University of Pennsylvania Dental Medicine

MRGPRX2 SIGNALING ON MAST CELL-MEDIATED PSEUDOALLERGY AND NEUROIMMUNE INTERACTION

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Give thanks to the Lord, for He is good; for his love endures forever.

(Psalm 107:1)
In addition to high affinity IgE receptor, human mast cells (MCs) express a newly identified receptor known as Mas-related G protein-coupled receptor X2 (MRGPRX2; mouse ortholog MrgprB2). This receptor is predominantly expressed on only one subtype of MCs and it can be activated by a diverse group of cationic agonists including host defense peptides, Food and Drug Administration (FDA)-approved drugs associated with pseudoallergy and neuropeptides secreted from sensory nerve endings. Not surprisingly, MRGPRX2 has been implicated in several MC-mediated health and disease, ranging from host defense and wound healing to drug-induced pseudoallergic reactions, neurogenic inflammation and pain. However, there is a controversy regarding its role on rocuronium-induced hypersensitivity. Furthermore, the molecular mechanisms underlying MRGPRX2 regulation remain largely unknown. In this dissertation, we first investigated the role of MRGPRX2 on rocuronium-induced hypersensitivity. The effect of MRGPRX2 mutations (M196I, L226P and L237P) identified in a patient with rocuronium hypersensitivity were also tested. We found that rocuronium induced degranulation in murine and human MCs via MrgprB2 and MRGPRX2, respectively, but with different affinities, indicating important functional differences between these receptors. This indicates that mice expressing MrgprB2 may not be a suitable model to study human MRGPRX2 function and highlights the need to develop better animal models.

It is now realized that activation of MCs by substance P (SP) via MRGPRX2 contributes to neurogenic inflammation, pain and itch. We sought to identify the mechanisms underlying MRGPRX2 signaling and regulation on SP-activated MC responses. Using pertussis toxin and YM-254890, we demonstrated that SP induces MRGPRX2-mediated Ca^{2+} mobilization and degranulation via both G\alpha_i and G\alpha_q. Next, we utilized information obtained from both structural modeling and naturally occurring MRGPRX2 missense variants to identify putative G protein
coupling regions. In addition, several gain- and loss-of-function missense single nucleotide polymorphisms (SNPs) in MRGPRX2 have been discovered.

Finally, we demonstrated that SP can activate β-arrestin recruitment and receptor internalization. A tyrosine residue in the highly conserved NPxxY motif of MRGPRX2 (Tyr279) is crucial for SP-induced β-arrestin recruitment and receptor internalization. This study reveals the novel findings that activation of MRGPRX2/B2 by SP is regulated by β-arrestins and that a highly conserved tyrosine residue is responsible for MRGPRX2 signaling and regulation.
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LIST OF ABBREVIATIONS

MC
Mast cell
MC<sub>TC</sub>
Mast cells containing tryptase and chymase
MC<sub>T</sub>
Mast cells containing tryptase
CTMCs
Connective tissue mast cells
MMCs
Mucosal mast cells
BMMCs
Bone marrow-derived mast cells
PMCs
Peritoneal mast cells
Ig
Immunoglobulin
F<sub>c</sub>εRI
High affinity immunoglobulin E receptor
GPCR
G protein-coupled receptor
MRGPRX2
Mas-Related G protein-coupled receptor X2
MrgprB2
Mas-Related G protein-coupled receptor B2
HDP
Host defense peptide
NP
Neuropeptide
FDA
Food and Drug Administration
SCF
Stem cell factor
TLR
Toll-like receptor
SP
Substance P
WT
Wild-type
NMBDs
Neuromuscular blocking drugs
POH
Perioperative hypersensitivity
BAT
Basophil activation test
IDT
Intradermal skin test
TM
Transmembrane
ECL
Extracellular loop
ICL
Intracellular loop
PTx
Pertussis toxin
MFI
Mean fluorescent intensity
LAMP-1
Lysosomal-associated membrane protein 1
RBL-2H3
Rat basophilic leukemia-2H3 cells
RBL-MRGPRX2
RBL-2H3 cells stably expressing MRGPRX2
HTLA
Engineered HEK-293T cells stably expressing a β-arrestin2–tobacco etch virus fusion gene
HTLA-MRGPRX2
HTLA cells stably expressing MRGPRX2-Tango
Tango assay
Transcriptional activation following arrestin translocation assay
SNPs
Single nucleotide polymorphisms
CHAPTER 1: Introduction

Mast cells (MCs) are multifunctional granulated immune cells of the myeloid lineage that are widely located at the host-environment interface such as the skin and mucosal tissues. While best known as effectors cells in anaphylaxis and allergic and inflammatory diseases, MCs also play important roles in diverse physiological processes including tissue homeostasis, host defense, and wound healing (1-3). They are widely distributed throughout the body in most vascularized tissues and are particularly found at the host-environment interfaces, such as the skin, respiratory tract, and gastrointestinal tract. Therefore, MCs are regarded as professional sentinels and first responders against infectious pathogens and other potential insults (3-5).

Classical MC activation occurs via the cross-linking of high affinity immunoglobulin E (IgE) receptor (FcεRI) with antigen. Besides FcεRI, MCs express a variety of receptors that enable them to respond to a broad range of stimuli. A major breakthrough in MC research has been the discovery of a novel G protein-coupled receptor (GPCR) known as Mas-related GPCR-X2 (MRGPRX2). Unique features of MRGPRX2 are that this receptor is expressed predominantly on one subtype of human MCs and that it is responsive to a wide spectrum of cationic ligands including host defense peptides (HDPs), neuropeptides (NPs) and many Food and Drug Administration (FDA)-approved drugs (6-10). Unsurprisingly, activation of MCs via MRGPRX2 has been implicated in multiple physiological and pathological conditions, including host defense against microbial infection, pseudoallergy, neurogenic inflammation, chronic inflammatory diseases, and pain (9-15). Better understanding of the molecular mechanisms underlying MRGPRX2 activation and regulation will not only enhance our fundamental knowledge of MC responses, but will also lead to the development of novel therapeutic approaches for the modulation of allergic and inflammatory diseases.
1.1. An overview of mast cells

1.1.1. Mast cell development

MCs are tissue-resident granulocytes derived from hematopoietic stem cells in the bone marrow (16). Unlike other immune cells, MCs do not terminally differentiate in the bone marrow, but circulate in the bloodstream as immature progenitors. They complete their differentiation and maturation in peripheral tissues under the influence of the cytokine milieu and tissue microenvironment (16-18). Mature MCs are long-lived and can undergo repeated rounds of activation in response to stimuli (19, 20), while other granulocytes, such as neutrophils and basophils, have short lifespan and undergo apoptosis after their recruitment and activation in the tissues (21).

Stem cell factor (SCF), the ligand for the CD117/c-Kit, is a major regulator of MC biology (22). Activation of c-Kit signaling through SCF is crucial for MC development. Furthermore, SCF/c-Kit signaling also play a role in the migration, proliferation, survival and activation of MCs (22, 23). Mouse strains bearing mutations in the genes for the c-Kit receptor (Kit<sup>W/W<sup>v</sub></sup> and Kit<sup>W/sh</sup>) or its ligand SCF (Sl/Sld) have profoundly deficient numbers of MCs, emphasizing an essential role of c-Kit and SCF in MC development (24, 25). In addition to SCF, various cytokines, such as interleukin (IL)-3, IL-4, IL-9, IL-10, transforming growth factor-β, and nerve growth factor, also modulate MC differentiation and proliferation (22, 26).

1.1.2. Mast cell heterogeneity and plasticity

MC maturation is largely influenced by microenvironmental conditions. As such, mature MCs display considerable heterogeneity as a direct consequence of different development patterns (18). Moreover, phenotypic and functional characteristics of MCs can be dynamically modulated or "tuned" by many genetic and environmental factors they encounter (27). For example, the expression profile of proteases in jejunal MCs can change during *Trichinella spiralis* infection, which results in the alteration of MC function (28).

In humans, MCs are generally categorized into two major subtypes based on the protease content of their secretory granules: MCs that contain both tryptase and chymase are known as
MC\textsubscript{TC}, and those that contain only tryptase are known as MC\textsubscript{T} (29, 30) (Table 1-1). MC\textsubscript{TC} are predominantly found in connective tissues such as the skin and gingiva, while MC\textsubscript{T} are more prominent in the mucosal tissues such as respiratory and gastrointestinal mucosa (17, 31, 32). In rodents, two subtypes of MCs have been described: connective tissue MCs (CTMCs) and mucosal MCs (MMCs). CTMCs are found in the skin and peritoneum, and express predominantly mouse MC protease (MCP)-4, -5, -6, and carboxypeptidase A, whereas MMCs reside in the lung and gut, and express MCP-1 and -2 (18, 29). Rodent CTMCs share characteristics with human MC\textsubscript{TC}, while MMCs resemble human MC\textsubscript{T} (17, 18) (Table 1-1).

MC\textsubscript{TC} and CTMCs are sometimes considered as innate/constitutive MCs as they are constitutively present in connective tissues and are generally unaffected by T cells. By contrast, MC\textsubscript{T} and MMCs are referred to as adaptive/induced MCs as their maturation are induced in a T-cell-dependent manner (31, 33). Intriguingly, while both MC subtypes in humans and mice can be activated by antigen/IgE via Fc\varepsilonRI, only MC\textsubscript{TC} and CTMCs are responsive to a diverse range of cationic substances, collectively called basic secretagogues, such as compound 48/80, mastoparan, peptidergic drugs, NPs, and HDPs (6, 8) (Table 1-1).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Mast Cell Subtypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human MCs</td>
<td>MC\textsubscript{TC}</td>
</tr>
<tr>
<td>Rodent MCs</td>
<td>CTMCs</td>
</tr>
<tr>
<td>Tissue localization</td>
<td>Skin, serosal cavities, and gingiva</td>
</tr>
<tr>
<td></td>
<td>Gastrointestinal and respiratory mucosa</td>
</tr>
<tr>
<td>Protease content</td>
<td>Human: Contain both tryptase and chymase</td>
</tr>
<tr>
<td></td>
<td>Rodent: Contain MCP-4, -5, -6, and</td>
</tr>
<tr>
<td></td>
<td>carboxypeptidase A</td>
</tr>
<tr>
<td></td>
<td>Human: Contain only tryptase</td>
</tr>
<tr>
<td></td>
<td>Rodent: Contain MCP-1 and -2</td>
</tr>
<tr>
<td>Type</td>
<td>Innate</td>
</tr>
<tr>
<td>T cell dependence</td>
<td>No</td>
</tr>
<tr>
<td>Stimulants</td>
<td>Antigens and basic secretagogues</td>
</tr>
<tr>
<td></td>
<td>(for example, NPs and HDPs)</td>
</tr>
</tbody>
</table>

Table 1-1. Major subtypes of MCs and their phenotypic/functional characteristics.
1.1.3. Mast cell activation and mediator release

Effector functions of MCs are dependent on their ability to release a plethora of biologically active mediators upon activation (1). MCs can be activated by a broad range of stimuli, such as antigens, infectious organisms, and endogenous inflammatory factors, via the receptors present on their cell surfaces. In addition to the classical and most studied FcεRI, MCs also express numerous receptors including immunoglobulin G receptors (FcγRs), Toll-like receptors (TLRs e.g. TLR2 and TLR4), complement receptors (e.g. C3aR and C5aR), and recently identified MRGPRX2, allowing MCs to recognize and react directly to pathogenic stimuli (4, 5, 8, 10, 34, 35).

Upon activation, MCs can release their preformed and de novo synthesized mediators in a phasic manner (4). An early phase of MC response involves degranulation, a process by which preformed mediators stored in their secretory granules can be immediately released into the extracellular environment following stimulation (36, 37). This process is mediated by intracellular calcium mobilization, which activates a cascade of downstream events that trigger exocytosis of secretory granules (38). Pre-stored mediators, such as histamine, proteases, vascular endothelial growth factor (VEGF), and tumor necrosis factor-alpha (TNF-α), have marked effects on endothelial cells and smooth muscle, and nerves, leading to increased vascular permeability, vasodilation, smooth muscle contraction, and stimulation of afferent neurons (1, 37, 39, 40). In addition to releasing preformed mediators, MC activation also leads to de novo production and secretion of lipid mediators, such as leukotrienes, prostaglandins and platelet-activating factor, as well as an array of cytokines and chemokines, which accounts as the late MC response (1, 4, 37). MC-derived cytokines and chemokines play critical roles in the differentiation and activation of several immune cells, contributing to the development and homeostasis of the immune system. Furthermore, they promote various cell recruitment to the sites of inflammation, contributing to the pathophysiology of inflammatory responses (4, 5, 41).
1.2. Mas-related G protein-coupled receptor X2 (MRGPRX2)

1.2.1. Discovery of MRGPRX2 as a mast cell receptor for basic secretagogues

Besides activation of MCs by antigen/IgE, connective tissue-type MCs can respond to basic secretagogues via an IgE-independent pathway. These cationic peptides were previously proposed to trigger MC degranulation in a receptor-independent manner by directly activating pertussis toxin-sensitive G proteins (Gαi2 and Gαi3) (42-44). Until in 2006, Tatemoto et al. (8) provided the first demonstration that MRGPRX2 (formerly known as MrgX2) is expressed at high level on human skin MC\textsubscript{Tc} but not on lung MC\textsubscript{T}, and is an endogenous receptor for basic secretagogues. A number of basic secretagogues, including compound 48/80, MC-degranulating peptide (MCDP), and substance P (SP), increased reporter gene expression in PC12h cells transiently transfected with MRGPRX2 and induced dose-dependent calcium mobilization in HEK-293 cells stably expressing MRGPRX2, but not parental cells (8). Furthermore, they demonstrated that MCDP and SP activate G proteins in membranes prepared from HEK-293 cells expressing MRGPRX2, suggesting that MRGPRX2 is a link between the activation of basic secretagogues and G proteins (8).

1.2.2. Identification of MrgprB2 as the mouse ortholog of human MRGPRX2

MRGPRs are recently identified GPCRs that belong to the rhodopsin-like class A GPCR family. They can be divided into nine subfamilies (A–H and X), with subfamily X is specific to primates including humans, while subfamilies A, B, C, and H exist only in rodents (45).

Despite many in vitro studies on MRGPRX2, no murine model for in vivo study was initially employed due to difficulty to identify the putative mouse ortholog of human MRGPRX2. Unlike the human genome, which encodes for only four MRGPRs (MRGPRX1 – 4), there are 22 potential coding genes for MRGPRs in mice (9, 10). In 2015, a landmark study by McNeil et al. (10) revealed that mouse peritoneal MCs (PMCs) solely express MrgprB2 mRNA and MrgprB2 expression is restricted to connective tissue-type MCs. By generating mice with a 4-base pair deletion in the MrgprB2 coding region (MrgprB2\textsuperscript{MUT} mice), MCs obtained from these mice show dramatic reduction of calcium mobilization and histamine release in response to MRGPRX2 ligands, including...
compound 48/80, SP, and peptidergic drugs, in vitro. Furthermore, MrgprB2MUT mice demonstrate remarkably reduced paw edema in vivo when compared to the wild-type (WT) mice (10). Of note, MrgrpB2 mutation in MCs does not impair their development and IgE-mediated response. These findings strongly indicate that MrgprB2 is the mouse basic secretagogue receptor and the ortholog of human MRGPRX2. This discovery paved the way for the in vivo studies of MrgprB2-mediated MC activation in physiology and diseases, which substantially advance our understanding of MRGPRX2’s functional roles. Since then, an ever-increasing number of MRGPRX2/B2 ligands, ranging from HDPs and bacterial quorum-sensing molecules (QSMs) to NPs and peptidergic drugs associated with pseudoallergic reactions, have been discovered (6, 10, 46-50) (Figure 1-1).

![Diagram](image)

**Figure 1-1.** Human MRGPRX2 and its mouse ortholog MrgprB2 can be activated by a diverse group of ligands, including endogenous NPs and HDPs, bacterial quorum-sensing peptides, and peptidergic drugs. MC activation by different MRGPRX2/B2 agonist results in distinct mediator release and biological responses. (Galli SJ et al., 2020).

It is worth noting that although MrgprB2 is regarded as the mouse counterpart of MRGPRX2, these two receptors share only ~53% sequence similarity and thus demonstrate significant species-specificity with respect to the concentrations of ligands required to activate each receptor (10, 51). EC50 values (concentration of ligand required to induce half-maximal response) of most ligands for MrgprB2 are significantly higher than those for MRGPRX2. For example, SP activates MrgprB2 and MRGPRX2 with an EC50 value of 54 μM and 152 nM, respectively. By contrast, rocuronium, a widely used neuromuscular blocking drug (NMBD), has an EC50 value of 22.2 μg/mL for MrgprB2, whereas an EC50 value for MRGPRX2 is 261 μg/mL (10).
1.3. Role of MRGPRX2 in drug hypersensitivity reactions

Anaphylaxis to FDA-approved drugs, including antibiotics, opioids, iodinated contrast media, and neuromuscular blocking drugs (NMBDs), have increased in recent years. These potentially life-threatening reactions represent a diagnostic challenge for allergists as the underlying mechanisms of allergic reactions to many drugs remain elusive. While it is generally thought to be mediated via a cross-linking of drug-specific IgE and FcεRI presented on the MC surfaces, some reactions are mediated independently of IgE. In 2015, McNeil et al. (10) demonstrated that certain drugs containing a tetrahydroisoquinoline (THIQ) motif or similar structure, such as NMBDs and fluoroquinolone antibiotics, induce MC degranulation and mediators release, subsequently resulting in allergic reactions, via MRGPRX2 and its mouse ortholog MrgprB2. MrgprB2\textsc{MUT} mice exhibited substantially reduced hind paw swelling and less drop in their body temperature compared to the WT mice after administration of peptidergic drugs. Of note, the deletion of the receptor in MrgprB2\textsc{MUT} mice did not alter the IgE-mediated pathway (10). These findings strongly indicate that MC activation through MRGPRX2/B2 is distinct from IgE-mediated pathway.

NMBDs, such as mivacurium, atracurium, cisatracurium, and rocuronium, while are routinely used during general anesthesia and surgery to reduce unwanted muscle movement, account for nearly 60% of anaphylactic reactions in a surgical setting (52). Of all the NMBDs, the incidence rates of anaphylaxis are found to be higher after rocuronium administration than those of the other NMBDs (53, 54). Spoerl et al. (55) reported three cases of rocuronium-induced hypersensitivity which displayed positive skin test reaction, despite having negative rocuronium-specific IgE and basophil activation test (BAT). Based on these findings, it was proposed that this rocuronium hypersensitivity is mediated via the direct activation of MRGPRX2 (56). On the contrary, a large clinical study conducted with 140 patients suspected of perioperative hypersensitivity to rocuronium demonstrated that hypersensitivity to rocuronium mainly results from IgE-mediated MC activation (57).
More intriguingly, the possibility that rocuronium activates human MCs via MRGPRX2 has been subject of controversy. While McNeil et al. (10) showed that rocuronium activates MRGPRX2 transfected in HEK293 cells with an EC$_{50}$ value of 261 µg/mL, Lansu et al. (51) could not reproduce this finding. Furthermore, rocuronium, even at high concentration (up to 2 mg/mL), did not induce degranulation in either human MC line LAD2 cells or CD34$^+$-derived human MCs that endogenously express MRGPRX2 (58, 59). This lack of effect of rocuronium on MRGPRX2 is surprising given that it induces massive degranulation in mouse PMCs via MrgprB2 and could reflect the differences between mouse MrgprB2 and human MRGPRX2 (10, 51).

1.4. Role of MRGPRX2 in neuroimmune interactions and skin homeostasis

The close relationship between MCs and nerves has been well established in most tissues including the skin, gastrointestinal mucosa, and respiratory tract. MCs are particularly found in close proximity to sensory nerve endings, and the frequency of anatomic associations between MCs and nerves is significantly increased at sites of inflammation (3). Furthermore, MCs and nerves demonstrate bidirectional communication that is mediated through shared ligands, such as cytokines and NPs, and their cognate receptors (3, 60). These close anatomic localization and functional interaction of MCs and sensory nerves provide a significant link between the immune and nervous systems and play important protective roles in maintaining tissue homeostasis and responding to external challenges. However, dysregulation of MC–nerve interaction has been suggested as a major contributor in the pathogenesis of neurogenic inflammation, pain, and allergic skin diseases (61-63).

Substance P (SP) is an 11-amino acid NP that belongs to the tachykinin family (64). It exerts a wide range of physiological as well as pathological functions, with well recognition for its roles in inflammation and pain perception (64, 65). SP is one of the most comprehensively studied NPs that mediates crosstalk between neurons and MCs. SP released from the sensory nerve endings can induce MC degranulation, resulting in the release of multiple pro-inflammatory mediators (64-66). In turn, MC-derived mediators, such as histamine, tryptase, and leukotrienes, can activate their specific receptors expressed on sensory nerves to cause further SP release (67-
Activation of MCs by SP has been implicated in the pathogenesis of a number of neuroinflammatory conditions such as sickle cell disease (70), chronic urticaria (6), and atopic dermatitis (15).

The neurokinin-1 receptor (NK-1R) is a canonical receptor for SP that was previously thought to be responsible for SP-induced MC activation. Murine MMCs such as bone marrow-derived MCs (BMMCs) do not generally express NK-1R, but when cultured in the presence of SCF and IL-4, they displayed significant expression of NK-1R (71). These SCF/IL-4-treated BMMCs was activated in response to SP and the activation was significantly diminished by an NK-1R antagonist (71), suggesting that SP may activate MCs via this receptor. The discovery that SP contributes to neurogenic inflammation and pain led to the development of multiple NK-1R antagonists as potential therapies for inflammatory conditions and pain over the past three decades. However, these compounds, while shown to be effective in animal models, have failed to demonstrate anti-inflammatory and analgesic effects in human clinical trials (72, 73).

In addition to NK-1R, human skin MCs express MRGPRX2 which has been shown to be a receptor for basic secretagogues including SP. Fujisawa et al. (6) demonstrated that SP-induced MC degranulation and prostaglandin D2 generation are significantly depleted in MRGPRX2-silenced human skin MCs. Additionally, conventional NK-1R antagonists have been shown an off-target effect on the mouse MrgprB2 (74). These findings therefore raise the possibility that the nociceptive and proinflammatory actions of SP may be mediated via alternative mechanisms, presumably MRGPRX2/B2, rather than the interaction with NK-1R.

The speculation that SP mediates neurogenic inflammation and pain via MRGPRX2/B2 has been recently confirmed by a well-integrated study by Green et al. (13). Using two inflammatory pain models, postoperative incision model and Complete Freund’s Adjuvant (CFA) model, they demonstrated that activation of MrgprB2 by SP is required for inflammatory mechanical and thermal hyperalgesia. MrgprB2-deficient (MrgprB2−/−) mice, but not NK-1R−/− mice, had significant reductions in swelling, pain hypersensitivity, and immune cell infiltration when compared to WT mice (13). They also showed that knockdown of MRGPRX2 expression in human MC line LAD2
cells results in substantially reduction of cytokines and chemokines, such as TNF-α, CCL2 and CCL3, released upon SP stimulation, whereas there is no decrease in LAD2 treated with an NK-1R antagonist (13). Taken together, these findings strongly indicate that MRGPRX2/B2 activation are responsible for the release of proinflammatory cytokines/chemokines and the recruitment of innate immune cells, including neutrophils and monocytes, to the site of injury following SP stimulation (Figure 1-2). Thus, targeting MRGPRX2 could therefore serve as novel therapeutic approach for the management of diseases associated with neurogenic inflammation and pain. However, the molecular mechanism of MRGPRX2 activation by SP has yet to be elucidated.

Figure 1-2. During tissue injury, substance P (SP) released from peripheral sensory nerve endings contributes to neurogenic inflammation through the activation of MCs via MRGPRX2/B2. Activation of MRGPRX2/B2 by SP leads to cytokine release and recruitment of immune cells, which facilitate inflammatory responses and peripheral nerve sensitization. (Meixiong J et al., 2020)

1.5. MRGPRX2 signaling and regulation: Implication from other GPCRs

MRGPRX2 is a member of class A GPCRs. Thus, it shares a common structure of seven transmembrane (TM) α-helices connected by three extracellular loops (ECLs) and three intracellular loops (ICLs), with an extracellular amino-terminal tail (N-terminus) and an intracellular carboxy-terminal tail (C-terminus). The extracellular part of the receptor is responsible for ligand binding, whereas the intracellular part is involved in binding downstream effectors such as heterotrimeric G proteins (Gαβγ) and β-arrestins (75) (Figure 1-3).
Figure 1-3. Snake diagram of secondary structure of MRGPRX2 obtained from the GPCR database (GPCRdb; www.gpcrdb.org) (76). Each circle represents amino acid residue with one letter code. The extracellular part, including extracellular loops (ECLs) and transmembrane domains (TMs), and N-terminus, is responsible for binding ligands. The intracellular part, including intracellular loops (ICLs) and TMs, and C-terminus, is involved in binding downstream effectors and initiating signaling cascades.

GPCRs, as their name implies, mainly interact with 'heterotrimeric G proteins' to translate the signal from extracellular ligands into intracellular responses. Binding of ligands at the "ligand binding cradle" on the extracellular region of GPCRs leads to conformational rearrangements of the cytoplasmic side to facilitate the binding of G proteins, leading to G protein activation and initiating downstream signaling cascades (75, 77). Closely related GPCRs exhibit high degree of conserved sequence motifs and a common activation pathway, especially in the regions implicated in ligand binding and G protein coupling (78).

Venkatakrishnan et al. (79) analyzed crystal structures of 27 class A GPCRs in inactive and active states to investigate the activation pathways across class A GPCRs and reported a common pattern of residue contact rearrangement involved TM3 (3x46), TM6 (6x37), and TM7 (7x53) in all class A receptors. (In this GPCR numbering system, the first number denotes the helix (1-7) and the second number indicates the residue position relative to the most conserved position, which is assigned the number 50. Thus, 7x53 denotes a residue in TM7, which is at 3 positions after the most conserved residue (7x50). Similarly, 3x46, denotes a residue in TM3, which is at 4 positions before the most conserved residue). In the inactive state, the residue at 6x37 is in contact with a conserved hydrophobic residue at position 3x46 (36). Upon activation, the residue at position
3x46 breaks the contact with 6x37 and forms a new contact with Tyr7x53 within the highly conserved NPxxY motif of TM7 (35-38) (Figure 1-4). Based on molecular modeling and naturally occurring missense mutations, studies from our and other labs led to the identification of the ligand binding cradle for a number of MRGPRX2 agonists (17-19). However, the possibility that residues 3x46, 6x37 and 7x53 in MRGPRX2 couple to G proteins to cause MC degranulation has not been investigated.

![Figure 1-4](image)

**Figure 1-4.** Pattern of residue contacts upon receptor activation in class A GPCRs. Dotted circles around 6x37 and 7x53 denote the movement of TM6 and TM7 upon activation. Figure is adapted from Venkatakrishnan et al., 2016.

There are four major subtypes of heterotrimeric G proteins according to the functional and structural homologies of the Gα subunits: Gαs, Gαi/o, Gαq/11, and Gα12/13. Interaction of GPCRs with different Gα subunits elicit distinct downstream signaling cascades and cellular responses (80). For example, Gαq/11 proteins activate phospholipase C, which hydrolyzes phosphatidylinositol phosphate to diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃), resulting in protein kinase C (PKC) activation and intracellular calcium mobilization (81). Previous studies on the mechanisms of MRGPRX2-mediated MC responses have shown that basic secretagogues cause MC degranulation via pertussis toxin (PTx)-sensitive G protein-dependent signaling pathway (8, 46, 48). PTx catalyzes the ADP-ribosylation of the α subunits of the heterotrimeric Gαi/o protein family (Gαo, Gαi1, Gαi2 and Gαi3) (82), of which MCs express Gαi2 and Gαi3 (44). However, while degranulation in response to MRGPRX2 agonists, such as cathelicidin LL-37 and human β-
defensin-3, is inhibited by PTx, calcium mobilization is not (46, 48). These findings suggest that MRGPRX2 signaling pathway is mediated via both PTx-dependent (Gαi) and -independent (presumably Gαq) G proteins in order to induce human MC activation and responses. However, the molecular mechanisms in which specific G protein mediates MRGPRX2 signaling to induce MC activation and degranulation has not been investigated.

In addition to G proteins, most GPCRs signal via an additional pathway that involves the recruitment of adapter proteins known as β-arrestins (83, 84). This pathway has been implicated in GPCR desensitization (uncoupling of the G protein from the cognate receptor), endocytosis, and internalization in order to prevent further G protein coupling and overactivation of the receptor (85). Furthermore, recent evidence shows that β-arrestins also play an important role in G protein-independent downstream signaling for cell migration, growth, and differentiation (86-88) (Figure 1-5).

![Figure 1-5](image)

**Figure 1-5.** In the canonical GPCR signaling paradigm, ligand binding to the receptor leads to G protein activation, resulting in the initiation of distinct downstream signaling cascades and cell responses such as the release of intracellular calcium from the endoplasmic reticulum (ER). This G protein signaling is regulated by adapter proteins, β-arrestins. Binding of β-arrestins can sterically block further G protein activation (desensitization) and lead to GPCR internalization/trafficking. Furthermore, β-arrestins serve as signal transducers in several G protein-independent signaling pathways.

Two isoforms of β-arrestins, β-arrestin1 and β-arrestin2, are ubiquitously expressed in all tissues and can differentially regulate GPCR signaling and trafficking (89-91). Our lab previously demonstrated that β-arrestin2 is expressed at high levels and contributes to GPCR desensitization

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in human MCs (91, 92). Furthermore, Roy et al., recently showed that mice with MC-specific deletion of β-arrestin2 display enhanced MC degranulation \textit{in vitro} and vascular permeability \textit{in vivo} in response to ciprofloxacin when compared to littermate controls (92). Of note, absence of β-arrestin2 had no effect on FcεRI/c-Kit cell surface receptor expression or MC number in these mice, indicating that β-arrestin2 has no effect on the development and maturation of MCs (92). These findings suggest that targeting β-arrestin2-associated pathway might represent a potential therapeutic strategy to modulate allergic and inflammatory diseases caused by MRGPRX2-mediated MC activation.

\textbf{1.6. Dissertation aims}

Since the discovery of MRGPRX2, this receptor has gained tremendous significance in MC-mediated health and disease. As the multiligand receptor that can be activated by various endogenous and exogenous stimuli, including SP and several FDA-approved drugs, MRGPRX2 has been suggested to modulate both pseudoallergic drug reactions and inflammatory responses. Thus, targeting MRGPRX2 might therefore represent promising therapeutic approaches for the prevention and management of drug hypersensitivity and inflammatory diseases. The specific aims of the works presented in this dissertation are as follows:

\textbf{Specific Aim 1}: To determine the role of MRGPRX2 in rocuronium-induced hypersensitivity reactions and anaphylaxis.

The demonstration that many NMBDs induce degranulation in MRGPRX2 transfected cells led to the hypothesis that this receptor contributes to clinically relevant NMBD-induced hypersensitivity reactions. However, other studies have raised doubts regarding the role of MRGPRX2 in rocuronium-induced hypersensitivity. In Aim 1, the ability of rocuronium to cause human MC degranulation was investigated. Furthermore, the potential effect of recently identified MRGPRX2 mutations on rocuronium-induced hypersensitivity was tested.

\textbf{Specific Aim 2}: To identify the MRGPRX2-G protein interaction in response to MC activation by SP.
In **Aim 2**, I first identified the specific G proteins ($\text{G}$$\alpha$$\text{i}$ and $\text{G}$$\alpha$$\text{q}$) and their interactions with MRGPRX2 that participate in SP-mediated MC responses. The specific amino acid residues on MRGPRX2 that are responsible for SP-induced MC activation and regulation were identified using the information obtained from both structural modeling and naturally occurring MRGPRX2 missense variants. The effect of these mutations on G protein-mediated MC activation in response to SP was tested.

**Specific Aim 3**: To investigate the MRGPRX2-$\beta$-arrestin2 interaction on modulating MC activation by SP.

In **Aim 3**, I first determined whether SP can induce $\beta$-arrestin-mediated signaling, including MRGPRX2 internalization and desensitization, and whether these responses are independent of G proteins. The biological role of $\beta$-arrestin2 on SP-induced MC degranulation was investigated. Finally, the structural component of MRGPRX2 that is responsible for receptor internalization and regulation was identified.
CHAPTER 2: MRGPRX2 Activation by Rocuronium: Insights from Studies with Human Skin Mast Cells and Missense Variants

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2.1. Abstract

Perioperative hypersensitivity (POH) to the neuromuscular blocking drug (NMBD) rocuronium was previously thought to be IgE and mast cell (MC)-mediated. However, the recent seminal observation that rocuronium induces degranulation in murine peritoneal MCs (PMCs) via Mas-related G protein-coupled receptor B2 (MrgprB2) led to the idea that POH to this drug involves the activation of MRGPRX2 (human ortholog of MrgprB2). Furthermore, based on the demonstration that a patient with POH to rocuronium displayed three missense mutations (M196I, L226P and L237P) in MRGPRX2's transmembrane domains, it was proposed that this hypersensitivity reaction resulted from aberrant activation of this receptor. We found that rocuronium at 20 µg/mL caused degranulation in mouse PMCs via MrgprB2 but required at least 500 µg/mL to induce degranulation in human MCs via MRGPRX2. Furthermore, RBL-2H3 cells transiently expressing M196I, L226P and L237P variants did not display enhanced degranulation in response to rocuronium when compared to the wild-type receptor. These findings provide the first demonstration that rocuronium induces degranulation in human MCs via MRGPRX2. Furthermore, the important differences between MrgprB2 and MRGPRX2 and the inability of rocuronium to induce enhanced response in cells expressing MRGPRX2 variants suggest that the mechanism of its POH is more complex than previously thought.

Keywords: MRGPRX2; MrgprB2; anaphylaxis; mast cells; missense mutation; rocuronium.
2.2. Introduction

Anaphylactic reactions to drugs used during general anesthesia are rare, yet seriously life-threatening conditions that can occur with a mortality rate ranging from 3% to 9% (93). Rocuronium is a widely used neuromuscular blocking drug (NMBD) that is frequently accounted for perioperative hypersensitivity (POH) (57). Recent evidence has shown that besides immunoglobulin E (IgE)/high-affinity IgE receptor (FcεRI)-dependent mast cell (MC) activation, rocuronium causes degranulation in murine peritoneal mast cells (PMCs) via the activation of a novel G protein-coupled receptor (GPCR) known as Mas-related GPCR-B2 (MrgprB2; human ortholog MRGPRX2) (10). Additionally, some patients with rocuronium-induced POH displayed positive skin tests to irritating concentrations of rocuronium (1 – 10 mg/mL), despite demonstrating negative specific-IgE (sIgE) and/or basophil activation test (BAT) (55, 94). Based on these findings, it has been proposed that MRGPRX2 plays a critical role in rocuronium-induced POH in humans (56).

Despite one report showing that rocuronium induces degranulation in mouse PMCs via MrgprB2, evidence that it induces degranulation in human MCs via MRGPRX2 is lacking. McNeil et al. (10) showed that rocuronium causes intracellular Ca\(^{2+}\) mobilization in transfected HEK293 cells with EC\(_{50}\) values of 22.2 µg/mL and 261 µg/mL, for mouse MrgprB2 and human MRGPRX2, respectively. Although these authors showed that rocuronium induces robust degranulation in mouse PMCs via MrgprB2, its effect on human MCs was not reported (10). More recent studies have shown that rocuronium, up to a concentration of 2 mg/mL, induces a small and transient Ca\(^{2+}\) mobilization in human peripheral CD34\(^+\) cell-derived MCs and a human MC line LAD2 cells via MRGPRX2 but this response does not provide sufficient signal for degranulation (58, 59, 95). The reason for the discrepancy between MrgprB2 and MRGPRX2 is not known but could reflect the fact that there is only ~53% sequence homology between these two receptors, resulting in different ligand binding affinities (9, 10). Although high concentrations of rocuronium causes irritative skin reactions, the possibility that this is mediated via the activation of MRGPRX2 in human skin MCs has not been determined.
Suzuki et al. (94) recently reported a patient who experienced severe POH to rocuronium. Intradermal injection of undiluted rocuronium (10 mg/mL) in this patient resulted in a positive skin reaction but a total IgE level was within normal limits and no sIgE to rocuronium was detected. Based on these findings, this POH reaction was diagnosed as rocuronium-induced non-IgE-mediated anaphylaxis. Sequence analysis of genomic DNA from the blood of this patient revealed the presence of three missense mutations (M196I, L226P and L237P) in MRGPRX2’s 5th and 6th transmembrane domains (94). Rocuronium did not induce degranulation in rat basophilic leukemia (RBL-2H3) cells transiently expressing MRGPRX2 (94). It was proposed that mutations found in MRGPRX2 of this patient with rocuronium-induced reaction would render the receptor more susceptible to activation. However, this hypothesis has not been tested experimentally.

The goals of the present study were to determine if rocuronium causes degranulation in mouse and human MCs and to test the hypothesis that missense mutations (M196I, L226P and L237P) in MRGPRX2’s transmembrane domains result in gain-of-function phenotype for MC degranulation by rocuronium. The data presented herein confirms the previous report that rocuronium induces degranulation in mouse PMCs via MrgprB2 but provides the first demonstration that it causes degranulation in human MCs via MRGPRX2 but requiring at least 25-fold higher concentration to do so. Furthermore, functional studies with MRGPRX2 mutants found in a patient with rocuronium-induced POH suggest that mechanism of its hypersensitivity is more complex than previously thought.

2.3. Materials and Methods

2.3.1. Reagents

All cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Amaxa transfection kit (Kit V) was obtained from Lonza (Gaithersburg, MD). Recombinant mouse interleukin-3 (IL-3) and stem cell factor (SCF) and recombinant human SCF (rhSCF) were purchased from Peprotech (Rocky Hill, NJ). Rocuronium bromide (CAS no. 119302-91-9, Item no. 23698) was purchased from Cayman Chemical (Ann Arbor, MI). p-nitrophenyl-N-acetyl-β-D-glucosamine (PNAG) was from Sigma-Aldrich (St. Louis, MO). Fluorescein isothiocyanate (FITC)-
conjugated anti-human LAMP-1 and phycoerythrin (PE)-conjugated anti-human MRGPRX2 antibodies were from BioLegend (San Diego, CA). Plasmid encoding hemagglutinin (HA)-tagged human MRGPRX2 in pReceiver-MO6 vector was obtained from GeneCopoeia (Rockville, MD).

2.3.2. Mice

C57BL/6 (wild-type; WT) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). MrgprB2−/− mice in C57BL/6 background generated using CRISPR-Cas9 mediated gene deletion of MrgprB2 were obtained from CRISPR-Cas9 core facility of the University of Pennsylvania (96). Mice were housed in pathogen-free cages on autoclaved hardwood bedding. Eight- to twelve-weeks-old male and female mice were used in this study. All experiments were approved by the Institutional Animal Care and Use Committee at The University of Pennsylvania.

2.3.3. Cell line cultures

The human MC line LAD2 was provided by Dr. A. Kirshenbaum and Dr. D. Metcalfe (Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) and was maintained in complete StemPro-34 medium supplemented with l-glutamine (2 mM), penicillin (100 IU/mL), streptomycin (100 μg/mL), and rhSCF (100 ng/mL). Hemidepletions were performed weekly with media containing rhSCF (97). RBL-2H3 cells were maintained as monolayer cultures in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 IU/mL) and streptomycin (100 μg/mL) at 37°C with 5% CO₂ (98). RBL-2H3 cells stably expressing MRGPRX2 (RBL-MRGPRX2) were used and maintained similarly in the presence of G-418 (1 mg/mL) (46, 99).

2.3.4. Human skin-derived mast cell isolation and culture

Surgical skin samples were collected from the Cooperative Human Tissue Network of the National Cancer Institute, as approved by the Internal Review Board at the University of South Carolina. Skin MCs were harvested and cultured from 3 human donors as previously described (100). Briefly, subcutaneous fat was removed by blunt dissection, and residual tissue was cut into 1- to 2-mm fragments and digested with type 2 collagenase (1.5 mg/mL), hyaluronidase (0.7
mg/mL), and type 1 DNase (0.3 mg/mL) in Hank’s Balanced Salt Solution (HBSS) for 2 h at 37°C. The dispersed cells were collected by filtering through a No. 80 mesh sieve and resuspended in HBSS containing 1% fetal calf serum (FCS) and 10 mM HEPES. Cells were resuspended in HBSS and layered over 75% Percoll in an HBSS cushion and centrifuged at 700 × g at room temperature for 20 min. Nucleated cells were collected from the buffer/Percoll interface. Percoll gradient-enriched cells were resuspended at a concentration of 1 × 10^6 cells/mL in serum-free X-VIVO 15 medium containing 100 ng/mL rhSCF. MCs were used after 6-10 weeks of culture, when purity was nearly 100%, as confirmed with toluidine blue staining.

2.3.5. Peritoneal mast cell isolation and culture

PMCs were purified from WT and MrgprB2−/− mice as described previously (92). Briefly, the peritoneal cavity was lavaged with 10 mL of HBSS supplemented with 3% FCS and 10 mM HEPES. The cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 10% FCS, murine IL-3 (10 ng/mL), and murine SCF (30 ng/mL). After 48 h, non-adherent cells were removed and adherent cells were cultured in fresh medium for an additional 10-14 days. Suspension cells were used for experiments as PMCs.

2.3.6. Mast cell staining

Toluidine blue and Alcian/Safranin staining were performed on the cultured PMCs (5 × 10^4) as described previously (101). Images were captured using a Nikon E600 microscope with a digital camera attached at 40X magnification and analyzed using Nikon NIS Elements software.

2.3.7. RNA isolation and PCR

RNA isolation and PCR was performed as described previously (92). Briefly, total mRNA was isolated from WT and MrgprB2−/− PMCs using the RNeasy Plus Mini Kit (Qiagen) as per manufacturer’s instructions. cDNA was prepared using High Capacity cRNA Reverse Transcriptase Kit (Applied Biosystem). PCR was performed using the MrgprB2 (Forward 5’-GTCACACCCAGTGTAAACACTTCC-3’ and Reverse 5’-CAGCCATAGCCAGGTGGAGAA-3’) and GAPDH (Forward 5’-CCATGACAACTTTGGCATTG-3’ and Reverse 5’-CCTGCTTCCACCATTTCTTG-3’) primers.
PCR product was run in 2% agarose gel and image was acquired in iBright™ 1500 Imaging System (Thermo Scientific).

2.3.8. Generation of RBL-2H3 cells transiently expressing MRGPRX2 and its variants

Q5 site-directed mutagenesis kit (New England BioLabs) was used to generate MRGPRX2 variants in pReceiver-MO6 vector. To confirm the correct nucleotide sequences, each mutant was verified by DNA sequencing prior to transfection. The forward and reverse primers used for new variants are listed below.

**M196I:** Forward: 5’-TTTTATTCATCGTTCTCTGTGGGTCC-3’
Reverse: 5’-AAATCAGCCACGCTGCAG-3’;

**L226P:** Forward: 5’-CTGACCATCCCGCTCACAGTG-3’
Reverse: 5’-GTACAGCCTGGTCAGTGG-3’;

**L237P:** Forward: 5’-CTCTGCGCCCGCCCTTTGGC-3’
Reverse: 5’-GAGGAACACCAGCAGTGTGAC-3’;

RBL-2H3 cells (2 x 10⁶) were transiently transfected with 2 µg of HA-tagged plasmid using the Amaxa Nucleofector Device and Amaxa Kit V according to the manufacturer’s protocol. Cells were used within 16 – 20 h after transfection.

2.3.9. MRGPRX2 expression and internalization using flow cytometry

To induce MRGPRX2 internalization, cells (5 x 10⁵) were stimulated with rocuronium (2 mg/mL) for 30 min at 37°C. To detect cell surface MRGPRX2 expression, cells were washed with FACS buffer (PBS containing 2% FCS and 0.02% sodium azide), incubated with the PE-conjugated anti-MRGPRX2 antibody for 30 min at 4°C in the dark, and fixed in 1.5% paraformaldehyde. The samples were acquired in a BD LSR II flow cytometer (San Jose, CA) and analyzed by WinList software. The adjusted mean fluorescent intensity (MFI) was calculated as MFI of sample/MFI of isotype control.

2.3.10. Degranulation measured by β-hexosaminidase release assay

RBL-2H3 cells (5 x 10⁴), LAD2 cells (1 x 10⁴), human skin-derived MCs (5 x 10³) and PMCs (1 x 10⁴) were seeded into a 96-well, white, clear-bottom cell culture plate in a total volume of 50
μl HEPES buffer containing 0.1% bovine serum albumin (BSA). Experimental groups were stimulated with different concentrations of rocuronium for 30 min at 37°C. Cells without treatment were designated as controls. To determine the total β-hexosaminidase release, unstimulated cells were lysed in 50 μl of 0.1% Triton X-100. Aliquots (20 μl) of supernatants or cell lysates were incubated with 20 μl of 1 mM p-nitrophenyl-N-acetyl-β-D-glucosamine (PNAG) for 1 h at 37°C. The reaction was stopped by adding 250 μl of stop buffer (0.1 M Na₂CO₃/0.1 M NaHCO₃). The β-hexosaminidase release was assessed by measuring absorbance at 405 nm using Versamax microplate spectrophotometer (San Jose, CA).

2.3.11. Degranulation measured by the surface expression of lysosomal-associated membrane protein 1 (LAMP-1)

Degranulation was also assessed by flow cytometric measurement of the surface expression of LAMP-1. Cells (5 × 10⁵) were stimulated with rocuronium (2 mg/mL) for 5 min, washed and exposed to FITC-conjugated anti-LAMP-1 antibody for 30 min at 4°C. Cell surface expression of LAMP-1 was determined by flow cytometry as described above.

2.3.12. Statistical analysis

Data shown are mean ± standard error of the mean (SEM) values derived from at least three independent experiments. Statistical significance was determined using t-test and one- or two-way ANOVA. Differences were considered as statistically significant at a value * p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001. Data were analyzed by GraphPad Prism version 6.07.

2.4. Results

2.4.1. Rocuronium activates murine PMCs via MrgprB2.

McNeil et al. (10) showed that EC₅₀ value for rocuronium-induced Ca²⁺ mobilization in HEK293 cells expressing MrgprB2 is 22.2 µg/mL. They also demonstrated that rocuronium at a concentration of 500 µg/mL induces substantial degranulation in mouse PMCs and that this response was abolished in cells obtained from MrgprB2⁻/⁻ mice (10). Due to the recent reports that rocuronium, even at a concentration of 2 mg/mL, does not induce degranulation in human MCs
(58, 59, 95), we first sought to validate the activity of rocuronium used in the present study by testing its ability to activate mouse MrgprB2. For this, we utilized MrgprB2−/− mice generated by CRISPR-Cas9-mediated gene deletion and confirmed that the absence of the receptor transcript in PMCs (Figure 2-1;A) had no effect on their differentiation and maturation as determined by Toluidine Blue and Alcian/Safranin staining (Figure 2-1;B). Rocuronium, at concentration of 20 μg/mL, induced ~30% degranulation in PMCs obtained from the wild-type (WT) mice but this response was absent in cells obtained from MrgprB2−/− mice (Figure 2-1;C). It is noteworthy that McNeil et al. (10) showed that 500 µg/mL of rocuronium induced >80% degranulation in mouse PMCs. Thus, the data presented in Figure 2-1 is consistent with previously reported EC50 value for rocuronium-induced Ca2+ mobilization in transfected HEK293 cells and degranulation in mouse PMCs.

2.4.2. Rocuronium induces MRGPRX2-mediated degranulation in human MCs.

Given that the EC50 for value for rocuronium-induced Ca2+ mobilization in HEK293 cells expressing MRGPRX2 is 261 μg/mL, we expected this concentration of the drug to induce degranulation in LAD2 cells naturally expressing the receptor (10). However, we found that the lowest concentration of rocuronium that induced degranulation, as measured by β-hexosaminidase release, was 500 μg/mL and maximal response was obtained at 2 mg/mL (Figure 2-2;A). We did not use rocuronium at concentration higher than 2 mg/mL because of its reported cytotoxic effect at higher concentrations (59). Rocuronium-induced degranulation in LAD2 cells was further confirmed by assessing the upregulation of LAMP-1 on the cell surface. Consistent with β-hexosaminidase release, rocuronium induced an increase in cell surface LAMP-1 expression on LAD2 cells as detected by flow cytometry (Figure 2-2;B,C). To confirm the biological relevance of our studies with LAD2 cells, we cultured MCs isolated from the human skin of 3 different donors. We found that rocuronium (2 mg/mL) triggered degranulation as measured by β-hexosaminidase release and LAMP-1 expression (Figure 2-2;D – F) but these responses were lower in magnitude than those observed with LAD2 cells.
Cell surface expression of MRGPRX2 on LAD2 cells and primary skin MCs was determined by flow cytometry using PE-conjugated anti-MRGPRX2 antibody. As shown in Figure 2-3;A,B, MRGPRX2 is expressed at a significantly lower level on skin MCs than LAD2 cells. This finding is consistent with lower level of degranulation in skin MCs when compared to LAD2 cells (Figure 2-2). Codeine induces degranulation in human skin MCs via MRGPRX2 and it also causes receptor internalization as measured by loss of cell surface expression by flow cytometry (102). To determine if rocuronium also causes MRGPRX2 internalization, LAD2 cells and skin MCs were exposed to rocuronium (2 mg/mL for 30 min) and cell surface receptor expression was determined by flow cytometry. As shown in Figure 2-3;C – F, rocuronium caused significant loss of cell surface receptor expression in both LAD2 and skin MCs. These findings suggest that similar to codeine (102), rocuronium induces degranulation in human MCs via MRGPRX2 and causes receptor internalization.

2.4.3. MRGPRX2 variants presented in a patient with POH to rocuronium do not display gain-of-function phenotype for rocuronium-induced MC degranulation.

Suzuki et al. (94) recently reported that a patient with severe rocuronium-induced POH harbors three missense mutations (M196I, L226P and L237P) in MRGPRX2’s 5th and 6th transmembrane domains (Figure 2-4;A). We have previously utilized RBL-2H3 cells transiently expressing missense variants of MRGPRX2 to determine the impact of single nucleotide polymorphism on receptor function in response to a variety of agonists (103, 104). To assess if rocuronium induces degranulation in RBL-2H3 cells, we utilized cells stably expressing MRGPRX2 (RBL-MRGPRX2) (46, 99). We found that similar to the situation in LAD2 cells (Figure 2-2;A), rocuronium caused dose-dependent degranulation (500 µg/mL – 2 mg/mL) in RBL-MRGPRX2 but not in untransfected cells (Figure 2-4;B,C).

To test the possibility that MRGPRX2 mutations reported in this patient render the receptor more susceptible to rocuronium-induced MC degranulation, we first constructed cDNAs encoding MRGPRX2 mutants; M196I, L226P and L237P, and generated separate transient transfectants expressing each variant in RBL-2H3 cells. Flow cytometry analysis demonstrated that while M196I
and L226P variants expressed on the cell surface at a level similar to WT MRGPRX2, L237P variant displayed reduced expression (Figure 2-4;D). Surprisingly, we found that cells expressing L226P and L237P variants showed loss-of-function phenotype for MC degranulation in response to rocuronium, while cells expressing M196I variant responded similarly to the WT receptor (Figure 2-4;E). Rocuronium at the concentration of 1 mg/mL evoked nearly peak response in cells expressing WT-MRGPRX2 (Figure 2-4;B), thus it would not be able to identify variants with enhanced activity. To further validate if M196I variant possibly displays a gain-of-function phenotype by rendering the mutated receptor more responsive at lower concentrations of the drug, we performed a dose response of rocuronium-induced degranulation in cells expressing M196I variant and the WT receptor. We found that rocuronium at different concentrations induces similar degranulation in cells expressing M196I variant and the WT receptor.

It is possible that the patient who displayed rocuronium-induced POH harbors more than one mutation in the same MRGPRX2 allele (94). Because we do not have any information on the MRGPRX2 status of the patient’s parents, we are unable to determine that possibility. However, we constructed cDNAs encoding double (M196I, L226P) and triple (M196I, L226P, L237P) variants, generated transient transfectants in RBL-2H3 cells and determined cell surface expression by flow cytometry. We found that the double mutant expressed on the surface of RBL-2H3 cells but the triple mutant did not (Figure 2-4;D). However, both double and triple mutants were resistant to rocuronium-induced degranulation (Figure 2-4;E). Thus, the demonstration that an individual with severe rocuronium-induced POH harbors MRGPRX2 mutations that renders loss-of-function phenotype for MC degranulation does not support the hypothesis that MRGPRX2 participates in rocuronium hypersensitivity in this patient.

2.5. Discussion

The seminal observation that rocuronium activates murine PMCs via MrgprB2 (10) led to the idea that POH to this drug involves the activation of MRGPRX2. However, attempts to demonstrate rocuronium-induced degranulation in MRGPRX2-expressing human MCs have not been successful to date (58, 59, 95). A number of patients with rocuronium-induced POH displayed
positive intradermal skin test (IDT) to rocuronium at irritative concentrations (1 mg/mL and 10 mg/mL), but were negative for both sIgE and BAT (55, 94). These individuals were diagnosed with non-IgE-mediated anaphylaxis and it was proposed that MRGPRX2 plays a critical role in rocuronium-induced POH without any evidence of MC degranulation by MRGPRX2. The novel findings of the present study are that rocuronium induces degranulation in human and murine MCs with different efficacies, and that missense mutations found in MRGPRX2 of a patient with rocuronium hypersensitivity do not render the receptor more susceptible to activation. These findings suggest that mechanism of rocuronium-induced POH is more complex than previously thought.

The data presented herein provides the first demonstration that irritating concentrations of rocuronium that were reported to induce skin reactions in patients with drug hypersensitivity (55, 94) induce degranulation in LAD2 cells, MRGPRX2-expressing RBL-2H3 cells and primary human skin MCs. The reason for the difference between the present study and previous reports is not clear but could reflect differences in receptor expression on different cell types and concentrations of rocuronium used. For example, Fernandopulle et al. (95) showed that while 200 µM rocuronium (~120 µg/mL) induces a small but significant Ca²⁺ response, this was not associated with degranulation in LAD2 cells. The demonstration in the present study that this concentration of rocuronium does not induce degranulation in LAD2 and transfected RBL-2H3 cells is consistent with previous report and suggest that small increase in Ca²⁺ mobilization does not provide sufficient signal for MC degranulation.

We found that although rocuronium caused degranulation in human skin MCs, the magnitude of the response was lower than that observed in LAD2 cells and transfected RBL-2H3 cells and this difference is associated with differences in cell surface receptor expression. Elst et al. (59) developed an MRGPRX2 knockdown strategy in primary human peripheral CD34⁺ cell-derived MCs to study MRGPRX2-mediated drug reactions in vitro. These MCs consist of almost equal numbers of MRGPRX2⁺ and MRGPRX2⁻ cells. The authors reported that high non-toxic concentration of rocuronium (1640 µM; ~1 mg/mL) induced a small but transient Ca²⁺ mobilization
via MRGPRX2 but this response was not associated with MC degranulation (59). This probably reflects low level of receptor expression, which provides sufficient signal for a small and transient Ca\(^{2+}\) mobilization but not for degranulation. The report by Navinés-Ferrer et al. (58) that rocuronium at a concentration of 2 mg/mL does not induce degranulation in LAD2 cells is inconsistent with our finding in this study and is difficult to explain. One possibility is that these authors utilized an inactive preparation of the drug as they did not perform control experiments to validate its activity using mouse PMCs or HEK293 cells expressing MRGPRX2 or MrgprB2.

The transfection and functional studies performed in this study were based on MRGPRX2 mutations present in one patient with rocuronium-induced POH. The hypothesis that these mutations would render the receptor more responsive to rocuronium was not realized (94). To more clearly define the roles of these MRGPRX2 mutations on the responsiveness to rocuronium, it will be important to generate MCs from CD34\(^+\) cells of this individual and to compare the expression of MRGPRX2 and their responsiveness to rocuronium with MCs derived from the CD34\(^+\) cells of normal individuals. For this patient, the diagnosis of rocuronium-induced POH was made based on a positive IDT with undiluted rocuronium (10 mg/mL). However, in the present study, we demonstrated that rocuronium at a concentration of 2 mg/mL induces degranulation in normal human skin MCs. These findings suggest that skin reactions induced by irritative concentrations of rocuronium are normal part of skin MC activation via MRGPRX2 and are unrelated to hypersensitivity. Furthermore, a negative rocuronium sIgE result does not rule out an IgE-mediated response to rocuronium or cross-reactivity with other drugs used in this patient (105, 106).

It is, however, possible that other mutations in MRGPRX2 can render the receptor more susceptible to activation by rocuronium and other drugs, making it an important target for hypersensitivity reactions. Examination of the GPCRdb database (GPCRdb.org) (107) reveals the presence of 72 missense MRGPRX2 variants with possible deleterious effects. It is therefore possible that one or more of these or other mutations positively modulate MRGPRX2 expression, ligand binding affinity and signaling to promote hypersensitivity in the absence of IgE (104, 108). Of interest, Lansu et al. (51) identified an MRGPRX2 mutation in its ligand binding pocket (E164D).
that increases the receptor affinity for drugs used in anesthesia. Sequence alignment predicts that MrgprB2’s E171 is the residue likely to “sits” in the MRGPRX2 E164 position (109). Taken together, these findings suggest that individuals harboring missense mutations in MRGPRX2’s ligand binding pocket that makes the receptor function similar to MrgprB2 may display rocuronium-induced POH via MRGPRX2. Therefore, analysis of MRGPRX2 polymorphisms in the genome of suspected patients together with studies to determine their expression level and susceptibility to degranulation may enable us to determine specific MRGPRX2 variants that are involved in POH to rocuronium and other drugs used during general anesthesia.

2.6. Conclusion

In summary, the present study demonstrated that rocuronium induces degranulation in murine and human MCs via MrgprB2 and MRGPRX2, respectively, but with different affinities, indicating important functional differences between these receptors. MRGPRX2 mutations recently reported in a patient with POH to rocuronium displayed loss-of-function phenotype, thus disputing the role of MRGPRX2 in rocuronium-induced POH and suggesting that mechanism of rocuronium-induced POH is more complex than previously thought.

2.7. Acknowledgments

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Figure 2-1. Rocuronium activates mouse PMCs via Mrgrp2. (A) PCR analysis was performed to determine the mRNA expression of Mrgrp2 in WT and Mrgrp2<sup>−/−</sup> PMCs. GAPDH was used for normalization. (B) WT and Mrgrp2<sup>−/−</sup> PMCs were stained with Toluidine blue and Alcian/Safranin. The images were acquired at 40X resolution. Bars = 10 and 20 µm. (C) WT and Mrgrp2<sup>−/−</sup> PMCs were stimulated with rocuronium (20 µg/mL) for 30 min, and β-hexosaminidase release was determined. Data are expressed as mean ± SEM. Statistical significance was determined by two-way ANOVA with Tukey post-hoc test. **** p < 0.0001 compared to the control, #### p < 0.0001 compared between WT and Mrgrp2<sup>−/−</sup> groups.
Figure 2-2. Rocuronium activates LAD2 cells and primary human skin MCs to cause degranulation. (A) LAD2 cells were exposed to buffer (control) or different concentrations of rocuronium for 30 min, and β-hexosaminidase release was determined. (B) Cells were stimulated with rocuronium (2 mg/mL) for 5 min, and LAMP-1 expression was determined by flow cytometry. Representative histograms of three independent experiments are shown. (C) The adjusted MFI levels of LAMP-1 expression are shown. Adjusted MFI was calculated as MFI of sample/MFI of isotype control. (D) Primary skin MCs were isolated and cultured from the human skin of 3 different donors. Skin-derived MCs were used to determine rocuronium-induced β-hexosaminidase release, and (E-F) LAMP-1 expression. All data points are the mean ± SEM of at least three experiments. For comparisons of two samples, two-tailed unpaired t-test was used. For comparisons of multiple samples to a control group, one-way ANOVA with Dunnett's post-hoc test was used. * p < 0.05, ** p < 0.01 and **** p < 0.0001.
Figure 2-3. Rocuronium induces MRGPRX2 internalization in LAD2 cells and primary human skin MCs. (A) Flow cytometry histograms for MRGPRX2 cell surface expression (solid black line) and isotype (dotted gray line) are shown. Histograms are representative of three independent experiments. (B) The comparison of MRGPRX2 expression levels between human MC line LAD2 cells and primary human skin MCs. (C) LAD2 cells were stimulated with rocuronium (2 mg/mL) for 30 min, and MRGPRX2 internalization was determined by flow cytometry. (D) The adjusted MFI levels of MRGPRX2 cell surface expression are shown. (E-F) Rocuronium-induced MRGPRX2 internalization and adjusted MFI levels of MRGPRX2 cell surface expression were determined in primary human skin MCs. All data points are the mean ± SEM of at least three experiments. Statistical significance was determined by two-tailed unpaired t-test. * p < 0.05.
Figure 2-4. MRGPRX2 mutations rendered the receptor unresponsiveness to rocuronium. (A) Snake diagram of MRGPRX2 indicating three missense mutations identified in the patient. (B) RBL-MRGPRX2 were stimulated with different concentrations of rocuronium for 30 min, and β-hexosaminidase release was determined. (C) Untransfected WT RBL-2H3 cells were stimulated with rocuronium (2 mg/mL) for 30 min, and β-hexosaminidase release was determined. (D) Cell surface expression of WT MRGPRX2 and its variants was determined by flow cytometry. (E) Cells expressing WT MRGPRX2 and its variants were exposed to buffer (control) or rocuronium (1 mg/mL) for 30 mins, and β-hexosaminidase release was determined. (F) Dose response of rocuronium-induced β-hexosaminidase release was determined in cells expressing WT MRGPRX2 and M196I. Data are the mean ± SEM of at least three experiments. For comparisons of two samples, two-tailed unpaired t-test was used. For comparisons of multiple samples to a control group, one-way ANOVA with Dunnett’s post-hoc test was used. ** p < 0.01, *** p < 0.001 and **** p < 0.0001.
CHAPTER 3: Identification of Gain and Loss of Function Missense Variants in MRGPRX2’s Transmembrane and Intracellular Domains for Mast Cell Activation by Substance P

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3.1. Abstract

The neuropeptide substance P (SP) contributes to neurogenic inflammation through the activation of human mast cells via MAS-related G protein-coupled receptor-X2 (MRGPRX2). Using pertussis toxin and YM-254890, we demonstrated that SP induces MRGPRX2-mediated Ca\textsuperscript{2+} mobilization and degranulation via both Gα\textsubscript{i} and Gα\textsubscript{q}. To determine the roles of MRGPRX2’s transmembrane (TM) and intracellular domains on SP-induced responses, we utilized information obtained from both structural modeling and naturally occurring MRGPRX2 missense variants. We found that highly conserved residues in TM6 (I225) and TM7 (Y279) of MRGPRX2 are essential for SP-induced Ca\textsuperscript{2+} mobilization and degranulation in transiently transfected rat basophilic leukemia (RBL-2H3) cells. Cells expressing missense variants in the receptor’s conserved residues (V123F and V282M) as well as intracellular loops (R138C and R141C) failed to respond to SP. By contrast, replacement of all five Ser/Thr residues with Ala and missense variants (S325L and L329Q) in MRGPRX2’s carboxyl-terminus resulted in enhanced mast cell activation by SP when compared to the wild-type receptor. These findings suggest that MRGPRX2 utilizes conserved residues in its TM domains and intracellular loops for coupling to G proteins and likely undergoes desensitization via phosphorylation at Ser/Thr residues in its carboxyl-terminus. Furthermore, identification of gain and loss of function MRGPRX2 variants has important clinical implications for SP-mediated neurogenic inflammation.

**Keywords:** Mast cells; MRGPRX2; Missense Variants; Substance P; Neurogenic inflammation
3.2. Introduction

Mast cells (MCs) are tissue-resident granulocytes of hematopoietic origin that play a pivotal role in the inflammatory processes due to their ability to release a wide array of proinflammatory mediators and recruit various immune cells upon stimulation (110-112). They are widely distributed throughout the body and found in close proximity to peripheral nerve endings in various tissues including skin, gastrointestinal mucosa, and respiratory tract (60). Besides close anatomic localization, accumulating evidence suggests bidirectional functional communication between MCs and neurons, providing a significant link between the immune and nervous system (3, 60). MC-derived mediators such as histamine and tryptase activate receptors on sensory nerve endings, resulting in the release of neuropeptides including substance P (SP) which, in turn, evokes further MC activation (3, 61, 66, 67). Activation of MCs by SP leads to their degranulation, resulting in vasodilation, plasma extravasation, and the recruitment of immune cells including lymphocytes, neutrophils, and macrophages (3, 63, 64). Immune cell recruitment further amplifies local inflammatory responses and facilitates peripheral nerve sensitization, which are critical characteristics of neurogenic inflammation (63). SP-induced neurogenic inflammation has been implicated in the pathogenesis of pain and many chronic inflammatory diseases such as sickle cell disease (70), atopic dermatitis (15), and chronic idiopathic urticaria (6).

The biological effects of SP were previously thought to be mediated via its canonical neurokinin-1 receptor (NK-1R) (64, 72, 113). Several antagonists of this receptor have been developed as potential therapies for a variety of conditions including chemotherapy-induced nausea, inflammation, and pain. While NK-1R antagonists are effective in the treatment of chemotherapy-induced nausea and vomiting, they fail to demonstrate significant anti-inflammatory and analgesic effects (72, 113). This raises the interesting possibility that the nociceptive and proinflammatory actions of SP may be mediated via alternative mechanisms. Recent studies have demonstrated that SP activates human and murine MCs via MRGPRX2 and Mrgprb2, respectively (8, 10). Expression of MRGPRX2 is upregulated in human skin MCs of patients with chronic idiopathic urticaria when compared to healthy individuals (6). Recent study by Serhan et al. (15)
demonstrated that SP released from nociceptors activates murine skin MCs and contributes to the development of allergic skin diseases, such as atopic dermatitis, through activation of Mrgrp2b. Furthermore, Green et al. (13) showed that inflammatory and thermal hyperalgesia requires Mrgrp2b-mediated recruitment of immune cells at the injury site. They also demonstrated that SP promotes the release of multiple pro-inflammatory cytokines and chemokines from human MCs via activation of MRGPRX2 (13). Taken together, these findings suggest that MRGPRX2/Mrgprb2 participate in neurogenic inflammation, chronic urticaria, atopic dermatitis, and pain (6, 13, 15, 114). However, the molecular mechanism by which MRGPRX2 is activated in response to SP has not been determined.

All G protein-coupled receptors (GPCRs) are structurally similar containing 7-transmembrane (TM) α-helices. Binding of ligands to the receptor from the extracellular site promotes the opening of TM6, which results in conformational changes in the cytoplasmic side of membrane, leading to allosteric activation of G proteins (75, 77, 79). Venkatakrishnan et al. (79) analyzed the pattern of contact between structurally equivalent residues from the crystal structures of 27 class A GPCRs. From this analysis, it became clear that, upon receptor activation, there is a highly conserved reorganization of residue contacts in TM3 (3x46), TM6 (6x37), and TM7 (7x53) (79). In this GPCR numbering scheme, the first number denotes the TM domains (1-7) and the second number indicates the residue position relative to the most conserved position, which is assigned the number 50 (76, 115). Thus, 3x46 denotes a residue in TM3, which is at 4 positions before the most conserved residue (3x50). Similarly, 7x53 denotes a residue in TM7, which is at 3 positions after the most conserved residue (7x50). Mutations of residues 3x46, 6x37, and 7x53 in a number of class A GPCRs result in significant reduction of G protein activation and downstream signaling, confirming the roles of these positions for the activation of different G proteins (79, 80). In addition to TM domains, conserved residues present in the 2nd intracellular loop (ICL2) of a number of class A GPCRs are involved in coupling to G proteins (84, 116). MRGPRX2 is a member of class A GPCR family, but the possibility that residues 3x46, 6x37 and 7x53 and conserved residues present in its ICL2 couple to G proteins to cause MC activation has not been tested.
In addition to G proteins, most class A GPCRs signal via another pathway that involves phosphorylation of the receptors at Ser/Thr residues in their carboxyl-terminus by GPCR kinases and the recruitment of adapter proteins known as β-arrestins (83, 117-119). This pathway has been implicated in the regulation of GPCR desensitization (uncoupling of the G protein from the cognate receptor), endocytosis, and internalization (119). GPCR agonists that preferentially activate G proteins are known as G protein biased and those activate β-arrestin are known as β-arrestin biased agonists. However, agonists that activate both pathways are known as balanced agonists (120). Our original studies using host defense peptide LL-37 as a ligand for MRGPRX2 demonstrated that the receptor is resistant to agonist-induced phosphorylation and desensitization, indicating that it acts as a G protein-biased agonist for the receptor (46). However, our more recent studies demonstrated that distinct ligands act as balanced or G protein biased agonists for MRGPRX2 (120). The carboxyl terminus of MRGPRX2 contains five Ser/Thr residues. However, the possibility that these potential phosphorylation sites contribute to receptor regulation by SP has not been determined.

Molecular modeling and mutagenesis studies led to the identification of ligand binding pocket for a number of MRGPRX2 agonists (51, 103, 109). We recently demonstrated that naturally occurring missense variants in MRGPRX2’s predicted ligand binding pocket result in loss of function phenotype of MC activation in response to a diverse groups of ligands including the neuropeptide SP (103). The goal of the present study was to utilize both structural information derived crystal structures of other GPCRs and naturally occurring MRGPRX2 missense variants to determine the roles of MRGPRX2’s TM and IC domains on MC activation by SP. The data presented herein identify a number of gain and loss of function of missense variants of MRGPRX2. These findings have important clinical implications with regard to resistance and susceptibility for developing MC-mediated neurogenic inflammation, pain, atopic dermatitis, and chronic urticaria (6, 13, 15, 114).
3.3. Materials and Methods

3.3.1. Materials

All cell culture reagents were obtained from Invitrogen (Gaithersburg, MD). Amaxa transfection kit (Kit V) was obtained from Lonza (Gaithersburg, MD). Q5 Site-Directed Mutagenesis Kit was from New England BioLabs (Ipswich, MA). Substance P (SP) was from AnaSpec (Fremont, CA). Pertussis toxin (PTx) was from List Biological Laboratories (Campbell, CA). YM-254890 compound was from Wako Chemicals USA (Richmond, VA). p-nitrophenyl-N-acetyl-β-D-glucosamine (PNAG) was from Sigma-Aldrich (St. Louis, MO). Fura-2 acetoxyethyl ester was from Abcam (Cambridge, MA). PE-conjugated anti-MRGPRX2 antibody was from BioLegend (San Diego, CA). MRGPRX2 plasmid encoding hemagglutinin (HA)-tagged human MRGPRX2 in pReceiver-MO6 vector was obtained from GeneCopoeia (Rockville, MD).

3.3.2. Cell culture

Rat basophilic leukemia (RBL-2H3) cells were maintained as monolayer cultures in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 μg/ml) at 37°C with 5% CO₂ (98). RBL-2H3 cells stably expressing MRGPRX2 (RBL-MRGPRX2) were maintained similarly in the presence of G-418 (1 mg/ml) (121).

3.3.3. Construction of MRGPRX2 variants

Q5 site-directed mutagenesis kit (New England BioLabs) was used to generate MRGPRX2 variants in HA-tagged plasmid. To confirm the correct nucleotide sequences, each mutant was verified by DNA sequencing prior to transfection. The forward and reverse primers used for each variant are listed below.

**V123A**: Forward: 5’-CTGAGCACGCGCCAGACCCGAG-3’
Reverse: 5’-CATGCTCAGGCTGCAAG-3’;

**V123F**: Forward: 5’-GCTGAGCACCTTCAGCACCGA-3’
Reverse: 5’-ATGCTCAGGCTGCAAGG-3’;

**Y137H**: Forward: 5’-GCCCATCTGGAATCCTGCAAGG-3’
Reverse: 5’-CACAGGACGGACAGGCAG-3’;
**R138C**: Forward: 5’-CATCTGGTATTGCTGCGCAGCCG-3’
Reverse: 5’-GGCCACAGGAGGACGG-3’;

**R140C**: Forward: 5’-GTATCGCTGCIGCCGCCCCAGCAGGC-3’
Reverse: 5’-CAGATGGGCACAGGAGG-3’;

**R141C**: Forward: 5’-TCGCTGCACTGTCACAGCT-3’
Reverse: 5’-TACCAGACAGGACGGAAG-3’;

**T224A**: Forward: 5’-GCTGTACCTGCTGGCCATCCTGCT-3’
Reverse: 5’-CTGGTCAGTGGCAGACCC-3’;

**I225A**: Forward: 5’-CTGGGTGTCATGGGCTCT-3’
Reverse: 5’-AGCCTGGTGGCAGG-3’;

**Y279A**: Forward: 5’-CCCCATCATTGCGTCTTCGG-3’
Reverse: 5’-TTGGCAGTCTGTTAAGAG-3’;

**V282M**: Forward: 5’-TTACTTCTACGCTGGCCAAG-3’
Reverse: 5’-ATGATGGGTTGGCACTG-3’;

**Q305R**: Forward: 5’-AGGGCTCTGCAGGACATTGCT-3’
Reverse: 5’-CTGGAGAGCCAGCTTGAG-3’;

**D311H**: Forward: 5’-TGCTGAGGTGCATCACAGTGAA-3’
Reverse: 5’-ATGTCCTGCAGAGCCCTC-3’;

**S325L**: Forward: 5’-CCGGAGATGTTGAGAAGCTCTG-3’
Reverse: 5’-GGTGCCCTGACGGAAGCA-3’;

**L329Q**: Forward: 5’-AGAAGCAGTCAGGTAGCTGAG-3’
Reverse: 5’-CGACATCTCCGGGGTGAG-3’

MRGPR2 phosphorylation-deficient mutant (Ser/Thr residues mutated to Ala; ΔST-MRGPR2) was generated by PCR. Construct was verified by DNA sequencing prior to transfection. The forward and reverse primers used are listed below.

**ΔST-MRGPR2**: Forward: 5’-ACATCCGGACCAGATGATCCCTAGCAGGATCCCAGACTAGCTGATCCACCACCCCCGCTGGAAGACGAA-3’
Reverse: 5’-ACATCTGGTACGCTTACACCAGGCGGCTCTCCTCCGACCTCCGCGCCTGGAAGACGAA-3’

**3.3.4. Generation of cells transiently expressing MRGPR2 and its variants**

RBL-2H3 cells transiently expressing MRGPR2 or its missense variants were generated as described previously (103). Briefly, cells (2 × 10⁶) were transfected with 2 μg of HA-tagged
plasmid using the Amaxa Nucleofector Device and Amaxa Kit V according to the manufacturer’s protocol. Cells were used within 16 – 20 h after transfection.

To detect cell surface MRGPRX2 and its variants expression, transfected RBL-2H3 cells (0.5 x 10^6) were incubated with PE-conjugated anti-MRGPRX2 antibody for 30 mins at 4°C in the dark, washed in FACS buffer (PBS containing 2 % FCS and 0.02% sodium azide), and fixed in 1.5% paraformaldehyde. Cells were acquired using a BD LSR II flow cytometer (San Jose, CA). Results were analyzed using WinList software, version 8.

3.3.5. Degranulation assay

The degranulation was measured by β-hexosaminidase release as described previously (98). Briefly, transfected RBL-2H3 cells (5 x 10^4 cells per well) were seeded into a 96-well, white, clear-bottom cell culture plate and incubated overnight in a 37°C incubator with 5% CO₂. To determine the inhibitory effects of PTx and YM-254890 on MC degranulation, cells were pretreated with PTx (100 ng/ml, 16 h) and/or YM-254890 (10 μM, 5 mins) prior to stimulation with SP. Cells were then washed twice and suspended in a total volume of 50 μl HEPES buffer containing 0.1% bovine serum albumin (BSA). Experimental groups were stimulated with SP for 30 mins at 37°C. Cells without treatment were designated as controls. To determine the total β-hexosaminidase release, unstimulated cells were lysed in 50 μl of 0.1% Triton X-100. Aliquots (20 μl) of supernatants or cell lysates were incubated with 20 μl of 1 mM p-nitrophenyl-N-acetyl-β-D-glucosamine (PNAG) for 1 h at 37°C. The reaction was stopped by adding 250 μl of stop buffer (0.1 M Na₂CO₃/0.1 M NaHCO₃). The β-hexosaminidase release was assessed by measuring absorbance at 405 nm using Versamax microplate spectrophotometer (San Jose, CA).

3.3.6. Calcium mobilization assay

Transfected RBL-2H3 cells (2 x 10^6) were loaded with 1 μM Fura-2 acetoxymethyl ester for 30 mins at 37°C, followed by de-esterification in HEPES-buffered saline for additional 15 mins at room temperature. Cells were washed, resuspended in 1.5 ml of HEPES-buffered saline containing 0.1% BSA, and then stimulated with SP. In some experiments, cells were treated with
PTx (100 ng/ml, 16 h) and/or YM-254890 (10 μM, 5 mins), and then stimulated with SP. Ca\textsuperscript{2+} mobilization was determined using a Hitachi F-2700 Fluorescence Spectrophotometer with dual excitation wavelength of 340 and 380 nm, and an emission wavelength of 510 nm.

3.3.7. Statistical analysis

Data shown are mean ± standard error of the mean (SEM) values derived from at least three independent experiments. GraphPad Prism scientific software version 6.07 was used for statistical analysis. Statistical significance was determined using two-tailed unpaired t-test and two-way ANOVA. Differences were considered as statistically significant at a value *p* < 0.05, **p* < 0.01, ***p* < 0.001, and ****p* < 0.0001.

3.4. Results

3.4.1. MRGPRX2 mediates SP-induced MC activation via both G\textsubscript{αi} and G\textsubscript{αq}.

In addition to SP, amphipathic peptides such as the cathelicidin LL-37 and human β-defensin-3 activate human MCs via MRGPRX2 (46, 48). We previously showed that while degranulation in response to these agonists is blocked by pertussis toxin (PTx), Ca\textsuperscript{2+} mobilization is not (46, 48). These findings suggest that MRGPRX2 may couple to both PTx-sensitive (G\textsubscript{αi}) and insensitive (G\textsubscript{αq}) G proteins. To determine the G protein specificity for SP-induced MRGPRX2-mediated responses, we utilized a pharmacological approach using a G\textsubscript{αi}-specific inhibitor (PTx) and a G\textsubscript{αq}-specific inhibitor (YM-254890) (122). Rat basophilic leukemia (RBL-2H3), a commonly used model for MC activation, does not endogenously express MRGPRX2. We therefore utilized RBL-2H3 cells stably expressing MRGPRX2 (RBL-MRGPRX2) to determine the effects of SP on MC activation (46, 48, 121).

SP has been shown to induce MRGPRX2-mediated MC degranulation in a dose-dependent manner (8). We found that at a low concentration of SP (0.1 μM), PTx caused substantial inhibition of MC degranulation. However, at higher concentrations of SP, only about 50% of MC degranulation was inhibited by PTx (Figure 3-1;A). A similar inhibitory profile was also observed for the G\textsubscript{αq} inhibitor, YM-254890, but the extent of inhibition was lower at high
concentrations of SP (1 and 10 μM) (Figure 3-1;A). However, SP-induced degranulation was abolished in cells treated with both PTx and YM-254890 (Figure 3-1;A) We also tested the effects of PTx and YM-254890 alone and in combination on SP-induced Ca\(^{2+}\) mobilization. Similar to degranulation, we found that PTx or YM-254890 caused partial inhibition of the SP response but a combination of both inhibitors resulted in almost complete inhibition of SP-induced Ca\(^{2+}\) response (Figure 3-1;B). Taken together, these findings suggest that MRGPRX2 utilizes both the G\(\alpha_i\) and G\(\alpha_q\) families of G proteins for SP-induced MC degranulation.

3.4.2. Mutations of the Highly Conserved Residues 3x46, 6x37, and 7x53 in MRGPRX2 Lead to a Significant Reduction in SP-Induced MC Activation.

Based on structural and computational studies, it was proposed that positions 3x46, 6x37, and 7x53 are conserved among class A GPCRs and likely participate in G protein coupling (79). Amino acids at these positions in MRGPRX2 were identified from the GPCR database (GPCRdb) (76). Residues at positions 3x46, 6x37, and 7x53 in MRGPRX2 are Val, Ile, and Tyr, respectively. Notably, these residues are either large hydrophobic or aromatic residues which are likely to fulfill the van der Waals criterion and facilitate contact formation during the receptor conformational rearrangement (79).

To determine if these residues in MRGPRX2 contribute to SP-induced MC activation, we first constructed single Ala substitution mutations at these positions, namely V123A, I225A, and Y279A, respectively (Figure 3-2;A,B). We then generated transient transfectants in RBL-2H3 cells. Flow cytometry analysis using phycoerythrin (PE)-conjugated anti-MRGPRX2 antibody showed that these point mutations did not adversely affect cell surface receptor expression (Figure 3-2;C). Interestingly, cells expressing V123A mutant responded normally to SP for Ca\(^{2+}\) mobilization but degranulation was inhibited by ~50% when compared to the wild-type (WT) receptor (Figure 3-2;D,E). Although the mutants I225A and Y279A expressed normally on the cell surface (Figure 3-2;C), they did not respond to SP for Ca\(^{2+}\) mobilization or degranulation (Figure 3-2;D,E).
3.4.3. Naturally Occurring Missense MRGPRX2 Variants at or Near the Conserved Residues, V123F and V282M, Display Loss of Function Phenotype for SP-Induced MC Activation.

Next, we searched the GPCRdb (76) to determine if there were any missense MRGPRX2 variants present in the human population with mutations at or near position 3x46, 6x36, or 7x53. We identified three MRGPRX2 variants, namely V123F (3x46), T224A (6x36), and V282M (7x56) (Figure 3-3;A,B). Allele frequency for each variant is shown in Figure 3-3;B. We used the site-directed mutagenesis approach to generate cDNAs encoding each of these variants, which were then transiently transfected in RBL-2H3 cells. Flow cytometry analysis demonstrated that MRGPRX2 and all its variants were expressed on the cell surface (Figure 3-3;C). SP-induced Ca$^{2+}$ mobilization was partially reduced in cells expressing the variant V123F when compared to the WT receptor, but degranulation was completely inhibited (Figure 3-3;D,E). However, cells expressing the variant T224A responded normally to SP for Ca$^{2+}$ mobilization and degranulation (Figure 3-3;D,E). By contrast, V282M variant was resistant to both SP-induced Ca$^{2+}$ mobilization and degranulation (Figure 3-3;D,E).

3.4.4. Naturally Occurring Missense MRGPRX2 Variants at the Second Intracellular Loop, R138C and R141C, Display Loss of Function Phenotype for SP-Induced MC Activation.

Apart from conformational changes in TM helices, recent crystallography and spectroscopy studies on GPCR-heterotrimeric G protein complexes have shown that intracellular loops of the receptors also interact with G proteins and are important for G protein activation (116, 123). Thus, we further searched for naturally occurring missense MRGPRX2 variants in the receptor’s intracellular loops and were able to identify four missense variants within ICL2 (Figure 3-4;A,B). cDNAs encoding these variants were generated and transiently transfected in RBL-2H3 cells. Flow cytometry analysis demonstrated that all four variants expressed on the cell surface (Figure 3-4;C). We found that Y137H and R140C variants responded to SP for Ca$^{2+}$ mobilization and degranulation similar to the WT receptor (Figure 3-4;D,E). By contrast, SP failed to activate these responses in cells expressing R138C and R141C variants (Figure 3-4;D,E).
3.4.5. Mutations in Potential Phosphorylation Sites of MRGPRX2 Leads to Enhanced MC Activation in Response to SP.

Phosphorylation of GPCRs by GPCR kinases provides an important mechanism for their desensitization (83, 117, 118). Human MRGPRX2 possesses five potential phosphorylation sites at its carboxyl-terminus. To determine the role of MRGPRX2 phosphorylation on SP-induced responses, we generated cDNAs encoding an MRGPRX2 mutant in which all Ser/Thr residues were replaced with alanine (ΔST-MRGPRX2) (Figure 3-5;A,B). Transiently transfected RBL-2H3 cells demonstrated reduced cell surface expression of ΔST-MRGPRX2, when compared to the WT receptor (Figure 3-5;C). Despite this, SP induced greater Ca²⁺ mobilization and degranulation in cells expressing ΔST-MRGPRX2 when compared to the WT receptor (Figure 3-5;D,E).

3.4.6. Naturally Occurring Missense MRGPRX2 Variants at its Carboxyl-Terminus, S325L and L329Q, Display Gain of Function Phenotype for SP-Induced MC Activation.

Search of the GPCRdb (76) led to the identification of four missense variants in the carboxyl-terminus of MRGPRX2 (Figure 3-6;A,B), of which one variant results in the replacement of Ser with Leu (S325L). Flow cytometry analysis of transfected RBL-2H3 cells demonstrated equivalent cell surface expression of all variants (Figure 3-6;C). Cells expressing Q305R and D311H variants responded similarly to SP for Ca²⁺ mobilization and degranulation when compared to the WT receptor (Figure 3-6;D,E). By contrast, S325L and L329Q variants displayed higher responses to SP for both Ca²⁺ mobilization and degranulation (Figure 3-6;D,E).

3.5. Discussion

Unique features of MRGPRX2 that differentiate it from other class A GPCRs are that it is expressed predominantly in one subtype of MCs and responds to a variety of cationic ligands, including SP (9, 13, 14, 50). Structure-based computational modeling and site directed mutagenesis approach have been used to show that negatively charged residues Glu164 (E164) in TM4 (4x60) and Asp184 (D184) in TM5 (5x36) are important for binding opioids and SP (51, 103, 109). We recently showed that missense variants in the MRGPRX2’s ligand-binding pocket (G165E and D184H) fail to respond to a variety of cationic ligands including SP, human β-defensin-3, and
icatibant (bradykinin B2 receptor antagonist) for receptor activation (103). In the present study, we utilized information derived from the comparison of crystal structures of a number of class A GPCRs, as well as naturally occurring missense variants in MRGPRX2’s predicted G protein coupling domains and potential phosphorylation sites to identify a number of gain and loss of function variants. These findings have important implications for SP/MRGPRX2-mediated conditions such as neurogenic inflammation, pain, atopic dermatitis, and chronic idiopathic urticaria (6, 13, 15, 114).

In the inactive state of class, A GPCRs, the residue at 6x37 is in contact with a conserved hydrophobic residue at position 3x46 (79). Upon receptor activation, this interaction is rearranged so that residue at 3x46 breaks contact with residue 637 and forms a new contact with a tyrosine residue, Tyr7x53, within the highly conserved NPxxY motif of TM7 (77, 79, 84). This rearrangement results in the activation of G proteins. Accordingly, Ala substitution of each of these residues (3x46, 6x37, and 7x53) of the vasopressin V2 receptor results in its uncoupling from Gαs and Gαq (79). We showed that MRGPRX2 coupled to both Gαi and Gαq families of G proteins for Ca2+ mobilization and degranulation in response to SP. Thus, PTx (a Gαi-specific inhibitor) in combination with YM-254890 compound (a Gαq-specific inhibitor) completely inhibited SP-induced MC activation. By contrast, using either PTx or YM-254890 alone was unable to abolish Ca2+ and degranulation responses to SP. Of note, many GPCRs have been shown to display distinct intracellular signaling and cellular responses depend on agonist concentrations (124). It is possible that low-dose SP induces MRGPRX2 to preferentially couple to either Gαi or Gαq, whereas a high concentration of SP mediates MRGPRX2 conformational change to couple to both G proteins. The data presented herein suggest that similar to other class A GPCRs, residues 3x46, 6x37, and 7x53 in MRGPRX2 contribute to coupling to Gαi and Gαq families of G proteins and that naturally occurring missense variants within or near some of these highly conserved residues may contribute to loss of function phenotype for MC activation by SP. One interesting finding of the present study was that while V123A (3x46) mutation resulted in partial inhibition of SP-induced degranulation, the missense variant V123F (3x46) failed to respond to SP for Ca2+ mobilization or degranulation. These findings
suggest that the presence of a bulky Phe group in the missense variant V123F less effectively breaks the interaction of 3x46 with 6x37 or blocks the formation of new contact Tyr residue at 7x53. Another interesting finding was that while cells expressing I225A mutation (6x37) were resistant to SP-induced Ca\(^{2+}\) mobilization and degranulation, a missense mutation T224A responded normally to SP. However, a missense V282M mutation three amino acids way from the Tyr\(^{7x53}\) in the conserved NPxxY motif resulted in complete loss of function phenotype for SP-induced MC degranulation. This finding likely emphasizes the importance of this region of MRGPRX2 for coupling to G proteins.

Additionally, we identified four missense MRGPRX2 variants at the predicted G protein coupling regions within its ICL2. Crystallography and cryogenic electron microscopy studies of GPCR-heterotrimeric G protein complexes have provided evidence that this ICL interacts with G\(\alpha\) subunit to promote GDP dissociation and subsequent GTP binding, resulting in activation of G proteins (116, 123). Mutations of ICL2 in \(\beta_2\) adrenergic receptor have been shown to impair G protein coupling (125). Here, we found that cells expressing R138C and R141C variants in this region displayed loss of function phenotype in response to SP. By contrast, other MRGPRX2 variants (Y137H and R140C) had no effect on SP-induced MC activation. Intriguingly, this region has also been identified as a cholesterol recognition amino acid consensus (CRAC) motif of MRGPRX2. Cholesterol-rich microdomains (lipid rafts) are membrane microdomains enriched in cholesterol and glycerophospholipids that mediate organization and function of many membrane receptors and biomolecules including GPCRs (126). The orientation and organization of membrane proteins present in the lipid raft allow greater efficiency and specificity of signal transduction by facilitating protein–protein interactions and preventing crosstalk between competing pathways (126). Given that MRGPRX2 contains the CRAC motif, it is possible that lipid rafts also contribute to MRGPRX2 activation and G protein coupling. Positively charged Arg residue of MRGPRX2 (R138 and R141) might be necessary to interact with negatively-charged hydroxyl group of cholesterol for proper MRGPRX2 functioning. Substitution of this amino acid with neutral amino acid Cys may disrupt the interaction with lipid raft domains, resulting in loss of function phenotype.
The interaction between MRGPRX2 and lipid rafts will be the subject of further investigation to delineate the role of lipid rafts in MRGPRX2 signaling.

While GPCR signaling is essential for regulating physiological function of cells, overstimulation can be deleterious and contributes to pathologic conditions. Thus, following their activation, GPCRs undergo desensitization via phosphorylation of Ser/Thr residues at their carboxyl-terminus (83, 84). Binding of β-arrestin to phosphorylated GPCRs has been implicated in receptor desensitization, endocytosis, and internalization (83, 84, 119). It also initiates a distinct downstream signaling pathway known as β-arrestin-mediated activation (83, 84, 119). Here, we showed that mutation of all possible phosphorylation sites of MRGPRX2 (ΔST-MRGPRX2) leads to significantly higher SP-induced MC activation. We further examined the effects of naturally occurring missense MRGPRX2 mutations within the carboxyl-terminus. Of these, we identified one missense variant in which a potential phosphorylation site is mutated, S325L. Interestingly, cells expressing this variant exhibited gain of function phenotype for MC degranulation in response to SP. These findings are consistent with previous studies in β2 adrenergic receptor that demonstrated the importance of distal phosphorylation residues for high-affinity β-arrestin binding and receptor desensitization (127). Distinct GPCR phosphorylation sites have been proposed to be targeted by different GPCR kinases and establish a specific barcode that imparts distinct conformations to the recruited β-arrestin, thus regulating different functional activities, such as desensitization, internalization, and downstream signaling (127). It is possible that the S325 of MRGPRX2 is responsible for receptor desensitization, thus mutation in this position leads to enhanced SP-induced responses due to impaired desensitization.

In addition, the carboxyl-terminus of MRGPRX2 contains a class I PDZ (PSD-95/Dlg/Zo1) recognition motif S/T-X-φ (where “φ” indicates hydrophobic amino acid and “X” indicates any amino acid). PDZ proteins have been implicated in regulating receptor desensitization, internalization, and signaling for several GPCRs such as β2 adrenergic receptor, parathyroid hormone receptor, and opioid receptors (128). For example, the PDZ protein, Na+/H+ exchanger regulatory factor 1 (NHERF1) has been shown to regulate type 1 parathyroid hormone receptor
signaling by anchoring the receptor to the plasma membrane, thus restricting its desensitization and internalization (129). Our lab previously demonstrated that these proteins also promote C3a-induced degranulation in human MCs (130). Given that MRGPRX2 possesses a class I PDZ motif, it is possible that PDZ proteins such as NHERF1 contributes to the regulation of MRGPRX2. It is also possible that missense mutations in the receptor’s PDZ motif may enhance the interaction with PDZ proteins, resulting in gain of function phenotype. Altogether, our findings herein indicate the significance of carboxyl-terminal residues for MRGPRX2 regulation and activation. The MRGPRX2 mutations at its carboxyl-terminus may lead to gain of function phenotype for SP-induced MC degranulation due to impaired receptor desensitization, enhanced interaction with PDZ proteins, or both.

Taken together, the data presented herein have identified mutations in MRGPRX2 at the regions involved in the receptor activation pathway. Missense MRGPRX2 mutations in the G protein-coupling regions in TMs and ICL2 fail to activate MC in response to SP, presumably due to impaired G protein coupling. By contrast, MRGPRX2 variants at the carboxyl-terminus, which is responsible for receptor phosphorylation and desensitization, lead to higher responses for MC activation. Thus, individuals with loss of function MRGPRX2 mutation, V123F, R138C, R141C, or V282M may display resistance to developing neurogenic inflammation and chronic inflammatory diseases. By contrast, individuals who harbor the gain of function variant, S325L or L329Q, may be more susceptible to develop these conditions.

3.6. Acknowledgements

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Figure 3-1. Effects of pertussis toxin (PTx) and YM-254890 on substance P (SP)-induced degranulation and Ca\textsuperscript{2+} mobilization in RBL-2H3 cells stably expressing MRGPRX2, (RBL-MRGPRX2). (A) Cells were cultured overnight in the absence or presence of PTx (100 ng/mL, 16 h), washed and incubated with or without YM-254890 (10 μM) for 5 min. Cells were then exposed to a buffer (control) or different concentrations of SP for 30 min, and β-hexosaminidase release was determined. All data points are the mean ± SEM of at least three experiments performed in triplicate. (B) Cells were cultured overnight in the absence or presence of PTx (100 ng/mL, 16 h), then loaded with Fura-2 and intracellular Ca\textsuperscript{2+} mobilizations in response to SP (1 μM) were determined. To determine the effect of G\textsubscript{q}, cells were incubated with YM-254890 (10 μM) for 5 min before stimulating with SP. Data shown are representative of three independent experiments. Statistical significance was determined by two-tailed unpaired t-test and two-way ANOVA. * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \), and **** \( p < 0.0001 \).
Figure 3-2. Effects of mutations at MRGPRX2’s highly conserved positions within transmembrane domains (V123A, I225A, and Y279A) on cell surface expression, SP-induced Ca²⁺ mobilization, and degranulation in transiently transfected RBL-2H3 cells. (A) Snake diagram of secondary structure of MRGPRX2. Each circle represents amino acid residue with one letter code. Solid red, yellow, and blue backgrounds denote the residues at positions 3x46 (V123), 6x37 (I225), and 7x53 (Y279), respectively. (B) Amino acid change for each MRGPRX2 mutant. (C) RBL-2H3 cells transiently expressing wild-type (WT)-MRGPRX2 and its mutants were incubated with phycoerythrin (PE)-anti-MRGPRX2 antibody and cell surface receptor expression was determined by flow cytometry. Representative histograms for WT/mutant (black line) and control untransfected cells (blue line) are shown. (D) Cells expressing WT-MRGPRX2 and its mutants were loaded with Fura-2 and intracellular Ca²⁺ mobilization in response to SP (1 μM) was determined. Data shown are representative of three independent experiments. (E) Cells were exposed to a buffer (control) or SP (1 μM) for 30 min, and β-hexosaminidase release was determined. All data points are the mean ± SEM of at least three experiments performed in triplicate. Statistical significance was determined by a two-tailed unpaired t-test. *** p < 0.001 and **** p < 0.0001.
Figure 3-3. Effects of naturally occurring MRGPRX2 variants at the receptor’s conserved transmembrane domains (V123F, T224A, and V282M) on SP-induced responses in transiently transfected RBL-2H3 cells. (A) Snake diagram of secondary structure of MRGPRX2. Each circle represents amino acid residue with one letter code. Solid red, yellow, and blue backgrounds denote the naturally occurring MRGPRX2 variants V123F, T224A, and V282M, respectively. (B) amino acid change for each MRGPRX2 variant with allele frequency. (C) cell surface expression of WT-MRGPRX2 and its variants was determined by flow cytometry using PE-anti-MRGPRX2 antibody. Representative histograms for WT/variant (black line) and control untransfected cells (blue line) are shown. (D) cells expressing WT-MRGPRX2 and its variants were loaded with Fura-2 and intracellular Ca$^{2+}$ mobilization in response to SP (1 μM) was determined. Data shown are representative of three independent experiments. (E) cells were exposed to a buffer (control) or SP (1 μM) for 30 min, and β-hexosaminidase release was determined. All data points are the mean ± SEM of at least three experiments performed in triplicate. Statistical significance was determined by a two-tailed unpaired t test. **** $p < 0.0001.$
Figure 3-4. Effects of naturally occurring MRGPRX2 variants at the receptor’s intracellular loops (Y137H, R138C, R140C, and R141C) on SP-induced responses in transiently transfected RBL-2H3 cells. (A) Snake diagram of secondary structure of MRGPRX2. Each circle represents amino acid residue with one letter code. Solid yellow, red, purple, and pink backgrounds denote the naturally occurring MRGPRX2 variants. (B) Amino acid change for each MRGPRX2 variant with allele frequency. (C) Cell surface expression of WT-MRGPRX2 and its variants was determined by flow cytometry using PE-anti MRGPRX2 antibody. Representative histograms for WT/variant (black line) and control untransfected cells (blue line) are shown. (D) Cells expressing WT-MRGPRX2 and its variants were loaded with Fura-2 and intracellular Ca$^{2+}$ mobilization in response to SP (1 μM) was determined. Data shown are representative of three independent experiments. (E) Cells were exposed to a buffer (control) or SP (1 μM) for 30 min, and β-hexosaminidase release was determined. All data points are the mean ± SEM of at least three experiments performed in triplicate. Statistical significance was determined by a two-tailed unpaired t-test. **** p < 0.0001.
Figure 3-5. Effects of Ser/Thr residues on MRGPRX2’s carboxyl-terminus on cell surface expression, SP-induced Ca\textsuperscript{2+} mobilization, and degranulation in transiently transfected RBL-2H3 cells. (A) Snake diagram of secondary structure of MRGPRX2. Each circle represents amino acid residue with one letter code. Solid red backgrounds denote Ser/Thr residues. (B) schematic representation of the carboxyl-terminus of MRGPRX2 (WT) and a phosphorylation-deficient mutant in which all Ser/Thr were replaced with Ala (ΔST-MRGPRX2). (C) cell surface expression of WT and ΔST-MRGPRX2 was determined by flow cytometry using PE-anti-MRGPRX2 antibody. Representative histograms for WT-MRGPRX2 (black line), ΔST-MRGPRX2 (red line), and control untransfected cells (blue line) are shown. (D) cells expressing WT and ΔST-MRGPRX2 were loaded with Fura-2 and intracellular Ca\textsuperscript{2+} mobilization in response to SP (1 μM) was determined. Data shown are representative of three independent experiments. (E) cells were exposed to a buffer (control) or SP (1 μM) for 30 min, and β-hexosaminidase release was determined. All data points are the mean ± SEM of at least three experiments performed in triplicate. Statistical significance was determined by a two-tailed unpaired t-test. * \( p < 0.05 \).
Figure 3-6. Effects of naturally occurring MRGPRX2 variants within the receptor’s carboxyl-terminus (Q305R, D311H, S325L, and L329Q) on SP-induced responses in transiently transfected RBL-2H3 cells. (A) Snake diagram of secondary structure of MRGPRX2. Each circle represents amino acid residue with one letter code. Solid purple, orange, pink, and red backgrounds denote the naturally occurring missense variants Q305R, D311H, S325L, and L329Q, respectively. (B) Amino acid change for each MRGPRX2 variant with allele frequency. (C) Cell surface expression of WT-MRGPRX2 and its variants was determined by flow cytometry using PE-anti MRGPRX2 antibody. Representative histograms for WT/variant (black line) and control untransfected cells (blue line) are shown. (D) Cells expressing WT-MRGPRX2 and its variants were loaded with Fura-2 and intracellular Ca^{2+} mobilization in response to SP (1 μM) was determined. Data shown are representative of three independent experiments. (E) Cells were exposed to buffer (control) or SP (1 μM) for 30 min, and β-hexosaminidase release was determined. All data points are the mean ± SEM of at least three experiments performed in triplicate. Statistical significance was determined by a two-tailed unpaired t-test. * p < 0.05 and ** p < 0.01.
CHAPTER 4: Substance P serves as a balanced agonist for MRGPRX2 and a single tyrosine residue is required for β-arrestin recruitment and receptor internalization

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4.1. Abstract

The neuropeptide substance P (SP) mediates neurogenic inflammation, pain and contributes to atopic dermatitis in mice through the activation of mast cells (MCs) via Mas-related G protein-coupled receptor (GPCR)-B2 (MrgprB2, human ortholog MRGPRX2). In addition to G proteins, certain MRGPRX2 agonists activate an additional signaling pathway that involves the recruitment of β-arrestins, which contributes to receptor internalization and desensitization (balanced agonists). We found that SP caused β-arrestin recruitment, MRGPRX2 internalization and desensitization. These responses were independent of G proteins, indicating that SP serves as a balanced agonist for MRGPRX2. A tyrosine residue in the highly conserved NPxxY motif contributes to the activation and internalization of many GPCRs. We have previously shown that Tyr^{279} of MRGPRX2 is essential for G protein-mediated signaling and degranulation. To assess its role in β-arrestin-mediated MRGPRX2 regulation, we replaced Tyr^{279} in the NPxxY motif of MRGPRX2 with Ala (Y279A). Surprisingly, we found that unlike the wild-type receptor, Y279A mutant of MRGPRX2 was resistant to SP-induced β-arrestin recruitment and internalization. This study reveals the novel findings that activation of MRGPRX2 by SP is regulated by β-arrestins and that a highly conserved tyrosine residue within MRGPRX2’s NPxxY motif contributes to both G protein- and β-arrestin-mediated responses.

**Keywords:** Mast cells; MRGPRX2; MrgprB2; Tyrosine; Substance P; Signaling; Internalization
4.2. Introduction

Mast cells (MCs) are key sentinel immune cells that are often found in close proximity to sensory nerve endings in various tissues, including the skin, gastrointestinal mucosa, and respiratory tract (3). The role of MC-neuron interaction as a regulatory unit in both physiology and disease has recently gained prominence (13, 15, 62, 131-133). Substance P (SP) has long been established as an inflammatory neuropeptide and is known to activate a variety of cell types via the neurokinin-1 receptor (NK-1R). Mouse bone marrow-derived MCs (BMMCs) express NK-1R and its expression level is upregulated upon FceRI stimulation (134). However, emerging evidence suggests that SP contributes to neurogenic inflammation and pain in mice through MC activation via a novel G protein-coupled receptor (GPCR) known as Mas-related GPCR-B2 (MrgprB2; human ortholog MRGPRX2) (13, 15). Activation of MrgprB2 by SP is required for regulating inflammatory hyperalgesia via the release of pro-inflammatory cytokines and chemokines, and the recruitment of immune cells at the injury site, which facilitates inflammatory responses and peripheral sensitization (13). Furthermore, Serhan et al. recently showed that SP released from nociceptors activates murine skin MCs and contributes to the development of atopic dermatitis, a type 2 allergic skin disease, through the activation of MrgprB2 (15). These findings challenge our current understanding of SP-mediated neuroinflammatory diseases and pain, and raise an interesting possibility that targeting MRGPRX2 might represent a promising therapeutic approach for the management of these neuroinflammatory associated conditions in humans. However, the mechanisms involved in the activation and regulation of MRGPRX2 by SP has yet to be fully elucidated.

As a member of class A GPCR family, MRGPRX2 shares a common structure of seven transmembrane (TM) α-helices. The extracellular part is responsible for ligand binding, whereas the intracellular part is involved in binding downstream effectors such as heterotrimeric G proteins (75). Besides G protein-mediated signaling, most GPCRs signal via an additional pathway that involves the recruitment of adapter proteins known as β-arrestins (83, 84). The recruitment of β-arrestins results in uncoupling of the receptor from G proteins and termination of receptor activation (desensitization) (83, 135). They further target the receptor internalization/endocytosis, which
downregulates signaling as the receptor is physically removed from the cell surface (86, 136, 137). In addition to receptor desensitization and internalization, it is now established that β-arrestins also play important roles in various G protein-independent downstream signaling to promote chemotaxis and to modulate inflammation (86-88). Thus, β-arrestins regulate nearly all aspects of receptor activity, including desensitization, downregulation, trafficking and signaling.

While most GPCR agonists target signaling pathways mediated by both G proteins and β-arrestins (“balanced agonists”), some agonists preferentially activate only particular pathway (“biased agonists”) (80, 138). GPCR agonists that preferentially activate G proteins are known as G-protein-biased and those activate β-arrestins are known as β-arrestin-biased agonists. For MRGPRX2, compound 48/80 and codeine activate both G proteins and β-arrestins (balanced agonists) and can cause receptor internalization, which is associated with functional desensitization (120, 139). On the contrary, host defense peptides, such as cathelicidin LL-37 and angiogenic peptide AG-30/5C, activate only G proteins but not β-arrestins (G protein-biased), and do not induce receptor desensitization and internalization (46, 120, 140). Biased signaling has gained important therapeutic implications in several GPCRs (80). However, whether SP acts as a balanced or biased agonist for MRGPRX2 remains unknown.

Closely related GPCRs exhibit high degree of conserved sequence motifs and a common activation pathway, especially in the regions implicated in ligand binding and G protein coupling (78). Recent computer-based structural modeling, sequence analysis and mutagenesis studies have led to the identification of residues in MRGPRX2 that are responsible for ligand binding and G protein coupling (51, 103, 104, 109). One of the most conserved GPCR sequences is the NPxxY motif located in TM7. A tyrosine residue Tyr^{753} in this motif is pivotal for receptor activation for all class A GPCRs (79). Structural modeling studies in β2 adrenergic receptor, rhodopsin and M2 muscarinic acetylcholine receptors revealed that upon ligand binding, Tyr^{753} undergoes substantial rotamer conformations and provides activation switch through formation of a water-mediated hydrogen bond (141, 142). This favors an outward movement in the cytoplasmic end of TM6 and allows the receptor coupling to G proteins and other signal transducers, which represents a hallmark of GPCR activation. Besides its importance in receptor activation, Tyr^{753} is suggested to
regulate agonist-induced receptor internalization in many GPCRs (143-146). In MRGPRX2, the corresponding tyrosine residue is Tyr^{279}. Our previous study has shown that this tyrosine residue is required for G protein-mediated MRGPRX2 activation in response to SP (104). However, the role of this tyrosine residue on β-arrestin-mediated MRGPRX2 signaling has not been determined.

The goals of the current study were to determine whether SP serves as a balanced or biased agonist for MRGPRX2 and to investigate the potential effects of MRGPRX2’s Tyr^{7x53} (Tyr^{279}) residue on β-arrestin recruitment and receptor internalization. The data presented in herein suggest that SP serves as a balanced agonist for MRGPRX2 and that Tyr^{7x53} contributes to both G protein-dependent signaling for degranulation and G-protein independent signaling for β-arrestin recruitment and receptor internalization.

4.3. Materials and Methods

4.3.1. Materials

All cell culture and Lipofectamine 2000 transfection reagents were obtained from Invitrogen (Gaithersburg, MD). Amaxa Nucleofector kit (Kit V) was purchased from Lonza (Gaithersburg, MD). Phycoerythrin (PE)-conjugated (Cat.#359004) and purified unconjugated anti-MRGPRX2 antibodies (Cat.#359002) were from BioLegend (San Diego, CA). Donkey anti-mouse Alexa Fluor 488 (Cat.#A21202) and 647 (Cat.#A31571) conjugated IgG secondary antibodies were from Invitrogen (Gaithersburg, MD). p-nitrophenyl-N-acetyl-β-D-glucosamine (PNAG) was from Sigma-Aldrich (St. Louis, MO). Fura-2 acetoxyethyl ester was from Abcam (Cambridge, MA). Pertussis toxin (PTx) was from List Biological Laboratories (Campbell, CA). Plasmid encoding hemagglutinin (HA)-tagged human MRGPRX2 in pReceiver-MO6 vector was obtained from GeneCopoeia (Rockville, MD). MRGPRX2 Y279A mutant in HA-tagged plasmid was reported previously (104). MRGPRX2-Tango plasmid (Addgene no. 66440) was a gift from Dr. Bryan Roth. MRGPRX2 Y279A mutant in Tango plasmid was generated by Penn Genomics Analysis Core (Philadelphia, PA).
4.3.2. Mice

C57BL/6 (wild-type; WT) and β-arrestin2 knockout (βArr2−/−) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in pathogen-free cages on autoclaved hardwood bedding. Eight-to-twelve-week-old male and female mice were used in this study. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

4.3.3. Cell culture

Rat basophilic leukemia (RBL-2H3) cells were maintained as monolayer cultures in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 IU/mL) and streptomycin (100 µg/mL) at 37°C with 5% CO₂ (98). RBL-2H3 cells stably expressing MRGPRX2 (RBL-MRGPRX2) were used and maintained similarly in the presence of G-418 (1 mg/mL) (46, 99).

HTLA (engineered HEK-293T cells stably expressing a β-arrestin2–tobacco etch virus fusion gene) cells and HTLA cells stably expressing MRGPRX2-Tango (HTLA-MRGPRX2) were maintained in DMEM supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml), hygromycin B (200 µg/ml), puromycin (5 mg/ml), and G-418 (500 µg/ml) (51, 147).

Peritoneal MCs (PMCs) were purified from WT and βArr2−/− mice as described previously (92). Briefly, the peritoneal cavity was lavaged with 10 mL of HBSS supplemented with 3% FCS and 10 mM HEPES. The cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 10% FCS, murine IL-3 (10 ng/mL), and murine SCF (30 ng/mL). After 48 h, non-adherent cells were removed and adherent cells were cultured in fresh medium for an additional 10-14 days. Suspension cells were then determined for MC receptor expression and function, and were used for experiments as PMCs.
4.3.4. Generation of cells transiently expressing WT MRGPRX2 and its variant

RBL-2H3 cells (2 x 10^6) were transiently transfected with 2 µg of HA-tagged plasmid using the Amaza Nucleofector Device and Amaza Kit V according to the manufacturer's protocol. Cells were used within 16 – 20 h after transfection (103).

For HTLA cells transiently expressing WT MRGPRX2 or its missense variant, cells (1 x 10^6 cells per well) were plated in a 6-well plate in antibiotic-free medium (DMEM supplemented with 10% FBS and L-glutamine) and incubated overnight at 37°C with 5% CO₂. The following day, cells were transfected with 2 µg of MRGPRX2 or its missense variants in Tango plasmids using the Lipofectamine 2000 DNA transfection reagent according to the manufacturer's protocol. Cells were incubated overnight at 37°C with 5% CO₂ in antibiotic-free medium and were used within 16 – 48 h after transfection (51).

4.3.5. Receptor expression and internalization using flow cytometry

Cells expressing either WT MRGPRX2 or its mutants (5 x 10^5) were stimulated with either SP or buffer for indicated time at 37°C. Cells were washed twice with ice-cold FACS buffer (PBS containing 2 % FCS and 0.02% sodium azide) and incubated with PE-conjugated anti-MRGPRX2 antibody for 30 min at 4°C in the dark. Cells were then washed with FACS buffer and fixed in 1.5% paraformaldehyde. Cells were acquired using a BD LSR II flow cytometer (San Jose, CA) and MRGPRX2 expression were analyzed using WinList software, version 8.

4.3.6. Degranulation

The degranulation was measured by β-hexosaminidase release as described previously (98). Briefly, RBL-2H3 cells (5 x 10^4 cells) or PMCs (1 x 10^4 cells) were seeded into a 96-well, white, clear-bottom cell culture plate and incubated overnight in a 37°C incubator with 5% CO₂. Cells were then washed twice and suspended in a total volume of 50 µl HEPES buffer containing 0.1% bovine serum albumin (BSA). Experimental groups were stimulated with SP for 30 min at 37°C. Cells without treatment were designated as controls. To determine the total β-hexosaminidase release, unstimulated cells were lysed in 50 µl of 0.1% Triton X-100. Aliquots (20 µl) of supernatants or cell
lysates were incubated with 20 µl of 1 mM p-nitrophenyl-N-acetyl-β-D-glucosamine (PNAG) for 1 h at 37°C. The reaction was stopped by adding 250 µl of stop buffer (0.1 M Na2CO3/0.1 M NaHCO3). The β-hexosaminidase release was assessed by measuring absorbance at 405 nm using Versamax microplate spectrophotometer (San Jose, CA).

4.3.7. Transcriptional activation following arrestin translocation (TANGO) assay

HTLA cells expressing either WT MRGPRX2 or its variants (5 x 10^4 cells per well) were plated into a 96-well plate in triplicates in a total volume of 160 µl antibiotic-free medium and incubated for 6 h at 37°C to allow attachment. After 6 h, the medium was aspirated, and cells were incubated with MRGPRX2 ligands in 160 µl antibiotic-free medium for additional 16 h at 37°C. The medium and ligands were then aspirated and 100 µl of Bright-Glo solution (Promega) was added to each well. Relative luminescence unit was measured in a Thermo Labsystems Luminoskan Ascent 392 Microplate Luminometer (51, 120).

4.3.8. β-arrestin translocation by live imaging confocal microscopy

RBL-2H3 cells were co-transfected with green fluorescent protein-tagged β-arrestin2 plasmid (βArr2-GFP) and WT-MRGPRX2 or its mutants with a ratio of 3:1 for β-arrestin2:receptor using Amaxa Kit V as described above. Transfected cells were plated onto a 35-mm glass bottom dish. Cell surface receptor was determined by incubating with purified anti-MRGPRX2 antibody, followed by Alexa Fluor 647-conjugated secondary antibody. Cells were then stimulated with SP and live images of β-arrestin2 translocation as indicated by green fluorescence images were collected using Nikon A1R confocal microscope.

4.3.9. Receptor trafficking using immunofluorescence microscopy

Receptor trafficking after SP stimulation was modified from previously described antibody feeding assay (148). RBL-2H3 cells expressing either MRGPRX2 or its variants were plated onto sterilized glass coverslips (2x10^5 cells/12-mm diameter coverslip in 24-well plate) and incubated overnight at 37°C with 5% CO₂ to allow attachment. Cells were rinsed with PBS and blocked with blocking buffer (PBS with 2% BSA) for 30 min at room temperature. Primary antibody incubation
was performed using purified anti-MRGPRX2 antibody (1:250 dilution) for 1 h at 4°C to label the cell surface expressed receptors. Cells were then stimulated with SP for 30 min at 37°C to allow internalization, followed by fixed with 4% paraformaldehyde for 15 min at 4°C. Labeled surface receptors were detected by incubating with saturated Alexa Fluor 647-conjugated secondary antibody (red) for 1 h at 4°C. Cells were then permeabilized by using 0.2% Triton X-100 in blocking buffer for 30 min and internalized receptors were detected by incubating with Alexa Fluor 488-conjugated secondary antibody (green) for 30 min at 4°C. Then, cells were mounted onto the glass slides using ProLong Gold Antifade mounting medium (Invitrogen) and images were visualized using a Nikon Eclipse Ni microscope.

4.3.10. Statistical analysis

Data shown are mean ± standard error of the mean (SEM) values derived from at least three independent experiments. GraphPad Prism scientific software version 6.07 was used for statistical analysis. Statistical significance was determined using unpaired two-tailed t-test and one- or two-way ANOVA. Differences were considered as statistically significant at a value * p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001.

4.4. Results

4.4.1. SP is a balanced agonist for MRGPRX2.

For many GPCRs, biased signaling has important therapeutic implications as it can elicit distinct physiological responses (80, 138, 149). Certain MRGPRX2 ligands, including compound 48/80, codeine and HDP AG-30/5C, have recently been identified as either balanced or G-protein-biased agonists (120, 139). SP is a well-known MRGPRX2 agonist; however, whether SP is a balanced or biased agonist for MRGPRX2 has yet to be investigated. We have previously shown that SP induces Ca²⁺ mobilization and degranulation in a G protein-dependent manner (104). Thus, we first sought to determine if SP can also trigger β-arrestin recruitment. For this, we utilized an assay known as transcriptional activation following arrestin translocation (TANGO) using HTLA cells (engineered HEK-293T cells stably expressing a β-arrestin2–tobacco etch virus fusion gene) stably expressing human MRGPRX2 (HTLA-MRGPRX2) (51, 120). Cells were exposed to either
buffer (control) or SP at different concentrations for 16 h and β-arrestin-mediated gene expression (indicative of β-arrestin recruitment) was measured. We found that SP (30 μM and 100 μM) significantly induced β-arrestin-mediated gene expression (Figure 4-1:A).

For most GPCRs, the recruitment of β-arrestins is involved in receptor internalization and desensitization. Compound 48/80 and codeine trigger robust β-arrestin recruitment, MRGPRX2 internalization and inhibition of degranulation in response to subsequent stimulation by the same ligand (desensitization) (120, 139). Given that SP induced β-arrestin recruitment, we hypothesized that it could also cause receptor internalization and desensitization. Rat basophilic leukemia (RBL-2H3) cell line, a commonly used model for MC activation, does not endogenously express Mrgrp receptors (46, 150). We therefore utilized RBL-2H3 cells stably expressing human MRGPRX2 (RBL-MRGPRX2) to determine the effects of SP on MRGPRX2 internalization and desensitization. For receptor internalization, RBL-MRGPRX2 cells were stimulated with different concentrations of SP for different time-points and incubated with PE-conjugated anti-MRGPRX2 antibody to determine cell surface receptor expression by flow cytometry. Consistent with β-arrestin recruitment, we found that SP triggered MRGPRX2 internalization and that this response was dose-dependent (Figure 4-1:B). Furthermore, preincubation of RBL-MRGPRX2 with 30 μM of SP overnight resulted in nearly complete inhibition of degranulation on second stimulation by the same ligand (Figure 4-1:C). Taken together, these studies suggest that SP serves as a balanced agonist for MRGPRX2.

4.4.2. SP-induced β-arrestin recruitment and MRGPRX2 internalization are G protein-independent.

SP has been shown to induce MRGPRX2-mediated Ca²⁺ mobilization and MC degranulation in a G protein-dependent manner (104). We therefore asked if β-arrestin recruitment and MRGPRX2 internalization in response to SP are also mediated in a G protein-dependent manner. We utilized a G protein inhibitor, pertussis toxin (PTx), and confirmed that pretreatment of RBL-MRGPRX2 cells with PTx (100 ng/ml, 16 h) attenuated degranulation in response to SP (Figure 4-2:A). By contrast, PTx had no effect on SP-induced β-arrestin-mediated gene expression.
and MRGPRX2 internalization (Figure 4-2;B,C). These findings indicate that β-arrestin recruitment and MRGPRX2 internalization in response to SP are mediated independently of G proteins.

4.4.3. β-arrestin2 regulates SP/MrgprB2-mediated MC degranulation.

MrgprB2 has been identified as the mouse ortholog of human MRGPRX2 [38]. Previous studies showed that SP activates murine MCs to cause degranulation and inflammatory responses via MrgprB2 [3,38]. Of note, there are significant differences in agonist affinities between mouse MrgprB2 and human MRGPRX2 receptors. While SP activates MRGPRX2 with an EC50 of 152 nM, it activates MrgprB2 with a higher EC50 value of 54 µM [38]. To investigate the biological role of β-arrestin2 on SP/MrgprB2-mediated MC responses, we utilized peritoneal MCs (PMCs) obtained from wild-type (WT) and β-arrestin2 knockout (βArr2−/−) mice [39]. Absence of β-arrestin2 had no effect on the development and maturation of MCs as shown by similar levels of cell surface FcεRI and c-Kit expression (Figure 4-3;A). However, degranulation in response to SP was significantly enhanced in PMCs generated from βArr2−/− mice when compared to cells obtained from WT mice (Figure 4-3;B). These findings suggest that β-arrestin2 expressed in MCs contributes to MrgprB2 desensitization in response to SP.

4.4.4. Mutation of a highly conserved tyrosine residue of MRGPRX2 (Y279A) abolishes SP-induced β-arrestin recruitment.

The highly conserved NPxxY motif is important for GPCR activation and regulation (78, 79). Consistent with the findings in other GPCRs (79, 151), the tyrosine residue located in the NPxxY motif of MRGPRX2 (Tyr279) has been previously shown to be essential for both SP-induced Ca2+ mobilization and degranulation (104). We sought to determine whether this residue also mediates β-arrestin signaling. Since we demonstrated that SP induced β-arrestin pathway independently of G proteins, our initial hypothesis was that it might not contribute to the β-arrestin signaling. To assess this, we first constructed Y279A mutant in the MRGPRX2-Tango plasmid and generated transient transfectants in HTLA cells. We found that Y279A mutant showed similar level of cell surface expression as the WT receptor (Figure 4-4;A). Surprisingly, however, Y279A
mutation resulted in complete loss of β-arrestin-mediated gene expression in response to SP (Figure 4-4;B).

One limitation of TANGO assay is that it measures β-arrestin-mediated gene expression after an overnight stimulation with agonist, thus not reflecting the typical β-arrestin recruitment which occurs rapidly within minutes (152). Therefore, we utilized a green fluorescent protein-tagged β-arrestin2 plasmid (βArr2-GFP) to detect changes in SP-induced β-arrestin recruitment by confocal microscopy. βArr2-GFP were co-expressed transiently with either WT-MRGPRX2 or Y279A mutant in RBL-2H3 cells. As shown in Figure 4-4;C, SP caused a rapid translocation (within a minute) of βArr2-GFP from cytoplasm to membrane in RBL-2H3 cells expressing the WT receptor. By contrast, this response was not observed in cells expressing Y279A mutant. These findings indicate that mutation of a highly conserved tyrosine residue of MRGPRX2 is required for SP-mediated β-arrestin recruitment.

4.4.5. Tyrosine residue in MRGPRX2 (Y279) is required for SP-mediated receptor internalization.

We next examined the effect of Y279A mutation on SP-induced MRGPRX2 internalization. HTLA cells expressing the WT-MRGPRX2 exhibited reduced cell surface receptor expression following SP stimulation as determined by flow cytometry, whereas SP was unable to trigger receptor internalization in cells expressing Y279A mutant (Figure 4-5;A,B).

We next performed immunofluorescence study to confirm and visualize the MRGPRX2 receptor internalization in RBL-2H3 cells. The differential labeling of cell-surface and internalized receptors technique was used (148). For this, MRGPRX2 expressed on cell surface were labeled with 1° antibody (unconjugated anti-MRGPRX2 antibody). Then, the receptor internalization was initiated by stimulating with SP for 30 min. The remaining surface receptors were labeled with Alexa Fluor 647-conjugated 2° antibody (red). Cells were then permeabilized and the internalized receptors were labeled with Alexa Fluor 488-conjugated 2° antibody (green) (Figure 4-6;A). We found that following SP stimulation, cell surface expression of WT-MRGPRX2 was significantly reduced and displayed punctate pattern, while there was an increase number of internalized
receptor, as indicated by an increased green signal. On the contrary, cells expressing Y279A mutant did not display receptor internalization and the expression of cell surface remained unchanged (Figure 4-6;B). Taken together, these findings indicate that the conserved tyrosine residue in NPxxY motif of MRGPRX2 plays an important role on the receptor regulation and internalization upon stimulation by SP.

4.5. Discussion

Activation of MCs by SP has been implicated in the pathogenesis of neurogenic inflammation, pain, and itch via MRGPRX2 and its mouse counterpart MrgprB2 (13, 15, 153). In addition to G proteins, most GPCRs have been shown to interact with β-arrestins for receptor desensitization and internalization. MRGPRX2 can undergo β-arrestin-mediated internalization in response to some, but not all agonists. For example, compound 48/80 and codeine act as balanced agonists for MRGPRX2 that activate both G proteins and β-arrestins, and thus can cause receptor internalization, which is associated with functional receptor desensitization (120, 139). On the contrary, host defense peptides (HDPs), such as cathelicidin LL-37 and angiogenic peptide AG-30/5C, activate only G proteins, but not β-arrestins (G protein-biased), and do not induce receptor desensitization and internalization (46, 120, 140). Biased signaling has gained important therapeutic implications for several GPCRs (80). However, whether SP is balanced or biased agonist for MRGPRX2 remains unknown. In this study, we demonstrated that SP serves as a balanced agonist for MRGPRX2 and that Tyr7x53 both contributes to G protein-dependent signaling for degranulation and promotes β-arrestin recruitment and MRGPRX2 internalization. Furthermore, β-arrestin2 negatively regulates MrgprB2-mediated MC degranulation in response to SP. Thus, targeting Tyr7x53 and β-arrestin2 could provide novel therapeutic modalities for modulating SP/MRGPRX2-mediated inflammatory diseases.

Based on the analysis of common activation pathway in 234 structures from 45 class A GPCRs, several conserved residues and key motifs have been identified that are involved in receptor activation and regulation (78). Of these, the NPxxY sequence at the cytoplasmic end of the TM7 domain is one of the most highly conserved motifs among class A GPCRs. A study by
Venkatakrishnan et al. suggested that Tyr\textsuperscript{7x53} in the NPxxY motif is essential for class A receptor activation and regulation (79). We previously demonstrated that substitution of the corresponding tyrosine residue (Tyr\textsuperscript{279}) in MRGPRX2 to Ala (Y279A) diminished SP-induced Ca\textsuperscript{2+} mobilization and degranulation responses (104). Besides G protein signaling, mutations in the NPxxY motif of \(\alpha_{1B}\)-adrenergic and \(\beta_2\)-adrenergic receptors have been associated with diminished agonist-mediated \(\beta\)-arrestin recruitment (154). Here, we found that \(\beta\)-arrestin recruitment following SP stimulation was abolished in Y279A mutant when compared with the WT receptor, indicating that this tyrosine residue is also important for \(\beta\)-arrestin-mediated signaling via MRGPRX2.

The NPxxY motif has also been suggested as a common endocytic motif for GPCRs. The role of the tyrosine residue in the highly conserved NPxxY motif in receptor endocytosis and regulation has been established in certain GPCRs, including \(\beta_2\)-adrenergic receptor (143), \(N\)-formyl peptide receptor (145), and NK-1R (146). For example, mutation of the corresponding tyrosine residue of the \(\beta_2\)-adrenergic receptor to alanine (Y326A) abolishes agonist-induced receptor phosphorylation, internalization, and desensitization (143, 155). Similarly, formyl peptide receptor mutation Y301A results in a complete loss of agonist-induced receptor internalization (145). However, while this conserved tyrosine residue appears to be essential for agonist-induced receptor internalization of some GPCRs, it is not required for the internalization of angiotensin II receptor (156, 157) or gastrin-releasing peptide receptor (158). Thus, it is possible that the role of a highly conserved tyrosine residue in the NPxxY motif on agonist-induced receptor internalization is receptor specific. Here, we found that MRGPRX2 Y279A mutation impaired receptor internalization in response to SP and caused retention of receptors at the cell surface. These findings suggest that the tyrosine residue in the NPxxY motif of MRGPRX2 is important for SP-induced receptor internalization. It is uncertain whether Tyr\textsuperscript{279} affects MRGPRX2 phosphorylation, and thus contributes to receptor internalization. It will be important to further investigate the effect of this tyrosine residue and NPxxY motif on the receptor phosphorylation. Furthermore, the mechanistic pathway responsible for MRGPRX2 internalization should be further identified.
It is worth noting that, in this study, we mostly utilized HTLA and RBL-2H3 cell lines transfected with human MRGPRX2 to investigate the effects of SP on MRGPRX2 regulation. While recent evidence demonstrates that ectopically expressed MRGPRX2 retains its authentic mechanisms for MC activation and degranulation as of human MCs endogenously expressed MRGPRX2 (150), further studies in human-derived MCs are warranted to confirm these findings.

In summary, this study extends our previous observations on the pivotal role of tyrosine residue Tyr\textsuperscript{279} in the NPxxY motif of MRGPRX2 on G protein activation (104). The data presented herein demonstrate the novel finding that this single Tyr\textsuperscript{279} residue contributes to both G protein and β-arrestin signaling in response to SP. Substitution of Tyr\textsuperscript{279} to alanine (Y279A) abolishes SP-induced MC degranulation, β-arrestin recruitment, and MRGPRX2 internalization. This tyrosine residue may contribute to the regulation of MRGPRX2 activation and internalization, presumably by maintaining receptor conformation, thus controlling G protein coupling and activation as well as the internalization process. These findings have an important clinical implication as individuals harboring this mutation may become resistant to developing neurogenic inflammation and inflammatory diseases.

4.6. Acknowledgements

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Figure 4-1. Substance P (SP) is a balanced agonist for MRGPRX2. (A) HTLA cells stably expressing MRGPRX2 (HTLA-MRGPRX2) were exposed to different concentrations of SP for 16 h, and β-arrestin-mediated gene expression was determined. (B) Rat basophilic leukemia (RBL) cells stably expressing MRGPRX2 (RBL-MRGPRX2) were stimulated with different concentrations of SP for the times indicated, and level of receptor internalization was determined by flow cytometry. (C) RBL-MRGPRX2 cells were cultured in the absence (untreated) or presence of SP (30 µM) for 16 h. Cells were subsequently stimulated with different concentrations of SP for 30 min, and percent degranulation was determined by β-hexosaminidase release assay. All data points are the mean ± SEM of at least three experiments. For comparisons of two samples, two-tailed unpaired t-test was used. For comparisons of multiple samples to a control group, one-way analysis of variance (ANOVA) with Dunnett's post-hoc test was used. *** p < 0.001 and **** p < 0.0001.
Figure 4-2. Pertussis toxin (PTx) inhibits SP-induced mast cell (MC) degranulation, but has no effect on β-arrestin recruitment and MRGPRX2 internalization. RBL-MRGPRX2 cells were cultured in the absence or presence of PTx (100 ng/ml, 16 h), and the effects of SP on (A) degranulation, (B) β-arrestin-mediated gene expression, and (C) MRGPRX2 internalization were determined. All data points are the mean ± SEM of at least three experiments. Statistical significance was determined by two-tailed unpaired t-test. **** p < 0.0001.
Figure 4-3. β-arrestin2 regulates MrgprB2-mediated MC degranulation in response to SP. (A) Peritoneal MCs (PMCs) obtained from wild type (WT) and βArr2−/− mice displayed similar levels of surface c-Kit and FcεRI expression as determined by flow cytometry. (B) Cells were exposed to either buffer (control) or SP (50 µM) for 30 mins, and β-hexosaminidase release was determined. All data points are the mean ± SEM of at least three experiments performed in triplicate. Statistical significance was determined by two-tailed unpaired t-test. *p < 0.05.
Figure 4-4. Y279A mutation of MRGPRX2 abolishes β-arrestin recruitment in response to SP. (A) HTLA cells were transiently transfected with cDNA encoding either WT-MRGPRX2 or its Y279A mutant, and cell surface receptor expression was determined by flow cytometry using PE-anti-MRGPRX2 antibody. (B) Cells were stimulated with SP (30 µM) for 16 h, and β-arrestin-mediated gene expression was measured. All data points are the mean ± SEM of at least three experiments. Two-tailed unpaired t-test was used. ** p < 0.01. (C) RBL cells co-expressing WT MRGPRX2 or Y279A and βAr2-GFP were stimulated with SP (30 µM) for 1 min and β-arrestin translocation was investigated by confocal microscopy. Scale bar = 10 µm.
Figure 4-5. Y279A mutation of MRGPRX2 impairs SP-induced receptor internalization. (A) HTLA transiently expressing WT-MRGPRX2 or its Y279A mutant were stimulated with SP (30 µM) for 30 min, and the receptor internalization was determined by flow cytometry. Representative histogram for cell surface receptor expression before (black line) and after SP stimulation (blue line) are shown. (B) The percentage of receptor internalization after SP stimulation was calculated. All data points are the mean ± SEM of at least three experiments. Two-tailed unpaired t-test was used. **p < 0.001.
Figure 4-6. Y279A mutation of MRGPRX2 displays resistance to SP-induced receptor trafficking. (A) Schematic showing the dual-color labeling of cell surface and internalized receptors (modified from Carrodus et al., 2014 (148)). (B) Change in receptor trafficking was observed in RBL-2H3 cells expressing WT MRGPRX2 and Y279A mutant after SP stimulation (30 µM; 30 min). Scale bar = 10 µm.
CHAPTER 5: Conclusion and Future Directions

In addition to the classical IgE-mediated activation, human MCs also respond to a diverse group of basic secretagogues, including host defense peptides (HDPs), neuropeptides (NPs), and a number of FDA-approved peptidergic drugs, via the newly identified MRGPRX2 receptor (mouse ortholog MrgprB2) (10). Recent studies have greatly emphasized the role of MRGPRX2 in both health and disease, ranging from host defense and wound healing to drug-induced pseudoallergic reactions, neurogenic inflammation, pain and itch (10, 13, 14, 50, 159). However, some studies have doubted the role of MRGPRX2 on neuromuscular blocking drug (NMBD)-induced hypersensitivity, especially for rocuronium (51, 57, 58, 106, 160). Furthermore, the molecular mechanisms involved in the regulation of MRGPRX2/B2 function remain largely unknown.

In CHAPTER 2, we addressed the discrepancy regarding the biological relevance of MRGPRX2 on rocuronium-induced hypersensitivity. Our study provided the first demonstration that rocuronium can induce degranulation in human MCs via MRGPRX2 and that the ability of rocuronium to induce degranulation depends on the level of MRGPRX2 expression. It is worth noting that the minimal concentration of rocuronium needed to stimulate human MC degranulation is significantly higher than that required for mouse MrgprB2 activation. These distinct differences between MrgprB2 and MRGPRX2 demonstrate the challenge in translating animal studies into humans. This highlights the need to develop better animal models to study MRGPRX2 function in vivo.

Furthermore, we tested the possibility whether MRGPRX2 mutations (M196I, L226P, and L237P) reported in a patient with rocuronium-induced perioperative anaphylaxis render the receptor more susceptible to rocuronium hypersensitivity (161). We found that cells expressing the mutated receptors (M196I, L226P, and L237P) either respond normally to rocuronium or display loss-of-function phenotype. These findings do not support the role of MRGPRX2 on rocuronium hypersensitivity in this patient. Given that MRGPRX2 is expressed predominantly in human skin MCs, it is plausible that erythema observed at injection site in this patient likely reflects normal degranulation process unrelated to anaphylaxis. On the other hand, anaphylaxis might be a result
from IgE/FcεRI-mediated activated of MCs that are present in other locations such as the gastrointestinal and respiratory tracts. However, it is worth noting that although MRGPRX2 expression is usually low in normal lung and colon MCs, it is upregulated in individuals with severe asthma and ulcerative colitis. Furthermore, MRGPRX2 expression is upregulated in cutaneous MCs of patients with skin inflammatory diseases, such as chronic spontaneous urticaria. Thus, it is possible that in those particular individuals, MRGPRX2 may also contribute to drug-induced pseudoallergy.

In CHAPTER 3 and 4, we delineated the mechanisms underlying MRGPRX2 signaling and regulation on SP-activated MC responses. It is now realized that SP released from sensory neurons also activate cutaneous MCs via MRGPRX2 to induce degranulation and chemokine/cytokine generation, contributing to neurogenic inflammation, pain, and itch. Thus, targeting MRGPRX2 and its signaling might therefore represent novel therapeutic approaches for the prevention and/or treatment of neurogenic inflammatory diseases and pain. In CHAPTER 3, we focused on the MRGPRX2-G protein interaction, while CHAPTER 4 the receptor-β-arrestin2 interaction was investigated.

First, we identified the specific G proteins that couple to MRGPRX2 in response to MC activation by SP. Previous studies on the mechanisms of MRGPRX2-mediated MC responses demonstrated that PTx inhibits MC degranulation in response to MRGPRX2 agonists, including compound 48/80, cathelicidin LL-37 and human β-defensin-3, but not calcium mobilization. These findings lead to the possibility that MRGPRX2 couple to both PTx-sensitive (Gαi) and insensitive (Gαq) families of G proteins, in order to induce human MC activation. To test if SP activates MRGPRX2 via both Gαi and Gαq to induce Ca^{2+} mobilization and MC degranulation, I utilized a pharmacological approach using a Gαi-specific inhibitor (PTx) and a Gαq-specific inhibitor (YM-254890). Using PTx or YM-254890 alone caused partial inhibition of Ca^{2+} mobilization and MC degranulation by SP, but a combination of both inhibitors abolished the responses. These findings suggest that, as expected, MRGPRX2 couples to both Gαi and Gαq families of G proteins to induce Ca^{2+} mobilization and degranulation in response to SP.
Next, the information obtained from both structural modeling (79) and naturally occurring MRGPRX2 missense variants were gathered to identify residues in the TM and intracellular domains of MRGPRX2 that regulate G protein-mediated MC activation by SP. Putative G protein coupling regions have been established. In addition, several gain- and loss-of-function missense single nucleotide polymorphisms (SNPs) in MRGPRX2 have been identified. We found that SNPs in MRGPRX2’s G protein-coupling regions (V123F, V282M, R138C and R141C) failed to activate MC in response to SP. By contrast, MRGPRX2 variants at the carboxyl-terminus that is responsible for receptor phosphorylation and desensitization (S325L and L329Q) showed higher SP-induced responses, presumably by preventing MRGPRX2 phosphorylation (104). The effects of these MRGPRX2 variants on modulating each G protein coupling and activation should be further investigated. Additionally, future studies will need to be conducted to determine impact of these SNPs on MRGPRX2/MC-mediated conditions.

Lastly, we provided the first demonstration that SP serves as a balanced agonist for MRGPRX2 and can induce β-arrestin-dependent signaling. A tyrosine residue in the highly conserved NPxxY motif of MRGPRX2, Tyr^{279}, has been identified as the residue not only important for G protein activation, but also for β-arrestin activation and receptor internalization. These findings altogether have an important clinical relevance as individuals with loss-of-function mutations in MRGPRX2 may display resistance to developing neurogenic inflammation and chronic inflammatory diseases. By contrast, individuals who harbor the gain-of-function variants may be more susceptible to develop these conditions. Determining the structure and understanding the molecular mechanisms of MRGPRX2 activation will not only enhance our fundamental knowledge of MRGPRX2-mediated MC responses, but will also provide great translational potentials for the development of novel therapeutics for SP-induced inflammatory conditions and pain.

Ultimately, future in vivo studies targeting either G protein or β-arrestin signaling of MRGPRX2 should be conducted in the context of pathological conditions. Previous studies in mice demonstrated that SP contributes to neurogenic inflammation through the activation of MCs via MrgprB2 (mouse counterpart of human MRGPRX2) (13). However, MrgprB2 shares only ~53%
sequence identity with MRGPRX2 (9). Thus, in vivo studies using MrgprB2−/− mice may not accurately reflect mast cell biological responses in humans. To overcome the translational challenge of using MrgprB2-modified mice, several approaches of humanized mice with MRGPRX2 expressing human MCs has been utilized to study the MRGPRX2 function (162, 163). Mice expressing human MRGPRX2 variants and/or β-arrestin knockout should be used to investigate the effects of MRGPRX2 signaling modulation on neurogenic inflammation in vivo.

Atopic dermatitis (AD), also known as atopic eczema, is a chronic and relapsing T-helper-2 (Th2)-associated inflammatory skin disease characterized by eczematous lesions and severe pruritus (164). Dysregulation between MC-neuron interaction have been implicated in the pathogenesis of AD (165-167). Recently, Serhan et al. provided a strong evidence that the MrgprB2+ MCs establish functional clusters in the skin with a subpopulation of SP-producing sensory neurons expressing the transient receptor potential cation channel subfamily V number 1 (TRPV1) and Tac1, the gene encoding the SP precursor (TRPV1+ Tac1+ nociceptors) (15). These MC-neuron clusters are required for the development and pathophysiology of AD. Hence, targeting MRGPRX2 and its signaling could be a promising therapeutic modality for the prevention and/or management of chronic itch and inflammation in human AD as well as other diseases mediated by this receptor.

MRGPRX2-expressing MCs are predominantly present in the skin as well as gingiva, and contribute to skin and gingival homeostasis. Our lab has previously shown that patients with severe periodontitis display higher number of MCs and increased MRGPRX2 expression. Oral medicine usually involves the diagnosis and management of patients with orofacial disorders and oral manifestations of systemic diseases. As several oral lesions represent the early/first signs of mucocutaneous disorders, it is possible that MRGPRX2 may be implicated in the pathogenesis/progression of these mucocutaneous disorders and the patients with those conditions may have upregulated MRGPRX2 expression. This putative role of MRGPRX2 on oral mucocutaneous lesions should be further investigated.
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