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Agonists of the *Mas*-Related Gene (Mrgs) Orphan Receptors as Novel Mediators of Mast Cell-Sensory Nerve Interactions

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IgE-dependent activation of mast cell activation is often associated with symptoms attributed to activation of sensory nerves. Depending on the tissues involved such symptoms include itching, sneezing, irritation, vasodilation, and reflex secretions. In the present study, we hypothesize that sensory neuroactive mediators released from mast cells may include agonists of recently discovered orphan receptors referred to as sensory nerve specific receptors or products of *mas* related genes. HEK-293 cells expressing MrgC11 receptors and wild-type HEK-293 cells were loaded with the calcium indicator Fura-2. A known stimulant of MrgC11 receptors the RF-amide, neuropeptide FF, evoked a rapid increase in cytosolic calcium in the MrgC11 expressing cells but not in the wild-type HEK-293 cells. IgE-dependent stimulation of either rat basophilic leukemia-2H3 cells (RBL-2H3 cells) or mouse bone marrow-derived mast cells, released a substance(s) that stimulated increases in cytosolic calcium in the MrgC11 expressing cells that far exceeded that seen in control cells. RT-PCR revealed that both mouse mast cells and RBL-2H3 cells express the RF-amide precursor gene proneuropeptide FF (A). Immunohistochemical analysis demonstrated RF-amide immunoreactivity in mouse skin mast cells in situ and in mast cells isolated from mouse skin. These data support the hypothesis that agonists of certain sensory nerve specific receptors or *mas* related genes may participate in mast cell sensory nerve interactions. *The Journal of Immunology*, 2008, 180: 2251–2255.

M any symptoms of allergic diseases such as itching, sneezing, coughing vasodilatation, increased secretions, and the reflex component of allergy-induced bronchospasm are consequences of activation of peripheral nerves. The fact that there is a close association between mast cells and peripheral nerves may help explain why allergic inflammation in particular is so adept at evoking such neuronal responses (1, 2). It is rather well established that allergen-induced activation of tissue mast cells is associated with alterations in the phenotype or physiology of nearby primary afferent (sensory) nerves (3–10). Some effect of mast cell activation on these nerves can be explained by defined mast cell mediators such as histamine, cys-leukotrienes, PGD2, and tryptase (3, 4, 10, 11). Other neuronal effects of mast cell activation, however, are unexplained by the release of these mediators (5). Even allergic itch, one of the most recognized consequences mast cell-sensory nerve interactions is only marginally inhibited by antagonists of histamine and other known mast cell mediators (12). Based on this, we hypothesized that a component of mast cell-mediated sensory neuromodulation may involve mast cell mediators that have not yet been characterized as such.

Recently, a novel family of sensory nerve specific orphan G protein coupled receptors (GPCRs) has been identified and termed Mrgs (*mas*-related genes) (13) or SNSR (sensory nerve specific receptor) (14). In the mouse, this family of receptors can be subdivided into MrgD and three subfamilies termed MrgA, MrgB, and MrgC based on homology analysis. In humans, there are seven Mrg receptors termed MrgX1-MrgX7 (13, 15, 16). Members of the rodent MrgA, B, C, and D subfamilies, as well as human MrgX2, 5, 7, and MrgD, appear to be selectively, if not specifically, expressed in sensory neurons with nociceptive phenotypes (13, 14, 17, 18).

MrgC11 produces a GPCR with 54% homology to the human MrgX1, and is localized to small diameter dorsal root ganglion neurons that typically bind isolecitin B4 (17). The MrgC11 is coupled to the Goq phospholipase C signaling pathway (17). This signaling pathway is likely to lead to increases in excitability of primary afferent nerves (19, 20). Mediators that act as MrgC11 (or other Mrg) agonists could therefore, in theory, serve as sensory neuromodulators in tissues. Indeed, MrgD activation has recently been shown to increase excitability of rat-isolated sensory neurons by inhibiting M-currents (21).

Candidates for the endogenous activators of MrgC11 include various RF-amide neuropeptides, including the prototypic molluscan peptide FMRFamide, but also mammalian RF-amides such as neuropeptide FF (NPFF) (17). In this study, we addressed the specific hypothesis that immunological activation of rat basophilic leukemia RBL-2H3 cells and mouse bone-marrow derived mast cells (BM-MCs) release mediators that activate sensory nerve specific MrgC11 receptors.

Abbreviations used in this paper: GPCR, G protein coupled receptor; Mrg, mas-related gene; SNSR, sensory nerve specific receptor; BMMC, bone-marrow derived mast cell; NPFF, neuropeptide FF; RBL-2H3 cells, rat leukemia-2H3 cell; HSA, human serum albumin.

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Materials and Methods

Cell culture

RBL-2H3 cells were maintained as monolayer cultures in Earl’s modified Eagle’s medium supplemented with 15% FBS, 2 mM glutamine, penicillin (1000 U/ml), and streptomycin (100 µg/ml).

BMMCs were prepared by flushing the tibia and femurs from mice using BMMC base media (without IL-3). On average, 3 to 5 × 10^6 BM cells were recovered per mouse. Cells were cultured initially at 5 × 10^5 cells/ml in BMMC base media containing 30% WEHI conditioned media as a source of IL-3. BMMC cultures (nonadherent cells) were passaged weekly at 2 to 5 × 10^5 cells/ml in fresh complete BMMC media. The surface phenotype of mature BMMC (4–8 wk old) revealed expression of Fce RI^\alpha^, c-Kit^+, CD45^+, and CCR3^+^ indicating that the cells were mast cells and not eosinophils or basophils. The granules were positive for heparin, β-hexosaminidase, and histamine, the histamine was detected by FACS staining and was >96%; and histamine content was normal, averaging 0.49 ± 0.04 pg/cell.

IgE-mediated degranulation

Anti-DNP IgE was added to 10 × 10^6 cells/ml rat basophilic leukemia (RBL)-2H3 cells or 20 × 10^6 cells/ml BMMC at the concentration of 50 ng/ml and shaken for 3 h, respectively, at 37°C. The cells were washed and resuspended with D-PBS (10% FBS), then stimulated by DNP-human serum albumin (HSA) (0.5 µg/ml) for 15 min at 37°C. The supernatant was obtained after centrifuging (1000 g for 2 min) and evaluated for Ag-induced degranulation. Degranulation was evaluated by the release of histamine from mast cells or β-hexosaminidase for RBL-2H3 cells, as described previously. DNP-HSA caused 10–45% release of the total β-hexosaminidase content, and histamine release >10 times the background.

Cytosolic free calcium measurement

HEK-293 cells stably expressing MrgC11 or MrgA1 were provided by Dr. David Anderson (CalTech, California). A description of their production can be found in Ref. 17 and wild-type HEK cells were transferred to culture dishes (0.6 × 10^6 cells in 2 ml/20 mm culture dish), loaded with Fura2-AM (4 µM) for 40 min at 37°C and then washed twice with D-PBS (10% FBS) just before recordings, as previously described (17). Cytosolic calcium measurements were performed under a microscope (Universal, Carl Zeiss,) equipped with epifluorescence. A field of cells was monitored by sequential dual excitation, 340 and 380 nm and emission at 510 nm and ratios of the images were obtained. The ratio images were acquired every 6 s. The recording fields usually contained 40–60 cells.

Coculture experiment

The Ag, DNP-HSA (50 ng/ml), was added to the incubation medium of the HEK-293 cells. IgE-sensitized RBL-2H3 cells (1 × 10^6 cells/100 ul) or BMMCs (2 × 10^6 cells/100 ul) were then added onto the recording fields. Therefore, the IgE-mediated reaction took place in the recording field. For control experiments, sensitized RBL or BMMC were applied to HEK-293 cells in the absence of DNP-HSA. In experiments with BMMCs the DNP-HSA was added to the mast cells 10 min before applying the cells to the HEK-293 cells. Ionomycin (1 µM) was used as positive control for checking Fura2 loading and the viability of cells. All experiments of calcium measurement were conducted at 34°C.

RT-PCR

Total RNAs of DRG, nodose ganglion, spinal cord, RBL-2H3, and BMMCs were extracted using TRIzol (Invitrogen Life Technologies). Reverse transcription was performed using SuperScriptII reverse transcriptase (Invitrogen). The PCR primers specific for NPPF precursor (5’ GGTTGAAA GACCAAGCTTTTGC 3’ and 5’ CTTCTCCTAAAGGGACTGAGG 3’), and MrgC11 (5’ CAGCAACATGTCACTTCAAC 3’ and 5’ ATGCCAATGAAAGGACAGAACC 3’) were obtained from MWG Biotech. To avoid genomic DNA contamination, the primer pairs were intron-spanning. The PCR products were confirmed by sequencing.

Immunohistochemistry

Mice were sensitized with 10 mg OVA (Grade V, Sigma-Aldrich) in 0.3 ml aluminum hydroxide gel adjuvant (Alhydrogel; Accurate) injected i.p. on two consecutive days. Control mice received Alhydrogel only. Beginning 3 wk after sensitization, the abdomen region was shaved, and skin was isolated. The skin was cut into small pieces (~3 × 3 mm). Skin segments were incubated (4 pieces per 1 ml tube) in Krebs bicarbonate buffer solution (37°C, gassed with a mixture of oxygen:carbon dioxide 95:5). The tissues were incubated for 60 min with the buffer replaced at 15-min intervals. The tissues were then exposed to OVA (100 µg/ml) or vehicle × 30 min. The supernatant solution was evaluated for histamine with an autotitrometric analyzer. Some segments were treated with 4% perchloric acid × 30 min to release tissue stores of histamine. Other segments were frozen in OCT at −80°C. Tissues were sectioned at 20 µm with a cryostat. In some studies, newborn skin was evaluated using a similar technique, although the skin was not immunologically challenged. The skin sections on slides or c-kit^+^ cells on coverslips were fixed with 4% paraformaldehyde at room temperature for 10 min. The slides were washed with PBS containing 0.1% Triton X-100 (PBT) for three times and blocked with 10% goat serum in PBT for 30 min. All sections were incubated with primary Abs diluted in blocking solution at 4°C for overnight. The primary Abs used were as follows: goat anti-rabbit (Alexa 488 conjugated; Molecular Probes) and anti-mouse IgG1 (A21124, Alexa 568 conjugated; Molecular Probes). Mast cells were identified with rhodamine-conjugated avidin (Vector; 1:1000). This method has been shown to effectively and selectively label mast cells in mouse skin tissue (8, 24). All secondary Abs were 1/500 diluted in blocking solution. Sections were washed with PBT and mounted with glycerol. Images were obtained using Zeiss LSM510 confocal microscope system.

Isolation of mouse skin c-kit^+^ cells

Skin from newborn mice was cut into small pieces and incubated in dissociation solution (5 mg/ml dispase; 1 mg/ml collagenase; 0.7 mg/ml hyaluronidase; 0.1% FBS in PBS) at 37°C for 45 min. Skin was dissociated by trituration and the dissociated tissues were filtered through nylon cell strainer (BD Falcon). c-kit^+^ cells were enriched by magnetic cell sorting developed by Miltenyi Biotec using CD117 (c-kit) MicroBeads. Enriched cells were plated on coverslips coated with poly-D-lysine for 2 h.

Results

We evaluated RBL-2H3 cells and mouse BMMCs to stimulate HEK-293 cells expressing MrgC11. For these studies we used a stable MrgC11 expressing HEK-293 cell line that was previously characterized (17).

RBL-2H3 cells

As expected, RBL-2H3 cells sensitized with anti-DNP IgE were effectively degranulated by Ag exposure as evidenced by DNP-HSA-induced hexosaminidase release that ranged between 10 and 45% of the total cell content.

The MrgC11-expressing HEK-293 cells strongly responded, with an increase in cytosolic free calcium, to antigenically (DNP-HSA) stimulated RBL-2H3 (peak Δ calcium increase = 33.1 ± 2.4 nM, n = 5). By contrast, wild-type HEK-293 cells failed to respond appreciably to antigenically stimulated mast cells (peak Δ calcium increase = 4.2 ± 2.1 nM, n = 7). The MrgC11 expressing HEK-293 cells also failed to respond appreciably to RBL-2H3 cells in the absence of antigenic stimulation (peak Δ calcium increase = 4.9 ± 3.6 nM, n = 5) (Fig. 1).

By contrast to the HEK-MrgC11 cells, HEK-MrgA1 cells (n = 3) did not respond to immunologically activated RBL-2H3 cells studied under identical conditions (peak Δ calcium increase = 1.3 ± 1.2 nM).

In an analogous set of studies, we stimulated IgE-sensitized RBL-2H3 cells (10/µl) with DNP-HSA, then evaluated the mast cell supernatant for its ability to activate wild-type HEK cells and the HEK cells expressing MrgC11. Unlike the coculture experiments, we noted that the wild-type HEK-293 cells responded to the Ag-stimulated mast cell supernatant (peak Δ calcium increase = 20 ± 10 nM). This distinction may be due to temporal differences in the two experimental designs. In the coculture study, the response is complete within the first 5 min, whereas in the supernatant study, 30 min is allowed for the release and accumulation of mediators before they are applied to the HEK cell preparation. Nevertheless, the MrgC11-expressing HEK-293 cells responded significantly (p < 0.01) stronger than wild-type cells to the Ag-stimulated mast cell supernatant (peak Δ calcium increase = 42 ± 8 nM, data not shown).
In parallel, we assessed the ability of the supernatant solution to stimulate HEK-MrgA1 cells (n = 6). The peak calcium increase in these cells averaged 17 ± 6 nM, which is not different from wild-type cells but significantly (p < 0.01) less than the response in the HEK-MrgC11 cells.

**Mouse BMMCs**

MrgC11-expressing HEK-293 cells also responded to Ag (DNP-HSA) exposed mouse mast cells that had been sensitized with anti-DNP IgE (Fig. 2). The wild-type HEK cells also responded modestly to the Ag-activated mast cells. Nevertheless, the peak increase in cytosolic calcium concentration in MrgC11-expressed cells was more than double that observed with wild-type HEK-293 cells (p < 0.05, peak response of wild-type vs MrgC11-expressing HEK-293 cells) (Fig. 2).

**NPFF stimulates MrgC11**

NPFF, a mammalian RF-amide neuropeptide mimicked the effect of Ag-challenged mast cells in stimulating MrgC11-expressing HEK-293 cells. In wild-type HEK cells, Ag stimulation did not result in a significant increase in cytosolic calcium (Fig. 3). NPFF did not affect wild-type HEK cells (n = 3). Another mammalian RF-amide, NPAF, did not activate MrgC11-expressing HEK cells (n = 4, data not shown). Consistent with previous findings using these cell lines (17), NPFF (1–10 μM) did not stimulate HEK-MrgA1 cells (n = 3, data not shown).

**NPFF in mast cells**

We used RT-PCR and subsequent sequence analysis to confirm that the gene for the common precursor peptide for NPFF (proNPFF(A)) is expressed in mouse mast cells and RBL cells (Fig. 4). Spinal cord mRNA was used as a positive control in these experiments.

Consistent with other reports, mast cells in mouse skin tissue were readily identified with rhodamine-labeled avidin (8, 24). A large percentage of mast cells in newborn mouse skin were labeled with the anti-RFamide Abs (Fig. 5A).

We evaluated the staining of mast cells with anti-RFamide Abs in adult skin sensitized and challenged with OVA ex vivo as described in Materials and Methods. Some skin fragments were used to evaluate the extent of histamine release to provide evidence that the tissue mast cells were indeed immunologically activated. The skin contained an average of 9.8 ± 0.3 μg/gm (wet weight). In two experiments, challenging the skin with OVA (100 μg/ml) caused mast cell degranulation as evidence by the release of 30 and 43% of the total histamine stores. Vehicle treatment resulted in <1% histamine release. Control and OVA-challenged skin segments were then prepared for immunohistochemical analysis. Most, if not all, avidin-stained mast cells stained positively with anti-RFamide Abs. As with the neonatal skin, there was little if any staining of other cell types in the skin with the RF-amide Ab. In control tissues, ~77 ± 8% of the mast cells appeared normal with no visual evidence of degranulation (n = 327 avidin stained mast cells). By contrast, 82 ± 2% of the mast cells in the Ag-challenged skin showed obvious signs of degranulation (n = 450 mast cells evaluated). The granule material leaving the mast cells was immunoreactive for RF-amide peptides (Fig. 5B).

**FIGURE 1.** Increases in cytosolic-free calcium in HEK-293 cells expressing MrgC11 or wild-type HEK-293 cells (○). The cells were exposed to RBL-2H3 cells passively sensitized with anti-DNP IgE. The peak responses in HEK-293 cells expressing MrgC11 was 33.1 ± 2.4 nM (n = 5) and 4.9 ± 3.6 nM (n = 5) in the presence (□) and absence (●) of the cross-linking Ag DNP-HSA, respectively. The peak response in wild-type HEK-293 cells with Ag stimulated RBL 2H3 cells was 4.2 ± 2.1 nM (n = 7). The asterisk denotes a significant difference (p < 0.001) between the peak response of MrgC11 expressing HEK cells exposed to activated mast cells and those of the two control groups (unstimulated mast cells in HEK-293 cells expressing MrgC11, or Ag stimulated mast cells in wild-type HEK-293 cells).

**FIGURE 2.** Increases in cytosolic-free calcium in HEK-293 cells expressing MrgC11 (□) or wild-type HEK cells (●). The cells were incubated in medium containing HSA-DNP (0.5 ng/ml) and exposed to mast cells (BMMCs) that were previously passively sensitized to anti-DNP IgE. The peak amplitudes of responses were 44.9 ± 7.7 nM (n = 8) and 26.7 ± 2.4 nM (n = 7) in MrgC11 expressing HEK cells and wild-type HEK cells, respectively. The asterisk denotes a significant difference (p < 0.05) between the peak response in the two groups of experiments.

**FIGURE 3.** Increases in cytosolic-free calcium in MrgC11-expressing HEK cells (■, n = 6) or wild-type HEK (○, n = 3) cells in response to increasing concentrations of NPFF. The EC50 was 67.5 nM in MrgC11 expressing HEK cells.

**FIGURE 4.** pro-NPFF mRNA is present in mast cells. RT-PCR of mRNA isolated from RBL-2H3 cells (RBL) and mouse BMMCs show that pro-NPFF mRNA is expressed in these cells. In each experiment, NPFF expression in spinal cord tissue (s.c.) was used as a positive control.
In a separate set of experiments, mast cells dissociated from the skin were identified using anti-c-kit Ab. As with the tissue sections, many of the dissociated mast cells (c-kit positive cells) were costained with the mast cell marker anti-c-kit Ab and either of the two anti-RF-amide Abs we used (Fig. 5C).

MrgC11 expression
Mast cells are commonly situated near vagal sensory C-fibers in the mucosa and submucosa of visceral tissues (25). We were therefore interested in whether MrgC11 is expressed by vagal afferent nerves. Although MrgC11 has been shown to be expressed in sensory neurons within the dorsal root ganglion, there is no information available on its expression in vagal sensory neurons. We found expression of MrgC11 mRNA in both dorsal root and vagal nodose mouse sensory ganglia (Fig. 6).

Discussion
The results of the present study provide support for the hypothesis that neural-active mediators secreted from IgE-dependent activation of mouse mast cells and RBL-2H3 cells may include stimulators of a family of sensory-specific orphan receptors termed Mrgs or SNSRs. In addition, the data provide circumstantial support for the hypothesis that one such mediator may be an RF-amide product of pro-NPFFa gene expression.

In the mouse, the sensory specific Mrgs have been categorized into MrgD and three families MrgA, MrgB, and MrgC (13). MrgC11 has homology to the human MrgX (17). The phenotype of sensory nerves that express MrgC11 in the mouse is consistent with nociceptive type nerves in that they are small diameter neurons that express isolectin B4 (17). In the vagus nerve, the majority of neurons project nociceptive C-fiber neurons; it is therefore not surprising that we noted in this study that, at least at the mRNA level, a subset of mouse vagal sensory neurons also expressed MrgC11.

Our data reveal that IgE-dependent activation of RBL-2H3 cells or mouse BMMCs acutely release mediator(s) capable of stimulating MrgC11. MrgC11 has been shown to couple to the Gq/11 pathway (17), and this pathway is associated with GPCR-mediated stimulation of nociceptors (19). It follows, therefore, that the MrgC11 activators released from mast cells may lead to the stimulation or increased excitability of somatosensory and visceral C-fibers.

The nature of the molecule(s) involved in mast cell-dependent MrgC11 activation remains to be worked out. Nevertheless, our data provide support for the hypothesis that one such mediator may be an RFamide neuropeptide. The first member of the RFamide
neuropeptide family was FMRFamide, isolated from ganglia of the clam *Macrocystis* *nimbus*). Several other members of this peptide family have been discovered each sharing carboxy-terminal arginine (R) and amidated phenylalanine (F) residues (26). Our pharmacological investigation, consistent with previous reports (17) show that the effect of mast cell secretion on MrgC11 expressing cells can be mimicked with the mammalian RF-amide NPFF. By contrast, the HEK-MrgA1 cells are relatively insensitive to NPFF (17), and these cells failed to respond to mast cell activation.

Adding additional evidence to the hypothesis that NPFF may be involved in the mast cell mediated activation of MrgC11 is the RT-PCR results showing that mast cells transcriptionally express the precursor of NPFF. In addition, we obtained histological evidence that NPFF or a related RF-amide peptide is present in neonatal and adult mouse skin mast cells and appears to be released during the process of degranulation. It should be kept in mind that the evidence implicating RF-amides as the key MrgC11 activators released from mast cells is circumstantial and there are other candidates for endogenous activators of MrgC11 including products of pro-opiomelanocortin that may be released from mast cells (14, 17, 27). Likewise, although the present study focused on MrgC11, several different Mrgs have been localized primarily to subsets of primary afferent nerves, and some of these are stimulated by NPFF (13).

Recent studies have shown that certain RFamides, including the prototypical FMRFamide, can stimulate action potential discharge in afferent terminals of rat skin-nerve preparations (28). In addition, the RF-amides increase peripheral pain responses consistent with nociceptor modulation (29). Some of these molecules may stimulate nociceptors by interacting with acid-sensing ion channels (30), but the algesic effect of RF-amides in rats, was not inhibited by pharmacological blockers of acid-sensing ion channels. It should be kept in mind that in addition to Mrg receptors, NPFF and other RFamides can also stimulate at least two other G-protein coupled receptors, NPFF1 and NPFF2 (31). It is not yet clear what the respective roles of Mrgs and other NPFF receptors are in the sensory nerve effects of NPFF.

It has recently been reported that human mast cells express an Mrg (MrgX2) and that basic compounds such as 48/80 and substance P may use this receptor to evoke cell activation (32). If a similar scenario exists in mouse mast cells, our data raise the possibility for an autocrine loop of a mast cell mediator feeding back to stimulate the cell via Mrg activation.

In summary, these data show for the first time that mast cells can secrete mediator(s) capable of stimulating the GPCR MrgC11 (and likely to stimulate the cell via Mrg activation.

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**Disclosures**

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