

Endogenous protein and enzyme fragments induce immunoglobulin E-independent activation of mast cells via a G protein-coupled receptor, MRGPRX2

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Abstract

Mast cells play a central role in inflammatory and allergic reactions by releasing inflammatory mediators through 2 main pathways, immunoglobulin E-dependent and E-independent activation. In the latter pathway, mast cells are activated by a diverse range of basic molecules (collectively known as basic secretagogues) through Mas-related G protein-coupled receptors (MRGPRs). In addition to the known basic secretagogues, here, we discovered several endogenous protein and enzyme fragments (such as chaperonin-10 fragment) that act as bioactive peptides and induce immunoglobulin E-independent mast cell activation via MRGPRX2 (previously known as MrgX2), leading to the degranulation of mast cells. We discuss the possibility that MRGPRX2 responds various as-yet-unidentified endogenous ligands that have specific characteristics, and propose that MRGPRX2 plays an important role in regulating inflammatory responses to endogenous harmful stimuli, such as protein breakdown products released from damaged or dying cells.

1 | INTRODUCTION

Mas-related G protein-coupled receptors (MRGPRs) belong to the G protein-coupled receptor (GPCR) family and were also known as sensory neuron-specific GPCRs.^{1,2} In mice, the MRGPR family comprises 32 distinct sequences.¹ In humans, there are 4 genes, MRGPRX1, MRGPRX2, MRGPRX3 and MRGPRX4,^{1,3} whereas the rat genome includes MrgA, MrgC and MrgD genes and 10 MrgB genes.⁴ Some of these MRGPRs are expressed in a specific subset of nociceptive sensory neurons, suggesting a role in regulating nociceptor function, including the sensation or modulation of pain¹; however, the physiological functions of this family of GPCRs remained largely unknown. In our previous work, we reported that MRGPRX2 is expressed in human mast cells and the activation of MRGPRX2 induces mast cell degranulation.⁵

Mast cells play a central role in inflammatory and allergic reactions through the release of stored or de novo-synthesized inflammatory mediators, such as histamine, proteinases, prostaglandins, leukotrienes and cytokines.⁶ The activation of mast cell is precisely controlled in multiple ways.⁷ Two main pathways have been identified for mast cell activation: immunoglobulin E (IgE)-dependent and IgE-independent pathways.^{6,8} The IgE-dependent pathway is mediated by the interaction of antigens with IgE bound to the high-affinity IgE receptor FcεRI.⁹ The IgE-independent activation of mast cells has also been studied,^{10,11} and mast cell degranulation has been revealed as mainly being induced by a variety of basic compounds collectively known as basic secretagogues which include peptides and amines, such as substance P, mast cell-degranulating peptide, neuropeptide Y, mastoparan and compound 48/80.⁶⁻⁸ We showed for the first time that all

known basic secretagogues tested activate MRGPRX2 and induce IgE-independent degranulation of mast cells,⁵ and this finding was confirmed in several other studies.^{12–15} It is interesting to note that only one receptor, MRGPRX2, responds to a broad range of ligands, compared with the activation pattern of many other GPCRs.

The exogenous mast cell secretagogues, such as mastoparan and compound 48/80, are not in contact with MRGPRX2 under normal physiological conditions. It is curious that many endogenous secretagogues, such as substance P, somatostatin and pituitary adenylate cyclase-activating polypeptide (PACAP), activate MRGPRX2 in much higher concentrations than the effective concentrations for their own receptor-mediated effects.^{5,16,17} We expected the possibility that there would be unidentified endogenous secretagogues that act on mast cells via MRGPRX2 with physiological significance. To test the possibility and to clarify the physiological role of MRGPRX2, we tried to isolate previously unidentified endogenous mast cell secretagogues from porcine brain and intestinal extracts, since these tissues are thought to be rich in bioactive compounds, and porcine proteins are similar to human proteins.

In this study, we discovered that some endogenous protein and enzyme fragments (such as chaperonin-10 fragment) activated MRGPRX2 and induced the degranulation of human mast cells. Considering the specific characteristics of ligands of MRGPRX2, we expect that many more unidentified endogenous protein fragments may act as ligands for MRGPRX2. We therefore propose that MRGPRX2 is a new type of GPCR that recognizes a variety of ligands and discuss the possibility that MRGPRX2 may recognize various endogenous protein fragments and breakdown products released from damaged or dying cells, to induce inflammatory responses via mast cell activation.

2 | MATERIALS AND METHODS

2.1 | Reporter gene assay

To measure the activation of MRGPRX2 by its ligands, early growth response protein 1 (EGR1) promoter was used as a reporter gene. Because EGR1 promoter possesses CRE and SRE, it can detect Gs and Gq signals from GPCRs in activation mode and Gi signal in suppression mode. By the addition of $G\alpha_{16}$, Gi signal can also be detected in activation mode. Therefore, the 3 main signals from GPCRs can be detected in activation mode by EGR1 promoter. The cDNA of MRGPRX2 was inserted into the expression vector pME18S under $SR\alpha$ promoter. The reporter plasmid pGL2-EGR1-luciferase was constructed by inserting rat EGR1 (known as *zif* 268) promoter (–526 to +97 from the transcription start site) into pGL2-basic vector (Promega).

Rat pheochromocytoma PC12h cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% horse serum and 5% foetal calf serum. The cells (1.5×10^5) were transfected with 1 μ g of pME18S-MRGPRX2 or pME18S (control), 100 ng of pGL2-EGR1-luciferase and 50 ng of pME18S- $G\alpha_{16}$ in 60 μ L of phenol red-free DMEM using 2 μ L of SuperFect (Qiagen). The transfected cells (1.5×10^4 cells/100 μ L) in phenol red-free DMEM supplemented with 0.5% horse serum and 0.25% foetal calf serum were cultured for 48 hours in a collagen-coated 96-well plate. After removing 50 μ L of the medium, test samples were dissolved in 50 μ L of phenol red-free DMEM and added to each well. After 6 hours of incubation, the cells were lysed and luciferase activity was measured using luciferase assay reagents (Promega) and a LB 96V luminometer (Berthold Technologies).

2.2 | Preparation of HEK-293 cells stably expressing MRGPRX2

HEK-293 cells were transfected using Lipofectamine 2000 (Invitrogen) with MRGPRX2 subcloned into the retroviral vector pLHCXI4, which is derived from pLNCX (Clontech) containing Hyg^R gene, CMV promoter, internal ribosomal entry site and truncated CD4 gene. HEK-293 cells stably expressing MRGPRX2 were isolated as a polyclonal population coexpressing CD4 using a CD4 cell selection kit (Miltenyi Biotec).⁵ The transfected HEK-293 cells were cultured in DMEM supplemented with 10% foetal calf serum, 100 IU/mL penicillin and 100 μ g/mL streptomycin.

2.3 | Measurements of intracellular calcium

After being washed once with calcium buffer (20 mmol/L HEPES, 115 mmol/L NaCl, 5.4 mmol/L KCl, 0.8 mmol/L $MgCl_2$, 1.8 mmol/L $CaCl_2$, 13.8 mmol/L glucose, 0.2% BSA, 2.5 mmol/L probenecid [Sigma], pH 7.4), HEK293 cells stably expressing human MRGPRX2 were incubated with the Fura2 loading buffer (calcium buffer additionally containing 2 μ mol/L Fura2 AM [Dojin], 0.02% Pluronic F127 [Molecular Probe], pH 7.4) for 1 hour at room temperature. The cells were washed with calcium buffer twice and suspended at a concentration of 1×10^6 cells/mL and dispensed into a 384-well plate (Nunc. 142761, 40 μ L each [40 000 cells/well]). Intracellular calcium mobilization was determined by injecting 10 μ L of each sample into the well and measuring fluorescent emission at 500 nm by excitation light at 340 and 380 nm (1.5 seconds interval for 3.5 minutes), using the fluorescence drug screening system FDSS3000 (Hamamatsu Photonics K. K.) to obtain the 340 nm excitation/380 nm excitation ratio.

2.4 | Membrane preparation and GTP-binding assay

HEK-293 cells stably expressing MRGPRX2 were suspended in ice-cold 15 mmol/L Tris buffer (pH 7.5) containing 2 mmol/L MgCl_2 , 0.3 mmol/L EDTA and 1 mmol/L EGTA then the suspension was homogenized using a glass homogenizer. The homogenized material was centrifuged at 50 000 *g* for 30 minutes at 4°C, and G protein activation in the membranes obtained was assessed using a DELFIA GTP-binding kit (Perkin-Elmer) as described previously.¹⁸ The fluorescence of europium bound on the filter was measured using a time-resolved fluorometer (Perkin-Elmer).

2.5 | Purification of the peptides activating MRGPRX2

We isolated the peptides activating MRGPRX2 from porcine brain and intestinal extracts using a reporter gene assay to follow their activity during the purification steps. Porcine brain or intestinal tissue (1 kg of each) was boiled in water, minced and extracted with 0.2 mol/L acetic acid. The extract was lyophilized and subjected to gel filtration on a Sephadex G-25 column in 0.1 mol/L acetic acid. The activity-containing brain fraction (23 g) or intestinal fraction (37 g) from gel filtration was purified by ion-exchange chromatography on a SP-Sepharose FF column (Amersham Biosciences). The brain (3 g) or intestinal fraction (8.9 g) eluted with 1 mol/L ammonium acetate buffer (pH 4.0) shown as having the activity was subjected to high-performance liquid chromatography (HPLC) steps. The successive sequential HPLC steps are described in the Results section and Figure 1.

2.6 | Human mast cell culture and measurement of degranulation

Human umbilical cord blood samples were obtained from Kyoto Senbai Hospital. The collection and use of human umbilical cord blood cells were approved by both the ethics committees of Central Pharmaceutical Research Institute of Japan Tobacco Inc. and Kyoto Senbai Hospital. Before the collection of umbilical cord blood, written informed consent was obtained from the donors.

Mononuclear cells were separated by density centrifugation over Ficoll-Paque (Amersham Biosciences), and CD34^+ cells were isolated from mononuclear cells using a CD34 cell isolation kit (Miltenyi Biotec). CD34^+ cells (1×10^5 cells/well) in 0.1 mL of Iscove's modified Dulbecco's medium (IMDM) supplemented with 1% insulin-transferrin, 50 mmol/L 2-mercaptoethanol, 1.0 $\mu\text{g/mL}$ stem cell factor, 0.5 $\mu\text{g/mL}$ interleukin-6, 0.01 $\mu\text{g/mL}$ interleukin-3 and 0.1% bovine serum albumin (BSA) were mixed with 0.9 mL of IMDM containing 0.9%

methylcellulose and incubated in a 24-well culture plate. Cells were harvested after 8 weeks.

For degranulation experiments, cells (6×10^3 cells/well) were incubated with 0.2 mL of IMDM containing various concentrations of ligand. After 30 minutes at 37°C, the supernatants (50 μL) were assayed for degranulation by measuring β -hexosaminidase activity. The supernatants were incubated with 1 mmol/L of the substrate, p-nitrophenyl N-acetyl- β -d-glucosamide (Sigma) (in 0.1 mol/L citrate buffer, pH 4.5) for 2 hours at 37°C, and treated with sodium carbonate buffer (pH 10), followed by measuring the absorbance at 405 nm. Percent degranulation was calculated from the enzyme activity in the supernatant compared with the total enzyme activity in the cells.^{19,20}

3 | RESULTS

3.1 | Purification of endogenous ligands for MRGPRX2 from porcine tissues

To identify endogenous ligands for MRGPRX2 other than the known basic secretagogues, we isolated MRGPRX2-activating peptides from porcine brain and intestinal extracts. The brain (3 g) or intestinal fraction (8.9 g) eluted with 1 mol/L ammonium acetate buffer (pH 4.0) found demonstrating the activity was subjected to high-performance liquid chromatography (HPLC) using a large reversed-phase C_{18} column (120 Å; YMC) with a gradient of acetonitrile in 0.1% trifluoroacetic acid (TFA). Two active peaks from the brain fraction and one active peak from the intestinal fraction were identified, and the fraction from each active peak was subjected to further HPLC purification. One brain fraction (13 mg) demonstrating the activity was purified by sequential steps of HPLC on a TSKgel CM-2SW column (Figure 1A), a C_8 column (300 Å) (Figure 1B), a C_{18} column (300 Å) (Figure 1C), and, finally, on a C_{18} column (120 Å) (Figure 1D). The remaining brain fraction (122 mg) demonstrating the activity was purified by sequential steps of HPLC on a TSKgel CM-2SW column (Figure 1E), a C_8 column (300 Å) (Figure 1F), and, finally, on a C_{18} column (300 Å) (Figure 1G). The intestinal fraction (20 mg) showing the activity was purified by HPLC on a C_8 column (300 Å) (Figure 1H), a TSKgel CM-2SW column (Figure 1I), a C_{18} column (120 Å) (Figure 1J), and, finally, on a C_{18} column (300 Å) (Figure 1K). In each case, after the final HPLC step, the activity was recovered as a single peak.

3.2 | Structures of endogenous ligands for MRGPRX2 from porcine tissues

To determine the amino acid sequences of the endogenous ligands for MRGPRX2, the purified peptide preparations

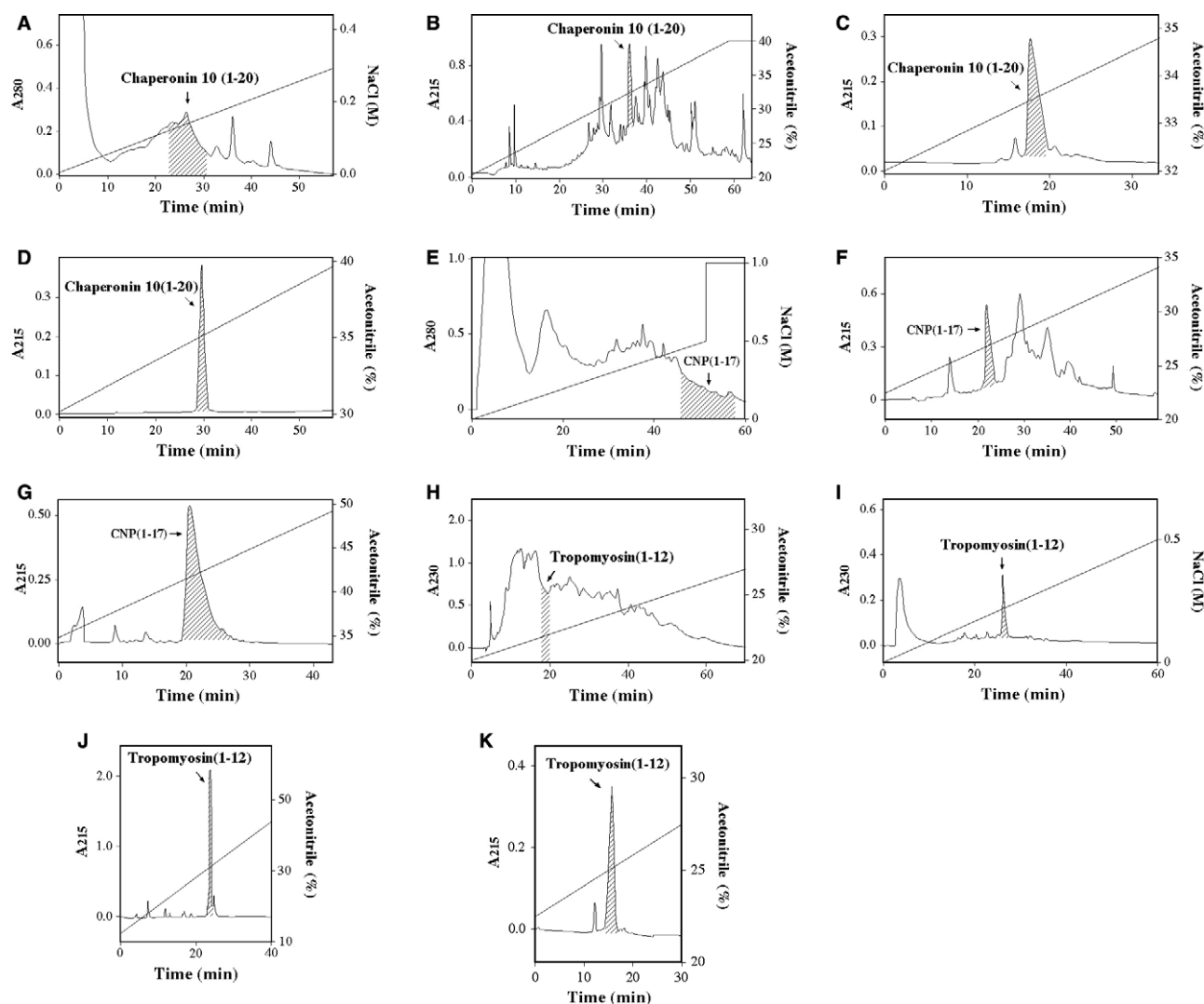


FIGURE 1 Purification of endogenous MRGPRX2 ligands from porcine tissues. (A) Ion-exchange HPLC on a TSKgel CM-2SW column (Tosoh, Tokyo, Japan) with a linear gradient of 0–1 mol/L NaCl in 5 mmol/L phosphate buffer (pH 6.5). (B) HPLC on a C₈ column (300 Å; YMC, Kyoto, Japan) with a linear gradient of acetonitrile in 0.1% TFA. (C) HPLC on a C₁₈ column (300 Å; YMC) with a linear gradient of acetonitrile in 0.1% TFA. (D) Final HPLC on a C₁₈ column (120 Å; YMC) with a gradient of acetonitrile in 5 mmol/L phosphate buffer (pH 6.5). (E) HPLC on a TSKgel CM-2SW column (Tosoh) with a linear gradient of 0–1 mol/L NaCl in 5 mmol/L phosphate buffer (pH 6.5). (F) HPLC on a C₈ column (300 Å; YMC) with a linear gradient of acetonitrile in 0.1% TFA. (G) Final HPLC on a C₁₈ column (120 Å; YMC) with a gradient of acetonitrile in 5 mmol/L phosphate buffer (pH 6.5). (H) HPLC on a C₈ column (300 Å; YMC) with a linear gradient of acetonitrile in 0.1% TFA. (I) Ion-exchange HPLC on a TSKgel CM-2SW column (Tosoh) with a linear gradient of 0–1 mol/L NaCl in 5 mmol/L phosphate buffer (pH 6.5). (J) HPLC on a C₁₈ column (120 Å; YMC) with a gradient of acetonitrile in 0.1% TFA. (K) Final HPLC on a C₁₈ column (300 Å; YMC) with a gradient of acetonitrile in 0.1% TFA

were digested with trypsin, and the resulting fragments were separated by HPLC using a reversed-phase C₁₈ column. A protein sequencer (Model 492; Applied Biosystems) was used to determine the amino acid sequences of the fragments, and peptide structures were confirmed by mass spectrometry using a Kompact MALDI III mass spectrometer (Shimadzu). The results of structural analyses indicated that the sequences of the 2 brain peptides were acetyl-AGQAFRKFLPLFDRVLVERS and acetyl-NRGFSRKSQTFLPKIFF and that of the intestinal peptide was acetyl-MDAIKKKMQMLK. These sequences

correspond to chaperonin-10 (1-20), 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNP) (1-17) and tropomyosin (1-12), respectively.

3.3 | Biological functions of the endogenous ligands for MRGPRX2

These 3 peptides, chaperonin-10 (1-20), CNP (1-17) and tropomyosin (1-12), and also substance P as a control, were synthesized using an automated synthesizer (Model 431A; Applied Biosystems) and the crude synthetic products were

purified by HPLC using a reversed-phase C18 column. The synthetic peptides including CNP (1-17) increased reporter gene expression as effectively as the natural peptides (Figure 2A); however, intact chaperonin-10 protein (also known as GroES) did not increase the expression (data not shown). These peptides also caused dose-dependent increases in $[Ca^{2+}]_i$ in HEK-293 cells stably expressing MRGPRX2 (Figure 2B). Chaperonin-10 (1-20) and CNP (1-17) caused dose-dependent activation of G proteins in the membranes of MRGPRX2-expressing HEK-293 cells (Figure 2C). Furthermore, the synthetic protein fragments degranulated cultured human cord mast cells which are connective tissue type (HCMC_{CT}) (Figure 3).

4 | DISCUSSION

In our previous paper, we reported for the first time that MRGPRs are expressed in human and rat mast cells and mediate the IgE-independent activation of mast cells.⁵ The presence of MRGPRX2 on mast cells was further confirmed utilizing immunohistochemical methods using specific antibodies against MRGPRX2.¹⁴ McNeil et al¹⁵ showed that mouse MrgprB2 is the orthologue of human MRGPRX2 by analysing MrgprB2-null mutant mice. Secretagogue-induced histamine release, inflammation and airway contraction are abolished in MrgprB2-null mutant mice; however, IgE-dependent mast cell activation is not abolished in these same mutant mice.¹⁵ Thus, MRGPRX2 was confirmed as being the major mast cell receptor that functions as a link between basic secretagogues and the activation of G proteins, leading to the IgE-independent activation.

In this study, we isolated 3 MRGPRX2-activating peptides from porcine brain and intestinal extracts. The isolated porcine peptides turned out to be chaperonin-10 (1-20), CNP (1-17) and tropomyosin (1-12). These are the N-terminal fragments of proteins that are abundant in the body. Surprisingly, these peptides exhibited potent degranulating activities in human mast cells that were comparable to those of well-known basic secretagogues, such as substance P and compound 48/80. Furthermore, these peptides caused the activation of G proteins and the mobilization of intracellular calcium upon binding to MRGPRX2. In contrast to chaperonin-10 (1-20), intact chaperonin-10 protein did not activate MRGPRX2 in our experiments. We also could not detect compounds capable of activating MRGPRX2 in gel filtration fractions containing large proteins and enzymes from the brain and intestinal extracts. Furthermore, the molecular weights of most known basic secretagogues are <4000 Da, suggesting that the breakdown products of proteins rather than the intact proteins are responsible for the activation of mast cells.

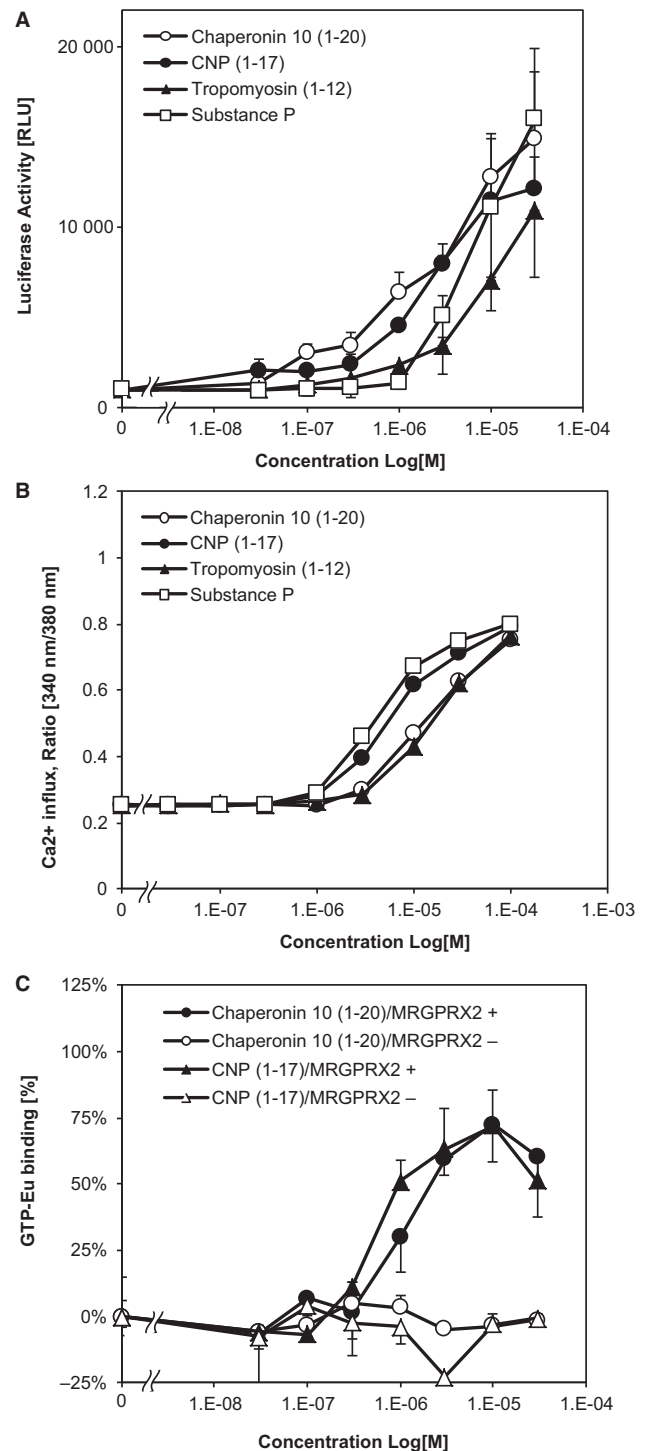


FIGURE 2 Effects of the endogenous MRGPRX2 ligands on MRGPRX2-expressing cells. (A) Endogenous MRGPRX2 ligand-induced reporter gene expression in PC12h cells transfected with MRGPRX2. (B) Endogenous MRGPRX2 ligand-induced intracellular calcium mobilization in HEK293 cells stably expressing MRGPRX2. The Y-axis represents the ratio of fluorescence emission at 500 nm with excitation light at 340 and 380 nm. (C) CNP (1-17) and chaperonin-10 (1-20) induced activation of G proteins in membranes prepared from MRGPRX2-expressing (MRGPRX2+) or parental HEK-293 (MRGPRX2-) cells. In all panels, values represent means $\pm$ SE (n = 4 in panel A; n = 2 in panel B; n = 3 in panel C)

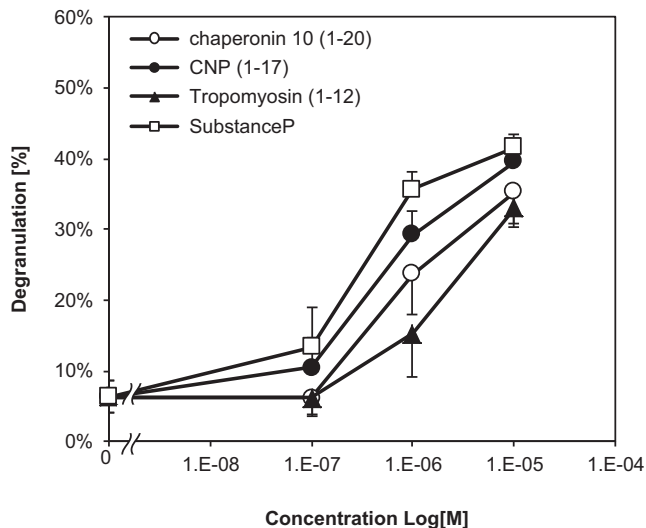


FIGURE 3 MRGPRX2 ligand-induced degranulation of HCMCCT. The degranulation of mast cells was assayed by measuring β -hexosaminidase activity. Values represent means $\pm$ SE ($n = 3$)

In addition to the known basic secretagogues (mainly peptides), we showed that some endogenous protein fragments also act as the ligands for MRGPRX2. Although these peptides do not have sequence homology each other, but they seem to have some simple and specific characteristics (a pattern) as follows: (1) having both a basic part (including plural basic amino acids) and a bulky hydrophobic part in the amino acid sequence, (2) having molecular weight of mostly 1000–4000 Da (10–40 amino acids). PACAP (6–27) having the pattern acts on PACAP receptor as an antagonist, but can activate MRGPRX2 and induces the degranulation of mast cells.⁵ Even a synthetic peptide, substance P antagonist which contains several D-amino acids in the sequence and has the above pattern, can activate MRGPRX2 and induces the degranulation of mast cells.⁵ It is important to emphasize that the pattern is not so rare in protein sequences. This leads us to an assumption that many more as-yet-unidentified endogenous MRGPRX2 ligands might be produced in the body. MRGPRX2 is thought to function as a nonselective and low-affinity receptor for the ligands. Clearly, these binding characteristics do not resemble those of typical GPCRs, such as neuropeptide receptors. We therefore indicate that MRGPRX2 is a new type of GPCR.

Pattern recognition receptors (PRRs) that include Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and Nod-like receptors (NLRs) recognize components of foreign pathogens referred to as pathogen-associated molecular patterns (PAMPs) and trigger innate immunity. There are indications that endogenous molecules produced by dying cells or in pathological conditions stimulate TLRs, resulting in the development or acceleration of inflammatory and autoimmune diseases.^{21,22} GPCRs of the MRGPR family are very different from PRRs in their molecular

structures and classification; however, we think it might be possible to expect that they have some similarities in their roles by recognizing a variety of ligands.

We think it is worthwhile to mention the possibility that MRGPRX2 is involved in pathogenesis. It is already shown that pseudoallergic reactions upon treatment with certain drugs are mediated by MRGPRX2.¹⁵ Fujisawa et al¹⁴ reported that skin mast cells in patients with severe chronic urticarial express increased levels of MRGPRX2. This suggests that MRGPRX2 pathway may contribute to the pathogenesis step of chronic urticarial. Many reports suggest the pathogenic role of mast cells in autoimmunity, but there is little information regarding mechanisms of mast cell activation in disease.²³ We think it is a challenging theme to investigate the relationship between autoimmune diseases and MRGPRX2.

We propose a hypothesis concerning CNP fragments. Mast cells are known to be involved in the pathogenesis of multiple sclerosis.^{23–29} CNP is a major myelin-associated enzyme in oligodendrocytes and Schwann cells closely associated with demyelinating diseases.³⁰ CNP activity in the cerebrospinal fluid of multiple sclerosis patients coincides with disease severity,³¹ and CNP is a major autoantigen in the pathogenesis of multiple sclerosis.³² Therefore, we hypothesize that abnormal accumulation of CNP fragments such as CNP (1–17) causes the release of inflammatory mediators from mast cells, resulting in inflammatory and autoimmune responses. To demonstrate the hypothesis, it is requested to detect the CNP fragments in patient's brain or cerebrospinal fluid.

In summary, we showed that in addition to the known basic secretagogues, some endogenous protein and enzyme fragments having the specific characteristics can act as the ligands for MRGPRX2 and induce degranulation of mast cells. We propose the possibility that many more as-yet-unidentified endogenous MRGPRX2 ligands are produced from proteins and enzymes in the body. It is also possible that such protein fragments may be derived from damaged or dying cells produced as a result of invasion by pathogens or other pathological conditions, and may activate MRGPRX2 on mast cells. We believe that the identification of such mast cell-activating protein fragments may be important in studying the role of MRGPRX2 in pathogenesis of various inflammatory and autoimmune diseases in which mast cells are involved, such as multiple sclerosis, atopic dermatitis and rheumatoid arthritis.

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