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Tachykinin-1 receptor antagonism suppresses substance-P- and compound 48/80-induced mast cell activation from rat mast cells expressing functional mas-related GPCR B3

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Abstract

Objective Mice and rats are important animal models for mast cell (MC) study. However, rat Mas-related-GPCR-B3 receptor (MRGPRB3) has been less studied than its mouse counterpart. Therefore, we aimed to characterize rat MRGPRB3.

Methods *Mrgprb3* mRNA expression was assessed in peritoneal cells (RPCs) and peritoneal MCs (RPMCs) of wild-type rats, RPCs of MC-deficient rats, and RBL-2H3 cells by reverse-transcriptase polymerase chain reaction (RT-PCR). RPMCs, *MRGPRX2*-transfected and non-transfected RBL-2H3 cells were activated by 15–30 min incubation with DNP-BSA, substance-P (SP), or compound-48/80. L732138 or CP96344 was used as a tachykinin/neurokinin-1-receptor antagonist. Histamine release from MCs was measured by HPLC fluorometry.

Results *Mrgprb3* mRNA expression was found in all cells, with the highest level in wild-type RPCs. All cells responded to DNP-BSA, but only *MRGPRX2*-transfected-RBL-2H3 cells and RPMCs responded to all activators. L732138 (0.1–10 μM) and CP96344 (1–100 μM) suppressed SP (10 μM)-induced RPMC activation. L732138 inhibition was dose independent, whereas CP96344 inhibition occurred in a dose-dependent manner. Additionally, only CP96344 suppressed SP (100 μM)- and compound-48/80 (10 μg/mL)-induced RPMC activation.

Conclusions RPMCs expressing functional MRGPRB3 response upon MRGPRX2 ligands to regulated MC-mediated activities. It`s provide novel insights for future pseudo-allergic studies in rodents.

Keywords MRGPRB3 · MRGPRX2 · Mast cells · Rat · Histamine

Introduction

In 2006, human mast cells (MCs) were reported to express Mas-related G protein-coupled receptor-X2 (MRGPRX2), a receptor responsible for MC activation by basic compounds and peptides [1]. This was in contrast to earlier reports, which suggested that basic compound and peptide activation of MC bypassed the agonist-receptor interaction [2–4]. The ability of MRGPRX2 to mediate MCs activation indicated that its receptor antagonist could attenuate inflammation or allergic conditions that failed to be attenuated by histamine receptor- [5, 6] or neurokinin receptor-antagonist alone [7]. The murine *Mrgpr* gene has the highest homology to *MAS1* (35% identity) and mas-related gene 1 (*MRG1*) (30–35%); of the 50 murine subfamilies (*MrgprA, MrgprB, MrgprC*), approximately 30% are pseudogenes [8]. In contrast, human *MRGPR* only has four closely related genes (*MRGPRX1–4*) and at least nine pseudogenes [8]. Although the human and mouse genes share strong sequence homology, they do not form clear orthologous pairs [9].

Early study of MRGPRX2 showed the ability of this receptor to mediate MC responses to substance-P (SP), cortistatin, proadrenomedulin, and adrenocorticotropin hormone (ACTH) [1, 10–12]. It was shown that MRGPRX2

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could be activated by common MC activators, including compound-48/80 and bee venom [13]. In mouse studies, MRGPRB2 demonstrated a potential role in mediating MC activation following stimulation with compounds that were reported to activate MC through MRGPRX2. The studies suggested that MRGPRB2 is an MRGPRX2 orthologue in mice [13, 14]. Muscle relaxants, antibiotics, and several peptide drugs have been shown to activate MCs through these receptors and mediate drug pseudo-allergic reactions in animal models [13, 14]. Peptides that have been reported to activate both MRGPRX2 and MRGPRB2 are known to have their own specific receptors. Thus, the interaction between MRGPRX2 and some of these peptide ligands are likely to be non-specific. For example, SP stimulates pharmacological activity through its specific binding with three different tachykinin/neurokinin receptors (TACR1–3) [15], whereas ACTH signals through multiple melanocortin receptors [16].

Unlike MRGPRX2 and MRGPRB2, few studies have focused on related receptors in rats, and limited reports about MRGPR in rat MCs are available. Of the ten MRGPR families in rats, MRGPRB3 and MRGPRB8 genes are expressed at high levels in rat peritoneal mast cells (RPMCs) [1]. Based on a luciferase gene-reporter and Ca^{2+} influx assay, MRGPRB3 was suggested to be the MRGPRX2 orthologue in rats [1]. However, because these experiments were only performed in cells transfected with MRGPRB3, additional studies are needed to characterize rat MRGPRB3.

Therefore, in this study, we characterized *Mrgprb3* expression in rats, which was previously thought to be selectively expressed in connective tissue type mast cells (CTMC) [1]. In addition, we also investigate pharmacological response of *Mrgprb3*-expressing RPMCs after stimulation with compounds previously reported to induce MC activation through MRGPRX2 or MRGPRB2.

Materials and methods

Chemicals and reagents

MEM medium, antibiotics (5000 unit/mL penicillin; 5000 µg/mL streptomycin), and fetal bovine serum (FBS) were purchased from *Gibco Life Technologies* (*Grand Island, NY, USA*). PIPES and Na_2-EDTA were purchased from *Dojindo* (*Kumamoto, Japan*). Solutions, including H_3PO_4, NaOH, *o*-phthalaldehyde, and KH_2PO_4, for HPLC fluorometry analysis of histamine and salts and glucose for PIPES buffer preparation and perchloric acid (PCA) were purchased from *Wako Chemical Company* (*Osaka, Japan*). Bovine serum albumin (Probumin®) was obtained from *Millipore* (*MA, USA*), and heparin was purchased from *Mochida Pharmaceutical* (*Tokyo, Japan*). Percoll solution, G418 antibiotic, tubocurarine, and compound-48/80 were purchased from *Sigma-Aldrich* (*St. Louis, MO, USA*). Dinitrophenylated bovine serum albumin (DNP_{24}-BSA) and monoclonal IgE against DNP-BSA were generated in our laboratory. Substance-P was purchased from *Peptide Institute Inc.* (*Osaka, Japan*), and ACTH was obtained from *Shionogi and Co Ltd.*, (*Osaka, Japan*). TACR1 antagonists L732138 and CP96344 were obtained from Tocris Bioscience (*Bristol, UK*) and Pfizer (*New York, NY, USA*), respectively.

Comparison of MRGPRX2, MRGPRB2 *and* MRGPRB3 *amino acid sequences and two-dimensional structures*

Comparison of MRGPRX2, MRGPRB2, and MRGPRB3 amino acid gene sequences was performed using MEGA 6.06 with Clustal W analysis. Amino acid sequences were retrieved from Pubmed (https://www.ncbi.nlm.nih.gov/pubmed) and Uniprot (https://www.uniprot.org) databases. For prediction of the two-dimensional structure of the protein and transmembrane region, three online analysis programs were used, including HMMTP version 2.0 (https://www.enzim.hu/hmmtop/) [17, 18], Protter version 1.0 (https://wlab.ethz.ch/protter/) [19], and RbDe program (https://icb.med.cornell.edu/services/rbde/) [20]. For analysis using Protter and RbDe, the following protein IDs were used: Q3KNA1 (mouse MRGPRB2), F1LLV4 (rat MRGPRB3), and Q96LB1 (human MRGPRX2). For HMMTP, the NCBI accession numbers were as follows: EDL22965 (mouse MRGPRB2), ACG60653 (human MRGPRX2), and XP_001077174.2 (rat MRGPRB3).

RBL-2H3 cell transfection with MRGPRX2-coding plasmid

RBL-2H3 cells were transfected with a *GFP* tagged-*MRGPRX2* plasmid *(Origene, MD, USA)* using RBL-2H3 cell Avalanche transfection reagent (*EZ biosystem, MD, USA*), according to the manufacturer's protocol. *MRGPRX2*-transfected RBL-2H3 (*MRGPRX2*-RBL-2H3) cells were maintained in MEM medium containing 1% penicillin–streptomycin (PenStrep), 400 µg/mL of G418, and 15% of FBS.

RPMC isolation by gradient centrifugation

Rats for RPMC isolation were obtained from Japan SLC, Inc. (Hamamatsu, Japan). Male Donryu rats (3–4 months) were given an intra-peritoneal injection of 20 mL Phosphate Buffer Saline (PBS) containing 0.1% bovine serum albumin (BSA) and 10 units/mL of heparin. The abdomen was then massaged for 2–3 min, swabbed with 70% ethanol, and the peritoneal cavity was opened carefully. Subsequently, the previously injected PBS was collected from the peritoneal cavity, followed by centrifugation

at 1000 rpm and 4 °C for 5 min. After removal of the supernatant, cell pellet was suspended in 2 mL PBS containing 0.1% BSA, placed over 4 mL of 60% Percoll in PBS solution, and centrifuged at 450–500 rpm, 4 °C for 20 min. The collected RPMCs were washed with 6 mL PBS containing 0.1% BSA solution by centrifugation at 1000 rpm, 4 °C for 5 min. Cell number was counted after staining with 0.05% toluidine blue solution. Cell isolation protocol from peritoneal cavity was performed following the guidelines of the Animal Care Committee of Ehime University. This protocol had been approved by the University Committee for Animal Research.

Mast cells activation assay

Mast cell activation was quantified by measuring histamine release after stimulation with peptides or basic compounds. Sample preparation followed the method as described in our previous study [21]. In brief, a suspension of 5×10^5/mL RBL-2H3 or *MRGPRX*2-RBL-2H3 cells (400 µL/well) was plated in MEM medium containing 1% PenStrep antibiotics, 15% FBS, and monoclonal IgE against DNP-BSA into a 24-well culture plate. After overnight incubation (5% CO_2, 37 °C), the medium was replaced with 200 µL PIPES buffer containing DNP-BSA (20 ng/mL), basic compounds, or peptides and incubated for 15–30 min at 37 °C in a water bath. Cell activation was stimulated by incubation with DNP-BSA for 30 min. For other compounds, cells were stimulated for only 15 min. For RPMCs, histamine release was assessed directly after RPMCs isolation using 4000 cells/well in a 96-well plate. When histamine release inhibition was analyzed, incubation with an TAC1R antagonist (L732138 or CP96344) was performed for 15 min, following the initial incubation with the MC activator (compound-48/80 or SP). Histamine release after cell activation was determined by HPLC fluorometry as described by Yamatodani et al. [22]. Histamine release percentage was calculated as previously described [21].

Total mRNA expression of *Mrgprb3*, *Tacr1*, *Tacr2*, and *Tacr3* receptors on rat MCs

Receptor expression was measured by RT-PCR of total mRNA from specified rat cells. Total mRNA was collected from wild type (Wt/Wt) and mast cells-deficient (Ws/Ws) rat peritoneal cells (RPCs), Wt/Wt RPMCs, and RBL-2H3 cells [as a type of mucosal type mast cell (MMC)] using ISOGEN (*Nippon Gene, Tokyo, Japan*) according to the manufacturer's protocol. Genomic DNA was eliminated using DNase I (*Nippon Gene, Tokyo, Japan*). The RT-PCR reaction was performed using 500 ng of total mRNA as a template with the Takara RNA PCR Kit (AMV) Ver 3.0 (*Takara Biotechnology, Shiga, Japan*). The RT conditions were as follows: 30 °C for 10 min, 60 °C for 30 min, 95 °C for 5 min, and 5 °C for 5 min, proceeded by the PCR reaction for 35 cycles of denaturation initiation for 2 min at 94 °C, denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C, elongation for 30 s at 72 °C, and final elongation at 72 °C. Primers for each gene amplification are shown in Table 1.

Statistical analysis

All graphical data in this study are presented as mean ± S.E.M. of triplicate experiments, unless otherwise stated. Statistical analysis was processed using one-way ANOVA multiple comparisons. *P* value lower than 0.05 ($P < 0.05$) was considered significant. Statistical analysis was processed using GraphPad Prism 8.

Results

Comparison of amino acid sequences of MRGPRX2, MRGPRB2, and MRGPRB3

Following alignment with MEGA Clustal W and Muscle, the amino acid similarity of MRGPRX2, MRGPRB2, and MRGPRB3 was 39% (Supplementary Table 1). By contrast, BLAST tools resulted in a lower sequence similarity of approximately 30% (Supplementary Table 2). When

Table 1 Primers for receptor gene detection

Gene	Forward primer	Reverse primer	Amplified size
Gapdh	CAA GTT CAA TGG CAC AGT CAA GGC T	AAC GGA TAC ATT GGG GGT AGG AAC A	609 bp
Mrgprb3	CCC CTG GAA TGT TCT TTT GTG TAG	ACA GTG AAA AAT GCA GGA ACT TGG	258 bp [1]
Tacr1	CCT CTT GCC CTA CAT CAA CCC	CTG TGT CTG GAG GTA TCG GG	231
Tacr2	CAT CAC TGT GGA CGA GGG GG	TGT CTT CCT CAG TTG GTG TC	490
Tacr3	CAT TCT CAC TGC GAT CTA CC	CTT CTT GCG GCT GGA TTT GG	324
Hrh4	TCA GTT TCA AAC GCT GTG CG	CTA GCG ACC TGG CTA GCT TC	560

comparing only two sequences of the receptors, MRGPRX2-MRGPRB2, MRGPRB2-MRGPRB3, or MRGPRX2-MRGPRB3, the percentage of sequence similarity was relatively identical at 50% (Supplementary Table 1). BLAST again showed lower similarity than MEGA (Supplementary Table 2). However, both tools showed a high similarity (30–50%) between MRGPRX2, MRGPRB2, and MRGPRB3.

Prediction of the protein transmembrane region using HMMTOP clearly showed that all receptors (EDL22965 for MRGPRB2, ACG60653 for MRGPRX2, and XP_001077174.2 for MRGPRB3) passed through the membrane seven times (Supplementary Fig. 1), confirming the GPCR characteristics. Similar results were obtained using Protter (Supplementary Fig. 2) and RbDe programs (Supplementary Fig. 3). From the two-dimensional structural prediction, differences in the intra-cellular loop 1–3 (IL 1–3) and extracellular loop 1–3 (EL 1–3) length of the receptors were observed. Rat MRGPRB3 had the same two-dimensional structure arrangement as human MRGPRX2 (Supplementary Figs. 2, 3). Mouse MRGPRB2 had a very short EL1 as compared to MRGPRB3 and MRGPRX2. The IL2 region in MRGPRB3 and MRGPRX2 was longer than their IL1 and IL3 regions. By contrast, the lengths of IL1–3 in MRGPRB2 were relatively identic. Collectively, the two-dimensional protein structure prediction results showed that the MRGPRB3 structure was more related to MRGPRX2 than MRGPRB2. However, there were no significant differences in the amino acid sequences between species.

Mrgprb3 expression specificity on CTMCs

Mrgprb3 has been suggested to be exclusively expressed in sensory neurons and MCs [1]. Here, we investigated *Mrgprb3* mRNA expression in several rat cell types, including RBL-2H3 cells, Wt/Wt RPC, Ws/Ws RPC, and Wt/Wt RPMCs (referred to as RPMCs). RBL-2H3 represented MMCs, RPMCs as CTMCs, and RPC represented RPMCs mixed with other peritoneal resident cells. The result showed *MrgprB3* expression in CTMCs, RBL-2H3 cells, and Ws/Ws RPCs (Fig. 1a). The expression level order from all of the samples was Wt/Wt RPC>RPMCs>Ws/Ws RPC>RBL-2H3 cells (Fig. 1b). Taken together, these results showed that all samples expressed different levels of *Mrgprb3*.

Activation of RBL-2H3 cells, *MRGPRX2*-RBL-2H3 cells, and RPMCs by basic compounds and peptides

To evaluate MRGPRB3 contribution to MCs activation, histamine release by RBL-2H3 cells, *MRGPRX2*-RBL-2H3 cells, and *Mrgprb3*-expressing RPMCs was measured after treatment with MC activators. Induction using 20 ng/mL of DNP-BSA showed identical dose–response curves in all cell groups (Fig. 2a). The calculated EC_{50} value was 2.13 ng/mL for RBL-2H3 cells and 1.74 ng/mL for *MRGPRX2*-RBL-2H3 cells, suggesting that transfection process of *MRGPRX2*-RBL-2H3 cells did not affect the responsiveness of RBL-2H3 cells towards antigen activation. In addition, the RPMC purification method could maintain the collected cells to response towards DNP-BSA (Fig. 2a).

Administration of compound-48/80, SP, and ACTH clearly induced histamine release from RPMCs and *MRGPRX2*-RBL-2H3 cells in a dose-dependent manner (Fig. 2b–d). Tubocurarine treatment could only induce histamine release at a high concentration (100 µM), which induced histamine release up to 20% and 25% in RPMCs and *MRGPRX2*-RBL-2H3, respectively (Fig. 2e). Substance-P induced histamine release in RPMCs at 10 µM, whereas on *MRGPRX2*-RBL-2H3 had the lowest effective concentration at 1 µM (Fig. 2c). Maximum RPMCs histamine release was reached when using 100 µM SP (Supplementary Fig. 4a). However, only 10 µM SP was required to release the maximum amount of histamine from *MRGPRX2*-RBL-2H3 cells. Compound-48/80 and ACTH also showed higher maximum histamine release in RPMCs compared to *MRGPRX2*-RBL-2H3 (Fig. 2b, d). At dose ranges used, all basic compounds and peptides failed to activate RBL-2H3 cells. However, high concentration of compound-48/80 induced a slight histamine release increase on RBL-2H3 cells (Supplementary Fig. 4). Taken together, these results showed that MRGPRX2 mediated RBL-2H3 cell activation by basic compounds and peptides. In addition, *Mrgprb3*-expressing RPMCs reacted to the same ligands as *MRGPRX2*-RBL-2H3 cells, with a slightly different potency.

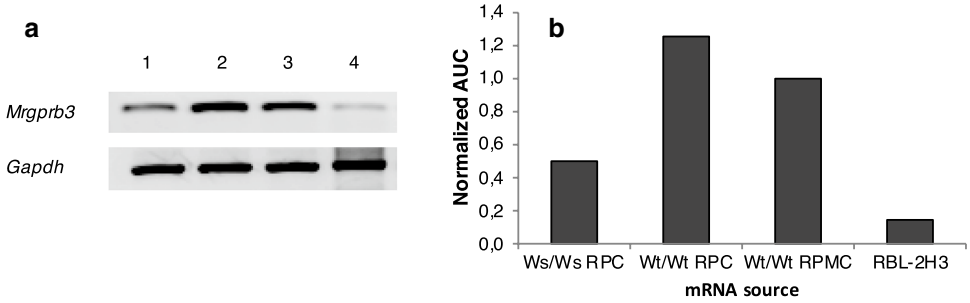

Fig. 1 *Mrgprb3* mRNA expression in rat cells, $n = 1$. Expression of *Mrgprb3* in Ws/Ws RPC (**a1**), Wt/Wt RPC (**a2**), Wt/Wt RPMC (**a3**), and RBL-2H3 cells (**a4**). Quantification of *Mrgprb3* RT-PCR band performed using Image-J software (**b**)

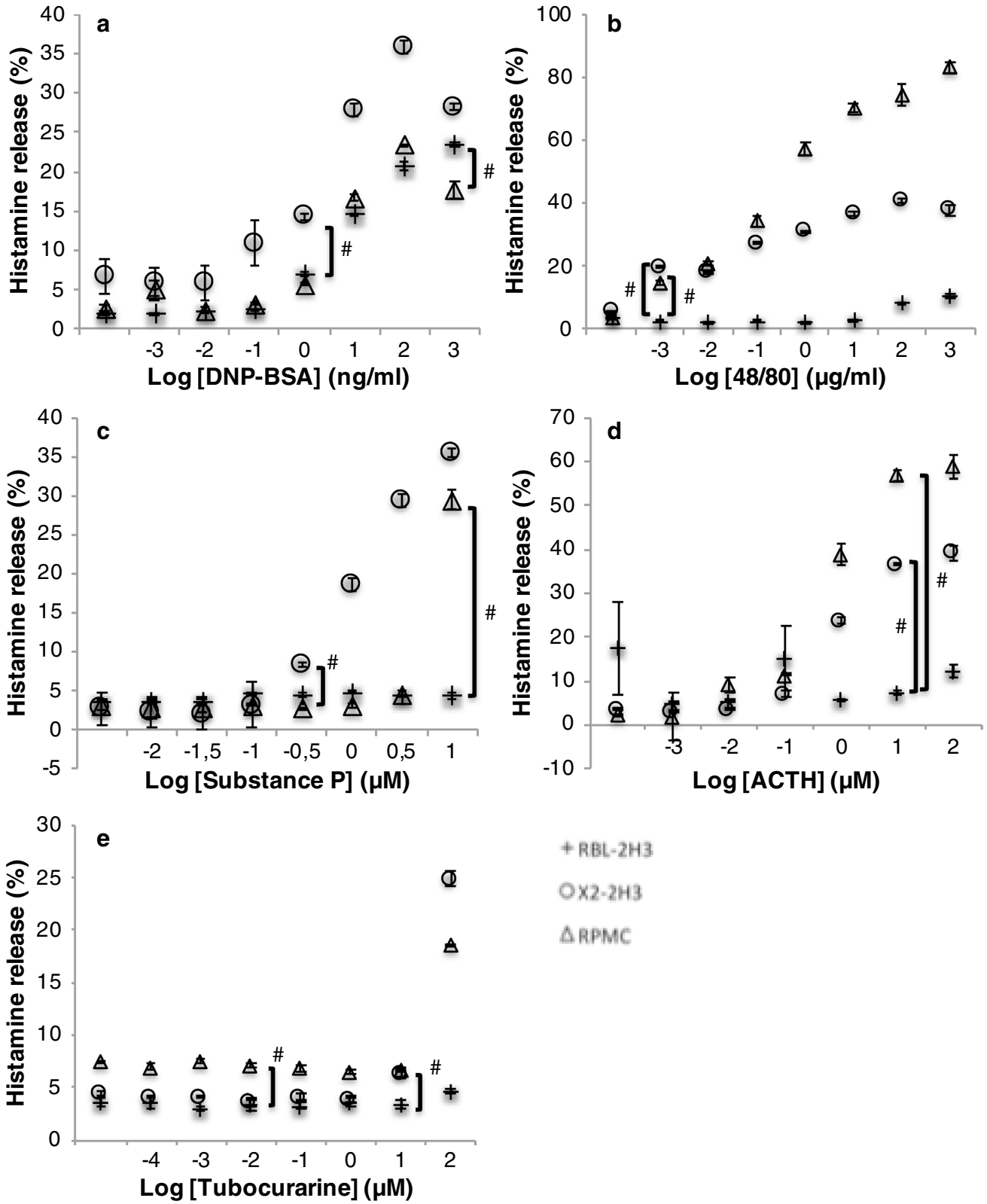

Fig. 2 Histamine release in RBL-2H3 cells, *MRGPRX2*-RBL-2H3 cells, and RPMCs. Histamine release was triggered using DNP-BSA (**a**), compound-48/80 (**b**), SP (**c**), ACTH (**d**), and tubocurarine (**e**). Data are expressed as mean ± S.E.M. from triplicate experiments. (#) indicates significant difference vs RBL-2H3 cell group, $P < 0.0001$

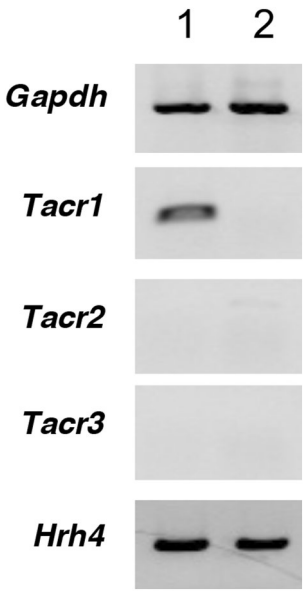

Fig. 3 Substance-P receptor (*Tacr1-3*) expression and effect of TACR1 antagonist on mast cells, $n = 1$. Expression of *Tacr1*, *Tacr2*, and *Tacr3* in RPMCs (1) and RBL-2H3 cells (2). Histamine receptor-4 (*Hrh4*) gene expression was used as a mast cell positive control

Incomplete inhibition of SP-mediated RPMC activation by TACR1 antagonist

Substance-P has been known to elicit its pharmacological effect through three different tachykinin receptors, TACR1-3. Therefore, we measured *Tacr1-3* expression in RBL-2H3 cells and RPMCs. Figure 3 demonstrated the specificity of *Tacr1* expression on RPMCs, but not RBL-2H3 cells. Further, *Tacr2* and *Tacr3* expression was not detected in either cell type. We further determined whether the effect of SP in RPMCs was mediated by TACR1 or other receptors through administration of the TACR1 antagonists, L732138 and CP96344. As MRGPRX2 and MRGPRB3 have unspecific ligands, the ability of L732138 and CP96344 to induce histamine release from RPMCs was investigated. Our data showed that the administration of L732138 and CP96344 (up to 10 µM and 100 µM, respectively) did not result in increased histamine release from RPMCs (Supplementary Fig. 5a, b). L732138 has an IC_{50} of 2.1 nM and is highly selective for TACR1. Therefore, 10 µM was considered effective for TACR1 inhibition without stimulating histamine release from RPMCs and was chosen as the maximum concentration used in our study. Due to limited in vitro data for CP96344, a high concentration of 100 µM was chosen for our study.

Three different concentrations of L732138 (0.1, 1, and 10 µM) failed to inhibit SP (100 µM)-induced MC activation (Fig. 4a). When a lower concentration of SP (10 µM) was used to induce MC activation, all L732138 concentrations inhibited histamine release with identical potency (Fig. 4a). Additionally, CP96344 inhibited histamine release induced by 10 µM SP in a dose-dependent manner (Fig. 4b). When the higher concentration of SP (100 µM) was used, CP96344 still inhibited histamine release, but without its dose dependency (Fig. 4b). Since a TACR1 antagonist was previously shown to act on MRGPRX2 [7], we tested the ability of L732138 and CP96344 to prevent histamine release in compound-48/80-activated RPMCs. When the cells were treated with compound-48/80 (10 µg/mL and 100 µg/mL), L732138 (0.1, 1, and 10 µM) failed to inhibit cell activation (Fig. 4c). Interestingly, CP96344 inhibited histamine release induced by 10 µg/mL of compound-48/80 (Fig. 4d), but failed at 100 µg/mL (Fig. 4d).

Together, these results showed that the TACR1 antagonist could only inhibit histamine release from MC when a low concentration of SP was used to activate the cells.

Discussion

Recently, studies on human MRGPRX2 and its orthologue in mice have increase significantly. However, studies in rats remain limited. Because rats are often used in MC studies, characterization of MRGPRB3, rat orthologue of human MRGPRX2, is essential. The use of both rats and mice in MC study is important, especially in translating in vivo work to human application. Mice are commonly used as animal models for many MC functional studies due to the availability of reagents, strains, and genetically modified mice for the specific condition [23, 24]. However, mice do not replicate all features present in the human disease condition [23, 25, 26]. In asthma, for example, rats more closely reflect the disease pathogenesis to humans [23, 25, 26]. Contrastingly, mice lack chronicity of the allergen response and tolerance after repeated allergen exposure [23]. In addition, the larger size of rats compared to mice is advantageous when body fluid (serum or mucus) are of interest [23], and they contain a greater number of cells per animal when working with PMCs. The study by Oka et al. also showed that cromolyn, a MC stabilizer that is effective in humans, was not effective in mice but effective in rats to stabilize MCs induced by IgE-antigen interaction [27].

Previously, it was shown that rat MRGPRB3 had the same function as MRGPRX2 in humans [1]. In the present study, *Mrgprb3* knock down in freshly isolated RPMCs successfully suppressed its expression. However, the cell condition was not conducive to measure histamine release as excessive histamine release occurred in this cell type without any stimulation (unpublished results). Hence, the present study considered MRGPRB3 to be the rat orthologue of MRGPRX2 based on two-dimensional structural comparison and *Mrgprb3* expression in cells. Due to the lack of an available

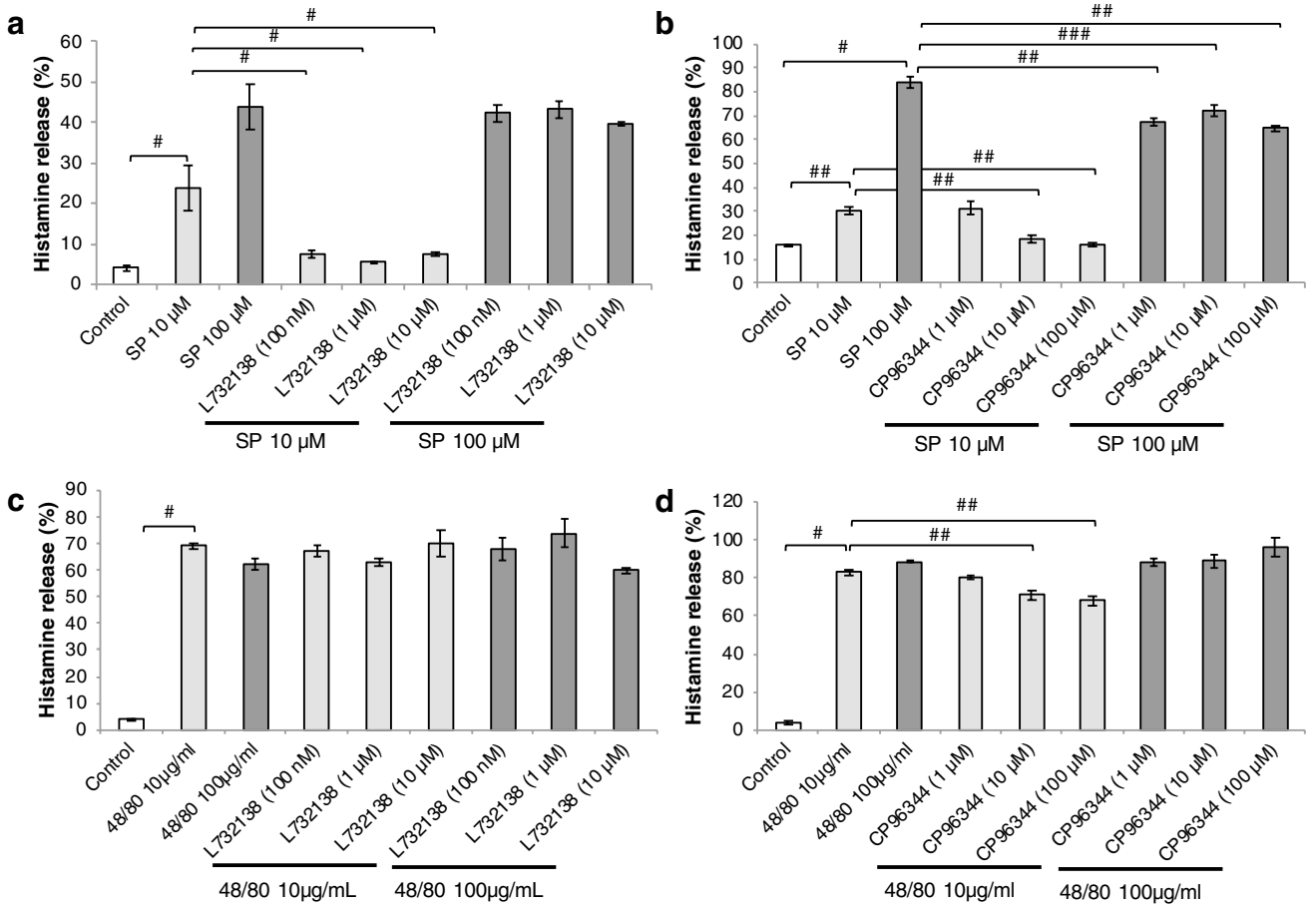

Fig. 4 Effect of TACR1 antagonist on SP and compound-48/80-induced histamine release from RPMCs. L732138 inhibited low concentration (10 μM) SP-induced histamine release from RPMCs, but no inhibition was observed with high concentration (100 μM) SP (**a**). CP-96344 inhibited MC activation by both low and high concentration SP (**b**). L732138 had no effect on cells activated with compound-48/80 (**c**). CP-96344 inhibited histamine release in cells treated with 10 μg/mL of compound-48/80. CP-96344 did not affect the histamine release in cells treated with 100 μg/mL of compound-48/80 (**d**). Data are expressed as mean ± S.E.M. from triplicate experiments. (#) indicates significant difference with $P < 0.0001$, (##) $P < 0.001$, (###) $P < 0.01$

commercial antibody, we were also unable to assess MRGPRB3 protein expression in this study. In future studies, heterologous overexpression of MRGPRB3 in RBL-2H3 cells will be conducted to provide additional evidence of MRGPRB3 pharmacological activity in rat MCs.

Mrgprb3 expression in Ws/Ws rat RPC was observed in this study, which suggested that the existence of cells other than MCs expressed this receptor. Cells that reside in adipose tissues within the peritoneal cavity could possibly contribute to the increased *Mrgprb3* expression in this sample. Previously, *MRGPRX2* expression in adipose tissue was confirmed [1]. In addition, even though Ws/Ws rats have been reported to develop MCs during infection [28], we can eliminate the contribution of infection-induced MC clones on *Mrgprb3* expression in this study because of two factors. First, infection in Ws/Ws rats results only in the development of MMC [28], which do not express *Mrgprb3* [1]. Second, toluidine blue staining of RPC from Ws/Ws rats during the RPC isolation process in this study did not show any existence of MC (data not shown). However, further investigation is needed to identify the adipose tissue residing cells or other peritoneal cavity resident cells that are responsible for *Mrgprb3* expression in the RPC of Ws/Ws rats.

Our results showed that the expression of functional *Mrgprb3* was specific to CTMCs. However, we were unable to determine whether *Mrgprb3* mRNA expression correlated with MRGPRB3 protein expression in all samples due to the unavailability of an anti-MRGPRB3 antibody. Analysis of the amino acid sequence and the prediction of two-dimensional transmembrane structure similarities highlighted the shared, conserved structure of MRGPRB3 and MRGPRX2. MRGPRB3 is important for MC activation by basic compounds (i.e., compound-48/80) and peptides (i.e., SP, ACTH). This receptor can also be activated by small chemicals, such as tubocurarine and ciprofloxacin (Supplementary Fig. 6). In this study, the molecules used to activate

MCs were cationic. Additionally, compound 48/80 (cyclized monomer) and tubocurarine shared a tetrahydroisoquinoline (THIQ) structure that is important for MC activation [13]. Surprisingly, a high concentration (1 mg/mL) of compound-48/80 stimulated an increase in histamine release by RBL-2H3 cells, which may be due to *Mrgprb3* expression on RBL-2H3 cells.

The comparison of *MRGPRX2* and *Mrgprb3* mRNA expression level in *MRGPRX2*-RBL-2H3 and RPMC, respectively, was not performed in this study. Thus, the correlation between the level of *MRGPRX2* and *Mrgprb3* expression to their sensitivity upon secretagogue application could not be made. However, it is very likely that the same receptor could result in different sensitivity levels due to species differences. A study by McNeil et al. [13] also showed that the sensitivity of substances used for MC activation varied between species. For example, the quinolone antibiotic family was more sensitive for human MRGPRX2 compared to mouse MRGPRB2 [13]. By contrast, the muscle relaxant rucronium was more effective on MRGPRX2, and atracurium was more effective on MRGPRB2 [13]. Thus, sensitivity variations in the histamine release responses observed in this study could also be the result of species differences. Yet, further comparison of *MRGPRX2* and *Mrgprb3* mRNA should be considered regarding these sensitivity differences.

In contrast with a previous study by Tatemoto et al. [1], we detected *Mrgprb3* expression in MMCs. The mucosal MC cell line representative, RBL-2H3, is a leukemic cell with chromosome instability [29] and is suggested to not fully represent the complete characteristics of MMCs [30]. Thus, it is possible that RBL-2H3 cells lack or express a low copy numbers of protein compared to native MMC. Conversely, it is also possible that RBL-2H3 cells express higher copy numbers of protein compared to native MMC. Our data clearly showed that the level of *Mrgprb3* expression in RBL-2H3 did not made this cell respond towards pharmacological doses of basic compounds or peptides. A previous study has shown an identical condition, in which several clones of RBL-2H3 cells with relatively equal IgE receptor expression demonstrated different histamine release abilities [29]. We speculate that at least three factors contributed to the unresponsiveness of *Mrgprb3*-expressing RBL-2H3 to basic compounds or peptides. First, the low copy number of *Mrgprb3* in RBL-2H3 was insufficient for optimal MC activation. Second, *Mrgprb3* mRNA was not translated to its functional MRGPRB3 receptor. Third, RBL-2H3 cells might be expressing non-functional MRGPRB3.

The ability of SP to activate *MRGPRX2*-RBL-2H3 cells and RPMCs, but not RBL-2H3, showed that SP acted on both MRGPRX2 or MRGPRB3 and TACR1. The lack of all *Tacr* in RBL-2H3 cells clearly suggested that in *MRGPRX2*-RBL-2H3 cells, SP activation occurred through MRGPRX2. There is still a possibility that SP-activated RPMCs through an undefined receptor besides TACR1 and MRGPRB3, since we failed to obtained histamine release data from the *Mrgprb3* knock down rat. The histamine release inhibition by TACR1 antagonist in SP (low concentration)-activated MC is in line with previous reports [7]. This condition also indirectly showed that TACR1 had a higher affinity to SP as compared to MRGPRB3. In a previous study, which supports this hypothesis, SP EC_{50} in TACR1, MRGPRB2, and MRGPRX2 were 4 nM [31], 54 µM, and 152 nM [13], respectively.

Effectiveness of CP96344 to inhibit histamine release from SP-activated RPMC was also assessed. We first hypothesized that CP96344 not active as a TACR1 antagonist. Previous study showed that CP96344 was ineffective in antagonizing TACR1 compared to its stereoisomer, CP96345 [32–35]. However, earlier studies on TACR1 antagonism showed affinity variation across species [7, 36]. For example, human TACR1 had an identical amino acid sequence with dog; however, its antagonist of aprepitant and CP 99994 was 5 to 10-fold more potent in dogs than in humans [36]. In addition, mouse and rat contain Val97 instead of Glu97 (in humans), which associate to TACR1 antagonist lower potency in rodents than human [36]. As the inactivity of CP96344 in antagonizing TACR1 is mostly observed in mice, it is highly possible that CP96344 is active in rats. Even though rat and mouse TACR1 is identical, human and dog identical sequence resulted in different responses towards TACR1 antagonist.

The histamine release levels between the CP96344 and L732138 groups (Fig. 4) are different and might lead to conclusion bias. Different cell pools between the two groups might have contributed to this condition. Single cell pools result in too many samples to process at one time that slightly affected the cell conditions and incubation duration between groups, which results in difficulties to obtain conclusion. The different histamine release levels between the groups obtained here is acceptable as the basal histamine release (without SP administration) level in the CP96344 group is higher than in L732138 (Fig. 4a, b). RPMC, with high basal histamine release, results in a higher histamine release level toward secretagogues treatment compared to cells with low basal histamine release.

As L732138 and an enantiomer of CP96344 is a competitive antagonist of SP [32, 37], it is possible for both L732138 and CP96344 to bind the SP binding site at MRGPRX2 or MRGPRB3 to inhibit MC activation. Unexpectedly, MC activation by compound-48/80 was inhibited by CP96344, but not with L732138, implying the possibility of CP96344 to have an overlapped binding site with SP and compound-48/80 at MRGPRB3. Another report using a tripeptide antagonist of SP (glutaminyl-D-tryptophylphenylalanine, QWF) supports the possibilities that a TACR1 antagonist could work in both MRGPRB3 and TACR1 [7].

However, further study is needed to confirm and elaborate the mechanism of L732138 and CP96344 in inhibiting SP- and compound-48/80-induced MC activation from RPMCs.

High concentration of L732138 and CP96344 showed significant histamine release compared to untreated cells (Supplementary Fig. 5). As MRGPRB3/MRGPRX2 was suggested to respond to a wide variety of ligands without a specific ligand, it is possible that both compounds also non-specifically activated these receptors. L732138 and CP96344 molecules are amine-containing molecules (Supplementary Fig. 7), which are basic in nature and can become cationic in slightly acidic conditions. In addition, both compounds also have THIQ-mimicking structure (Supplementary Fig. 7). These two factors might be responsible for the high histamine release profile at high concentration of L732138 and CP96344. Nevertheless, there is still a strong possibility that this compound induced histamine release through another pathway.

Taken together, our results show that *Mrgprb3*-expressing RPMCs have pharmacological activity that mimics the activity mediated by MRGPRX2 in *MRGPRX2*-RBL-2H3 cells. The mRNA expression of *Mrgprb3* was not strictly distributed to CTMC, because MMC (i.e., RBL-2H3 cells) and Ws/Ws RPC also expressed *Mrgprb3* mRNA. Further investigation should be performed to observe the degranulation event in *Mrgprb3* knock down rats and to elaborate the finding of MRGPRB3 antagonizing by TACR1 antagonist found in this study. An antibody against MRGPRB3 should also be produced to obtain a better understanding of MRGPRB3 and to clarify its function and protein expression in MMC and Ws/Ws RPC. Finally, rat MRGPR3 study will results in better translation of animal data for human application of allergic inflammation condition.

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Compliance with ethical standards

Conflict of interest We do not have conflict of interest to disclose.

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