min. The phosphorylation levels of SNAP-23 and syntaxin-4 were analyzed by Western blotting. (A) Upper panels for each protein show the amount of phosphorylated protein eluted from the phosphoprotein column. Lower panels for each protein show the amount of total protein. (B) Relative optical density was normalized to β -actin. $n = 4$ for SNAP-23, $n = 5$ for syntaxin-4 (** $p < 0.01$, * $p < 0.05$ compared with the absence of cationic liposomes).

non-treated mice, as shown in Fig. 7. This suggested that the cationic liposomes suppressed the elevation in vascular permeability induced by mast cell activation, which is an important symptom of immediate-type allergic reaction in mice.

4. Discussion

Cationic liposomes are used as vectors for effective means of introducing foreign genes to target cells; a function that is aided by their low immunogenicity and toxicity, and the advantages offered by easy preparation and targeting [1–7]. Because cationic liposomes are known to affect immunocompetent cell function [11–14], we investigated their influence on mast cell activation *in vitro* and allergic reaction *in vivo*. On the basis of our previously obtained data [32–35], the cationic liposomes used in this study consisted of DOPE and a cholesterol derivative containing a hydrophilic amino head group, OH-Chol. The cationic liposomes attached to the plasma membrane of mast cells (Fig. 1) as previously observed during gene transfection experiments using the same liposomes and target cells such as NIH-3T3 cells. The attached liposomes affected neither the binding of IgE and Fc ϵ RI nor antigen-induced cross-linking of Fc ϵ RI, suggesting that the events on the plasma membrane involved in activation of mast cells occurred in liposome-attached cells in a similar manner to non-treated cells.

Despite antigen-induced cross-linking of Fc ϵ RI, degranulation was significantly inhibited in mast cells pretreated with cationic liposomes in a dose-dependent manner (Fig. 2A). The cationic liposomes also suppressed degranulation induced by costimulation with thapsigargin and PMA, which led to mast cell activation without cross-linking of Fc ϵ RI by directly increasing $[Ca^{2+}]_i$ and activating PKC, respectively (Fig. 2B). From these results, we hypothesized that suppression of degranulation by the cationic

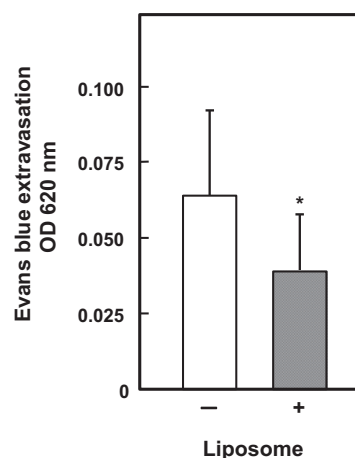


Fig. 7. Inhibition of increased vascular permeability in mice by injection of the cationic liposomes. Evans blue was intravenously administered to mice 1 h after subcutaneous injection of the cationic liposomes into the abdomen. Compound 48/80 was then subcutaneously injected into the abdomen to induce allergic reaction. The absorbance (at 620 nm) of Evans blue in skin harvested 30 min after compound 48/80 administration is shown. Data are presented as extravasated Evans blue dye normalized for protein amount ($n = 9$). * $p < 0.05$ compared with values obtained in the absence of cationic liposomes.

liposomes was not attributable to inhibition of a signaling process prior to $[Ca^{2+}]_i$ elevation and PKC activation. To address the step in the degranulation process that was inhibited by the cationic liposomes, we investigated the effect on $[Ca^{2+}]_i$ mobilization. Measurement of Ca^{2+} release from ER and subsequent SOCE (Ca^{2+} entry through the plasma membrane in response to the decrease in Ca^{2+} concentration in the ER) revealed that the cationic liposomes inhibited Ca^{2+} release from SOCE but not from ER. SOCE is known to play an essential role in degranulation of antigen-stimulated mast cells because degranulation is attenuated in mast cells stimulated in the absence of extracellular Ca^{2+} [43–46]. These results suggested that the cationic liposomes suppressed degranulation by inhibiting SOCE in activated mast cells.

Furthermore, we investigated the effect of the cationic liposomes on the phosphorylation of SNARE proteins that are essential for exocytotic release in non-neuronal secretory cells [47,48]. In mast cells, phosphorylation of SNARE proteins, such as SNAP-23 and syntaxin-4, also played significant roles in membrane fusion between secretory granules and the plasma membrane [26,49–51]. We found that the antigen-induced phosphorylation of SNAP-23 and syntaxin-4 was inhibited by cationic liposomes (Fig. 6). We previously showed that the exocytotic release involved in SNARE proteins required a sustained increase in $[Ca^{2+}]_i$ for membrane fusion between secretory granules and the plasma membrane [52]. The inhibition of SNARE phosphorylation by the cationic liposomes was considered to result from the decrease in the sustained level of $[Ca^{2+}]_i$ by inhibition of SOCE. As shown in Fig. 6, phosphorylation of SNAP-23 and syntaxin-4 were detected in RBL-2H3 stimulated by antigen with or without cationic liposome pretreatment. Previous study also showed that phosphorylation of SNAP-23 and syntaxin-4 in RBL-2H3 or mast cells were detected when cells were not stimulated [50,53]. Thus we thought that there might be components in cell culture medium to phosphorylate the SNAP-23 and syntaxin-4 without stimulation.

Adherence of the cationic liposomes to the plasma membrane appeared to diminish the function of store-operated Ca^{2+} channels (SOC) on the plasma membrane. However, the mechanism of SOC inhibition by the cationic liposomes remains to be elucidated. The cationic liposomes, which are positively charged on the surface, are able to attach to negatively charged cell surfaces. Several studies have shown that cationic liposomes could bind to membrane

protein components, such as proteoglycans and glypican, and to negatively charged extracellular matrix cell components [54,55]. Although more detailed studies are required, the cationic liposomes may inhibit SOC function by physically masking channel components or by distorting channel structure. For example, plasma membrane lipid raft domains, which contains the caveolin involved in the generation of caveolae lipid raft, anchors STIM1 (stromal interaction molecule 1) at specific ER-plasma membrane junction, regulating the SOCE [56]. Meanwhile, several studies showed that some cationic liposomes could enter cells via caveolae-mediated pathways [57–59]. Therefore cationic liposomes have the possibility to inhibit the SOCE by adhesion of cationic liposomes to the plasma membrane lipid raft domain.

Finally, we investigated whether the cationic liposomes could inhibit allergic reaction mediated by mast cell activation *in vivo*. Some inflammatory mediators released by degranulation, such as histamine, enhance blood vessel permeability [60]. We measured vascular protein leakage using Evans blue, which can bind to albumin leaked from blood vessels when vascular permeability is increased by mast cell activation [61]. The cationic liposomes showed suppression of vascular protein leakage to the skin in response to mast cell activation (Fig. 7). This suggested that the cationic liposomes are able to inhibit at least the vascular leakage component of the allergic reaction mediated by mast cells *in vivo*.

In this study, we showed that cationic liposomes composed of DOPE and OH-Chol had the ability to inhibit activation of mast cells *in vitro* and to diminish induced vascular permeability. The cationic liposomes suppressed SOCE, leading to a decrease in the sustained level of $[Ca^{2+}]_i$ and inhibition of SNARE phosphorylation consequently leading to suppression of mast cell degranulation. To our knowledge, the inhibitory effect on allergic reaction demonstrates a novel property of cationic liposomes. The potential of cationic liposomes as anti-allergic drug carriers possessing anti-allergic action is a novel feature of cationic liposomes demonstrated in this study, which may lead to the development of innovative treatments for allergic diseases.

In conclusion, these findings suggest that the cationic liposomes used in this study exert an anti-inflammatory effect by inhibiting mast cell degranulation *in vitro* and *in vivo*. These data reveal novel properties of cationic liposomes and have the potential for development into efficient liposome-based therapeutic approaches to allergy diseases.

References

- [1] Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, et al. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci USA* 1989;84:7413–7.
- [2] Farhood H, Bottega R, Epan RM, Huang L. Effect of cationic cholesterol derivatives on gene transfer and protein kinase C activity. *Biochem Biophys Res Commun* 1992;1111:239–46.
- [3] Lasic DD. Novel applications of liposomes. *Trends Biotechnol* 1998;16:307–21.
- [4] Nishiyama M, Iriyama A, Jang WD, Miyata K, Itaka K, Inoue Y, et al. Light-induced gene transfer from packaged DNA enveloped in a dendrimeric photosensitizer. *Nat Mater* 2005;4:934–41.
- [5] Ko YT, Bhattacharya R, Bickel U. Liposome encapsulated polyethylenimine/ODN polyplexes for brain targeting. *J Control Release* 2009;133:230–7.
- [6] Schäfer J, Höbel S, Bakowsky U, Aigner A. Liposome-polyethylenimine complexes for enhanced DNA and siRNA delivery. *Biomaterials* 2010;31:6892–900.
- [7] Manjappa AS, Chaudhari KR, Venkataraju MP, Dantuluri P, Nanda B, Sidda C, et al. Antibody derivatization and conjugation strategies: application in preparation of stealth immunoliposome to target chemotherapeutics to tumor. *J Control Release* 2011;150:2–22.
- [8] O'Hagan DT, Rappuoli R. Novel approaches to vaccine delivery. *Pharm Res* 2004;21:1519–30.
- [9] Barnier QC, Elsharkawy A, Romeijn S, Kros A, Jiskoot W. Cationic liposomes as adjuvants for influenza hemagglutinin: more than charge alone. *Eur J Pharm Biopharm* 2012;81:294–302.
- [10] Christensen D, Korsholm KS, Andersen P, Agger EM. Cationic liposomes as vaccine adjuvants. *Expert Rev Vaccines* 2011;10:513–21.
- [11] Aramaki Y, Takano S, Arima H, Tsuchiya S. Induction of apoptosis in WEHI 231 cells by cationic liposomes. *Pharm Res* 2000;17:515–20.
- [12] Aramaki Y, Takano S, Tsuchiya S. Induction of apoptosis in macrophages by cationic liposomes. *FEBS Lett* 1999;460:472–6.
- [13] Yan W, Chen W, Huang L. Reactive oxygen species play a central role in the activity of cationic liposome based cancer vaccine. *J Control Release* 2008;130:22–8.
- [14] Iwaoka S, Nakamura T, Takano S, Tsuchiya S, Aramaki Y. Cationic liposomes induce apoptosis through p38 MAP kinase-caspase-8-ZBID pathway in macrophage-like RAW264.7 cells. *J Leukoc Biol* 2006;79:184–91.
- [15] Jacquet A, Vanderschrick JF, Vandenbranden M, Elouahabi A, Magi M, Garcia L, et al. Vaccination with the recombinant allergen ProDer p 1 complexed with the cationic lipid DiC14-amidine prevents allergic responses to house dust mite. *Mol Ther* 2005;11:960–8.
- [16] Tanaka T, Legat A, Adam E, Steuve J, Gatot JS, Vandenbranden M, et al. DiC14-amidine cationic liposomes stimulate myeloid dendritic cells through Toll-like receptor 4. *Eur J Immunol* 2008;38:1351–7.
- [17] Vangasseri DP, Cui Z, Chen W, Hokey DA, Falo Jr LD, Huang L. Immunostimulation of dendritic cells by cationic liposomes. *Mol Membr Biol* 2006;23:385–95.
- [18] Yan W, Chen W, Huang L. Mechanism of adjuvant activity of cationic liposome: phosphorylation of a MAP kinase, ERK and induction of chemokines. *Mol Immunol* 2007;44:3672–81.
- [19] Ouali M, Ruysschaert JM, Lonz C, Vandenbranden M. Cationic lipids involved in gene transfer mobilize intracellular calcium. *Mol Membr Biol* 2007;24:225–32.
- [20] Abramson J, Pecht I. Regulation of the mast cell response to the type 1 Fcε receptor. *Immunol Rev* 2007;217:231–54.
- [21] Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, et al. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B. *Curr Biol* 1997;7:261–9.
- [22] Holgate ST. The epidemic of allergy and asthma. *Nature* 1999;402:2–4.
- [23] Turner H, Kinet J. Signalling through the high-affinity IgE receptor FcεRI. *Nature* 1999;402:24–30.
- [24] Blank U, Rivera J. The ins and outs of IgE-dependent mast-cell exocytosis. *Trends Immunol* 2004;25:266–73.
- [25] Paumet F, Le Mao J, Martin S, Galli T, David B, Blank U, et al. Soluble NSF attachment protein receptors (SNAREs) in RBL-2H3 mast cells: functional role of syntaxin 4 in exocytosis and identification of a vesicle-associated membrane protein 8-containing secretory compartment. *J Immunol* 2000;164:5850–7.
- [26] Puri N, Kruhlak M, Whiteheart S, Roche P. Mast cell degranulation requires N-ethylmaleimide-sensitive factor-mediated SNARE disassembly. *J Immunol* 2003;171:5345–52.
- [27] Puri N, Roche P. Mast cells possess distinct secretory granule subsets whose exocytosis is regulated by different SNARE isoforms. *Proc Natl Acad Sci USA* 2008;105:2580–5.
- [28] Sander LE, Frank SP, Bolat S, Blank U, Galli T, Bigalke H, et al. Vesicle associated membrane protein (VAMP)-7 and VAMP-8, but not VAMP-2 or VAMP-3, are required for activation-induced degranulation of mature human mast cells. *Eur J Immunol* 2008;38:855–63.
- [29] Guo Z, Turner C, Castle D. Relocation of the t-SNARE SNAP-23 from lamellipodia-like cell surface projections regulates compound exocytosis in mast cells. *Cell* 1998;94:537–48.
- [30] Ryan JJ, Bateman HR, Stover A, Gomez G, Norton SK, Zhao W, et al. Fullerene nanomaterials inhibit the allergic response. *J Immunol* 2007;179:665–72.
- [31] Tahara K, Tadokoro S, Yamamoto H, Kawashima Y, Hirashima N. The suppression of IgE-mediated histamine release from mast cells following exocytic exclusion of biodegradable polymeric nanoparticles. *Biomaterials* 2012;33:343–51.
- [32] Okayama R, Noji M, Nakanishi M. Cationic cholesterol with hydroxyethylamino head group promotes significantly liposome-mediated gene transfection. *FEBS Lett* 1997;408:232–4.
- [33] Inoh Y, Kitamoto D, Hirashima N, Nakanishi M. Biosurfactants of MEL-A increase gene transfection mediated by cationic liposomes. *Biochem Biophys Res Commun* 2001;289:57–61.
- [34] Inoh Y, Kitamoto D, Hirashima N, Nakanishi M. Biosurfactant MEL-A dramatically increases gene transfection via membrane fusion. *J Control Release* 2004;94:423–31.
- [35] Inoh Y, Furuno T, Hirashima N, Kitamoto D, Nakanishi M. The ratio of unsaturated fatty acids in biosurfactants affects the efficiency of gene transfection. *Int J Pharm* 2010;398:225–30.
- [36] Teshima R, Suzuki K, Ikebuchi H, Terao T. Possible involvement of phosphorylation of a 36,000-dalton protein of rat basophilic leukemia (RBL-2H3) cell membranes in serotonin release. *Mol Immunol* 1986;23:279–84.
- [37] Passante E, Frankish N. The RBL-2H3 cell line: its provenance and suitability as a model for the mast cell. *Inflamm Res* 2009;58:737–45.
- [38] Nakamura R, Furuno T, Nakanishi M. The plasma membrane shuttling of CAPRI is related to regulation of mast cell activation. *Biochem Biophys Res Commun* 2006;347:363–8.
- [39] Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 1985;260:3440–50.
- [40] Hiramatsu H, Tadokoro S, Hirashima N. Latrotoxin induces degranulation in RBL-2H3 cells transfected with latrophilin. *Toxicol* 2010;56:1372–80.
- [41] Arisaka M, Takano K, Negishi Y, Arima H, Aramaki Y. Involvement of lipid rafts in macrophage apoptosis induced by cationic liposomes. *Arch Biochem Biophys* 2011;508:72–7.
- [42] Lonz C, Vandenbranden M, Ruysschaert JM. Cationic lipids activate intracellular signaling pathways. *Adv Drug Deliv Rev* 2012;64:1749–58.

- [43] Rivera J. Molecular adapters in FcεRI signaling and the allergic response. *Curr Opin Immunol* 2002;14:688–93.
- [44] Galli SJ, Kalesnikoff J, Grimbaldston MA, Piliponsky AM, Williams CMM, Tsai M. Mast cells as tunable effector and immunoregulatory cells: recent advances. *Annu Rev Immunol* 2005;23:749–86.
- [45] Takekawa M, Furuno T, Hirashima N, Nakanishi M. Mitochondria take up Ca^{2+} in two steps dependently on store-operated Ca^{2+} entry in mast cells. *Biol Pharm Bull* 2012;35:1354–60.
- [46] Calloway N, Vig M, Kinet JP, Holowka D, Baird B. Molecular clustering of STIM1 with Orai1/CRACM1 at the plasma membrane depends dynamically on depletion of Ca^{2+} stores and on electrostatic interactions. *Mol Biol Cell* 2009;20:389–99.
- [47] Wheeler MB, Sheu L, Ghai M, Bouquillon A, Grondin G, Weller U, et al. Characterization of SNARE protein expression in beta cell lines and pancreatic islets. *Endocrinology* 1996;137:1340–8.
- [48] Reed GL, Hough A, Fitzgerald ML. Human platelets contain SNARE proteins and a Sec1p homologue that interacts with syntaxin 4 and is phosphorylated after thrombin activation: implications for platelet secretion. *Blood* 1999;93:2617–26.
- [49] Hepp R, Puri N, Hohenstein AC, Crawford GL, Whiteheart SW, Roche PA. Phosphorylation of SNAP-23 regulates exocytosis from mast cells. *J Biol Chem* 2005;280:6610–20.
- [50] Morita Y, Tadokoro S, Sasai M, Kitamoto D, Hirashima N. Biosurfactant mannosyl-erythritol lipid inhibits secretion of inflammatory mediators from RBL-2H3 cells. *Biochim Biophys Acta* 2011;1810:1302–8.
- [51] Nanamori M, Chen J, Du X, Ye RD. Regulation of leukocyte degranulation by cGMP-dependent protein kinase and phosphoinositide 3-kinase: potential roles in phosphorylation of target membrane SNARE complex proteins in rat mast cells. *J Immunol* 2007;178:416–27.
- [52] Tadokoro S, Nakanishi M, Hirashima N. Complexin II facilitates exocytotic release in mast cells by enhancing Ca^{2+} sensitivity of the fusion process. *J Cell Sci* 2005;118:2239–46.
- [53] Salinas E, Quintanar-Stephano A, Córdova LE, Quintanar JL. Antioxidant enzyme activity and malondialdehyde concentration in the plasma and erythrocytes of patients with urticaria induced by nonsteroidal anti-inflammatory drugs. *J Invest Allergol Clin Immunol* 2008;18:366–71.
- [54] Wiethoff CM, Smith JG, Koe GS, Middaugh CR. The potential role of proteoglycans in cationic lipid-mediated gene delivery. *J Biol Chem* 2001;276:32806–13.
- [55] Liang Y, Haring M, Roughley PJ, Margolis RK, Margolis RU. Glypican and biglycan in the nuclei of neurons and glioma cells: presence of functional nuclear localization signals and dynamic changes in glypican during the cell cycle. *J Cell Biol* 1997;139:851–64.
- [56] Pani B, Ong HL, Liu X, Rauser K, Ambudkar IS, Singh BB. Lipid rafts determine clustering of STIM1 in endoplasmic reticulum-plasma membrane junctions and regulation of store-operated Ca^{2+} entry (SOCE). *J Biol Chem* 2008;283:17333–40.
- [57] Rejman J, Oberle V, Zuhorn IS, Hoekstra D. Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. *Biochem J* 2004;377:159–69.
- [58] Hoekstra D, Rejman J, Wasungu L, Shi F, Zuhorn I. Gene delivery by cationic lipids: in and out of an endosome. *Biochem Soc Trans* 2007;35:68–71.
- [59] Caracciolo G, Callipo L, De Sanctis SC, Cavaliere C, Pozzi D, Laganà A. Surface adsorption of protein corona controls the cell internalization mechanism of DC-Chol-DOPE/DNA lipoplexes in serum. *Biochim Biophys Acta* 2010;1798:536–43.
- [60] Hart PH. Regulation of the inflammatory response in asthma by mast cell products. *Immunol Cell Biol* 2001;79:149–53.
- [61] Hamer PW, McGeachie JM, Davies MJ, Grounds MD, Evans Blue Dye as an in vivo marker of myofibre damage: optimising parameters for detecting initial myofibre membrane permeability. *J Anat* 2002;200:69–79.