

BRIEF CONCLUSIVE REPORT

Novel MRGPRX2 antagonists inhibit IgE-independent activation of human umbilical cord blood-derived mast cells

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Abstract

Human MCs are primary effectors implicated in immune surveillance and defense by secreting histamine and various inflammatory mediators, a mechanism termed as degranulation. MCs can be activated by two pathways: IgE-dependent classical pathway and the IgE-independent pathway that utilizes various cationic molecules including substance P (SP) and pituitary adenylate cyclase-activating polypeptides, which are host defense peptides collectively known as basic secretagogues. Our pharmacological study investigated whether or not IgE-independent MC activation is mediated via MRGPRX2. We identified two novel MRGPRX2 antagonists, which completely inhibited the degranulation of human cord blood-derived MCs (hCMCs) induced by basic secretagogues and pseudoallergic drug, icatibant, but IgE- or A23187-challenged hCMCs were resistant to MRGPRX2 antagonists. The MRGPRX2 antagonists markedly inhibited the de novo synthesis of SP-induced prostaglandin D₂ in hCMCs. Moreover, the antagonists were able to inhibit p42/44 mitogen-activated protein kinase signal in hCMCs activated by SP. This study strongly suggests that MRGPRX2 antagonists may be a promising drug to prevent the IgE-independent allergic reactions, and thus, MRGPRX2 antagonist development may lead to a promising therapeutic medication for the IgE-independent allergic reactions.

KEYWORDS

basic secretagogue, degranulation, GPCR, inflammation, MAPK, PGD₂, pseudo-allergy

1 | INTRODUCTION

MCs produce biologically active mediators that have a critical role in hypersensitivity and other allergic inflammatory reactions.¹ Specific antigen crosslinking of IgE antibodies is one of the most extensively studied mechanisms of MC degranulation. However, other nonimmune MC triggers have been reported, including anaphylatoxins, cytokines, and neuropeptides, that include complement fragments and bacterial products, which are collectively termed as basic secretagogues.² In addition to high-affinity IgE receptors, MCs express a novel GPCR known as Mas-related GPCR X2 (MRGPRX2), which was identified as a human GPCR for several peptides including cortistatin, somatostatin, substance P (SP), and proadrenomedullin peptide.^{3,4} We previously

reported novel MC basic secretagogues, including compound 48/80 (c48/80) as well as cationic neuropeptides such as SP, VIP, and pituitary adenylate cyclase-activating peptide (PACAP) (6-27), which induced degranulation in MCs and functioned as ligands for human MRGPRX2.⁵ The reactivity of basic secretagogues toward MRGPRX2 was correlated with the inductive activities of degranulation in MCs.⁵ Recently, antimicrobial peptides (LL-37 and β -defensins)^{6,7} and several drugs (morphine and icatibant)⁸⁻¹⁰ involved in pseudoallergic reactions were also identified as MRGPRX2 agonists. Curiously, MRGPRX2 ligands do not have common characteristics other than basic property. Nevertheless, it has been reported that diverse MRGPRX2 ligands use common sites on receptor to induce MC degranulation.^{9,11} These findings suggest that the MRGPRX2 receptor may act as a promiscuous receptor with respect to ligands. This feature is clearly different from typical GPCRs such as neuropeptide receptors.

The physiological functions of MRGPRX2 were proposed to include nociception and catecholamine secretion from the adrenal glands on the basis of tissue distribution studies of MRGPRX2 gene expression

Abbreviations: c48/80, compound 48/80; hCMC, human cord blood-derived mast cell; M2R, muscarinic acetylcholine M2 receptor; MRGPRX2, Mas-related G protein-coupled receptor X2; NK-1R, neurokinin-1 receptor; PACAP (6-27), pituitary adenylate cyclase-activating peptide (6-27); PGD₂, prostaglandin D₂; rHL, Recombinant human interleukin; SP, substance P; SPA, [D-Pro²,D-Trp^{7,9}]-Substance P (1-11); SPB, [D-Pro⁴,D-Trp^{7,9,10}]-Substance P (4-11).

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in dorsal root ganglia and adrenal chromaffin cells.^{3,4,12} In addition to small diameter neurons in the dorsal root and trigeminal ganglia, connective tissue MCs are unique immune cells that constitutively express MRGPRX2, which is upregulated on the surface of MCs during pathological conditions such as chronic urticaria. Moreover, Fujisawa et al. reported the high expression of MRGPRX2 on skin-derived cultured human MCs and human cord blood-derived MCs (hCMCs), but a low expression of MRGPRX2 on lung-derived cultured hMCs.¹³ MCs derived from the bone marrow migrate into host tissues at strategic points in capillaries close to nerve endings containing neuropeptides.^{14–16} This close anatomical relationship in various tissues, including the skin, intestine, Dura mater, and airway mucosa, suggests MCs are involved in the neurogenic component of inflammatory conditions.^{17,18} For example, SP, VIP, and PACAP are a family of neuropeptides stored in small caliber sensory C-fiber nerve endings and which are implicated in inflammation and the modulation of hypersensitivity reactions.^{19–21} It was reported that MCs isolated from mice deficient in *Mrgprb2*, the murine orthologue of human MRGPRX2, were not activated by basic secretagogues and that the drug-induced symptoms of anaphylactoid responses were reduced.

MRGPRX2 is a unique GPCR because it has broad reactivity to a wide variety of peptides and compounds. Owing to its promiscuity, it is questionable whether small molecules can be identified as MRGPRX2 antagonists. Even though several studies using specific short-interfering RNA knockdown techniques support the idea that MRGPRX2 is involved in hMC activation,^{6,7} it is still not clear whether or not MRGPRX2 is an intriguing target for small molecules that fall within the Lipinski's rule of five.²² We believe that MRGPRX2 antagonists can clarify the intact action of the receptor to determine whether or not it is a potential target for the treatment of pseudoallergy in humans. To date, no MRGPRX2 antagonists with molecular mass less than 500 Da have been reported. In this study, we demonstrate the inhibitory effects of novel small-molecule MRGPRX2 antagonists on the IgE-independent hCMCs activation by measuring degranulation, the de novo synthesis of prostaglandin D₂ (PGD₂), and p42/44 MAPK signals.

2 | RESULTS AND DISCUSSION

2.1 | Positive correlation between the inductive activities of degranulation in hCMCs and MRGPRX2 signals by cationic peptides

We assessed the relationship between the inductive activities of degranulation in hCMCs and that of MRGPRX2 signals by cationic peptides, including SP, PACAP (6-27), SPA, and SPB. SP, SPA, and SPB are the NK-1 antagonists. SP, SPA, and PACAP (6-27) induced degranulation in hCMCs and increased the intracellular Ca²⁺ in MRGPRX2-expressing HEK293 cells in a concentration-dependent manner except for SPB (Fig. 1A). Cationic peptides induced degranulation in hCMCs with a maximum effect at approximately 3 μ M (approximately 30% degranulation, Fig. 1B). Moreover, an increase of intracellular Ca²⁺ in

hCMCs by cationic peptides was observed at the same concentration range as the induction range of hCMC degranulation (Fig. 1C). The effects of SP, SPA, and PACAP (6-27) on the intracellular Ca²⁺ increase in MRGPRX2-expressing HEK293 cells pretreated with PTX were reduced by 80% (Fig. 1D). The higher potency observed in hCMCs than that in HEK293 cells might be related to differences in the cell types used. Since degranulation in MCs induced by basic secretagogues was reported to be sensitive to PTX, we assessed whether PTX-sensitive G proteins were also involved in downstream of the signal transduction of MRGPRX2 by stimulations of especially SP and SPA. Pretreatment of hCMCs with PTX (100 ng/mL, 18 h) abolished the ability of cationic peptides to induce degranulation (Fig. 1E). Others and ours studies indicate that MRGPRs mediate the IgE-independent activation of MCs by basic secretagogues such as SP, VIP, and PACAP (6-27). Recently, we showed that several endogenous enzyme-fragmented peptides (CNP fragments and chaperonin-10 fragments) were MRGPRX2 ligands and could induce degranulation in MCs.²³ McNeil et al. found that *Mrgprb2*, which is the murine orthologue of human MRGPRX2, triggered drug-induced allergy-like reactions.⁸ Several studies reported that basic secretagogues, including antimicrobial peptides (LL-37), also acted as MRGPRX2 ligands and activated MCs. MRGPRX2 ligands seem to have simple and specific characteristics, such as basic sequence, including plural basic amino acids and bulky hydrophobic amino acids, in their sequences. However, MRGPRX2 seems to recognize its ligands even though other peptides might harbor similar characteristics. Indeed, a synthetic neurokinin-1 receptor (NK-1R) antagonist peptide SPA containing D-amino acids in its sequence activated MRGPRX2, but a similar synthetic peptide NK-1R antagonist SPB did not (Fig. 1A). Therefore, it is likely that the mechanism of MRGPRX2 activation is different from typical GPCRs.

2.2 | MRGPRX2 antagonists

Our in-house chemical library, which consists of approximately 12,000 commercially available compounds, was screened in the SP-induced intracellular Ca²⁺ mobilization assay by use of a MRGPRX2 stable-expression system in HEK293 cells. A screening campaign led us to identify two different heterocyclic compounds: compound 1 (1-(5H-10 λ ²-phenazin-5-yl)ethan-1-one, MW. 223.26) and compound 2 (3-(pyridine-2-ylmethyl)-2-thioxo-2,3-dihydro-4H-1 λ ²-quinazolin-4-one, MW. 268.31) whose structures are shown in Fig. 2A. Both compounds could be dissolved in phosphate buffered saline up to 1 mM. All experiments using with antagonists could be performed in the presence of 0.1% DMSO without any effect of DMSO. Both compounds obstructed MRGPRX2-mediated intracellular Ca²⁺ mobilization induced by basic secretagogues and SPA in a concentration manner with IC₅₀ values in the lower single-digit micromolar range (Fig. 2B). Since Robas et al.³ reported that cortistatin-14 was a potent agonist for MRGPRX2, we assessed the inhibitory effects of compounds 1 and 2 on MRGPRX2-mediated intracellular Ca²⁺ mobilization induced by 1 μ M cortistatin-14. We observed IC₅₀ values of 1.6 and 2.5 μ M, respectively. Furthermore, both compounds were effective at preventing the binding of SP to human MRGPRX2

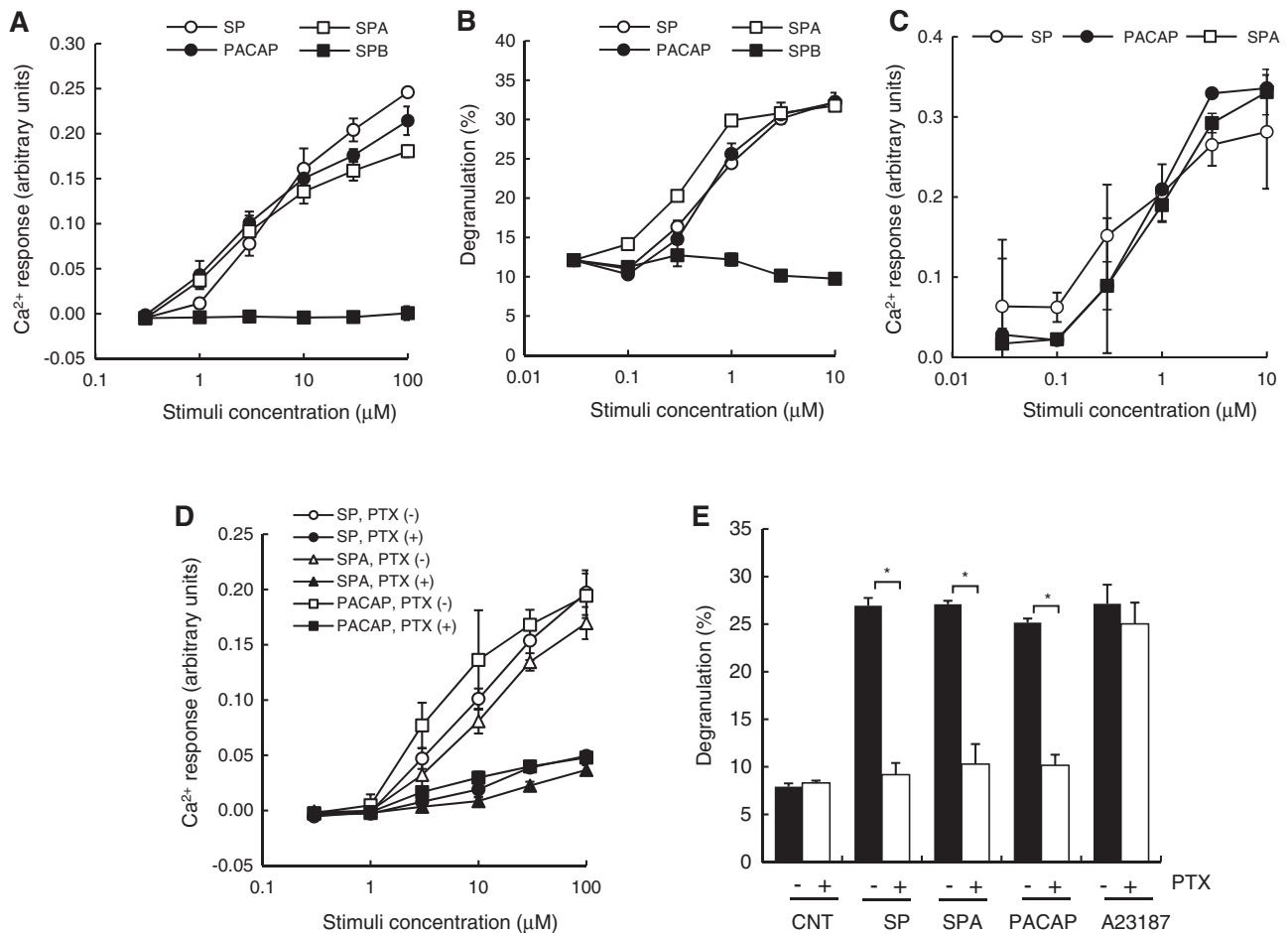


FIGURE 1 Human CMC degranulation induced by SP, its derivatives, and PACAP (6-27) is correlated with Ca²⁺ mobilization and is sensitive to PTX. (A) MRGPRX2-expressing HEK293 cells (1×10^4 cells) loaded with Fura-2-AM were stimulated with the indicated concentrations of SP, PACAP (6-27), SPA, or SPB. (B) For analysis of the activities of peptides on hCMCs, hCMCs (1×10^4 cells) were incubated with the indicated concentrations of stimuli for 30 min. Degranulation of hCMCs was assessed by the activities of β -hexosaminidase in supernatants from cells stimulated with the indicated concentrations of SP (closed circle), PACAP (6-27) (closed circle), SPA (open square), or SPB (closed square). (C) hCMCs (1×10^4 cells) loaded with Fura-2-AM were stimulated with the indicated concentrations of stimuli. (D) MRGPRX2-expressing HEK293 cells preincubated with or without PTX (100 ng/mL) for 18 h were stimulated with the indicated concentrations of SP, SPA, and PACAP (6-27). (E) hCMCs were preincubated with or without PTX (100 ng/mL) for 18 h. Human CMCs were incubated with 1 μ M of each stimuli for 30 min. The results (mean $\pm$ SD, $n = 3$) of a representative experiment are depicted. Parametric analysis (ANOVA, followed by Tukey's-Kramer post-hoc test) was used to compare the effects between groups. The level of statistical significance used was $*P < 0.05$. SP, substance P; hCMC, human cord blood-derived mast cell; MRGPRX2, Mas-related G protein-coupled receptor X2; PACAP, pituitary adenylate cyclase-activating peptide; SPA, [D-Pro²,D-Trp^{7,9}]-Substance P (1-11); SPB, [D-Pro⁴,D-Trp^{7,9,10}]-Substance P (4-11)

(Fig. 2C). As we previously reported that SP induced GTP- γ S binding to membrane fractions prepared from MRGPRX2-expressing cells in a dose-dependent manner,⁵ we also ensured that the capacity of both compounds blocks the GTP- γ S binding activities of a G α protein just downstream of MRGPRX2 (Fig. 2D). These findings led us to conclude that both compounds were competitive antagonists toward MRGPRX2 receptor. Molecular modeling and docking approaches have proposed that the negatively charged residues, such as Glu164 (E164) and Asp184 (D184), in the fourth and fifth transmembrane domains of MRGPRX2 are involved in the activation of MRGPRX2.¹¹ It was also reported that the naturally occurring missense MRGPRX2 variants, G165E and D184H, failed to respond to MRGPRX2 ligands.⁹ It will be interesting to investigate the interaction between MRG-

PRX2 antagonists and amino acid residues around E164 or D184 in MRGPRX2. Azimi et al. reported that Boc-Gln-D-Trp(Forlyl)-Phe benzyl ester trifluoroacetate, which is an antagonist for classical SP receptor, NK-1R, also inhibit MRGPRX2.²⁴ To investigate the characterization of our compounds, we tested the compounds in intracellular Ca²⁺ mobilization assays for NK-1R and muscarinic acetylcholine M2 receptor (M2R), a Gi-protein coupled receptor.^{25,26} The compounds did not block NK-1R or M2R-mediated intracellular Ca²⁺ mobilization (Fig. 2E). It has been proposed elsewhere that SP, a representative MRGPRX2 ligand and basic secretagogue, activates MCs through the direct activation of PTX-sensitive G proteins in the inner face of the plasma membrane,²⁷⁻²⁹ because of (1) the requirement of high concentrations of SP to activate human MCs, (2) the failure of

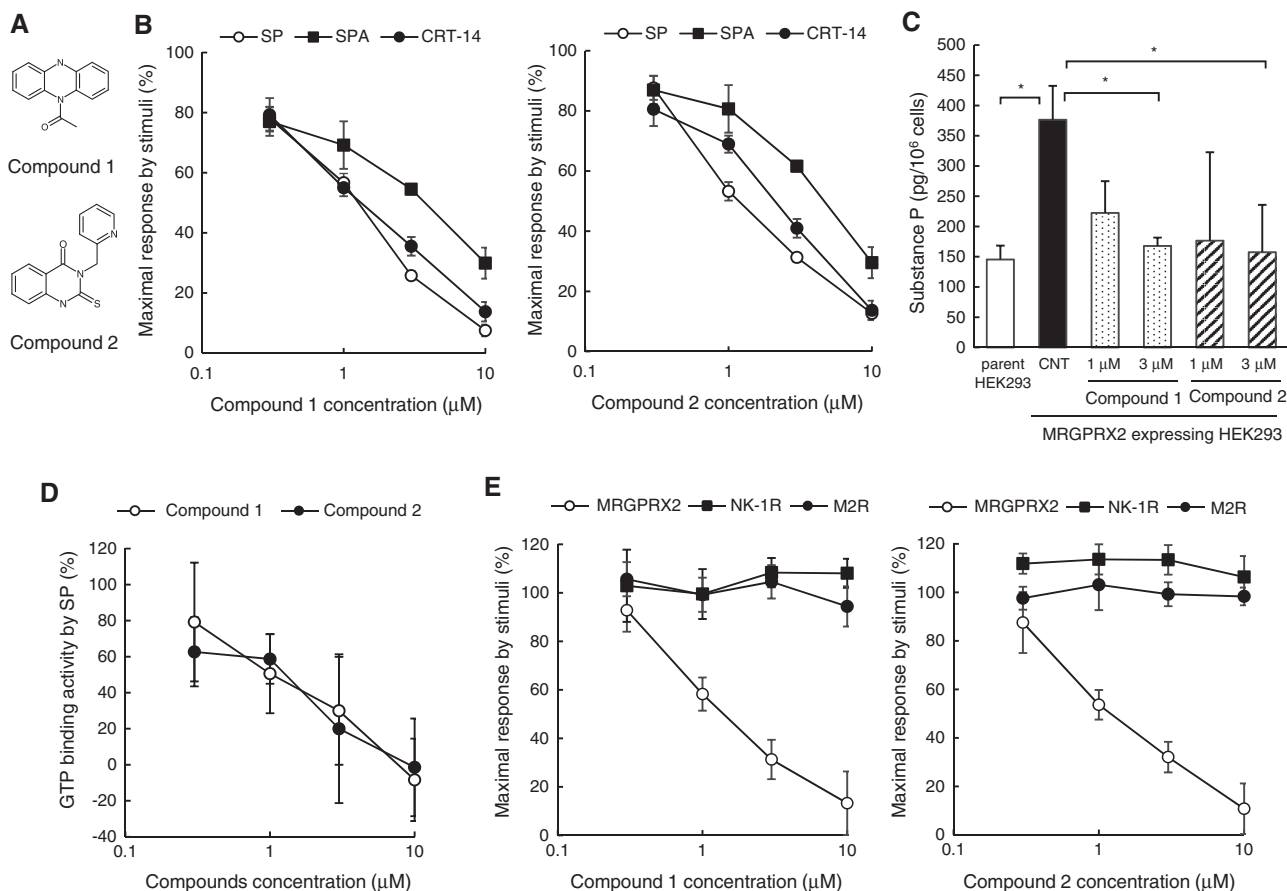


FIGURE 2 Inhibitory activities of MRGPRX2 antagonists on Ca^{2+} mobilization, ligand binding, and GTP- γ S binding, but not on NK-1R and M2R activities. Chemical structures of compounds 1 and 2 are shown in panel (A). (B) MRGPRX2-expressing HEK293 cells loaded with Fura-2-AM were stimulated with 10 μM SP, SPA, or 1 μM cortistatin-14 (CRT-14) in the presence of the indicated concentration of compounds 1 or 2. (C) MRGPRX2 antagonists inhibit the binding of SP to MRGPRX2. Parent and MRGPRX2-expressing HEK293 cells were incubated with 3 μM SP in the presence of the indicated concentration of compounds 1 or 2. ELISA was used to quantify the binding of SP to cells. (D) The inhibitory effects of compounds to GTP- γ S binding activities stimulated with 3 μM SP in MRGPRX2 expressing membranes. (E) The effects of compounds to MRGPRX2, NK-1R, and M2R were assessed by stimulation with 10 μM SP, 3 nM SP, and 30 μM carbachol, respectively. The results (mean $\pm$ SD, $n = 3$) of a representative experiment are depicted. Parametric analysis (ANOVA, followed by Tukey's-Kramer post-hoc test) was used to compare the effects between groups. The level of statistical significance used was $*P < 0.05$. SP, substance P; SPA, [D-Pro²,D-Trp^{7,9}]-Substance P (1-11); CRT-14, cortistatin-14; MRGPRX2, Mas-related G protein-coupled receptor X2; NK-1R, neurokinin-1 receptor; M2R, muscarinic acetylcholine M2 receptor

inhibition by conventional NK-1R antagonists,^{29,30} and (3) the potent inhibition by pretreatment with PTX.^{30,31} The failure to inhibit M2R led us to the conclusion that both compounds did not directly affect the PTX-sensitive $\text{G}\alpha$ -proteins. To the best of our knowledge, this is the first study to identify small-molecule MRGPRX2 antagonists without inhibitory activity to NK-1R or another Gi-coupled GPCR such as M2R.

2.3 | Human CMCs activation via MRGPRX2

MCs can be activated by many nonimmune mechanisms, including acute stress, and can participate in a variety of inflammatory diseases in the nervous system, skin, joints, as well as cardiopulmonary, intestinal, and urinary systems.³² Although MCs are considered to play a central role in the spectrum of diseases, the development of therapeutic agents has been difficult because the mechanism of

MC activation by neuropeptides is poorly understood. Real-time quantitative PCR analysis demonstrated the high expression of MRGPRX2 mRNA in the skin, adipose tissue, colon, and bladder, all of which contain MCs.⁵ MRGPRX2 ligands exist in the brain, heart, lung, pancreas, skin, bladder, and gastrointestinal tract, some of which are involved in neurogenic inflammation.²¹ We examined the effects of MRGPRX2 antagonists (compounds 1 and 2) on degranulation in hCMCs stimulated with cationic peptides including neuropeptides. Both compounds blocked SP-induced degranulation in hCMCs in a concentration-dependent manner (Fig. 3A). To investigate whether or not the compounds were selective for the degranulation of hCMCs stimulated with cationic peptides, the compounds were assessed in two degranulation assays distinct from basic secretagogues: (1) Ca^{2+} ionophore, A23187; and (2) IgE challenge. Both types of degranulation in hCMCs were resistant to both compounds at concentrations up to 10 μM (Figs. 3A and B). Thus, these results indicated that the inhibitory

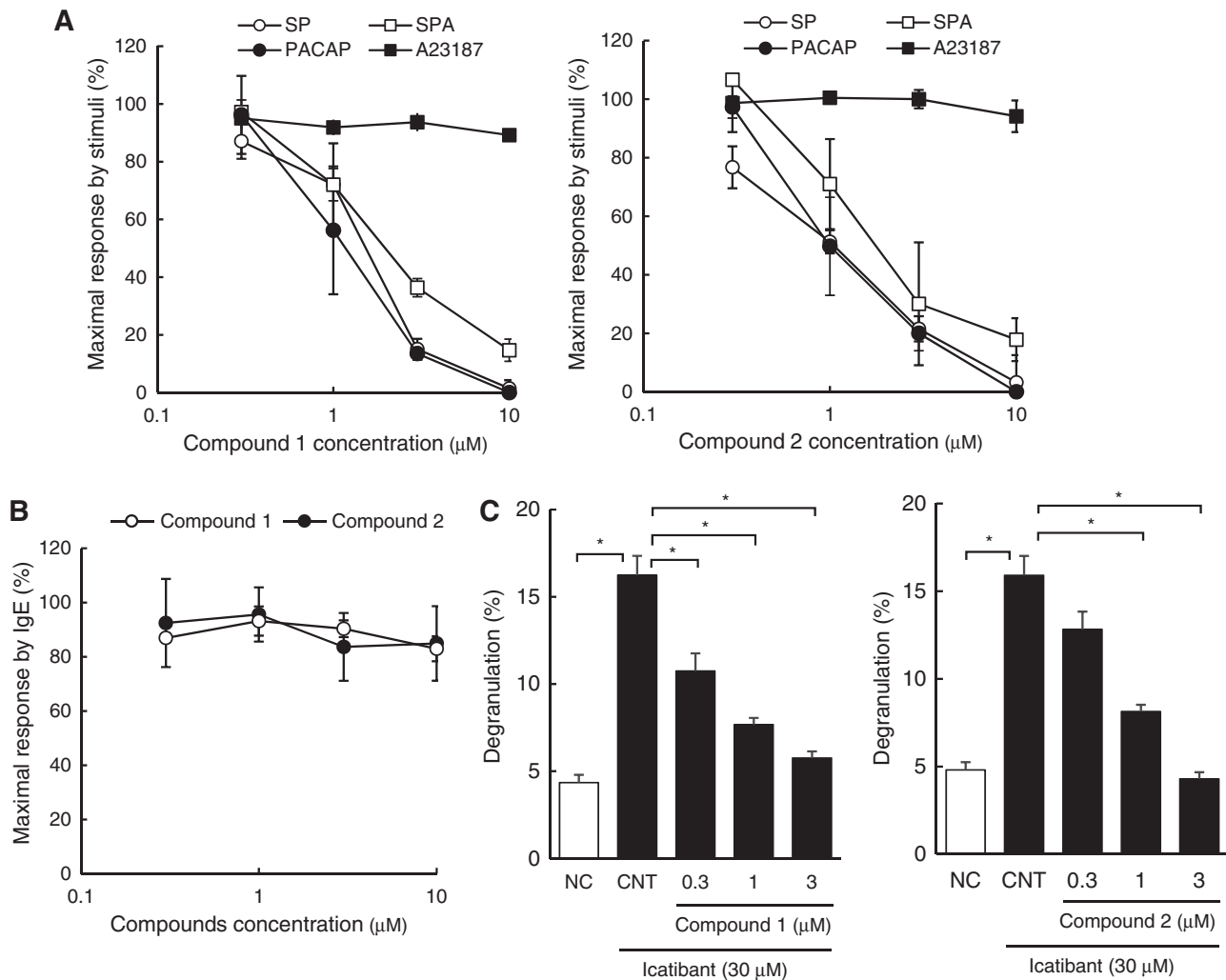


FIGURE 3 MRGPRX2 antagonists inhibit hCMC degranulation induced by basic secretagogues and icatibant. (A) Human CMCs were stimulated with 1 μM SP (closed circle), PACAP (6-27) (closed circle), SPA (open square), or A23187 (closed square) for 30 min in the presence of the indicated concentrations of compounds 1 or 2, or (B), with 10 μg/mL of anti-human IgE antibody in the presence of the indicated concentrations of compounds 1 or 2. (C) Human CMCs were stimulated with 30 μM icatibant for 30 min in the presence of the indicated concentrations of compounds 1 or 2. Degranulation of hCMCs was assessed by the release of β -hexosaminidase in the supernatants. A value of 100% is given to cells without antagonists. The results (mean $\pm$ SD, $n = 3$) of a representative experiment are depicted. Parametric analysis (ANOVA, followed by Tukey's-Kramer post-hoc test) was used to compare the effects between groups. The level of statistical significance used was * $P < 0.05$. MRGPRX2, Mas-related G protein-coupled receptor X2; hCMC, human cord blood-derived mast cell; SP, substance P; PACAP, pituitary adenylate cyclase-activating peptide; SPA, [D-Pro², D-Trp^{7,9}]-Substance P (1-11); Me₂SO, dimethyl sulfate; NC, negative control; CNT, activated control

effects of both compounds were selective for the degranulation of hCMCs via an MRGPRX2-mediated pathway. Recently, it has been reported that MRGPRX2 contributes to pseudoallergic reactions by many small-molecule drugs.^{8,9} We examined the effects of MRGPRX2 antagonists on degranulation in hCMCs stimulated with icatibant. As we expected, both compounds blocked icatibant-induced hCMC degranulation in a concentration-dependent manner (Fig. 3C). Therefore, our findings will suggest that MRGPRX2 is a suitable therapeutic target for not only a variety of IgE-independent inflammatory diseases in the nervous system but also drug-induced pseudoallergies.

Activation of MCs causes the release of preformed granule-associated mediators and initiates the de novo synthesis of lipid-derived substances.¹ Benyon et al.³³ reported eicosanoids released

from MCs in response to SP. Then, we evaluated the production of newly formed mediators (PGD₂) in response to increasing concentrations of SP. Figure 4 shows that SP released PGD₂ from hCMCs. The amount of PGD₂ in a 0.5% Nonidet P-40-treated hCMC lysate was detected the same as that in the supernatants of nonstimulated hCMCs, suggesting that PGD₂ released in the supernatants was synthesized de novo in hCMCs. Consistent with the degranulation data, we observed a strong suppression of PGD₂ production by MRGPRX2 antagonists (Fig. 4). Therefore, we concluded that the activation of MRGPRX2 by SP induced the de novo synthesis of PGD₂, as well as degranulation in hCMCs.

It was previously demonstrated that the p42/44 MAPK signal pathway was involved in the SP or c48/80-stimulated secretion and

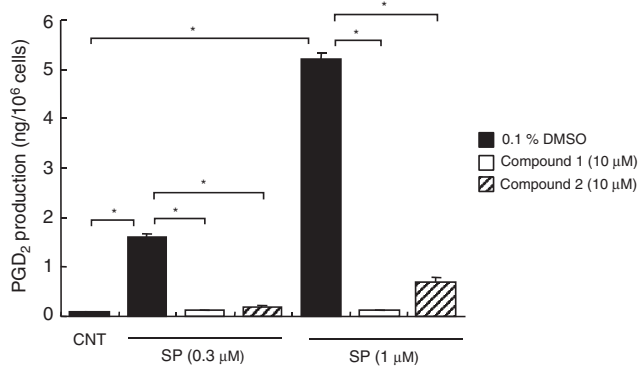


FIGURE 4 MRGPRX2 antagonists inhibit PGD₂ production induced by SP. PGD₂ production was assessed after 30 min of incubation with SP at the indicated concentrations in the presence of compounds 1 and 2 (10 μM). Data represent the mean ± standard deviation of triplicate determinations. Parametric analysis (ANOVA, followed by Tukey's-Kramer post-hoc test) was used to compare the effects between groups. The level of statistical significance used was **P* < 0.05. MRGPRX2, Mas-related G protein-coupled receptor X2; PGD₂, prostaglandin D₂; SP, substance P

production of inflammatory mediators in MCs including histamine, TNF-α, and arachidonic acid.^{34–38} We attempted to determine the SP-induced phosphorylation of p42/44 MAPK in MRGPRX2-expressing HEK293 cells by western blot analysis using antibodies directed

against the activated, phosphorylated forms of each kinase (phosphor) in comparison with the total kinases used as a control (total). As shown in Fig. 5A, the phosphorylated forms of p42/44 MAPK in MRGPRX2-expressing HEK293 cells were higher than that in parent HEK293 cells. We concluded that the SP-induced activation of p42/44 MAPK was mediated via MRGPRX2 because MRGPRX2 antagonists also inhibited the SP-induced phosphorylation of p42/44 MAPK in a dose-dependent manner (Fig. 5B). Next, we examined whether or not the activation of p42/44 MAPK in hCMCs by SP was also mediated through MRGPRX2. Figure 5C shows that SP (1 μM)-treated hCMCs had increased amounts of the phosphorylated form of p42/44 MAPK. As shown in Fig. 5C, 10 μM of MRGPRX2 antagonists completely abolished the phosphorylation of p42/44 MAPK stimulated by SP. Although, p42/44 MAP kinase activation in MCs induced by basic secretagogues was reported to be dependent on clathrin-mediated endocytosis but independent of the receptor,³⁷ our results suggest that MRGPRX2 is involved in the SP-induced activation of the p42/44 MAPK signal pathway. We anticipate MRGPRX2-mediated p42/44 MAPK signal may be involved in the secretion and production of inflammatory mediators such as TNF-α and arachidonic acid. GPCR engages β-arrestins, which are scaffolding proteins. Typically, β-arrestin recruitment inhibits further G protein coupling, promotes receptor internalization by coupling receptors to endocytic machinery, and then triggers signaling by recruiting scaffolded signaling

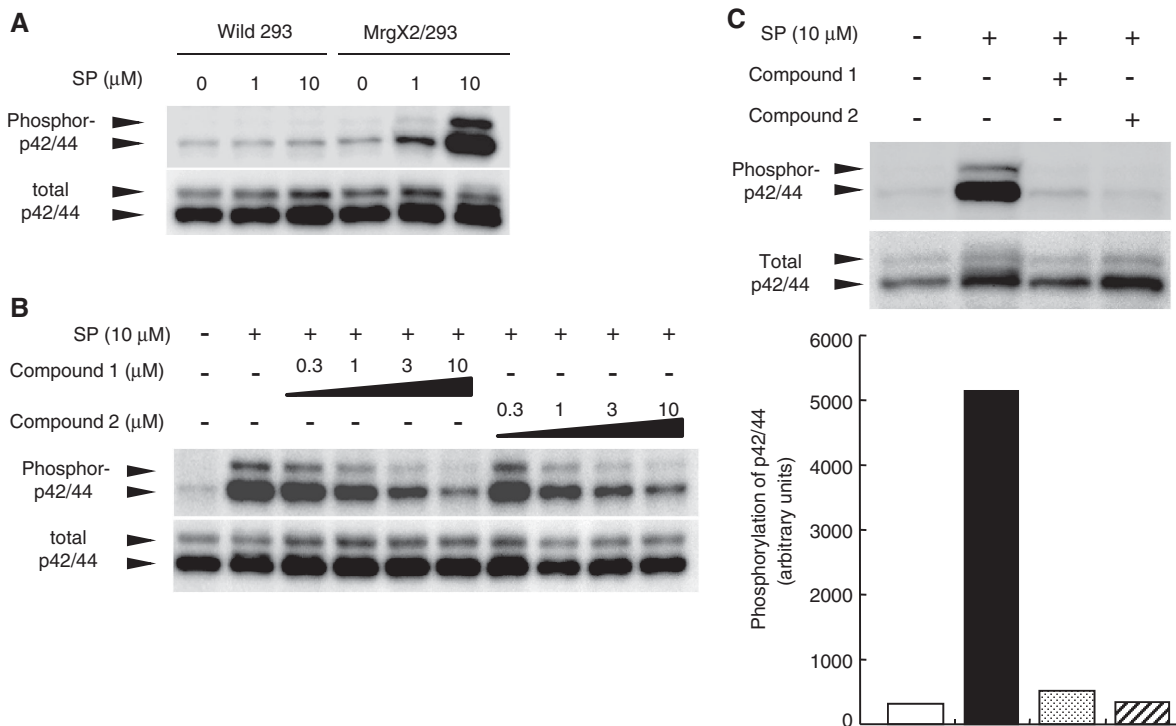


FIGURE 5 SP induces the phosphorylation of p42/44 MAPK through MRGPRX2. (A) MRGPRX2-expressing HEK293 cells or parent HEK293 cells (3×10^5 cells) were incubated with the indicated concentrations of SP. (B) MRGPRX2-expressing HEK293 cells (3×10^5 cells), and (C), hCMCs (3×10^5 cells) were stimulated by SP (10 μM) in the presence of the indicated concentrations of compounds 1, 2, or 0.1% DMSO (control). After 10 min incubation, cells were sedimented, washed, and lysed. Cells extracts were resolved by SDS-PAGE (4–20% gradient gel) and probed with antiactive p42/44 kinase (phosphor-p42/44) antibodies. The membrane was then reprobed with anti-p42/44 kinase (total p42/44) antibodies. SP, substance P; MAPK, mitogen-activated protein kinase; MRGPRX2, Mas-related G protein-coupled receptor X2; hCMC, human cord blood-derived mast cell

proteins to the receptor.³⁹ It remains unclear whether or not the MRGPRX2-mediated activation of p42/44 MAPK results from β -arrestin recruitment or clathrin-mediated endocytosis. Clarification of the MRGPRX2 downstream pathway using MRGPRX2 antagonists might provide new insights into MRGPRX2 signaling.

Murine MrgprB2 is homologous to human MRGPRX2. Unfortunately, neither compound 1 nor 2 could be used in *in vivo* models to define the physiological roles of MRGPRX2 in neurogenic inflammation, because the antagonists had no inhibitory crossreactivity to degranulation in mouse MCs induced by SP (Supplemental Fig. 1). We previously suggested that MrgprB3 is another murine orthologue of the human MRGPRX2 gene, because some MRGPRX2 ligands, except for SP and cortistatin-14, act as MrgB3 ligands.⁵ However, the murine orthologues, MrgB2 and B3, have low similarity to MRGPRX2 (53 and 46%, respectively). To verify the pharmacological function of the receptor in mouse MCs *in vivo*, and to develop therapeutic agents, MRGPRX2 antagonists should be modified to crossreact with murine MRGPRX2 orthologues.

In summary, we identified two novel MRGPRX2 antagonists. These antagonists inhibited hCMC activation induced by basic secretagogues and small-molecule drug associated with pseudoallergy. The discovery of a specific antagonist for human MRGPRX2 and murine orthologues will allow us to further elucidate the involvement of MRGPRX2 in IgE-independent allergic reaction of MCs *in vivo*. Now we have been able to open up new avenues for the therapeutic implications of human MRGPRX2 antagonists on the IgE-independent activation of MCs in inflammatory diseases.

3 | MATERIALS AND METHODS

3.1 | Materials

SP and [D-Pro²,D-Trp^{7,9}]-Substance P (1-11) (SPA) were purchased from Peptides Laboratories, Osaka, Japan. Human IgE and anti-human IgE Ab were purchased from Chemicon (Temecula, CA). Recombinant human interleukin-6 (rhIL-6), stem cell factor (rhSCF), rhIL-4, and rhIL-3 were purchased from PeproTech (Rocky Hill, NJ). A GTP binding kit (Delfia) was purchased from Perkin-Elmer (Waltham, MA). Anti-p44/42 MAPK antibodies and anti-phospho-p44/42 MAPK antibodies were purchased from Cell Signaling (Danvers, MA). [D-Pro⁴,D-Trp^{7,9,10}]-Substance P (4-11) (SPB) was synthesized using automatic solid-phase methods using standard Fmoc chemistry as described.⁵

3.2 | Human cord blood-derived MCs

Human cord blood CD34⁺ cells were separated from human cord blood MNCs (Stemcyte, Covina, CA) by a CD34⁺ progenitor cell isolation kit (Miltenyi Biotec, North Rhine-Westphalia, Germany). Human cord blood-derived MCs were induced from cord blood CD34⁺ cells as described previously.⁵

3.3 | DNA construction and MRGPRX2-expressing cells

Human MRGPRX2, human NK-1R, and M2R-stably expressing HEK293 cells were established as described.⁵

3.4 | Activation of hCMCs and PGD₂ EIA assays

Human CMCs cultured were incubated with stimuli for 30 min. In experiments with pertussis toxin (PTX), hCMCs were subsequently activated with the indicated concentration of stimuli for 30 min after preincubation with 100 ng/mL PTX for 18 h. For the IgE challenge, cells were preincubated for 5 days in IMDM containing 0.1% BSA, 100 U/mL penicillin, 100 μ g/mL streptomycin, 1% Insulin-Transferrin-Selenium, 2-ME, and supplemented with 100 ng/mL rhSCF, 50 ng/mL rhIL-6, 1 ng/mL rhIL-3, 1 μ g/mL human IgE antibodies, and 10 ng/mL rhIL-4 (R&D Systems, Minneapolis, MN). The percentage of degranulation was assessed as described previously.⁵ The generation of PGD₂ in the supernatants was measured with an EIA for PGD₂ (Cayman Chemical, Ann Arbor, MI).

3.5 | Intracellular calcium measurement and GTP γ S binding assay

Cells were loaded with 2 μ M Fura-2 AM (Dojindo laboratories, Kumamoto, Japan) for 30 min at room temperature. The intracellular calcium concentration was measured in response to activation by stimuli using a Functional Drug Screening System (Hamamatsu Photonics, Hamamatsu, Japan) as described previously.⁵ GTP binding for MRGPRX2 expressing membranes was assessed as described previously.⁵

3.6 | SP binding assay by ELISA

SP binding for human MRGPRX2-expressing HEK293 cells was assessed as Azimi et al. described.²⁴ Briefly, MRGPRX2-expressing HEK293 cells treated with 3 μ M of SP with or without MRGPRX2 antagonists for 1 h at room temperature. Parent HEK293 cells were also treated with 3 μ M of SP. After wash with PBS twice, cells were suspended in 200 μ L of PBS and lysed by two freeze-thaw cycles. The amount of SP in the lysates was assessed using SP competitive enzyme immunoassay kit (Enzo Life Science, NY).

3.7 | Western blot analysis

Cells were stimulated with SP (1 or 10 μ M) for 10 min with or without MRGPRX2 antagonists. Cellular extracts were separated by SDS-PAGE, followed by immunoblotting with primary antibodies. Specific bands were detected with an ECL chemiluminescence system (Amersham-Pharmacia Biotech, Piscataway, NJ). The chemiluminescent intensities of phospho-p42/44 and p42/44 were measured using a LAS-3000 system (Fuji Film, Tokyo, Japan).

AUTHORSHIP

H.O. directed and performed experiments for all biological assays. M.F. and K.E. carried out chemical library screening for MRGPRX2

antagonists. M.N. directed all experiments and led the project as a representative. All authors contributed to the manuscript writing. H.O. wrote the manuscript with input from all coauthors. Hiroyuki Ogasawara and Masato Noguchi contributed equally to this work.

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DISCLOSURE

The authors declare no competing interests.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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Supplemental information

Supplemental Method:

Balb/c mice were purchased from Charles River laboratories Japan. Procedures were conducted in accordance with the ARRIVE guidelines. Freshly dissected spleens were isolated from Balb/c mouse. Spleen cells were spun at 8000 rpm by centrifugation at 4°C into PBS, and red blood cells were lysed in ammonium chloride/potassium bicarbonate lysis buffer for 10 min on ice. After centrifugation, cells were resuspended in PBS, passed through a cell strainer, and counted. Spleen cells were cultured for more than 8 weeks in 10% FBS α -MEM supplemented with 50 ng/ml mouse SCF (PeproTech, NJ, USA). Cells (2×10^4 cells) were aliquoted in 96-well culture plates. Cells were activated by the indicated concentration of stimuli for 30 min with or without MRGPRX2 antagonists. Total release was established by adding distilled water to obtain complete lysis of cells. After 2 h incubation of the supernatant containing 1 mM p-Nitrophenyl-N-acetyl- β -D-glucosamine (pN-GlcNAc, Sigma, St. Louis, MO) in 0.1 M citrate buffer (pH 4.0), the reaction was stopped by adding 0.1 M carbonate buffer (pH 10). Colorimetric absorbance was measured using a multi-well plate reader at 405 nm. The percentage of degranulation was calculated as: $((a - b)/(t - b)) \times 100$, where a is the amount of β -hexosaminidase released from stimulated cells, b is the blank, and t is the total cellular content.

Supplemental figure:

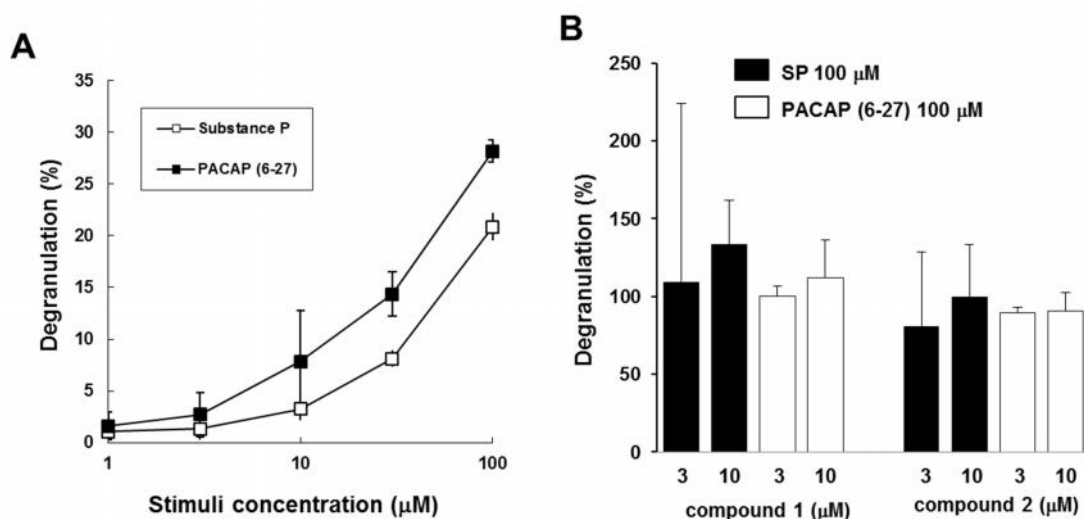


Figure S1. Degranulation in mouse MCs derived from spleens were not inhibited

by MRGPRX2 antagonists. Spleen cells were cultured for more than 8 weeks in 10% FBS α -MEM supplemented with 50 ng/ml mouse SCF. (A) Mouse MCs were stimulated with the indicated concentrations of SP or PACAP (6-27) for 30 min. (B) Mouse MCs were stimulated with 100 μ M SP or PACAP (6-27) for 30 min in the presence of the indicated concentrations of MRGPRX2 antagonists. Data represent the mean $\pm$ standard deviation of triplicate determinations. Degranulation of hCMCs was assessed by the release of β -hexosaminidase in the supernatants.

MC, mast cell; MRGPRX2, Mas-related G protein-coupled receptor X2; SCF, stem cell factor; PACAP, pituitary adenylate cyclase-activating peptide; hCMC, human cord blood derived mast cell.