Dual action of neurokinin-1 antagonists on Mas-related GPCRs

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Introduction

The neurokinin-1 receptor (NK-1R) and its peptidic ligand, substance P (SP), have been implicated in mouse models of human conditions, including emesis (1), asthma (2), and migraine (3). Antagonists to NK-1R were developed and found to be effective in animal models of diseases. This success in animals led to NK-1R antagonists being evaluated in many clinical trials over the past two decades. NK-1R antagonists were found to be effective in the treatment of nausea and vomiting associated with chemotherapy but failed to improve inflammation and nociception (1, 4–7). The discrepancy surrounding the efficacy of NK-1R antagonists in animal models and their failure in clinical trials has not been understood (4, 7).

Mast cells express NK-1R and contribute to inflammation and allergic reactions via IgE-independent and IgE-dependent mechanisms. With respect to the IgE-independent mechanism, a pivotal study revealed that basic secretagogues, which include SP, compound 48/80, cationic peptide antibiotics, and drugs associated with pseudoallergic reactions, activate MRGPRX2 and the mouse ortholog, MrgprB2, to induce mast cell degranulation (8). In contrast, IgE-dependent responses are not mediated through Mas-related GPCRs (Mrgprs) (8).

Here, we suggest that a differential effect of NK-1R antagonists on human versus mouse Mrgprs may solve the mystery of the differential efficacy of NK-1R antagonists in animal models versus clinical trials. We investigated the activity of the structurally related NK-1R antagonists L733060, used widely in mouse studies, and aprepitant, an approved drug, on human MRGPRX2 and mouse MrgprB2. Neither of these antagonists effected the activation of human MRGPRX2 by SP. However, they inhibited the activation of mouse MrgprB2 by SP. We therefore asked if an unrelated tripeptide NK-1R antagonist might serve a dual function as an antagonist of human MRGPRX2. This tripeptide both suppresses itch in mice and inhibits degranulation from the LAD-2 human mast cell line elicited by basic secretagogue activation of MRGPRX2. Antagonists of Mrgprs may fill the void left by the failure of NK-1R antagonists.

Results

SP activates human MRGPRX2, mouse MrgprB2, and mouse MrgprA1 in addition to NK-1R. In rodents, as compared with primates, Mrgprs are widely expanded (10, 11). A single human Mrgpr could thus be
homologous to several mouse Mrgprs. It has been reported that mouse MrgprB2 is the ortholog of human MRGPRX2. However, ligands that activate both human MRGPRX2 and mouse MrgprB2 are more active on the human MRGPRX2. The EC\textsubscript{50} of SP for hMRGPRX2 is approximately 150 nM, while the EC\textsubscript{50} of SP for mMrgprB2 is approximately 50 μM (8). MRGPRX2 has been reported to be expressed on mast cells and dorsal root ganglions (DRGs) in humans and primates (12–15), whereas MrgprB2 is expressed exclusively on mouse mast cells. This line of reasoning leads to the suggestion that a distinct Mrgpr on mouse sensory nerves may also serve as an ortholog to human MRGPRX2. Previous studies revealed that MrgprA1 is exclusively expressed at functional levels (16) on mouse DRGs but not mast cells (8, 11, 16). We evaluated the interaction of SP with several mouse Mrgprs and found that SP activates mouse MrgprA1 (Figure 1 and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/jci.insight.89362DS1). The EC\textsubscript{50} of SP on MrgprA1 was 10 times better than on MrgprB2 (Figure 1). These results indicate that the mouse receptors MrgprA1 and MrgprB2 are each homologous to human MRGPRX2.

**NK-1R antagonists are also antagonists of mouse MrgprB2.** NK-1R antagonists are effective in mouse models, and SP is an agonist of both NK-1R and the mouse MrgprB2. We therefore asked if established NK-1R antagonists could block the capacity of SP to activate MrgprB2. We found that the NK-1R antagonists L733060 and aprepitant each prevented activation of mouse MrgprB2 by SP (Figure 2). This observation is critical, as it can explain the inconsistencies among clinical trials of NK-1R antagonists in humans and mouse models of inflammatory disease, as described further in the Discussion, and as it shows that mouse MrgprB2 and human MRGPRX2 are important for inflammation. L733060 is effective in mouse models, most likely by antagonizing both NK-1R and, as reported here, MrgprB2. Aprepitant has limited effectiveness in humans because it is an antagonist of NK-1R but not MRGPRX2.
Identification of QWF as a dual NK-1R and Mrgpr antagonist. We next asked if an unrelated competitive NK-1R antagonist would serve additionally as an antagonist of the human receptor MRGPRX2. We selected the tripeptide NK-1R antagonist Gln-D-Trp(Formyl)-Phe benzyl ester, abbreviated with the single amino acid letter code as QWF. QWF was effective at preventing SP from activating NK-1R, mouse MrgprB2, mouse MrgprA1, and human MRGPRX2, as measured by calcium imaging of receptor activation (Figure 2). QWF was also effective at preventing the binding of SP to NK-1R, mouse MrgprB2, mouse MrgprA1, and human MRGPRX2 (Figure 3A). The capacity of QWF block the activation of MRGPRX2 was also evaluated (Figure 3B). We also evaluated the interaction of QWF with human MRGPRX1, mouse MrgprA3, PAR2, histamine-1 receptor, and melanocortin-1 receptor in the presence of their ligands. QWF did not affect the activation of these receptors by their ligands (Supplemental Figure 3).

QWF inhibits activation of human MRGPRX2 by basic secretagogues and by medications associated with pseudoallergic drug reactions. Basic secretagogues and drugs that are associated with pseudoallergic reactions interact with human MRGPRX2 to induce IgE-independent mast cell degranulation (8). We therefore asked if QWF could inhibit this process. QWF indeed inhibited activation of human MRGPRX2 by compound 48/80; atracurium, a neuromuscular blocking agent; and ciprofloxacin, an antibiotic, as determined by Fura-2 calcium imaging in heterologous cells (Supplemental Figures 3 and 4). These studies with QWF were extended to mouse MrgprB2 with similar results (Supplemental Figures 3 and 4).

QWF inhibits IgE-independent degranulation from human LAD2 mast cells. We next asked if QWF would inhibit mast cell degranulation induced by basic secretagogues and drugs associated with pseudoallergic reactions. QWF indeed inhibited degranulation by SP, compound 48/80, atracurium, and ciprofloxacin.
These results suggested that human MRGPRX2 antagonists could potentially prevent pseudoallergic drug reactions.

**QWF is an antagonist of compound 48/80–induced Mrgpr activation and scratching in mice.** We next asked if the in vitro findings above could be extended in vivo. We selected scratching behavior in mice, a correlate to itching in humans. We evaluated the capacity of QWF to affect receptor activation in vitro and scratching behavior in vivo by compound 48/80, an established pruritogen. Compound 48/80 is a polymer that induces mast cell degranulation and provokes itch in humans and mice (17, 18). It had been thought that compound 48/80 provokes itch via mast cell degranulation and was thus histamine dependent. It was subsequently determined that compound 48/80, and SP, provoke similar numbers of scratching bouts in WT and mast cell–deficient mice (17). These results revealed that mast cells were not critical for itch provoked by these compounds in mice. It was suggested that mast cells may thus play a modulatory role (17). In addition, compound 48/80 directly interacts with sensory nerves, potentially through Mrgprs (19). Since QWF antagonizes the interaction of compound 48/80 with Mrgprs, we examined the capacity of QWF to block compound 48/80–provoked itch. Compound 48/80 activates human MRGPRX2, mouse MrgrpB2 (expressed on mouse mast cells), and mouse MrgrpA1 (expressed on mouse DRGs). QWF antagonized the activation of these Mrgprs by compound 48/80 and significantly inhibited scratching provoked by compound 48/80 (Figure 5). Since MrgrpA1 is expressed on nerves and is activated by compound 48/80, our results could explain the observations with compound 48/80 and SP in mast cell–deficient mice.

**SP–induced itch is not decreased in NK-1R KO mice.** We next sought to begin to clarify the links among SP, NK-1R, itch, and urticaria, with a consideration of the role of SP and Mrgprs in these conditions. Injection of SP into the skin of humans induces urticarial (20), and patients with chronic urticaria exhibit enhanced wheal reactions to SP as compared with healthy controls (21). These observations are important, as the etiology of up to 50% of patients suffering from chronic urticaria remains unknown, antihistamines are typically not effective, an association with IgE-mediated degranulation can not be established (22), and a recent study of patients with severe chronic urticaria demonstrated upregulated expression of human MRGPRX2 (23). A separate study demonstrated that NK-1R antagonists do not block SP–induced degranulation of human mast cells (24). Several NK-1R antagonists have been tried for treating itch with conflicting results (25, 26), and many of these antagonists have entered clinical trials without, to our knowledge, evaluation in NK-1R–/– mice (7).
We compared SP-induced itch between WT and NK-1R−/− mice. Remarkably, SP-induced itch was not decreased in NK-1R−/− mice (Figure 6A). As SP activates Mrgprs, and Mrgprs have been implicated in itch, we evaluated the effect of QWF on SP-induced itch (Figure 6, B and C). Since QWF is a dual NK-1R/Mrgpr inhibitor, we coinjected QWF with SP in NK-1R−/− mice. SP-induced itch was significantly decreased in these mice. To confirm and extend these findings, we evaluated the effect of QWF on SP-induced itch in WT mice. QWF significantly decreased SP-induced itch in WT mice. These data indicate that SP-induced itch may be mediated by Mrgprs rather than NK-1R.

Discussion

A plethora of studies over the past decade have implicated Mrgprs in nociception and inflammation (27–30). While the expression of most Mrgprs is restricted to sensory dorsal root ganglion neurons, human MRGPRX2 and the mouse ortholog MrgprB2, are expressed on mast cells (8, 31). Human MRGPRX2 and mouse MrgprB2 mediate IgE-independent mast cell degranulation induced by basic secretagogues, including SP, implicating Mrgprs not only in nociception but also in inflammation (8).

Given that human MRGPRX2 and mouse MrgrpB2, rather than NK-1R, mediate SP-induced mast cell degranulation (8), it is possible that the antiinflammatory properties observed with NK-1R antagonists in animal models may have been the result of the interaction of these drugs with mouse MrgprB2. Several studies have demonstrated a role for mast cells in inflammatory conditions, including asthma (32) and migraine (33), for which NK-1R antagonists were expected to work. We suggest that these antagonists were not effective in clinical trials because NK-1R does not mediate SP-induced mast cell degranulation and because NK-1R antagonists, as reported here, do not interact with human MRGPRX2. Put another way, a previously unidentified off-target effect of NK-1R antagonists on Mrgprs mediates their effects in mice, and NK-1R itself may not be responsible for the proinflammatory properties of SP on mast cells.

MRGPRX2-specific antagonists have not yet been reported. The NK-1R antagonists evaluated here additionally block mouse MrgrpB2. The effects of these agents in animal models thus can not be differentiated in WT mice. The use of these dual antagonists in NK-1R KO mice can help to clarify the contribution of Mrgprs to inflammatory models but does not eliminate a role for NK-1R. The separate contributions of NK-1R and MrgprB2 await the availability of MrgprB2 KO mice or receptor-specific antagonists.

Our findings, that SP-mediated scratching in mice correlates with Mrgrp activation as opposed to NK-1R activation, are the first to our knowledge to indicate a physiologic consequence of Mrgrp activation to an endogenous ligand. It was reported previously that scratching provoked by cathepsin S was mediated by cleavage of MrgrpC11 (28). SP activates MrgrpB2, MrgrpA1, and MRGPRX2 in addition to NK-1R; Mrgprs have otherwise been considered orphan receptors. MRGPRX2 can be activated by a number of peptides, including cortistatin-14, the proadrenomedullin C-terminal peptides PAMP-12 and -20, and LL-37, but physiological consequences have not been identified (12, 13, 34).

The similar number of SP-induced scratching bouts reported in WT and mast cell–deficient mice (17) can now be explained as follows: MrgrpB2 is expressed on mast cells and thus absent in mast cell–deficient mice (16). MrgrpA1 is expressed at functional levels on nerves and MrgrpB2 is not (11, 16). As mouse MrgrpA1 is 10-fold more sensitive to SP than mouse MrgrpB2, scratching in WT and mast cell–deficient mice is driven by the interaction between SP and mouse MrgrpA1. Studies in human subjects reveal that subcutaneous pre-
treatment with compound 48/80 inhibits SP-provoked itch (20). This observation has been confirmed in mice (17, 35). Given that mast cells are not the major mediators of SP- or compound 48/80–provoked itch in mice, the simple explanation that pretreatment with compound 48/80 depletes histamine from mast cells is not satisfactory (17, 36). The direct interaction of compound 48/80 and SP with a common receptor on mouse sensory nerves, presumably MrgprA1, and desensitization of this receptor by 48/80 can explain the observation. Compound 48/80 does not activate NK-1R, which was previously thought to mediate SP-provoked itch. We emphasize that mouse MrgprB2 is much more sensitive to compound 48/80 as compared to SP (8), therefore mast cells may play a prominent role in compound 48/80–induced itch in mice as compared with SP.

Several conclusions can be drawn from the data reported here. First, L733060 and aprepitant turn out to be dual antagonists of NK-1R and mouse MrgprB2, and mouse MrgrpA1 by compound 48/80. (G) Compound 48/80–induced itch is significantly decreased after coinjection with QWF (P = 0.002). Studies in A–F were performed at least twice. The study in panel G was performed twice using 2-tailed unpaired Student’s t test was used. *P ≤ 0.05, **P ≤ 0.01.

Figure 5. QWF is an antagonist of compound 48/80–induced Mrgrp activation and itch provocation. (A–F) QWF inhibits activation of human MRGPRX2, mouse MrgrpB2, and mouse MrgrpA1 by compound 48/80. (G) Compound 48/80–induced itch is significantly decreased after coinjection with QWF (P = 0.002). Studies in A–F were performed at least twice. The study in panel G was performed twice using 2-tailed unpaired Student’s t test was used. *P ≤ 0.05, **P ≤ 0.01.
here suggest that antagonism of human MRGPRX2 may be relevant to the treatment of SP-associated itch. While it is tempting to suggest that QWF may have a therapeutic role as a result of its antagonism of NK-1R and MRGPRX2, the tripeptide is rapidly metabolized in plasma (37), limiting its efficacy. The implications of the use of human MRGPRX2 antagonists may extend to the treatment of itch and, thus, beyond urticaria and pseudoallergic drug reactions. There are limitations to this study, including that the mouse cheek model of acute itch is not known to resemble a disease condition in humans. In addition, evaluation of the specific roles of NK-1R, MrgprB2, and MrgprA1 in itch or inflammation would benefit from the identification of MrgprA1- or MrgprB2-specific antagonists and the availability of MrgprA1 or mMrgprB2 KO mice. In conclusion, antagonists of MRGPRX2 may have a therapeutic benefit in conditions in which NK-1R antagonists have shown promising results in animal models but failed in humans.

**Methods**

**Peptides and chemicals.** SP and SLIGRL were obtained from GenScript and dissolved in PBS. Chloroquine was obtained from Sigma-Aldrich. The NK-R antagonists, L733060 and QWF (Boc-Gln-D-Trp(Formyl)-Phe benzyl ester trifluoroacetate), were obtained from Sigma-Aldrich, while aprepitant was purchased from Selleckchem. Aprepitant and QWF were dissolved in DMSO at 10 mM and diluted into PBS at 1 mM or lower concentrations.

**Cell culture.** HEK293 and HeLa cells were obtained from ATCC.

**Animals.** C57BL/6 mice were obtained from JAX. *NK-1R−/−* mice on a C57BL/6 background were provided by Norma Gerard, Children's Hospital Boston.

**cDNA clones.** Human MRGPRX2 was isolated by PCR from human genomic DNA using the forward and reverse primers, CTCGAGAGCATGGATCCAACCACC and AAGCTTCTCTACACCAGCTGCTTCCTCG, and cloned into pcDNA3.1(-). The mouse MrgprB2 coding sequences were amplified from mouse genomic DNA using the primer pair, CTCGAGAACATGAGTGGAGATTTCCTAATCAAG and AAGCTTTCAGCTGCAGCTCTGAACAGTTTCCAG, and cloned into pcDNA3.1(-). Human *NK-1R* cDNA was obtained from Life Technologies, PCR amplified with Xho I-Hind III ends, and cloned into pcDNA3.1(-). All other Mrgprs were cloned by PCR from genomic DNAs and inserted into pcDNA3.1(-).

**Calcium imaging of transfected HeLa cells.** 10 μg of expression vector (human MRGPRX2, mouse MrgprB2, or NK-1R) and 10 μl of Lipofectamine 2000 transfection reagent were diluted into 0.5 ml of DMEM and separately left at room temperature for 5 minutes. They were then mixed and incubated at room temperature for 20 minutes prior to being added to HeLa cells. HeLa cells were grown to confluence and trypsinized, and 1 × 10⁶ cells were pelleted in a 15-ml tube by centrifugation at 250 g for 5 minutes. The DMEM–Lipofectamine 2000–DNA mixture (1 ml) was then added to the cell pellet, suspended, and incubated at room temperature for 5 minutes. Two ml of complete DMEM with 10% FBS without antibiotics was added to the tube, mixed by inverting the tube, plated into a 96-well glass bottom plate at 50,000 cells/well, and placed in a 37°C CO₂ incubator for 3 hours. HeLa cells transfected with salmon sperm DNA were plated as a control. The medium was changed after 3 hours and left in the incubator. Twenty-four hours after transfection, the medium in the wells was removed and 100 μl of complete DMEM containing 2 μM of Fura-2 was added to each well and left at room temperature in the dark for 1 hour.

**Figure 6.** Scratching from substance P is maintained in *NK-1R−/−* mice and is blocked by QWF. (A) Itch induced by substance P (SP) (500 μM) is not decreased in *NK-1R−/−* mice compared with WT mice. (B) SP-induced itch is significantly decreased (*P* = 0.0040) in *NK-1R−/−* mice after coinjection with QWF (500 μM). (C) SP-induced itch is significantly decreased (*P* = 0.0134) in WT mice after coinjection with QWF (500 μM). These studies were performed twice using 2-tailed unpaired Student’s *t* test. *P* ≤ 0.05, **P** ≤ 0.01.
with Fura-2, the medium was removed, washed with PBS, and replaced with 90 μl of HEPES-buffered saline (20 mM HEPES, 115 mM NaCl, 5.4 mM KCl, 2 mM CaCl₂, 0.8 mM MgCl₂, 13.8 mM glucose, pH 7.4). Calcium imaging was performed immediately after loading cells with Fura-2 using a Zeiss Axiovert 200M microscope platform equipped with a flipping filter wheel for ratiometric imaging. Axiovision software, version 4.6, was used for calcium image analysis of the cells excited at 340 nm and 380 nm. Agonists were added at 15 seconds after the start of the excitation procedure, as indicated in Figure 2, 5, and all the Supplemental Figures. Antagonists were added 5 minutes before agonists. Images were taken every 5 seconds, including at 0 time, during a 90-second period, or longer if required. The software later analyzed all images taken during each excitation period. Ratiometric changes were measured in 10–20 cells in each individual experiment. An average of the fluorescence of the cells in each image was calculated, represented as F340/380 in the figures, and plotted against time in seconds. Colored curves in the figures represent responses of representative individual cells.

**Behavioral studies.** The mouse cheek model was used to evaluate scratching behavior (38). Mice were habituated for 30 minutes/day for 3 days prior to the study and for 15 minutes on the day of the study. 10 μl of each compound was delivered by a 31-gauge needle to the cheek. Mice were not shaved prior to injections to minimize irritation. All experiments were performed at consistent times during the day (9:00 am to 2:00 pm) and under the same conditions and in groups of 7 mice (4 male, 3 female), the number of mice demonstrated via power calculation to generate significance in behavioral studies. Mice were 2–3 months old and 20–30 g. Mice were videotaped in a soundproof environment to minimize distraction. Recordings were scored for the number of scratching bouts that occurred over 1-minute intervals during 25-minute observation periods, and the investigator was not aware of the group allocation during scoring the study and behavioral analysis. A scratching bout was initiated by lifting of the hind paw to the area of injection and ended by returning of the hind paw to the floor or to the mouth. Compound 48/80 (500 μM), SP (500 μM), and QWF (500 μM) were injected compounds. The concentrations of SP and compound 48/80 were standard for behavioral studies.

**Human LAD2 mast cell culture, degranulation assay, and calcium imaging.** The human LAD2 mast cell line (from D. Metcalfe, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Maryland, USA) was cultured in StemPro-34 SFM medium (Life Technologies) supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 ng/ml recombinant human stem cell factor (PeproTech). The cells were maintained at 0.25 × 10⁷ to 5 × 10⁷ cells/ml at 37°C and 5% CO₂ and hemidepleted each week. Degranulation was measured as described previously (39). In brief, LAD² cells were washed before incubation with various concentrations of QWF (1 μM, 10 μM, 100 μM) for 10 minutes before activation by SP (1 μM). 100 μM QWF was effective at decreasing SP-induced mast cell degranulation, and this concentration was used for studies involving basic secretagogues (1.5 μM compound 48/80, 100 μM atrucurium, and 100 μM ciprofloxacin). The level of mast cell degranulation was assessed by the release of β-hexosaminidase in mast cell granules, as quantified by the level of its substrate, p-nitrophenyl N-acetyl-β-D-glucosamide, digested in a colorimetric assay. For calcium studies, methods were identical to those using HeLa cells, except that LAD2 cells were incubated for 24 hours on imaging plates coated with poly-D-lysine to improve the attachment of the cells.

**SP-binding assay by ELISA.** HEK293 MRGPRX2 Cells (0.5 × 10⁶) were transferred to 2 tubes in a volume of 500 μl PBS. One tube was treated with SP to a final concentration of 1 μM for 1 hour at room temperature, and the other tube was first incubated with QWF (100 μM) for 10 minutes followed by SP (1 μM) for 1 hour. Similar procedure was carried with HEK293 cells transfected with pcDNA3.1(-)mMrgprB2. Control HEK293 cells (0.5 × 10⁶) were also treated with SP (1 μM) in a similar manner. Cells were pelleted and washed twice with PBS in a volume of 500 μl. Supernatants were discarded. Cell pellets were suspended in 300 μl of PBS and lysed by two freeze-thaw cycles using liquid nitrogen. The lysates were spun and supernatants were assayed in triplicate using an R&D Systems SP competitive enzyme immunoassay (catalog KGE007). The readings from SP-untreated HEK293 cells as controls were subtracted from the final values.

**Statistics.** For statistical comparison, 2-tailed unpaired Student's t test was used to determine significance. Differences were considered to be statistically significant at P < 0.05. Group data are presented as mean with SEM. Data analysis was performed using Prism 6. All in vitro studies were performed at least 3 times.

**Study approval.** The present studies in mice were reviewed and approved by the Institutional Animal Care and Use Committee at Massachusetts General Hospital, Boston, Massachusetts, USA.
Author contributions
EA, VBR, and EAL conceived the overall design of the study. EA performed behavioral studies. VBR performed in vitro studies and helped analyze them with EA and EAL. KTCS and RMA performed mast cell studies in conjunction with EA and EAL. ST and PJSP helped with in vitro studies and the structure of the manuscript. All authors contributed to the writing and review of the manuscript.

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