

Extracellular Ca²⁺ aggravates IgE-induced allergic reaction in mast cells through GPRC6A, a novel family C G-protein-coupled receptor

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Abstract

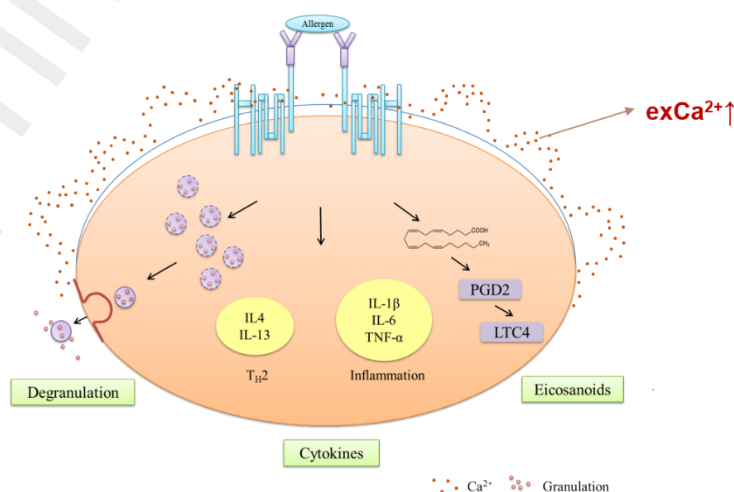
Aims: As an important indicator of allergic reactions, mast cell (MC) activation involves FcεRI-mediated signaling and the release of allergic mediators. In FcεRI signaling, Ca^{2+} are located at the intersection of multiple cellular signaling pathways. However, the effect of extracellular Ca^{2+} (exCa^{2+}) on MCs during anaphylaxis remain unclear, along with its exact mechanisms. Therefore, here we sought to determine whether and how elevated exCa^{2+} amplifies allergic reactions.

Main methods: *In vitro* experiments used immunoglobulin E (IgE)/antigen (Ag)-induced activation of rat and mouse MCs *in vitro*. The levels of MC degranulation mediators were used to evaluate the effect of exCa^{2+} . *In vivo* experiments used MC-mediated passive systemic anaphylaxis (PCA) Balb/c mice. Anaphylaxis indexes such as rectal temperature and allergic symptom score were detected after stimulation.

Key findings: *In vitro* experiments revealed that exCa^{2+} is a stimulus signal for the aggravation of allergic reactions in MCs. When antagonists or siRNA inhibited GPRC6, MCs released fewer inflammatory mediators. Moreover, *in vivo* experiments confirmed *in vitro* results. Allergic symptoms were alleviated by antagonists NPS2143 in PCA mice, demonstrating that exCa^{2+} aggravates allergic reactions through GPRC6A.

Significance: Our study provides an important theoretical basis for targeting Ca^{2+} and GPRC6A as a therapeutic option for allergies.

Graphic Abstract



Abbreviations

BMMC: bone marrow-derived mast cells; Ca^{2+} : calcium ion; $[\text{Ca}^{2+}]_i$: Intracellular Ca^{2+} concentration; exCa^{2+} : Extracellular Ca^{2+} ; $\text{ex}[\text{Ca}^{2+}]$: Extracellular Ca^{2+} concentration; CaSR: calcium-sensing receptor; GPRC6A: G Protein-Coupled Receptor Class C Group 6 Member A; IgE: immunoglobulin E; MC: mast cell; PCA: passive system anaphylaxis; siRNA: small interfering RNA

*In this article, calcium-sensing receptor specifically refer to CaSR, and calcium sensing receptors includes both CaSR and GPRC6A calcium sensing receptors.

Key words: Allergic diseases; mast cells (MCs); extracellular calcium; G protein-coupled calcium sensing receptors; G Protein-Coupled Receptor Class C Group 6 Member A (GPRC6A)

1 Introduction

The prevalence of allergic diseases has increased annually, affecting more than 20% of the world's population [1]. Mast cells (MCs) are anaphylaxis modulators [2,3] and effective therapeutic targets for allergic diseases [4]. Multiple studies indicate that Ca^{2+} is at the center of multiple cellular signaling pathways involved in MC activation. During MC release of histamines [5-7], Ca^{2+} disassembles the F-actin ring and allows for secretory vesicles to fuse with the plasma membrane [8-10]. Ca^{2+} mobilization from the endoplasmic reticulum is necessary and sufficient for histamine release [11]. Ca^{2+} is also necessary for activating the calcineurin/Nuclear factor of activated T-cells (NFAT) pathway and cytokine production during antigen activation of MCs [12-15]. Moreover, Ca^{2+} is essential for eicosanoid generation in MCs as a response to stimuli in the extracellular regulated protein kinase (ERK)1/2- and Phosphoinositide-3 kinase (PI3K)/Bruton's tyrosine kinase (Btk)-dependent pathways [16-19]. These allergic mediators stimulate B cell proliferation and differentiation, increasing immunoglobulin E (IgE) binding to Fc ϵ RI (a high-affinity IgE receptor) on MCs in a positive feedback loop [20,21].

Ca^{2+} affects MC activation through different signaling pathways. Pandey et al. [22] found that calcium transients persisted for hours in the presence of IgE, resulting in continued translocation of the transcription factor nuclear factor of activated T cells (NFAT) to the nucleus; removing IgE reversed this process. Hence, Ca^{2+} may be important in the IgE-Fc ϵ RI signaling pathway. Moreover, extracellular Ca^{2+} (exCa^{2+}) acts as “first messenger” through calcium sensing receptors (on the cell membrane [23]). For example, increased exCa^{2+} activates the Nod-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome through two G protein-coupled calcium-sensing receptors: calcium-sensing receptor (CaSR) and G protein-coupled receptor class C group 6 member A (GPRC6A) [24]. Several studies have also reported that calcium sensing receptors are widespread in cells and participate in maintaining systemic Ca^{2+} homeostasis [25]. However, most such studies focused on excitable cells, such as neurocytes [26], gland cells [27,28], and myocytes [29,30].

Yet calcium sensing receptors are also expressed on immune cells, such as human peripheral blood T lymphocytes, and contribute to cytokine secretion through mitogen-activated protein kinases (MAPKs) or nuclear factor kappa-light-chain-enhancer of activated B cell (NF- κ B) pathways [31]. Therefore, we hypothesize that exCa^{2+} may also participate in MC activation through calcium-sensing receptors.

Under homeostatic conditions, exCa^{2+} concentration ($\text{ex}[\text{Ca}^{2+}]$) is tightly regulated in interstitial fluid [32]. At infection sites, $\text{ex}[\text{Ca}^{2+}]$ increases to activate effector cells, including tissue MCs and blood basophils [14]. However, we know little regarding how elevated $\text{ex}[\text{Ca}^{2+}]$ affects MC activation in allergies. We hypothesized that increased $\text{ex}[\text{Ca}^{2+}]$ from necrotic cells conveys danger signals to surrounding cells and triggers MC or basophil activation, exacerbating allergic responses. Here, we used *in vitro* and *in vivo* models to demonstrate that exCa^{2+} is a stimulatory signal for MCs, acting through GPRC6A to aggravate hypersensitivity. Our results should provide insight into treatments for allergic diseases.

2 Methods

2.1 Mice

Balb/c mice were used at 8-10 weeks of age (Vital River Laboratories, Inc., Beijing, China). The study was conducted in the specific pathogen-free (SPF) animal laboratory of the College of Food Science and Nutritional Engineering, China Agricultural University (Beijing, China). All experiments were carried out following the Guide for the Animal Experimental Welfare and Ethics in the Food Science and Nutritional Engineering College of China Agricultural University and were approved by the Animal Experimental Welfare and Ethical Inspection Committee at China Agricultural University.

2.2 Mast cells

RBL-2H3 cells. The rat basophil leukemia cell line RBL-2H3, obtained from the National platform of experimental cell resources (Beijing, China) were cultured in MEM medium (Corning, USA) supplemented with 15% fetal bovine serum (FBS,

Gemini Bio-Products, CA) and 1×10^5 U/L penicillin/streptomycin (Corning, USA) at 37 °C in a humidified 5% CO₂ incubator.

Bone marrow mast cells (BMMCs). Mouse BMMCs were generated from the bone marrow of Balb/c mice described by Andrade et al. [13] with minor modifications. Briefly, mice were sacrificed, and intact femurs were removed. The sterile endotoxin-free medium was repeatedly flushed through the bone shaft using a needle and syringe. The suspension of bone marrow cells was cultured at a density of 1×10^5 /mL cells in RPMI1640 medium (Corning, USA) supplemented with 10% FBS, 50 μ M/L non-essential amino acid (Corning, USA), 10 ng/mL recombinant mouse IL-3 (PeproTech EC Inc., UK) and 10 ng/mL recombinant mouse SCF (PeproTech EC Inc., UK) at 37°C in a humidified atmosphere containing 5% CO₂. The culture medium was changed weekly. After culturing for 6-8 weeks, the cell numbers of surface co-expression of CD117 and Fc ϵ RI- α were 94.20% as assessed by flow cytometry, which can be used for further research.

2.3 Measurement of [Ca²⁺]_i with Fluo-3AM

Cells were first incubated with 5 μ M Fluo-3AM (Sigma-Aldrich, China) at 37°C for 30 min in the dark, then washed three times with a Ca²⁺-free tyrode's solution to remove the remaining dye, and the dying cells were further incubated in Ca²⁺-free tyrode's solution. Following a 60 s baseline recording in Ca²⁺-free tyrode's solution, CaCl₂ concentration in the Ca²⁺-free Tyrode's solution was increased gradually to 1.8 mM (CaCl₂ concentration in the MEM) or 0.42 mM (CaCl₂ concentration in the RPMI1640), 2.5 mM, 5 mM and 7.5 mM, and the intracellular fluo-3 fluorescence measurements continued for 240 s. In some experiments, cells were exposed to Gd³⁺ (300 μ M, Sigma-Aldrich, China) and then recorded for 80 s at 6-s intervals. In some groups, cells were first preincubated with 10 μ M NPS2143 (antagonist of GPRC6A, Sigma-Aldrich, China) for 30 min before the Ca²⁺ (5 mM) challenge. FI (fluorescence intensity) of fluo-3 in cells was recorded using the Thermo Scientific Varioskan Flash (Thermo, USA) with excitation at 488 nm and emission at 525 nm. FI₀ was set at the initial fluorescence intensity when cells were incubated in Ca²⁺-free tyrode's solution

and $[Ca^{2+}]_i$ changes were expressed as fluorescence intensity representing FI and normalized to initial fluorescence intensity (FI_0).

2.4 Effect of $ex[Ca^{2+}]$ on the cell viability using CCK-8

Cell Counting Kit-8 (CCK-8, Beyotime, China) was used to analyze the effect of increased $ex[Ca^{2+}]$ concentration on cellular growth. First, 1×10^5 RBL-2H3 or 1×10^6 BMMCs (100 μ L/well) were inoculated in a 96-well plate and exposed to different $ex[Ca^{2+}]$ concentrations in the Ca^{2+} -free tyrode's solution for 4 h. Then 10 μ L of CCK-8 was added to each well after removing the supernatant and washing the cells three times and incubated for another 2 h (37°C, 5% CO_2). Finally, the absorbance was measured at 450 nm by the Thermo Scientific Varioskan Flash (Thermo, USA). The absorbance of cells in the Ca^{2+} -free tyrode's solution was taken as 100% viability.

2.5 Sensitization and stimulation

RBL-2H3 and BMMCs were cultured for 24 hours (37°C, 5% CO_2) until the degree of cell convergence was 70% ~ 80%. The cells were washed twice with PBS buffer and then sensitized with 1 μ g/mL anti-DNP IgE antibody (Sigma-Aldrich, China) for 2 h (37°C, 5% CO_2). The antibody was dissolved in Ca^{2+} -free tyrode's solution containing different $ex[Ca^{2+}]$. The sensitized cells were then washed three times with a Ca^{2+} -free tyrode's solution and stimulated with 100 ng/mL of DNP-HSA (Sigma-Aldrich, USA) at 37°C for 30 min.

2.6 Measurement of β -hexosaminidase release

1.5×10^5 cells (100 μ L/well) were inoculated into a 96-well plate, and were cultured, sensitized and stimulated as described in part 2.5. The cells were put into an ice box to terminate the reaction after stimulation. Then 30 μ L of the culture supernatant were transferred to another 96-well plate and incubated with 50 μ L of 1 mM 4-nitrophenyl N-acetyl- β -D-glucosaminide (Sigma, China) in 0.1 M citrate buffer (pH 4.5) at 37°C for 1 h. The reaction was stopped by adding a 200 μ L stop solution (0.1 M $Na_2CO_3/NaHCO_3$, pH 10.0). The absorbance was measured at 405 nm by the Thermo

Scientific Varioskan Flash (Thermo, USA). The total β -hexosaminidase release was determined by adding 250 μ L of 0.1% Triton X-100 in PBS, and the spontaneous release was evaluated by adding 50 μ L of Tyrode's solution to each well. The β -hexosaminidase release ratio was calculated by the following equation to ensure the effectiveness of the increased $\text{ex}[\text{Ca}^{2+}]$ to the RBL-2H3 degranulation.

The β -hexosaminidase release ratio (%) = (Absorbance of test samples - absorbance of Tyrode's solution) / (Absorbance of 0.1% Triton X-100 - absorbance of Tyrode's solution) $\times$ 100%

2.7 Measurement of histamine, cytokines and eicosanoids using ELISA

1×10^6 cells (2 mL/dishes) were inoculated into 3.5 cm cell Petri dishes. The culture supernatant was obtained as described in part 2.6. Cells Other allergic mediators and cytokines secreted into the supernatant, such as rat and mouse histamine, IL-4, IL-6, IL-13, IL-1 β , TNF- α , LTC4 and PGD2, were measured by the commercially available enzyme immunoassay kits (Dogesce, China) following the manufacturer's protocol.

2.8 Analysis of IgE-Fc ϵ RI binding capacity using Flow Cytometry

To detect whether the IgE-Fc ϵ RI binding capacity was affected by the increased $\text{ex}[\text{Ca}^{2+}]$, we incubated RBL-2H3 cells with PE-conjugated anti-mouse IgE (eBioscience, China) or PE-conjugated rat IgG2b κ isotype control (eBioscience, China) for 30 min at 4°C in the dark followed by sensitization, as described in part 2.5. Cells were then washed twice and analyzed with the BD FACSCalibur (BD Bioscience, CA).

2.9 Determine the expression of Calcium sensing receptors using reverse transcription PCR (RT-PCR) and Western Blotting

RT-PCR. Species-specific CaSR and GPRC6A primers were designed to detect its mRNA expression in RBL-2H3 and BMMCs using reverse transcription PCR (RT-PCR). More specifically, total RNA was prepared using Trizol reagent (TransGen Biotechnology, China) and cDNA was transcribed using the TransScript[®] One-Step

gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotechnology, China). PCR was performed using the 2×TSINGKE Master Mix (blue) (TsingKe biological technology, China) for CaSR, GPRC6A and β -actin with primers as shown in table 1. The PCR reaction protocol was as follows: initial denaturation at 95 °C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, with an additional extension at 72 °C for 7 min. The RT-PCR products were analyzed by 1% agarose electrophoresis with a TAE buffer (40 mM Tris, 2 mM EDTA disodium salt dihydrate, 20 mM acetic acid) and sent to Sangon Bioengineering Ltd. Co. (Beijing, China) for sequencing using forward and reverse primers by ABI3700 automatic sequence analyzer. The sequencing primers used were the same as the PCR primers.

Table 1 Primers used in RT-PCR

	Gene name	Primer sequence (5'-3')	Product (bp)	Genbank Accession No.
		F:		
	CaSR	GGACTGTAGGTGTATTTGGAGGG	170	NM_016996.2
		R: GCGTGCTACCGTCTGAAGTT		
Rat	GPRC6A	F: TGGTCATTTGCACGCTTTGG	181	NM_001271106.1
		R: AGCTTCCTCCCCTTGAATGC		
	β -actin	F: GCAGGAGTACGATGAGTCCG	74	NM_031144.3
		R: ACGCAGCTCAGTAACAGTCC		
	CaSR	F : CACAGTTGCCTTGTGATCCT	245	NM_013803.3
		R : GAGGTGTAGGGTGGTTGGTC		
Mouse	GPRC6A	F : TCTGCCACGCCCTTAACCTT	332	NM_153071.1
		R : TGACCCCTCCTCACATTCCA		
	β -actin	F : GCAGGAGTACGATGAGTCCG	74	NM_007393.5
		R : ACGCAGCTCAGTAACAGTCC		

Western Blotting. The cell samples were homogenized in a lysis buffer with protease inhibitors. The protein concentration of the supernatant was determined using a BCA Protein in Assay Kit (CWBIO, China). 40 μ g of protein samples were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes, blocked in TBST solution containing 5% BSA for 1 h at room temperature, and incubated overnight at 4°C with antibodies against CaSR, GPRC6A and β -actin (MultiSciences Biotech,

China). After washing 8 times with TBST, the membranes were next incubated with HRP-conjugated secondary antibody (Cell Signaling Technology, USA) for 1 h at room temperature. The signal was visualized by enhanced chemiluminescence and exposure to an X-ray film (Sage creation Mnin Chemi II, China).

2.10 Knockdown of GPRC6A with siRNA

siRNA treatment. RBL-2H3 and BMMC cells were transfected with siRNA oligonucleotides using the riboFECT™ CP Reagent (RiboBio Co., Ltd, China) for 72 h in 6-well plates following the manufacturer's protocol. Both the rat or mouse *GPRC6A* siRNA and the scrambled control siRNA were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). We selected one of the siRNA sequences from three candidates based on the highest knockdown efficiency, as confirmed by qPCR and Western Blotting. The target sequences of these siRNAs are listed in table 2.

Table 2 GPRC6A siRNA sequences

No.	Name	Target sequences
siG170621103158	si-r-Gprc6a_001	GCTGGCTACTCTGAAATAT
siG170621103232	si-r-Gprc6a_002	CCAATGCATTTCCAACAT
siG170621103340	si-r-Gprc6a_003	CCTCTCAGATAATACCATT
siG170721091222	si-m-Gprc6a_001	CCAGAAATGTGCTGGCTTT
siG170721091301	si-m-Gprc6a_002	GCTCGTTTCTGCCTGTAAA
siG170621103340	si-m-Gprc6a_003	CCTCTCAGATAATACCATT

Cells grouping treatment: Cells were divided into three groups: (i) exCa^{2+} group: exCa^{2+} treatment (3 mM Ca^{2+} for RBL-2H3; 4 mM Ca^{2+} for BMMC), sensitization and stimulation as described in 4.5; (ii) NPS2143+ exCa^{2+} group: preincubated with 10 μM NPS2143 (Sigma-Aldrich, China) for 30 min before the same treatment as the exCa^{2+} group; (iii) GPRC6A-siRNA+ exCa^{2+} group: same treatment as the exCa^{2+} group was performed after the treatment described above with the siRNA. Measurement of β -hexosaminidase release, histamine, cytokine and eicosanoids was performed as described in 4.6 and 4.7.

2.11 Establishment of passive systemic anaphylaxis model in Balb/c mice

Eighty Balb/c mice (8 weeks old, weighing 20-25 g, half male and half female) were divided into four groups (n=20, 5/cage) with an initial body weight difference of $\pm 20\%$ after 3 days of acclimation: control group, allergy group, Ca^{2+} group and NPS2143 group. First, 50 μM NPS2143 was administrated to mice in the NPS2143 group by tail vein injection to inhibit the Ca^{2+} -sensing function of GPRC6A. Next, Mice in the control group were intraperitoneally injected with 200 μL saline solution containing 50 mM Ca^{2+} ; the allergy group were intraperitoneally injected with 20 μg anti-DNP IgE dissolved in 200 μL saline solution; the Ca^{2+} group and NPS2143 group were intraperitoneally injected 200 μL saline solution with 50 mM Ca^{2+} and 20 μg anti-DNP IgE. After 24 h, each mouse was injected with 100 μL DNP-HSA by tail vein to induce passive systemic anaphylaxis, as shown in Fig. 8A.

2.12 Detection of allergic symptoms in passive systemic anaphylaxis mice

Rectal temperature. Rectal temperature was measured before and after 90 min of the DNP-HSA stimulation at 10-min intervals using a WI88375 probe (Beijing Science and Technology, Beijing, China).

Allergic symptoms score. Symptoms were observed within 40 min after post-challenge in a blinded manner by two independent researchers. The standard of Balb/c mice passive systemic anaphylaxis shock grade was shown in table 3 according to the existing evaluation criteria from Ma and Beaven [16]. The clinical allergic symptoms were scored as previously described by Robinson et al.[17] and shown in table 4.

Table 3 Evaluation standard of Balb/c mice passive systemic anaphylaxis shock grade

Degree of PSA	Decrease of rectal temperature (Δt_{max})	Deaths
Grade 1 no shock	$+1^{\circ}\text{C} > \Delta t_{\text{max}} \geq 1^{\circ}\text{C}$	No
Grade 2 Moderate shock	$-1^{\circ}\text{C} > \Delta t_{\text{max}} \geq -4^{\circ}\text{C}$	No
Grade 3 Severe shock	$-4^{\circ}\text{C} > \Delta t_{\text{max}}$	Maybe

Table 4 Evaluation standard of Balb/c mice anaphylaxis clinical symptoms grade

Grade	Clinical symptoms	Description
Degree 0	No signs	
Degree 1	Scratch	Between 4 and 10 times over 15 min
Degree 2	Scratch, reduced activity, bristled fur	More than 10 times over 15 min
Degree 3	Reduced activity, watery diarrhea, difficulty walking, bristled fur and sometimes labored respiration	Similar to degree 2 but stronger
Degree 4	Cyanosis around the mouth and tail	Similar to degree 3 but stronger
Degree 5	Death	

Evans blue extravasation in mouse footpad. After the challenge, 4 mice in each group were injected by tail vein with 0.5% Evans Blue (100 μ L), and Evans Blue extravasation in the footpad was recorded pre- and post-challenge by a Canon EOS camera to qualitatively analyse the vascular permeability.

Level of albumin in peritoneal lavage fluids. After the challenge, mice were euthanized and disinfected the abdominal skin. Then, we performed a 2 cm midline abdominal incision, made the peritoneum expose and slowly injected 2 mL of 0.9 % NaCl and 8 mL of air into the peritoneal cavity using a 27G needle. Finally, we gently massaged the abdomen for 3 min, recovered the peritoneal lavage fluid using a 22G needle and measured the level of albumin in PLF using a BCA Protein Assay Kit (CWBIO, China).

Level of histamine in serum. Blood samples were collected from the orbital venous plexus of mice's eyes. After coagulation overnight at 4 °C, blood samples were centrifuged for 15 min at 3500g at 4 °C to obtain the serum. Serum was stored at 80 °C before measuring histamine by commercially available enzyme immunoassay following the manufacturer's protocol (Dogesce, China).

2.13 Statistical analysis

Statistical significance was analyzed by the two-tailed Student's t-test with GraphPad Prism 5.01 (GraphPad Software, Inc.). All data were presented as mean values $\pm$ standard deviation (SD) and the statistical level was set at a P value <0.05 .

3 Results

3.1 Extracellular Ca^{2+} aggravates IgE-mediated allergic reaction in mast cell

To test the hypothesis that exCa^{2+} is a stimulatory signal aggravating MC hypersensitivity, we stimulated MCs *in vitro* with elevated $\text{ex}[\text{Ca}^{2+}]$. CaCl_2 was added in excess to raise $\text{ex}[\text{Ca}^{2+}]$ in serum-supplemented cell culture media. Using fluorescent indicator Fluo-3AM and CCK-8 assays, we determined that adding 0–5 mM Ca^{2+} significantly increase intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) without affecting cell viability (Figure 1).

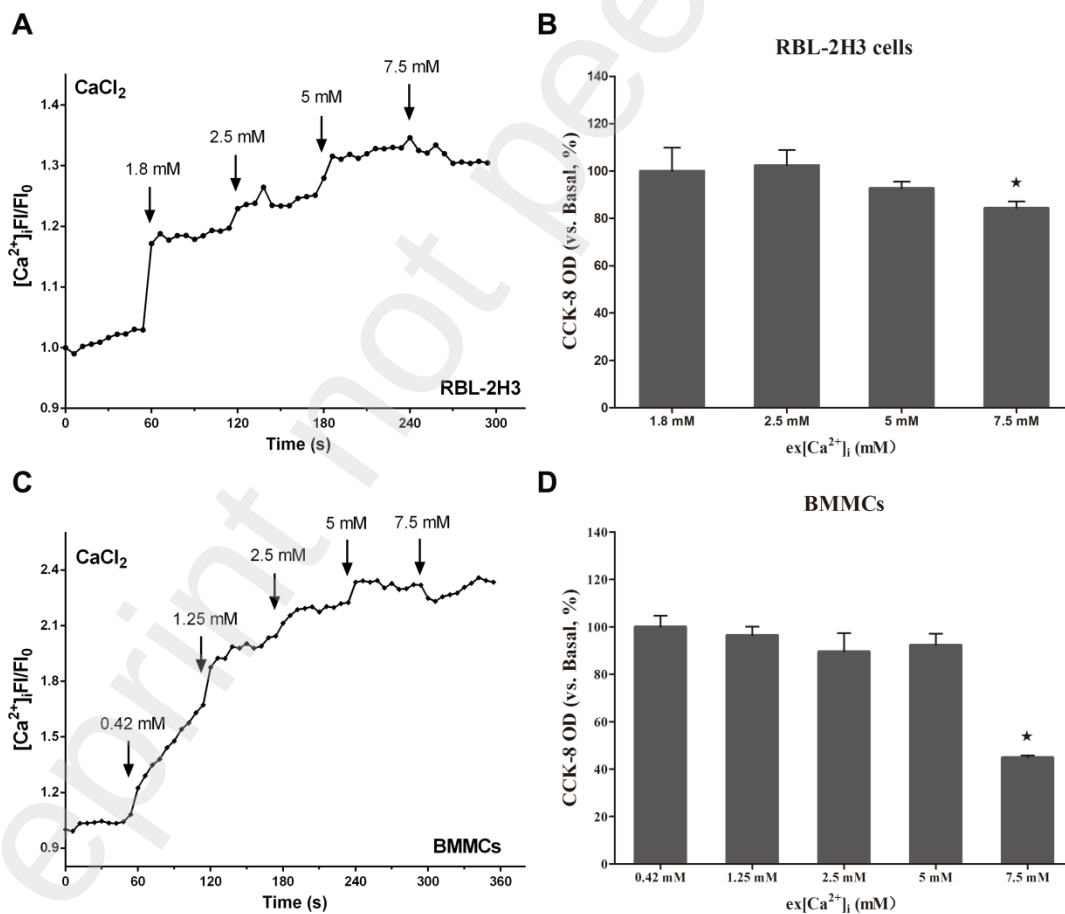


Figure 1 Determine the maximum concentration of extracellular Ca^{2+} and its influence on the cell vitality. A and C, The FI/FI₀ of different $\text{ex}[\text{Ca}^{2+}]$

concentrations on the intracellular calcium ($[Ca^{2+}]_i$) levels in RBL-2H3 or BMMCs. B and D, Effect of different $ex[Ca^{2+}]$ on the cell viability. Data are representative of three independent experiments performed in triplicate. * $P < 0.05$ versus MEM group ($ex[Ca^{2+}] = 1.8$ mM) or RPMI1640 group ($ex[Ca^{2+}] = 0.42$ mM) group.

The mechanism of MC activation comprises two steps: sensitization and effect. During sensitization, antigen-specific IgE (sIgE) binds to FcεRI on the surface of MCs [33]. Next, MCs enter the effect stage when an antigen or anti-IgE antibody binds to the IgE-FcεRI complex [34,35]. During the acute effect phase, activated MCs instantaneously release pre-formed (e.g., histamine and β-aminohexosaminidase) or newly synthesized mediators (arachidonic acid metabolites, e.g., leukotriene [LTC4] and prostaglandin [PGD2]) [33,36]. These compounds can cause allergic symptoms, such as erythema, edema, and increased vascular permeability [37]. Subsequently, MCs release proinflammatory cytokines, including T helper 2 (Th2) cytokines (interleukin [IL]-4, IL-5, IL-13) as well as inflammation-related cytokines and chemokines (IL-1β, IL-6, tumor necrosis factor [TNF] -α, mouse mast cell protease [mMCP]-1) [37]. These cytokines recruit eosinophils, basophils, and T lymphocytes to the inflammatory site, contributing to the development of chronic inflammation [38]. Based on this existing knowledge, we used cell-based *in vitro* immunological detection to determine whether increased $ex[Ca^{2+}]$ aggravated IgE-mediated MCs activation. Three variables were measured: degranulation, cytokines, and nicotine analogs. Because the culture media already contained Ca^{2+} , we added 0–3 mM Ca^{2+} and 0–4 mM Ca^{2+} to incomplete MEM medium and incomplete RPMI 1640 medium. These additions ensured that $ex[Ca^{2+}]$ was within the tolerable concentration range of rat mast cell line RBL-2H3 (1.8–4.8 mM) and mouse bone marrow-derived mast cells (BMMCs) (0.42–4.42 mM), respectively.

For RBL-2H3, as $ex[Ca^{2+}]$ increased, β-hexosaminidase release and histamine level rose significantly from 9.32% to 70.11% and 8.06 ng/mL to 13.84 ng/mL, respectively (Figure 2A). The increase was also significant in BMMCs, from 19.32% to 71.06% and 10.54 ng/mL to 16.82 ng/mL, respectively (Figure 3A). Unsensitized

groups treated with different $\text{ex}[\text{Ca}^{2+}]$ did not differ in β -hexosaminidase release or histamine levels ($P \geq 0.05$, Figure 2A and 3A).

$\text{T}_\text{H}2$ -type (IL-4, IL-6, and IL-13) and inflammatory cytokines (IL-1 β and TNF- α) also increased significantly as $\text{ex}[\text{Ca}^{2+}]$ increased, but Ca^{2+} alone did not induce any effects (Figure 2B and 2C, Figure 3B and 3C). Notably, 1.5 mM of $\text{ex}[\text{Ca}^{2+}]$ significantly increased all cytokine levels in RBL-2H3, whereas only 0.5 mM Ca^{2+} was needed in BMMCs. Because BMMCs are primary cells from mouse bone marrow, they may be more sensitive and susceptible to anti-DNP IgE and $\text{ex}[\text{Ca}^{2+}]$.

The results for eicosanoids were in line with those for degranulation and cytokines. Eicosanoids LTC₄ and PGD₂ increased significantly as $\text{ex}[\text{Ca}^{2+}]$ rose in both RBL-2H3 and BMMCs ($P < 0.05$), but the corresponding Ca^{2+} level group did not yield a significant difference (Figure 2D and Figure 3D).

Here, we demonstrated that increasing $\text{ex}[\text{Ca}^{2+}]$ during sensitization aggravates IgE-induced mast cell activation, including degranulation, cytokine secretion, and eicosanoid production. $\text{Ex}[\text{Ca}^{2+}]$ is tightly regulated in interstitial fluid, ranging from 0.9 to 1.2 mM [24], but increases at infection sites [39] and around activated cells [14]. Therefore, $\text{ex}[\text{Ca}^{2+}]$ may act as positive feedback in the effect stage of allergies, although we do not know the underlying mechanisms.

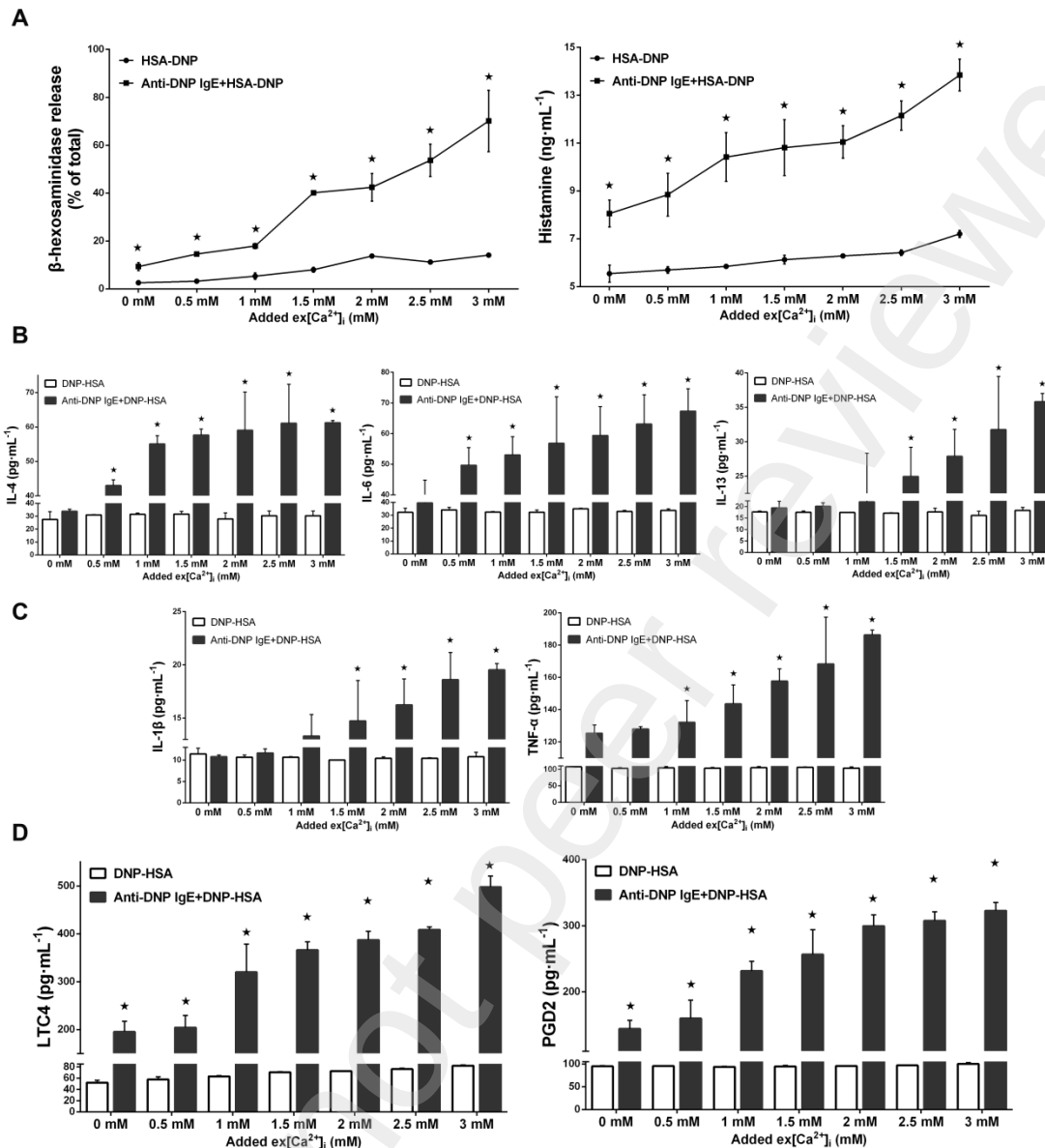


Figure 2 Ex[Ca²⁺] aggravate the IgE-mediated allergic reaction in RBL-2H3. (A) Degranulation including β -hexosaminidase and histamine release in response to increasing ex[Ca²⁺] plus anti-DNP IgE or ex[Ca²⁺] alone and subsequent cross-linking with DNP-HSA. (B) T_H2 type cytokine secretion including IL-4, IL-6 and IL-13. (C) Inflammatory cytokine secretion including IL-1 β and TNF- α . (D) Productions of eicosanoids including LTC₄ and PGD₂. Data are representative of three independent experiments performed in triplicate. * $P < 0.05$ versus the corresponding group without anti-DNP IgE sensitization at the same ex[Ca²⁺] level.

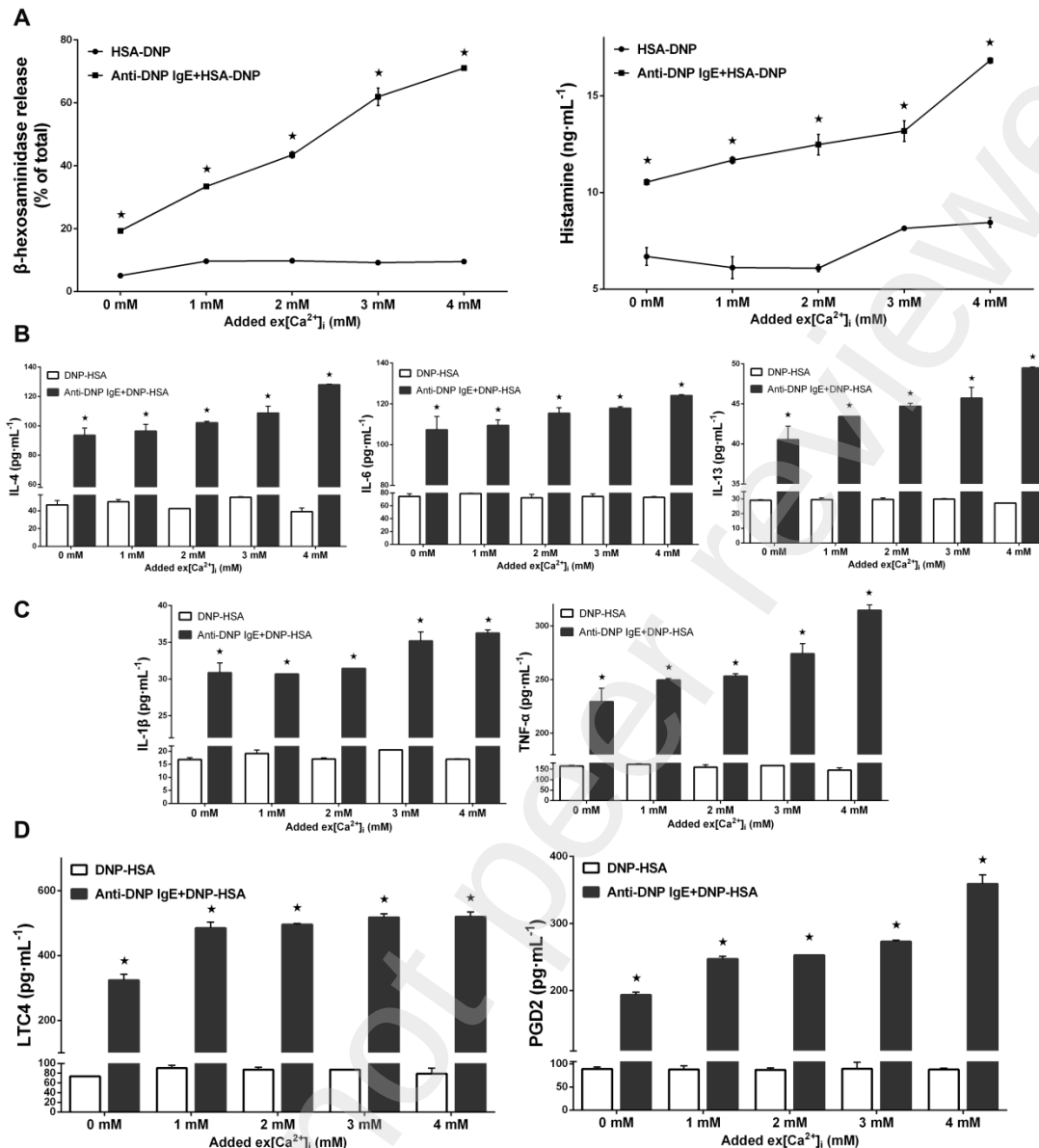


Figure 3 Ex[Ca²⁺] aggravate the IgE-mediated allergic reaction in BMMCs. (A) Degranulation including β -hexosaminidase and histamine release in response to increasing ex[Ca²⁺] plus anti-DNP IgE or ex[Ca²⁺] alone and subsequent cross-linking with DNP-HSA. (B) T_H2 type cytokine secretion including IL-4, IL-6 and IL-13. (C) Inflammatory cytokine secretion including IL-1 β and TNF- α . (D) Productions of eicosanoids including LTC₄ and PGD₂. Data are representative of three independent experiments performed in triplicate. * $P < 0.05$ versus the corresponding group without anti-DNP IgE sensitization at the same ex[Ca²⁺] level.

3.2 Extracellular Ca²⁺ does not aggravate allergic reactions through Fc ϵ RI signaling

Mobilization of exCa^{2+} is required for sustained $\text{Fc}\epsilon\text{RI}$ -dependent activation [40]. Thus, we speculated that increased $\text{ex}[\text{Ca}^{2+}]$ aggravated MC activation through promoting IgE binding to $\text{Fc}\epsilon\text{RI}$. To test our hypothesis, we incubated RBL-2H3 cells with PE-conjugated anti-mouse IgE, followed by sensitization with anti-DNP IgE. We measured changes in fluorescence intensity at different $\text{ex}[\text{Ca}^{2+}]$ during sensitization. Immediately after sensitization, we detected a strong fluorescence signal, indicating that anti-DNP IgE bound to $\text{Fc}\epsilon\text{RI}$. However, as $\text{ex}[\text{Ca}^{2+}]$ increased, fluorescence intensity did not continue to increase (Figure 4). Therefore, we excluded the hypothesis that $\text{ex}[\text{Ca}^{2+}]$ affect MCs activation through the IgE- $\text{Fc}\epsilon\text{RI}$ pathway. Instead, we considered that $\text{ex}[\text{Ca}^{2+}]$ may aggravate allergic reactions by activating calcium sensing receptors on MCs.

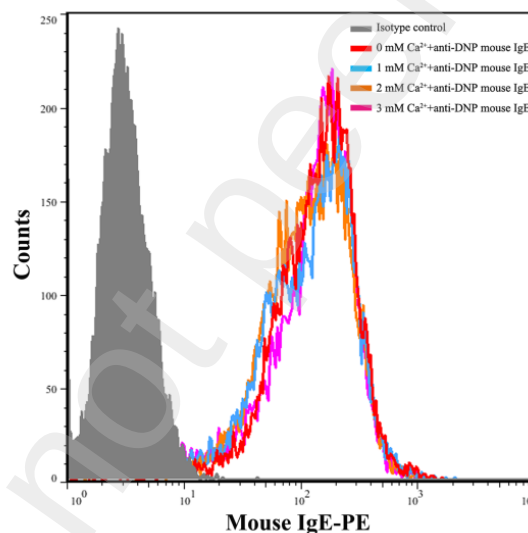
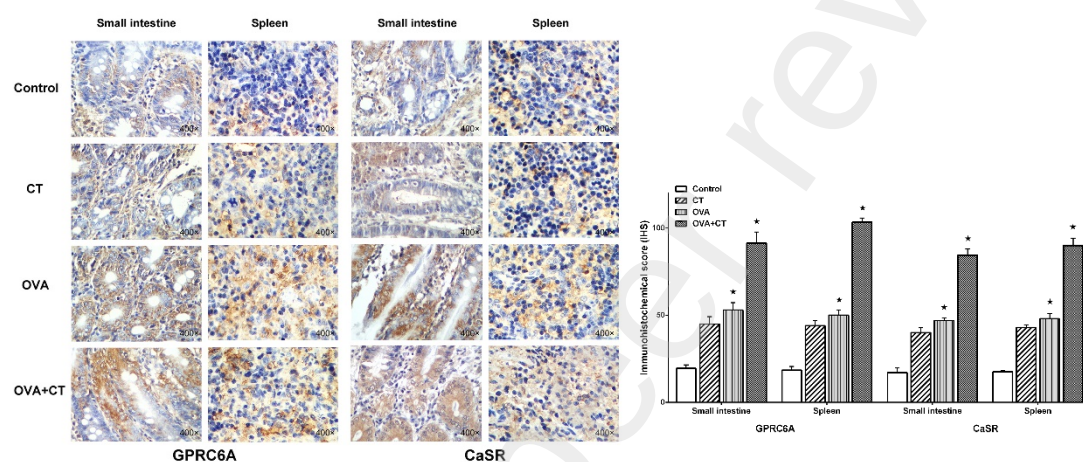


Figure 4 ExCa^{2+} does not affect the binding of IgE to $\text{Fc}\epsilon\text{RI}$ on the surface of mast cells. IgE- $\text{Fc}\epsilon\text{RI}$ binding capacity of RBL-2H3 cells in response to increasing $\text{ex}[\text{Ca}^{2+}]$ plus IgE antibody analyzed by flow cytometry.

In vivo and *in vitro* studies have shown that increasing $\text{ex}[\text{Ca}^{2+}]$ in monocytes and macrophages can heighten the inflammatory response via activating two calcium sensing receptors, CaSR and GPRC6A [24]. In addition, we previously observed increased CaSR and GPRC6A expression as allergic reactions strengthened in the small intestine and spleen of OVA- or OVA-cholera toxin (CT)-induced food allergy mouse models (Supplement Figure 1, unpublished data). We thus investigated the role of GPRC6A and CaSR in exCa^{2+} -induced MC activation.



Supplement Figure 1 Immunohistochemical expression of GPRC6A and CaSR in the small intestine and spleen tissues of food allergic mice. Data are representative of three independent experiments performed in triplicate. * $P < 0.05$ versus the control group.

3.3 Extracellular Ca^{2+} participates in MC activation through GPRC6A and not CaSR

First, we determined mRNA and protein expression of GPRC6A and CaSR in RBL-2H3 and BMMCs. A GPRC6A band (RBL-2H3:181 bp; BMMCs:250 bp) was detected with RT-PCR (Figure 5A). Sequencing and BLAST searches confirmed that the band was GPRC6A. However, we did not observe any CaSR bands. Consistent with these RT-PCR results, western blotting only detected the GPRC6A protein band (105 kDa, Figure 5B) in RBL-2H3 and BMMCs. Therefore, we speculated that GPRC6A was the main calcium sensing receptor mediating exCa^{2+} activation of MCs.

We further confirmed the exCa^{2+} sensing capacity of GPRC6A by preincubating MCs with receptor-specific antagonist NPS2143 [41] and cation activator gadolinium trichloride (GdCl_3) [42]. The fluorescence value of control cells without exCa^{2+} stimulation was always at the baseline ($\text{FI}/\text{FI}_0 = 1.0$, black lines; Figure 5C). After Ca^{2+} stimulation, fluorescence values representing $[\text{Ca}^{2+}]_i$ in RBL-2H3 or BMMCs increased significantly to 1.70 and 2.49, respectively. Furthermore, NPS2143 significantly lowered exCa^{2+} -induced $[\text{Ca}^{2+}]_i$ increase in RBL-2H3 and BMMCs, while GdCl_3 elevated $[\text{Ca}^{2+}]_i$ by 0.93 and 1.74 times, respectively. These results indicate that GPRC6A expressed on RBL-2H3 cells and BMMCs has an exCa^{2+} -sensing function. Additionally, exCa^{2+} sensing is inhibited by NPS2143 and promoted by GdCl_3 .

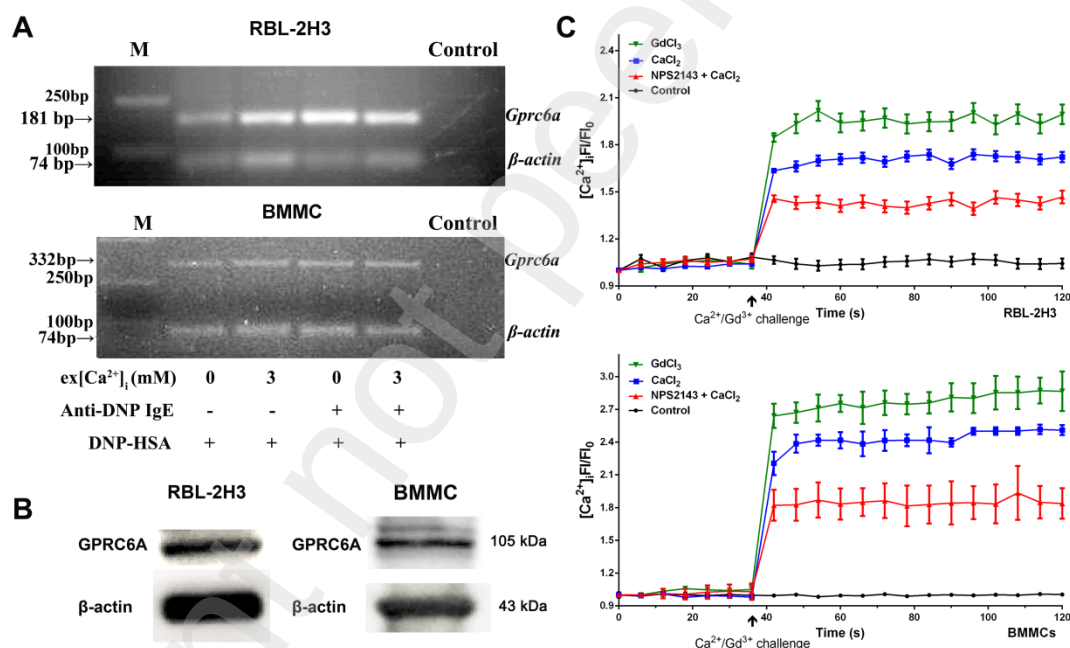
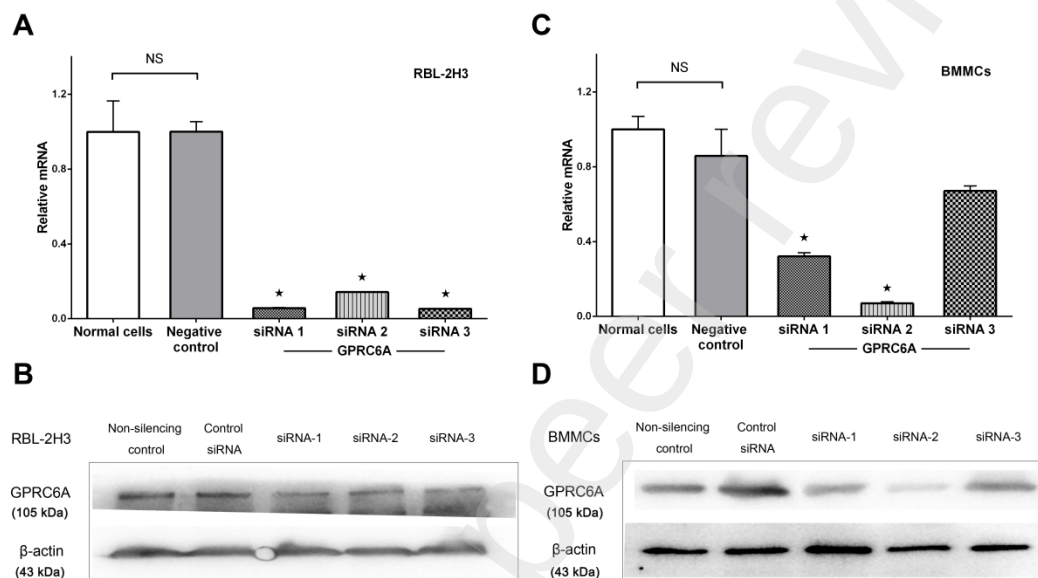


Figure 5 The functional expression of Calcium sensing receptor GPRC6A in RBL-2H3 and BMMCs. (A) mRNA expression and (B) Protein expression of GPRC6A in RBL-2H3 and BMMCs. (C) Effects of NPS2143 (GPRC6A antagonist) and GdCl_3 (GPRC6A alternative agonists) on the $[\text{Ca}^{2+}]_i$ in RBL-2H3 and BMMCs. Data are representative of three independent experiments performed in triplicate.

2.4 GPRC6A inhibition attenuates the stimulatory effect of exCa^{2+} on MC activation

We previously observed that GPRC6A inhibitors inhibit exCa^{2+} influx. To confirm that GPRC6A is the target of exCa^{2+} , we designed three GPRC6A-specific small interfering RNAs (siRNAs) to silence GPRC6A expression and explore the effect on MC activation. After preliminary testing with qPCR and western blotting, we chose GPRC6A-r-siRNA 1 and GPRC6A-m-siRNA 2 for subsequent experiments (Supplement Figure 2).



Supplement Figure 2 The effect of siRNA on the levels of GPRC6A expression in RBL-2H3 and BMMCs. (A) mRNA expression and (B) Protein expression of GPRC6A in RBL-2H3. (C) mRNA expression and (D) Protein expression of GPRC6A in BMMCs.

Both NPS2143 and siRNA treatment significantly inhibited the increase in IgE-induced degranulation, cytokine secretion, and eicosanoid production in RBL-2H3 cells and BMMCs (Figures 6 and 7). These data demonstrated that exCa^{2+} targets GPRC6A to enhance MC activation.

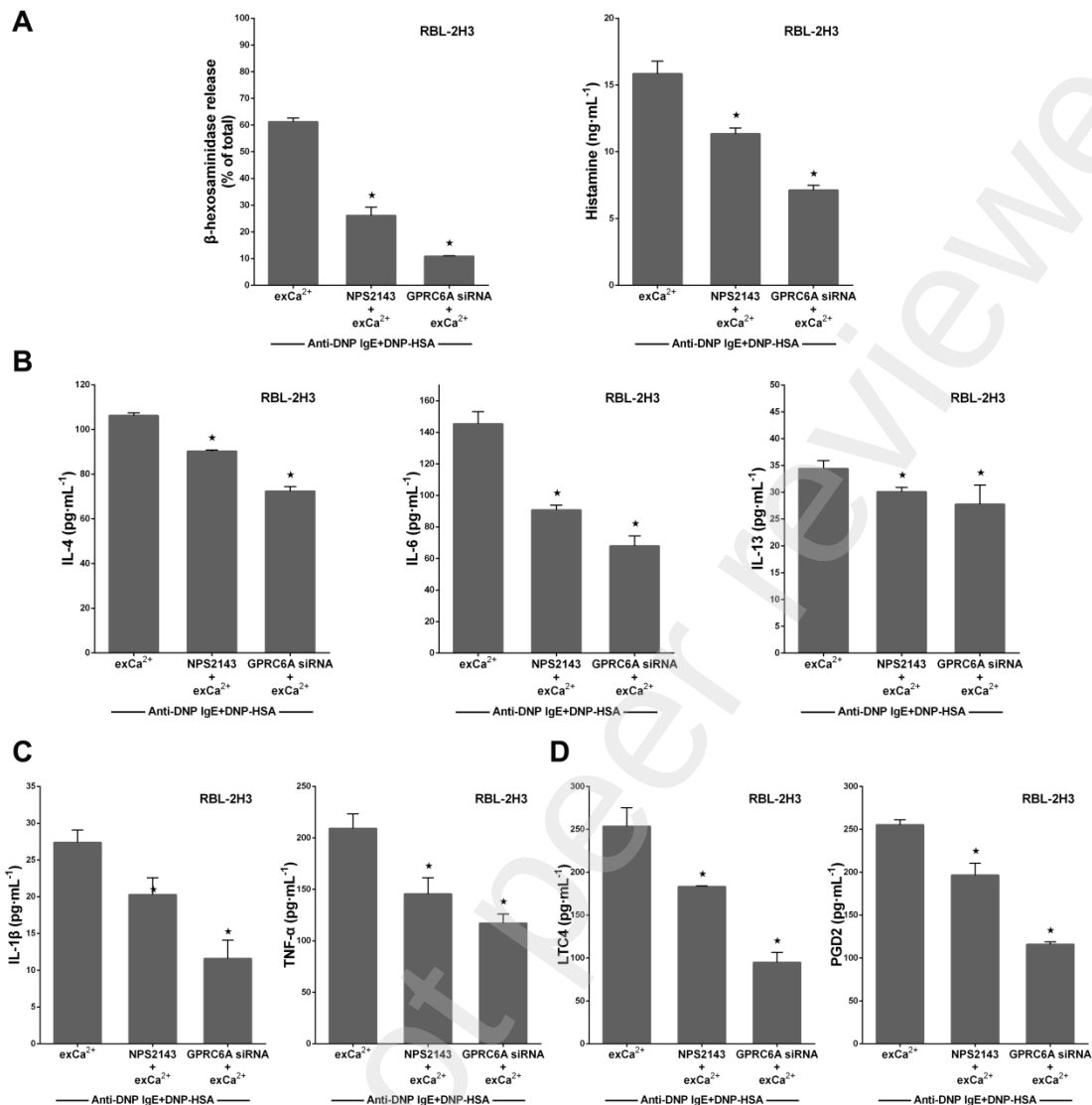


Figure 6 Inhibition of GPRC6A can attenuate the aggravative effect of exCa²⁺ on the IgE/Ag-induced RBL-2H3 activation. (A) Degranulation, (B) T_H2 and (C) inflammatory-related cytokines secretion and (D) eicosanoids production in RBL-2H3 after inhibiting GPRC6A. Data are representative of three independent experiments performed in triplicate. **P*<0.05 versus the exCa²⁺ group.

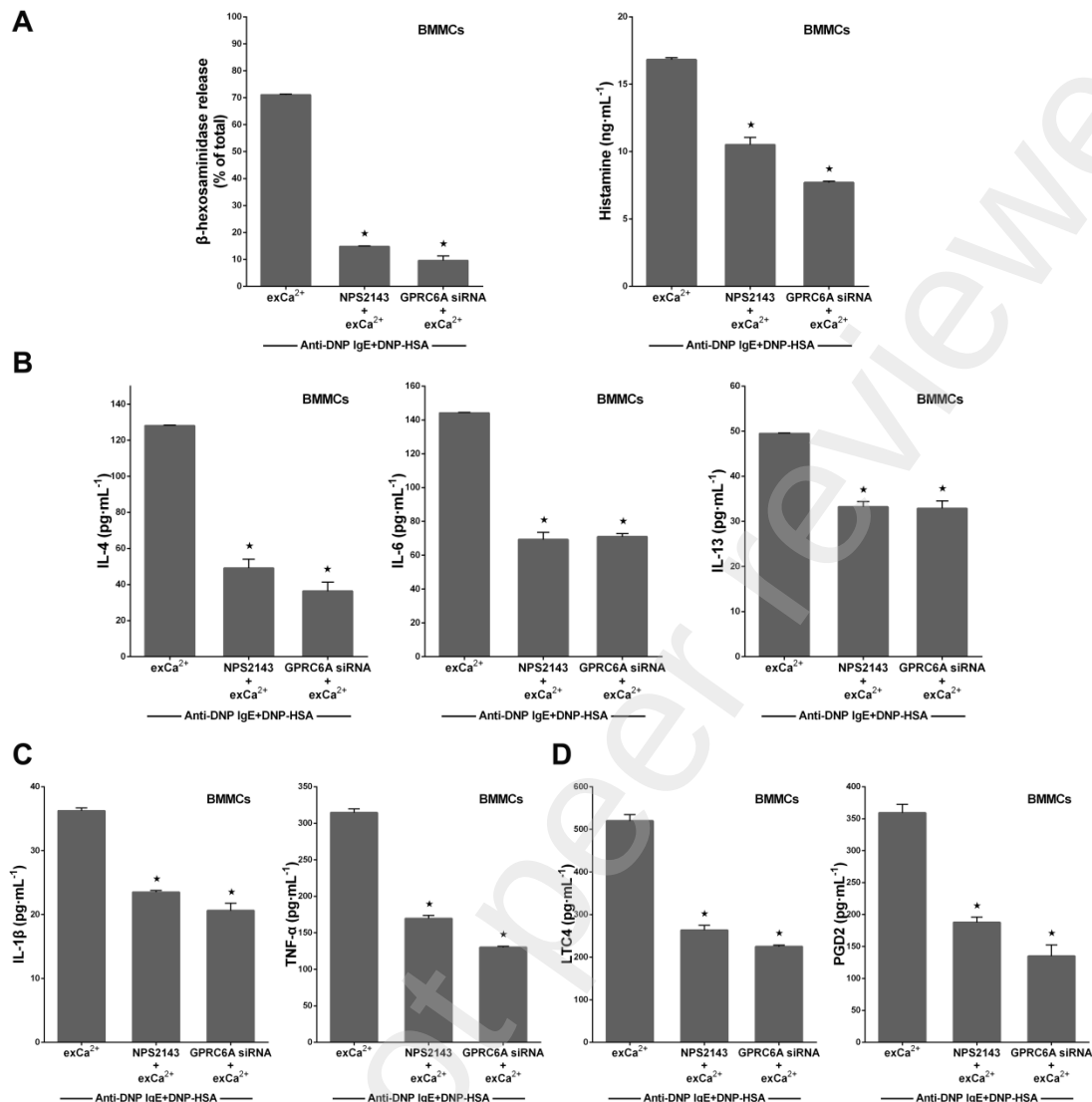


Figure 7 Inhibition of GPRC6A can attenuate the aggravative effect of exCa²⁺ on the IgE/Ag-induced BMMCs activation. (A) Degranulation, (B) T_H2 and (C) inflammatory-related cytokines secretion and (D) eicosanoids production in RBL-2H3 after inhibiting GPRC6A. Data are representative of three independent experiments performed in triplicate. **P*<0.05 versus the exCa²⁺ group.

3.5 Extracellular Ca²⁺ aggravate passive system anaphylaxis of Balb/c mice, which can be inhibited by NPS2143

We determined that exCa²⁺ aggravates MC activation through GPRC6A *in vitro*, but remained uncertain regarding its effect *in vivo*. Therefore, we used a mouse model of passive systemic anaphylaxis (PCA) for verification (Figure 8A). This model was

selected because PCA occurrence in these mice totally depends on MCs [41] and thus is more comparable to results from cell-based immunology research.

The allergy group, Ca^{2+} group (additional 50 mM Ca^{2+} without sensitization and stimulation), and NPS2143 group decreased in rectal temperature from control (50 mM Ca^{2+} only) and were scored as exhibiting Grade 2 moderate shock (according to the Balb/c mice PCA shock scale, Supplementary Table 3) (Figure 8B). Although rectal temperature and PCA shock grade did not differ significantly across the three groups (allergy, Ca^{2+} , and NPS2143), we obtained notable results for other variables.

We recorded allergic symptoms 30 min after DNP-HAS injection and scored mice according to the Balb/c mice anaphylaxis clinical symptom grade (Supplementary Table 4). Compared with the allergy group (score of 1), mice in the Ca^{2+} group scratched more often (> 10 times/15 min), shook more, and reached a score of 2 (Figure 8C). Importantly, NPS2143 treatment inhibited the aggravation effect of ex Ca^{2+} on allergic reactions (decreased average clinical allergic score to 1.25 ± 0.46 , Figure 8C).

Next, to assess vascular permeability (key indicator of allergic reactions [43]), half of the mice in each group were injected through the tail vein with 0.5% Evans blue dye after DNP-HSA stimulation. Macroscopic observation revealed that regions with abundant blood vessels and thin skin (nose and footpad) appeared blue after sensitization and stimulation. In contrast, blue dye extravasation was absent in the control group. Together, these findings indicate that the vascular permeability of mice increased significantly under PCA. Moreover, footpads of mice in the Ca^{2+} group were bluer than those in the allergy group, indicating that ex Ca^{2+} significantly aggravates albumin exudation in allergic mice. NPS2143 treatment decreased Evans blue extravasation (Figure 8D).

Results of a BCA protein assay on peritoneal irrigation fluids showed that intraperitoneal injection of Ca^{2+} increased peritoneal albumin exudation ($P < 0.05$), while NPS2143 decreased it ($P < 0.05$, Figure 8E). Serum histamine levels exhibited the same pattern. Sensitization and stimulation increased histamine levels in mouse

serum ($P < 0.05$), whereas exCa^{2+} treatment alone had no effect. Moreover, additional exCa^{2+} increased histamine levels (Figure 8F, Ca^{2+} group versus allergy group, $P < 0.05$), while NPS2143 partially (Figure 8F, NPS2143 group versus allergy group, $P < 0.05$) but significantly inhibited exCa^{2+} effects (Figure 8F, NPS2143 group versus Ca^{2+} group, $P < 0.05$). Our results demonstrated that exCa^{2+} aggravates PCA in BALB/c mice through activating GPRC6A.

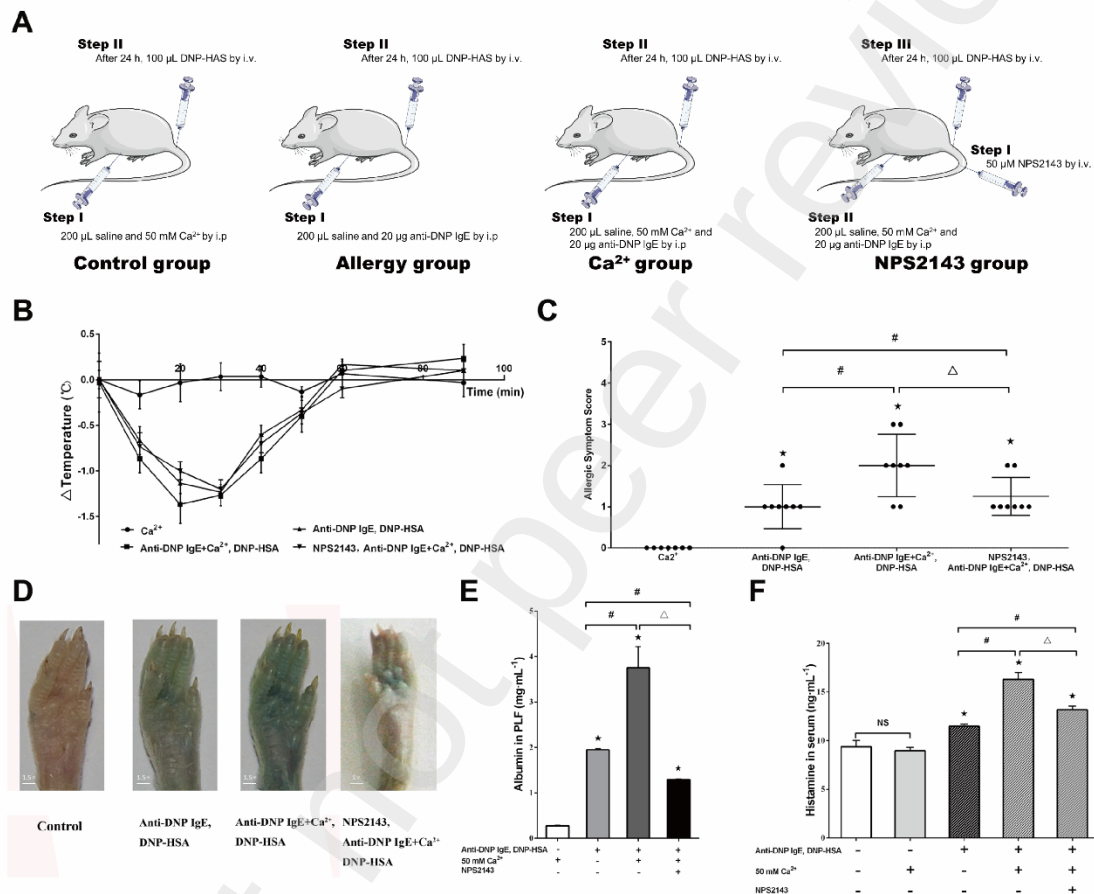


Figure 8 Extracellular Ca^{2+} aggravate passive system anaphylaxis of Balb/c mice by activating GPRC6A. (A) Schematic drawing representing the passive systematic anaphylaxis model protocols and doses used in this work. (B) The variation of rectal temperature ($^{\circ}\text{C}$). (C) Allergic symptom score. (D) Qualitative and (E) quantitative detection of the vascular permeability after Evan's Blue Dye/DNP-HSA administration. (F) Histamine level in serum. Data are representative of three independent experiments performed in triplicate. $*P < 0.05$ versus the control group. $\#P < 0.05$ versus the allergy group. $\Delta P < 0.05$ versus the NPS2143 group.

4 Discussion

Mast cell-driven allergic reactions are mainly a consequence of allergic mediators released after antigen-induced aggregation of the IgE-FcεRI complex on the MC surface [44]. To determine the effect of ex[Ca²⁺] variation on allergic reactions, we measured degranulation, cytokines, and eicosanoids after treating two MC lines (RBL-2H3 from rats and BMMCs from mice). We obtained the same results for both cell lines, indicating that increasing ex[Ca²⁺] during sensitization significantly enhances MC-mediated allergic reactions in a dose-dependent manner. Our *in vitro* experiments demonstrated that increased ex[Ca²⁺] promotes degranulation, including β-hexosaminidase and histamine release; increases secretion of T_H2-type and inflammatory-related cytokines (e.g., IL-4, IL-13, IL-1β, IL-6, TNF-α); and induces eicosanoid production (e.g., LTC₄ and PGD₂). *In vivo* experiments using the PCA mouse model yielded similar findings. Intraperitoneal injection of 50 mM Ca²⁺ significantly aggravated allergies in Balb/c mice, manifesting as increased clinical scores for allergic symptoms, vascular permeability, and serum histamine secretion. Next, we explored the mechanism of exCa²⁺ effects on MC activation. Considering the importance of sIgE and FcεRI binding in MC activation and the role of Ca²⁺ in FcεRI signaling, we first hypothesized that exCa²⁺ aggravates MCs through upregulating IgE binding to FcεRI. However, flow cytometry results showed that the fluorescence intensity of bound IgE did not change with increasing ex[Ca²⁺]. We next hypothesized that exCa²⁺ acts as a ligand to activate a receptor-mediated signaling pathway in MCs. We found that GPRC6A, rather than CaSR, was expressed in MCs and acted as a ligand-activated receptor for exCa²⁺. GPRC6A is a novel family C G-protein-coupled receptor, comprising a Venus flytrap (VFT) domain, cysteine-rich domain (COD), and classic seven-transmembrane domain that anchors to the plasma membrane [45]. A prior study used immunofluorescence localization to verify that GPRC6A is expressed on the cell membrane and plays a role in sensing [Ca²⁺] variation [46]; this data was strong evidence that exCa²⁺ ligates with GPRC6A to aggravate MC activation. Calcium sensing receptor-specific inhibitor NPS2143 and GPRC6A-specific siRNA both inhibited the effect of exCa²⁺ on MC activation,

confirming that GPRC6A regulates Ca^{2+} mobilization. In addition, injecting PCA mice with NPS2143 revealed that inhibiting GPRC6A activation significantly weakened exCa^{2+} aggravation of IgE-mediated PCA, further confirming that GPRC6A is the target of exCa^{2+} in allergic effector cells.

Although we have provided evidence exCa^{2+} and GPRC6A involvement in allergic diseases, this study has several unresolved questions that can be addressed in future research. The inhibitory effect of siRNA treatment on MC activation was significantly greater than that of NPS2143 (Figure 6). Therefore, while a GPRC6A inhibitor like NPS2143 may be adequate *in vitro*, it probably has several targets besides MCs *in vivo*. Thus, our study cannot definitively conclude that the effects of NPS2143 on anaphylaxis are due to inhibiting GPRC6A expression. The use of GPRC6A^{-/-} mice or reconstitution of MC-deficient mice with GPRC6A^{-/-} MCs would properly address this issue. In particular, we consider it necessary to use GPRC6A^{-/-} mice for investigating downstream signaling pathways (e.g., cAMP production, G protein coupling, and phosphorylation) and determining whether GPRC6A and FcεRI signaling pathways merge to enhance MC activation. In addition, necrotic cells release intracellular Ca^{2+} and increase Ca^{2+} levels in body fluid when homeostasis is disrupted. Thus, it is essential to use human samples and perform clinical experiments that can clarify the specific effect of necrotic cells on Ca^{2+} levels under allergic inflammation, as well as confirm the roles of exCa^{2+} and GPRC6A. Finally, calcium is widely used in conjunction with vitamin D to prevent osteoporosis, and calcium supplementation is also promoted for its potential benefits in lowering the risk of metabolic syndromes and cancers [47]. However, calcium as a treatment for allergic diseases remains controversial. Therefore, exploring the causal link between calcium and various health outcomes, including allergic diseases, will provide an empirical basis for intake recommendations in patients with allergies.

5 Conclusion

In conclusion, our study showed that increased exCa^{2+} aggravates allergic reactions via activating the calcium-sensitive receptor GPRC6A on effector cell membranes.

Specifically, exCa^{2+} promotes degranulation, histamine release, cytokine secretion, and eicosanoid synthesis in effector cells. In PCA mice, exCa^{2+} inhibits Evans blue exudation in footpads, lowers albumin content in the abdominal cavity, and decreases histamine levels. Controlling exCa^{2+} influx is an important strategy for allergy prevention and treatment, and our findings indicate that GPRC6A is a suitable therapeutic target.

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Conflicts of interest

The authors have no conflicts of interest to disclose.

Author contributions

S.W.H, G.R.L and H.L.C designed the study. G.R.L, S.W.H, S.S.J and Y.C.J performed the experiments. S.W.H, G.R.L, Y.C.J and N.S contributed to the data analysis, interpretation of the data and statistics. G.R.L and S.W.H drafted the article, which was critically revised and edited by S.S.J and H.L.C. All authors revised the manuscript and approved the final version.