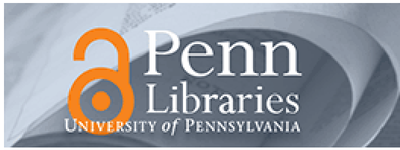


University of Pennsylvania
ScholarlyCommons

Dental Theses

Penn Dental Medicine

Summer 8-4-2017

The Role of Mas-Related G-Protein Receptor X2 (MRGPRX2) on Neuropeptide Induced Reponses in Human Mast Cells

Wichayapha Manorak
University of Pennsylvania School of Dental Medicine, wmanorak@upenn.edu

Follow this and additional works at: http://repository.upenn.edu/dental_theses

Part of the Dentistry Commons

Recommended Citation

Manorak, Wichayapha, "The Role of Mas-Related G-Protein Receptor X2 (MRGPRX2) on Neuropeptide Induced Reponses in Human Mast Cells" (2017). *Dental Theses*. 24.
http://repository.upenn.edu/dental_theses/24

This paper is posted at ScholarlyCommons. http://repository.upenn.edu/dental_theses/24
For more information, please contact repository@pobox.upenn.edu.

The Role of Mas-Related G-Protein Receptor X2 (MRGPRX2) on Neuropeptide Induced Reponses in Human Mast Cells

Abstract

Rationale: Substance P (SP) and hemokinin-1 (HK-1) are neuropeptides (NPs) that promote inflammatory responses by signaling through the neurokinin-1 receptor (NK-1R). Antagonists of NK-1R are highly effective in allergic inflammation and airway hyperresponsiveness in mice but lack efficacy in humans. The reason for this difference is unknown. Human mast cells express both the NK-1R and Mas-related G protein receptor X2 (MRGPRX2) which are both activated by SP. The objective of this study was to determine if HK-1 activates human mast cells via MRGPRX2. Moreover, since MRGPRX2 contains a cholesterol recognition amino acid consensus (CRAC) domain the interaction between MRGPRX2 and cholesterol in lipid rafts likely contributes to MRGPRX2 function. Another objective in this study is to investigate whether lipid rafts are associated with MRGPRX2 function.

Materials and Methods: Flow cytometry was used to determine the expression of NK-1R and MRGPRX2 in a human mast cell line (LAD2). MRGPRX2 function was investigated by using SP and HK-1 to induce degranulation with a selective NK-1R antagonist (CP96345). Methyl-b-cyclodextrin (MbCD) was used for cholesterol depletion in this study. In addition, mutants of the CRAC domain on MRGPRX2 were used to compare the functions of the receptor on degranulation and Ca^{2+} mobilization in response to SP, HK-1, and other compounds (ciprofloxacin, HOE 140 and compound 48/80). Confocal microscopy was used to determine the localization of lipid raft compartments and MRGPRX2.

Results: LAD2 cells expressed both MRGPRX2 and NK-1R. HK-1 and SP induced degranulation in LAD2 cells and RBL-2H3 cells stably expressing MRGPRX2, but this response was resistant to inhibition by an NK-1R inhibitor (CP96345). However, SP and HK-1 induced degranulation in RBL cells transiently expressing NK-1R and this response was inhibited by CP96345. Depleting cholesterol by using MbCD decreased the degranulation response. Furthermore, confocal microscopy showed that the lipid rafts and MRGPRX2 colocalized in both WT and the CRAC domain mutants. However, CRAC domain mutants of MRGPRX2 did not respond to SP in Ca^{2+} mobilization and degranulation assays.

Discussion: This study provides a potential explanation for the previous observation that NK-1R antagonist are highly effective in allergic responses in mice but fails in human. Our findings suggest that unlike the situation in mice where the effects of neuropeptides are mediated via NK-1R, these effects are mediated by MRGPRX2 in humans. Moreover, lipid rafts may not be associated with MRGPRX2 function. However, the CRAC domain mutant appears to be defective in coupling G protein.

Conclusion: This finding suggests that MRGPRX2 may serve as a novel target for modulating asthma and other neuropeptide/mast cell-mediated diseases.

Degree Type
Thesis

Degree Name
MSOB (Master of Science in Oral Biology)

This thesis is available at ScholarlyCommons: http://repository.upenn.edu/dental_theses/24

Primary Advisor
Dr. Hydar Ali

Keywords
Neuropeptide, SP, HK-1, MRGPRX2, human mast cell, lipid rafts

Subject Categories
Dentistry

This thesis is available at ScholarlyCommons: http://repository.upenn.edu/dental_theses/24

University of Pennsylvania Dental Medicine

The role of Mas-related G-protein coupled receptor X2 (MRGPRX2) on neuropeptide induced responses in human mast cells

THESIS

Presented to the Faculty of Penn Dental Medicine in Fulfillment of the Requirements for the Degree of Master of Science in Oral Biology

Hydar Ali, PhD
Principal Thesis Advisor

Gary H. Cohen, PhD
Thesis Committee Member

Panagiota Stathopoulou, PhD
Thesis Committee Member

Yan Yuan, PhD
Thesis Committee Member

Acknowledgement

I would like to express my appreciation to my advisor, Dr. Hydar Ali, for the honorable opportunity to work in this laboratory. His initiation and creativity greatly contributes to my work.

I would also like to thank all of the members of Dr. Ali's lab especially Dr. Kshitij Gupta for his dedication, patience, and engagement that contributed greatly to my progress.

I am grateful to all of the faculty and staff of the University of Pennsylvania School of Dental Medicine Periodontics Department, particularly Dr. Dana T Graves and Dr. Panagiota Stathopoulou for their encouragement and support throughout this program.

Finally, I would like to express my appreciation to the MSOB committee members, Dr. Gary H. Cohen, Dr. Yan Yuan and Dr. Panagiota Stathopoulou for providing encouragement and valuable guidance during this project.

I would like to thank the PDM flow cytometry and confocal microscopy core facilities for their assistance with this project. This project was funded by NIAID grant # R01-AI-124182.

Abstract

Rationale: Substance P (SP) and hemokinin-1 (HK-1) are neuropeptides (NPs) that promote inflammatory responses by signaling through the neurokinin-1 receptor (NK-1R). Antagonists of NK-1R are highly effective in allergic inflammation and airway hyperresponsiveness in mice but lack efficacy in humans. The reason for this difference is unknown. Human mast cells express both the NK-1R and Mas-related G protein receptor X2 (MRGPRX2) which are both activated by SP. The objective of this study was to determine if HK-1 activates human mast cells via MRGPRX2. Moreover, since MRGPRX2 contains a cholesterol recognition amino acid consensus (CRAC) domain the interaction between MRGPRX2 and cholesterol in lipid rafts likely contributes to MRGPRX2 function. Another objective in this study is to investigate whether lipid rafts are associated with MRGPRX2 function.

Materials and Methods: Flow cytometry was used to determine the expression of NK-1R and MRGPRX2 in a human mast cell line (LAD2). MRGPRX2 function was investigated by using SP and HK-1 to induce degranulation with a selective NK-1R antagonist (CP96345). Methyl-β-cyclodextrin (MβCD) was used for cholesterol depletion in this study. In addition, mutants of the CRAC domain on MRGPRX2 were used to compare the functions of the receptor on degranulation and Ca^{2+} mobilization in response to SP, HK-1, and other compounds (ciprofloxacin, HOE 140 and compound 48/80). Confocal microscopy was used to determine the localization of lipid raft compartments and MRGPRX2.

Results: LAD2 cells expressed both MRGPRX2 and NK-1R. HK-1 and SP induced degranulation in LAD2 cells and RBL-2H3 cells stably expressing MRGPRX2, but this

response was resistant to inhibition by an NK-1R inhibitor (CP96345). However, SP and HK-1 induced degranulation in RBL cells transiently expressing NK-1R and this response was inhibited by CP96345. Depleting cholesterol by using MβCD decreased the degranulation response. Furthermore, confocal microscopy showed that the lipid rafts and MRGPRX2 colocalized in both WT and the CRAC domain mutants. However, CRAC domain mutants of MRGPRX2 did not respond to SP in Ca^{2+} mobilization and degranulation assays.

Discussion: This study provides a potential explanation for the previous observation that NK-1R antagonist are highly effective in allergic responses in mice but fails in human. Our findings suggest that unlike the situation in mice where the effects of neuropeptides are mediated via NK-1R, these effects are mediated by MRGPRX2 in humans. Moreover, lipid rafts may not be associated with MRGPRX2 function. However, the CRAC domain mutant appears to be defective in coupling G protein.

Conclusion: This finding suggests that MRGPRX2 may serve as a novel target for modulating asthma and other neuropeptide/mast cell-mediated diseases.

Table of contents

Acknowledgement	**2**
Abstract	**3**
Table of Contents	**5**
List of abbreviation	**6**
Chapter 1 Introduction	**8**
Chapter 2 Objective	**17**
Chapter 3 Materials and Methods	**18**
Chapter 4 Results	**23**
Chapter 5 Discussion	**26**
Chapter 6 Conclusion	**30**
Chapter 7 Figures	**31**
Chapter 8 Figures legend	**44**
Chapter 9 Bibliography	**47**

List of Abbreviations

1. AMPs	Antimicrobial peptide
2. Anti-Tac1r	Anti-neurokinin receptor 1 antibody
3. CNS	Central Nervous System
4. CRAC	Cholesterol Recognition Amino Acid Consensus
5. CST	Cortistatin
6. DMEM	Dulbecco's Modified Eagle's Medium
7. FBS	Fetal Bovine Serum
8. FcεIR	High-affinity receptor for the Fc region of immunoglobulin E (IgE)
9. GPCRs	G protein coupled receptors
10. hBDs	Human β-defensins
11. HK-1	Hemokinin-1
12. IgE	Immunoglobulin E
13. IL-6	Interleukin-6
14. MβCD	Methyl-β-cyclodextrin (cholesterol depletion)
15. MCDP	Mast Cell Degranulating peptide
16. MCs	Mast cells
17. MC_T	Mast cells contain only tryptase
18. MC_{TC}	Mast cells contain both tryptase and chymase
19. MHC	Major Histocompatibility Complex
20. MRGPRs	Mas-Related G Protein Coupled Receptors
21. MRGPRX2	Mas-Related G Protein Coupled Receptor X2
22. NK-1R	Neurokinin-1 Receptor

23. NKs	Neurokinins
24. NPs	Neuropeptides
25. PNS	Peripheral Nervous System
26. RBL-2H3	Rat Basophilic Leukemia
27. SP	Substance P
28. SPR	Substance P receptor
29. TACR1	Tachykinin receptor-1
30. VIP	Vasoactive intestinal polypeptide

Chapter 1

Introduction

The immune system is the host defense response which is comprised of innate and adaptive immunity. Innate immunity (non-specific) recognizes and destroys the foreign antigen to protect cells and then adaptive immunity (specific) which is mediated by B and T cells, comes after to create immune memory. When there are injuries or foreign antigens enter the human body, the immune system will create an inflammatory response.

1. Mast Cells (MCs)

MCs are multifunctional immune cells that are important in allergic and inflammatory diseases including anaphylaxis and asthma [1, 2].

MCs development and differentiation

MCs are leukocytes which are derived from hematopoietic progenitor cells [3]. The hematopoietic lineage for MCs development differs from other myeloid-derived cells in that MCs leave the bone marrow as progenitors rather than as circulating end-stage cells [4]. They circulate in the immature form in the blood stream before migrating to vascularized tissues, where they undergo final differentiation and maturation with the help of stem-cell factor and other cytokines secreted by endothelial cells and fibroblasts [5].

After their development from bone marrow-derived progenitor cells that are primed with stem cell factor, MCs continue their maturation and differentiation in peripheral tissue, developing into two well-described types: MC_T and MC_{TC} cells [6]. These cells can also be distinguished on the basis of their tissue location, dependence on T lymphocytes protease composition and their secretory granule contents. In human,

most MCs are MC_{TC} which are found in connective tissues such as the skin and contain both tryptase and chymase [7]. In contrast to MC_T which are found in lung and contain only tryptase. In mice, connective tissue MCs resemble MC_{TC} cells while mucosal MCs resemble MC_T cells [8].

MCs functions

MCs are best known for their role in responding to a mediating allergic disease [3]. They are critical regulators of innate immunity and play important roles in allergic and hypersensitive disease [3, 4, 9, 10], regulating inflammation, host defense, and innate immunity. MCs are one of the first immune cells to interact with pathogens. They are also involved in maintaining a healthy physiology by promoting innate immunity, angiogenesis, and wound healing [11].

By virtue of their location and mediator production, MCs may play an active role in many diseases, such as allergy, parasitic diseases, atherosclerosis, malignancy, asthma, pulmonary fibrosis, and arthritis [3, 10]. Recent data shows that MCs play a vital role in host defense against pathogens by secretion of tumor necrosis factor alpha. MCs also express the Toll-like receptor, which may further accentuate their role in the immune-inflammatory response [10]. MCs also promote the initiation and generation of the adaptive immune response by expressing both major histocompatibility complex (MHC) class I and class II proteins by which they present antigens to T cells [12-14].

MCs activation and mediator production

MCs can be activated by antigens/allergens, superoxides, complement proteins, NPs, and lipoproteins. After activation, MCs express mediators such as histamine, leukotrienes, and prostanoids, as well as proteases, and many cytokines and chemokines

[10]. Mediators such as histamine and leukotriene can induce bronchoconstriction, mucus secretion, and mucosal edema which are all features of asthma.

MCs are the participants in allergic inflammation, expressing a potent array of inflammatory mediators and large numbers of the high affinity IgE receptor (FcεIR) [15]. IgE-dependent activation of MCs leads to the secretion of mediators. However, MCs can also be activated to perform important effector and immunomodulatory functions by mechanisms that are independent of IgE, and amount of the of stimulus can determine the kinetics, amounts and/or spectrum of mediators that are released [6].

The activation of mast cells is via stimulation of the FcεIR. First, IgE binds to the FcεIR on tissue MCs. The cross-linking of bound IgE to FcεRI by allergen then triggers MC degranulation [7, 16]. This results not only in the release of performed MC mediators, such as histamine and tryptase, but also in the synthesis and release of newly generated lipid mediators [15].

MCs and the nervous system

MCs are found localized near nerve endings at several organs and organ systems including the skin, lungs, intestinal mucosa, and central nervous system. Histamine, tryptase, and serotonin released from MCs have an impact on the activity of sensory neurons, and conversely, MCs are activated by NPs such as SP which are released by terminal nerve endings of sensory neurons [17]. SP plays a key role in stress response, neurogenic inflammation, and pain [17, 18]. Human MCs have been shown to degranulate and release mediators in response to SP [19-21]. Other NPs, which activate MCs include vasoactive intestinal polypeptide (VIP) and neuropeptide Y [20, 22, 23]. A very rich supply of sensory nerve endings is found on the skin, which releases SP and

other NPs leading to MC degranulation in response to the continuous exposure to physical as well as emotional stimuli. Therefore, the interaction between MC and NPs results in the promotion of MC-driven inflammation and granulocyte infiltration [18, 24].

2. MRGPRX2

G-Protein Coupled Receptors (GPCRs) are the largest and most diverse family of transmembrane proteins. They are activated by a wide variety of stimuli including biogenic amines peptides, bioactive intracellular signaling events [25, 26]. Mas-Related G-Protein Coupled Receptors (MRGPRs) belong to the GPCR family and are divided into several subfamilies. The MRG gene family has 32 murine and 4 human gene (MRGPRX1-MRGPRX4) [27]. Not only is MRGPRX2 a member of the Mas-related gene that is primarily expressed in human dorsal root ganglia and mast cells but, MRGPRX2 is a receptor expressed on human skin mast cells. Human mast cell line (LAD2) express MRGPRX2 responsive to LL-37 [2].

MRGPRX2 has been shown to function as the non-selective, high affinity binding site for ligands such as Substance P (SP), cortistatin (CST), somatostatin, mast cell degranulating peptide (MCDP), neuropeptide Y, compound 48/80 and VIP [2, 17, 25, 28]. Although these peptides are structurally unrelated, they are all amphipathic small peptides, which induce dose-dependents degranulation in human MCs via the activation of MRGPRX2. This induction is associated with an increase in the intracellular Ca^{2+} concentration [25, 28].

A unique feature of MRGPRX2 is that it is activated by antimicrobial peptides (AMPs) such as the cathelicidin, LL-37, human β-defensins (hBDs), and NPs such as SP which is produced by mast cells, lung epithelial cells and nerve endings. However,

MRGPRX2 is not expressed in mice. Therefore, many of the peptides that induce degranulation in human MCs via MRGPRX2 do not activate murine MCs.

Subramanian et al. also showed that hBDs and the cathelicidin LL-37 activate human MCs via MRGPRX2 [29, 30]. In their study, LL-37 and the Cortistatin induced sustained MRGPRX2 functions as a non-selective binding site that links these basic peptides to G proteins, leading to the secretion of histamine and other mediators by MCs [31].

3. Neurokinin-1 Receptor (NK-1R)

The protein is the product of the *TACR1* gene [32]. Tachykinin receptor 1 (TACR1), also known as neurokinin-1 receptor (NK-1R) or SP receptor (SPR), is a GPCR found in the central and peripheral nervous systems. The endogenous ligand for this receptor is SP, although it has some affinity for other tachykinins. MCs express the NK-1R which can respond to antigens with immune function and leads to inflammation and allergic reactions via IgE and non-IgE-mediated mechanism [33].

4. Neuropeptides (NPs)

SP and HK-1 are NPs in the tachykinin family that are amplified inflammatory response [34] by signaling through the NK-1R [35]. In addition to FcεRI, MCs express numerous GPCRs, which are the most common targets of drug therapy. SP is released from the peripheral nerve endings of sensory neurons. While the newly NPs, HK-1 is the only tachykinin peptide that is produced outside the neuronal tissue [36]. A recent study by Sumpter et al., [37] showed that FcεRI activation of murine BMMCs results in enhanced expression of both HK-1 and NK-1R without modifying SP levels. Furthermore, it was found that FcεRI-mediated MC degranulation and TNF/IL-6

production is substantially inhibited in NK-1R$^{-/-}$ BMMCs when compared to wild-type MCs. These findings suggest that HK-1, via its action on NK-1R, acts as an autocrine/paracrine factor for FcεRI-mediated MC mediator release. As described below, the HK-1/NK-1R axis has a profound impact on IgE-mediated experimental anaphylaxis and MC-dependent model of chronic atopic airway inflammation. These are important feature of asthma [38].

5. Role of MRGPRX2 in asthma

There are differences between the mechanisms through which NPs activate murine and human MCs. Although normal human lung MCs are the MC_T type, severe asthma is dominated by the presence of MC_{TC} in the airway submucosa and epithelium. It is now well documented that MC_{TC} type MCs express MRGPRX2, which is activated by SP.

Fujisawa et al. [39] showed that skin MC_{TC} express MRGPRX2 and that its expression is upregulated in patients with severe chronic urticaria. They also showed that isolated human lung mast cells (MC_T) do not express cell surface MRGPRX2. Another study was done by Balzar et al. which showed MC phenotype, location, and activation in severe asthma. They found that there is a shift in phenotype from MC_T in normal lung to MC_{TC} in severe asthma. This study led to the study by Idahosa et al. [40] in the title of "MRGPRX2 in health and disease". They used immunohistochemistry and immunofluorescence techniques to determine the expression of MRGPRX2 in normal and diseased human tissue. They analyzed control samples as well as skin, gingiva and lung samples from patients with atopic dermatitis, rosacea, chronic periodontitis and asthma. It was found that MCs in healthy and diseased skin, gingiva and lungs express MRGPRX2. There was a significant increase in number of MCs, MRGPRX2-positive

cells, as well as MRGPRX2-positive MCs in asthmatic lung tissue compared to healthy lung tissue. Idahosa et al. concluded that expression of MRGPRX2 is upregulated in chronic asthma, suggesting that participates in the pathogenesis of asthma.

6. Murine and human MCs

Sumpter et al. [37] showed that the HK-1 and its cognate GPCR, NK-1R are upregulated in FcεIR-activated murine bone marrow-derived MCs. *In vivo* studies demonstrated that the autocrine HK-1 acts via NK-1R on MCs and functions as an adjuvant for IgE-medicated anaphylaxis and lung inflammation in a mast cell-dependent model of chronic asthma. However, previous studies showed that while NK-1R antagonists block experimental allergic responses in mice, they lack efficacy in humans. This discrepancy can be challenge in translating findings from animal models to the clinic. NK-1R not only participates in experimental allergic responses as discussed above, but it also plays an important role in human disease. However, a number of NK-1R antagonists, which are highly effective in animal models of allergic asthma and inflammation lack efficacy in humans [41-44].

7. Lipid Raft

Cell membranes are phospholipid bilayers, crowded with proteins occupying around 20% of the bilayer area. Membrane proteins alter their lipid environment not only by binding specific lipids but also by influencing their surrounding lipid environment [45]. Lipid rafts can be found in all eukaryotic cells. They are compose of high levels of cholesterol, sphingolipids, and gangliosides [46-48]. Lipid rafts are highly organized and probably exist in a liquid-ordered phase which is different from the rest of the plasma membrane that consists mainly of phospholipids in a liquid-disordered phase.

Lipid raft are implicated in the function of diverse signaling pathways such as those mediated by growth factors, morphogens, integrins, and antigen receptors on immune cells including mast cells. The structural basis for the association of FcεRI with lipid rafts is partially understood and appears to involve the transmembrane segments of FcεRI.

8. Cholesterol recognition amino acid consensus (CRAC)

Cholesterol is a component of cell membranes, which modulates the physical state of membrane phospholipid bilayers, including membrane fluidity and membrane permeability [49]. At the cell membrane interface, there are segments of integral membrane proteins that facilitate interactions with cholesterol-binding proteins or that have partitioned into cholesterol-rich domains, characterized by the presence of a **"cholesterol recognition amino acid consensus"** sequence, otherwise known as the CRAC domain which is defined as a sequence pattern, -L/V-(X)(1-5)-Y-$(X)_{(1-5)}$-R/K-, in which $(-X-)_{(1-5)}$ represents between one and five residues of any amino acid [50-52]. Li et al. [50] showed that first protein studied with a CRAC domain was the peripheral-type benzodiazepine receptor now known as the translocator protein (TSPO). The CRAC domain of the TSPO is located at its C-terminus. TSPO transfers cholesterol across the membrane. In this process, the CRAC domain is critical for cholesterol binding [53].

CRAC peptides have been synthesized and investigated in artificial bilayer lipid membranes (BLM). Epand et al. [54] found that the cholesterol-binding peptide, LWYIK, was able to stimulate the formation of cholesterol-rich domains in BLM, which is comprised of phosphatidylcholine and cholesterol. The CRAC domain is a primary structure pattern used to identify regions that may be responsible for preferential cholesterol binding in many proteins [55].

9. CRAC domain in GPCRs

GPCRs are the largest molecule involved in signal transduction across the membrane [56-58]. Membrane cholesterol plays an important role in the function of GPCRs. Several structural features of proteins are involved in cholesterol have been recognized. CRAC sequence represents such a motif. Many proteins that interact with cholesterol have been reported to contain the CRAC motif in their sequence. Interestingly, the function of GPCRs has been previously shown to be dependent on membrane cholesterol [59]. The presence of CRAC domains in GPCRs indicates that interaction of cholesterol with GPCRs could be specific in nature [59-61]. Since MRGPRX2 contains a CRAC domain, the interaction of the receptor with cholesterol in lipid rafts likely contributes to its function.

SP and HK-1 are NPs that promote inflammatory response by signaling through NK-1R. Antagonists of NK-1R are highly effective in allergic inflammation and airway hyperresponsiveness in mice, but lack efficacy in humans. However, the reason for this difference is unknown. The objective of this study was to 1) verify the presence of NK-1R and MRGPRX2 on human MCs and to test if HK-1 activates MCs via MRGPRX2. Since MRGPRX2 contains a CRAC domain, the interaction between the receptor with cholesterol in lipid rafts will likely contribute to receptor function. Finally, this study aims 2) to investigate the role of lipid rafts and the CRAC domain in MRGPRX2 function.

Chapter 2

Objective

There are two objectives of the present study

1) To verify the presence of NK-1R and MRGPRX2 on human MCs and to test if HK-1 activates MCs via MRGPRX2.
2) To investigate the role of lipid rafts and the CRAC domain in MRGPRX2 function.

Chapter 3

Materials and Methods

3.1 Materials

Reagents:

All cell culture reagents were purchased from Invitrogen (Gaitherburg, MD). Amaxa transfection kit (Kit V) was purchased from Lonza (Gaitherburg, MD).

Antibody:

Antibody PE anti-human MRGX2 Clone : K125H4 (Cat:359004) was obtained from BioLegend (San Diego, CA) and Anti-Tac1r (Rabbit polyclonal Anti-Neurokinin Receptor 1 antibody) TA329063 was obtained from Origene (Rockville, MD). Goat anti-rabbit IgG-FITC sc-2012 was obtained from Santa Cruz Biotechnology (Dallas, TX).

Plasmid:

MRGPRX2 plasmid encoding hemagglutinin (HA)-tagged human MrgX2 in pReceiver-MO6 vector was obtained from GeneCopoeia (Rockville, MD). NK-1R plasmid was obtained from cDNA resource center (Bloomsburg, PA). Wild type and CRAC domain of MRGPRX2 (VLWPIWYRCRRPR) was made by single point mutation: Mutant 8 (M8) (VLWPIWPRCRRPR) and Mutant 10 (M10) (VLWPIWYRCRRPA) were obtained from Penn Genomics Analysis Core (Philadelphia, PA).

Inhibitor:

A selective and potent non-peptide NK-1 receptor antagonist (CP 96345) (CAS 132746-6-2) ((2S,3S)-cis-2-(Diphenylmethy)-N-[(2-methoxyphenyl)methyl]-1-

azabicyclo[2.2.2]octan-3-amine) cat# 135911-02-3 was purchased from Chem Cruz (Dallas, TX)

Neuropeptides:

Substance P (SP) and human hemokinin-1 (HK-1) cat# SP-89065-5 was obtained from Alpha diagnostic international (San Antonio, TX)

Cholesterol removal:

Methyl-β-cyclodextrin (MβCD) cat#332615 was purchase from Sigma (St. Louis, MO)

Compound:

Ciprofloxacin cat# 449620050 was purchased from Acros (NJ, USA), HOE 140 was purchased from Anaspec (Fremont, CA) and compound 48/80 cat# 94724-12-6 was purchased from Santa Cruz (Dallas, TX).

3.2 Methods

3.2.1 Cell culture

3.2.1.1 Rat Basophilic Leukemia (RBL-2H3) cells were grown in complete Dulbecco's modified Eagle's medium supplemented (DMEM) containing 10% fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml).

3.2.1.2 LAD2 cells were used to represent human MCs. Cells were maintained in StemPro-34 medium containing nutrient supplements (Invitrogen) supplemented with 2 mM L-glutamine 100 IU/ml penicillin, 100 μg/ml streptomycin, and 100 ng/ml rhSCF. Half of the cell culture medium was replaced weekly with fresh culture medium.

3.2.2 Transfection

MRGPRX2, NK-1R, WT, M8 and M10 – RBL-2H3 cells were detached with 0.05% trypsin-EDTA (1x) was purchased from Gibco by Life Technologies, washed with DMEM, and 10^6 cells were transfected with plasmids encoding HA-tagged MRGPRX2, NK-1R and CRAC mutant plasmid, using the Amaxa nucleofector device and Amaxa kit V according to the manufacturer's protocol. After nucleofection, MRGPRX2 cells were cultured in the presence of G418 (1 mg/ml) and cells expressing equivalent receptors were sorted using an anti-HA-specific antibody 12CA5/fluorescein isothiocyanate-conjugated anti-mouse-IgG and used for studies on degranulation. NK-1R, WT, M8 and M10 cells were seeded into 96 well-plates for studies on degranulation.

3.2.3 Flow Cytometry - analysis of receptor expression

LAD2 and NK-1R (1 x 10^6 cells) were washed twice with cold FACs buffer (PBS containing 2% FBS) and stained with Anti-Tac1r at 4 °C for 30 min. Cells were washed twice with cold FACs buffer and stained with Goat anti-rabbit IgG-FITC sc-2012 at 4 °C for 30 min. Cells were washed twice with cold FACs buffer and fixed in 300 µl of 1.5% formaldehyde. Samples were acquired and analyzed with BD LSR II flow cytometry. MRGPRX2, WT, M8 and M10 cells (1 x 10^6) were washed twice with cold FACs buffer (PBS containing 2% FBS) and stained with Antibody PE anti-human MRGX2 Clone:K125H4 (Cat:359004) at 4 °C for 30 min. Cells were washed twice with cold FACs buffer and fixed in 300 µl of 1.5% formaldehyde, and samples were acquired and analyzed with BD LSR II flow cytometry.

3.2.4 Degranulation

LAD2 cells (1 x 10^4 cells) NK-1R, MRGPRX2**,** WT, M8 and M10 cells (5 x 10^4 cells) were seeded into 96-well plates in a total volume of 50 µl HEPES buffer containing

0.1% BSA (Sigma) in control group and treated with 10 µM of CP 96345 for 10 mins at 37 °C in test group then exposed to 10 µM of HK-1 and 1 µM of SP for 30 mins at 37 °C. Moreover, WT and mutants were exposed to 100 µg/ml of ciprofloxacin, 25 µg/ml of HOE 140 and 10 µg/ml of 48/80 compound for an hour at 37 °C. For 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 for an hour at 37 °C. The reaction was stopped by adding 250 µl of a 0.1 M Na_2HCO_3 buffer and absorbance was measured at 405 nm.

3.2.5 Cholesterol depletion - MRGPRX2 (5×10^4 cells) cells were seeded into 96-well plates in a total volume of 200 µl overnight. Cells were rinsed twice with HEPES buffer containing 0.1% BSA to remove excess medium. 50 µl of MβCD (10 mM) was added for 45 mins in the test group while only HEPES buffer containing 0.1% BSA was added in control group. Cells were rinsed twice times with HEPES buffer containing 0.1% BSA. 45 µl of HEPES buffer containing 0.1% BSA was added to each well and cells were incubated for 5 mins in a 37 °C water bath with constant shaking. Cells were exposed to 1 µM of SP for an hour at 37 °C. Cells were then used for the degranulation assay.

3.2.6 Confocal microscopy to examine localization of lipid raft compartments and MRGPRX2 [62]– MRGPRX2, WT and M8 (1×10^6 cells) were sensitized by SP (1 µM). Cells were then collected and fixed in 4% paraformaldehyde (Sigma-Aldrich). Cells were resuspended in 0.15% triton-X (Sigma-Aldrich) in PBS containing 1% FBS (Invitrogen) and probed with Cholera toxin subunit B, Alexa Fluor 488 conjugate antibody (1:100) (Invitrogen) and Antibody PE anti-human MRGX2 (1:100) for 30 mins

on ice to detect lipid rafts and MRGPRX2. Cells were washed twice with PBS, and then place onto slides and air dried. Slides were sealed with Pro-Long antifade reagent (Invitrogen). Confocal images of cells were obtained using a Plan Fluor 100x oil objective and Nikon ECLIPSE TE200 microscope (Nikon). Ez-C1 Nikon software with a channel-series approach was used to prevent spectral overlap between fluorescent signals.

3.2.7 Calcium mobilization –WT and M8 (1.5 x 10^6 cells) were loaded with 1 μM indo-1 AM (Invitrogen) and 1 μL of IgE for 30 min at room temperature. Cells were washed and resuspended in 1.5 ml of HEPES buffer containing 0.1% BSA, Ca^{2+} mobilization was measure in a Hitachi F-2500 spectrophotometer with an excitation wavelength of 355 nm and an emission wavelength of 410 nm. Mean Ca^{2+} ratios were calculated by averaging the ratios at individual time points following stimulation with 1 μM of SP and 100 ng/ml of DNP/BSA.

Statistical analysis

GraphPad Prism scientific software was used for statistical analysis. Differences in percent of β-hexosaminidase release were evaluated by using unpaired t-Test (2 groups) and Anova-Williams test (multiple comparisons). Moreover, $P<0.05$ was considered statistically significant.

Chapter 4

Results

1. NPs activate human MCs (MRGPRX2 and NK-1R)

- **Human MCs**

Using flow cytometry, we found that LAD2 cells which we used to represent human mast cells express both MRGPRX2 and NK-1R (Fig.1). In this study, the NPs which we have used are HK-1 (10 μM) and SP (1 μM). They induced degranulation in LAD2 cells. We found that in both control and NK-1R antagonist groups. HK-1 and SP induced degranulation: 20-25% and 30-35% β-hexosaminidase release respectively. However, this response was resistant to inhibition by an NK-1R antagonist CP93645 (Fig.2). There are no significant ($P<0.05$) differences between control and NK-1R antagonist group.

- **NK-1R**

To test the effectiveness of NK-1R antagonist by using RBL-2H3 cells transiently expressing NK-1R. Flow cytometry analysis showed that cells were expressing NK-1R (Fig.3). NK-1R responded to both SP and HK-1 for degranulation. When using HK-1, we found that there is 25% β-hexosaminidase release in the control group and only 10% degranulation in the NK-1R antagonist group. For SP, there is 25% β-hexosaminidase release in the control group and 15 % β-hexosaminidase release in the NK-1R group. This shows that CP93645 caused significant ($P<0.05$) inhibition of this response (Fig.4). This NK-1R antagonist has high effectiveness for inhibition.

- **MRGPRX2**

To investigate the role of MRGPRX2 on HK-1 induced mast cell degranulation, we used RBL-2H3 cells stably expressing MRGPRX2. We determined that there is MRGPRX2 expression by using flow cytometry (Fig.5). We found that both SP (1 μM) and HK-1 (10 μM) induced degranulation. In both groups, there is 50% β-hexosaminidase release by using HK-1 and 75-90% β-hexosaminidase release by using SP. However, this response was resistant to inhibition by CP93645 (Fig.6). There are no significant ($P<0.05$) differences between control and NK-1R antagonist group.

2. **NPs induced lipid raft part response in human MCs (MRGPRX2)**

- **Cholesterol depletion**

Recent studies have shown that MRGPRX2 contains the CRAC domain and we know that MβCD depletes cholesterol, which reduces receptor activity. In this study we were using SP (1 μM) to induce degranulation in MRGPRX2. There is 30% β-hexosaminidase release in the control group (without MβCD). However, there is only 15 % β-hexosaminidase release in MβCD group, which suggests that MβCD caused significant ($P<0.05$) inhibition of this response (Fig.7).

- **WT and CRAC mutants**

Flow cytometry showed that receptor expression level of the CRAC mutants (M8 and M10) is greater than 90% (Fig.8 and 9). We found that both SP (1 μM) and HK-1 (10 μM) induced degranulation in the control group (WT) 30-35% β-hexosaminidase release. The degranulation was significantly decreased ($P<0.05$) in mutants in which we only found 10% β-hexosaminidase release (Fig.10). Furthermore, there is 40% β-hexosaminidase release induced by ciprofloxacin (100 μg/ml), HOE 140 (25 μg/ml) and

48/80 (10 μg/ml) in WT. However, there is less than 10% β-hexosaminidase release in mutants which is significantly decreased ($P<0.05$) when compared with control group (Fig.11).

- **The association between lipid rafts compartment and MRGPRX2 by confocal microscopy**

Using PE anti-human MRGX2 antibody, we found that MRGPRX2 present MRGPRX2 in red (Fig.12A) and cholera toxin subunit B, Alexa Fluor 488 conjugate antibody, we found that MRGPRX2 present the lipid raft compartment in green (Fig.12B). Overlay of MRGPRX2 (red) and cholera toxin (green) showed that there is colocalization (orange) (Fig.12C). Similar to how CRAC domain mutant presents the MRGPRX2 (red) (Fig.12D) and lipid raft part (green) (Fig.12E). Overlay of MRGPRX2 (red) and cholera toxin (green) showed that there is colocalization (orange) (Fig.12F).

- **Ca^{2+} mobilization**

To test the CRAC domain mutant defective in coupling to G protein by using Ca^{2+} mobilization. We found that SP (1 μM) resulted in enhanced Ca^{2+} mobilization in MRGPRX2 treated with this IgE and also antigen (100 ng/ml DNP/BSA) increasing Ca^{2+} mobilization (Fig.13A). Mutant is associated with a significant reduction in SP-induced Ca^{2+} mobilization, however, the antigen (100 ng/ml DNP/BSA) is inducing Ca^{2+} mobilization (Fig.13B).

Chapter 5

Discussion

MCs are the immune cells that are best known for allergic disease such as asthma. MCs response to endogenous and exogenous stimuli that promote degranulation. While both human MC subtypes (M_{TC} and MC_T) are activated via FcεIR, only MC_{TC} respond to SP [63, 64], which is reduced by NK-1R antagonist or in cells obtained from NK-1R$^{-/-}$ mice. These findings suggest that activation of NK-1R in murine MCs by SP results in increased vascular permeability and inflammatory cell aggregation. NK-1R is the classic GPCR for neurokinins such as SP. It now appears MRGPRX2 that is also a neurokinin receptor [39] found on human MCs. Given the recent study [40] which reports that expression of MRGPRX2 on human MCs is upregulated in asthmatic lung suggests it participates in the pathogenesis of asthma. SP and HK-1 are NPs which belong to the tachykinin family. SP is released from nerve ending while HK-1 is produced by FcεRI-activated MCs. Recent studies [37] have showed that HK-1 acts on NK-1R in an autocrine manner to facilitate IgE-mediated anaphylaxis and lung inflammation. However, NK-1R antagonists, which are highly effective in modulating experimental allergic inflammation in mice, does not inhibit MCs response in humans. This discrepancy exemplifies the challenges in the translating findings from animal models to human model. Although SP induces degranulation in human skin MC_{TC} via the activation of MRGPRX2 [39]. However, the effect of HK-1 in human MCs has not been reported. We hypothesized that HK-1 can activate human MCs. This study demonstrates how HK-1 activates human MCs (MRGPRX2) and which receptor is induced by HK-1. We found that LAD2 cells, which represent human MCs, express both MRGPRX2 and NK-1R. NPs

(HK-1 and SP) induced degranulation in LAD2 cells, but this response was resistant to inhibition by an NK-1R inhibitor CP93645. Thus, despite the presence of NK-1R in LAD2 cells, SP and HK-1 preferentially utilize MRGPRX2 to induce degranulation. To determine if the NK-1R antagonist (CP93645) is effective in inhibition, we used RBL-2H3 cells transiently expressing NK-1R responded to both SP and HK-1 for degranulation and that CP93645 caused significant inhibition of this response. This suggests that CP93645 is highly efficient inhibitor. We also tested the role of MRGPRX2 on SP and HK-1-induced mast cell degranulation, we used RBL-2H3 cells stably expressing MRGPRX2. We found that both SP and HK-1 induced degranulation, but this response was resistant to inhibition by CP93645. Previous studies demonstrated that NK-1R antagonists are highly effective in modeling allergic responses in mice but fail in humans. This study provideds a potential explanation for this difference. Unlike the situation in mice where the effects of neuropeptides are mediated via NK-1R, our findings suggest that in humans, these effects are mediated via MRGPRX2. Nevertheless, the limitation of our study is that it is difficult to control the receptor expression level to be the same in all cell types. Therefore, the stably expressing receptor can be more reliable than the transiently transfected cells.

GPCRs are the largest molecules involved in signal transduction [56-58]. Membrane cholesterol plays an important role in the function of a member of GPCRs such as MRGPRX2. Many proteins that interact with cholesterol have been reported to contain the CRAC domain in their sequence which is presented in MRGPRX2. Lipid rafts are part of membrane which can bind to protein, lipid and signaling substrate [65]. There are many studies that have shown that lipid rafts promote MC activity [66, 67]. It is

possible that we can also modulate MRGPRX2. Our hypothesis was that MRGPRX2 might be associated with MRGPRX2. Because MRGPRX2 contains a CRAC domain, the interaction of the receptor with cholesterol in lipid rafts likely contributes to its function. This study showed that depleting cholesterol with MβCD reduced the receptor activity, which was measured by using SP induces degranulation in MRGPRX2. There is significant reduction of this response in MGPRX2 with MβCD compared with the control group (without MβCD). This suggests that the cholesterol on MC membrane is associated with MRGPRX2 function. In addition, we induced point mutations in the CRAC domain sequences of MRGPRX2 to explore the function of the receptor by changing its DNA sequence in the CRAC domain (mutants). The CRAC domain mutant expressed the same level as the receptor WT; however, NPs (HK-1 and SP) induced degranulation in the control group, but this response was significantly reduced in CRAC domain mutants. To further test how the cells function by using other compounds such as ciprofloxacin (antibiotic drug), and HOE 140 (selective B2 bradykinin receptor antagonist), which are used to treat asthma patients, and compound 48/80, which is widely used in animal or tissue models as a selective mast cell activator to induce degranulation. Even though the CRAC domain mutant expressed at similar levels to the WT, receptor do not function. In addition to discover the association between lipid rafts compartment and MRGPRX2. Our hypothesis was that WT might colocalize with lipid raft compartments in contrast to CRAC mutant should be difference by using confocal microscopy, the lipid raft compartment and MRGPRX2 were stained by fluorescent cholera toxin subunit B [62] and antibody PE anti-human MRGX2 respectively to investigate the association between lipid rafts compartment and MRGPRX2. Our confocal microscopy data suggests that the

lipid raft colocalized with MRGPRX2 in both control (MRGPRX2) and CRAC domain mutants. These findings demonstrate that lipid rafts may not be associated with MRGPRX2 and CRAC mutant appear to be defective in coupling to G protein. Given the results of Ca^{2+} mobilization to test if the CRAC domain mutant is defective in coupling to G protein. There was no Ca^{2+} mobilization in CRAC domain mutant.

SP and HK-1 cause mast cell degranulation via MRGPRX2 in humans. In addition to this receptor, lipid rafts may not be involved in MRGPRX2 function; however, the CRAC mutant appears to be defective in coupling to G protein which affects MCs function. In future studies, we may be able to develop antagonists for MRGPRX2 or we may try to inhibit the CRAC domain of this receptor for asthma therapy. This finding suggests that MRGPRX2 may serve as a novel target for modulating asthma and other neuropeptide/mast cell-mediated diseases.

Chapter 6

Conclusion

MCs are multifunctional immune cells. Their responses contribute to allergic and inflammatory disease such as anaphylaxis and asthma. MC activation start when they are activated by ligands binding to their receptors. In addition to FcεRI, MCs express numerous GPCRs including MRGPRX2 and NK-1R. This study has shown that human MCs express MRGPRX2 and NK-1R on the cell surface. NPs (HK-1 and SP) cause MC degranulation via MRGPRX2 while NPs activate murine MCs through NK-1R. However, in human MCs it prefers to go to MRGPRX2. Since MRGPRX2 contains a CRAC domain, the interaction of the receptor to the cholesterol in lipid rafts could potentially contribute to its function. This study demonstrated that cholesterol depletion reduced the MRGPRX2 receptor activity. CRAC domain mutants express the receptor at similar level to WT, but the receptors do not function. The data presented here supports that the CRAC domain may not be associated with MRGPRX2 and the CRAC domain mutant appears to be defective in coupling to G protein. Future directions for this study include the development of small molecule MRGPRX2-specific antagonists, or inhibiting the CRAC domain of MRGPRX2, both of which may be new targets for the treatment of MC-mediated allergic and inflammatory disease.

CHARPTER 7

Figures

Figure 1. Receptor expression level in LAD2 (human MCs).

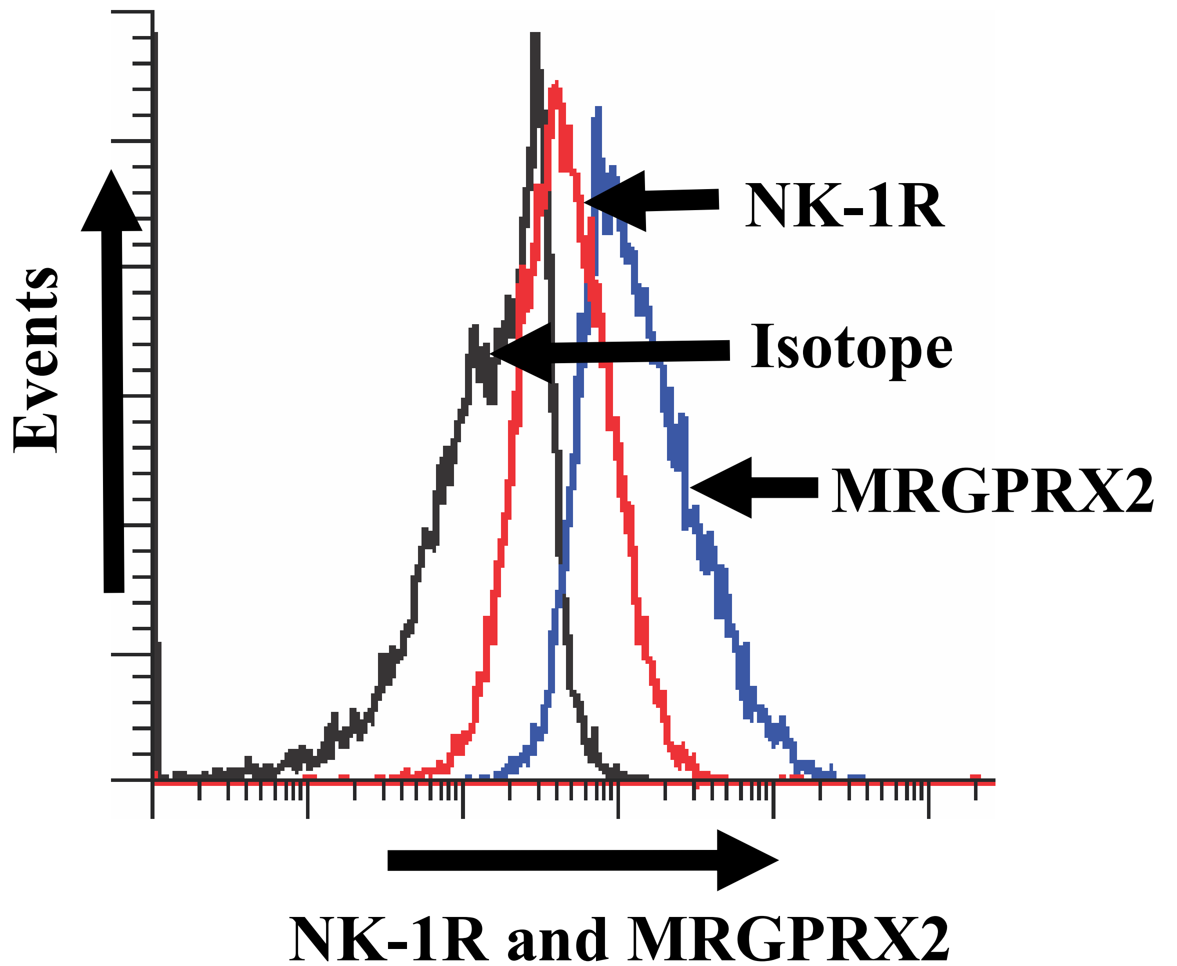


Figure 2. NPs induced degranulation in LAD2

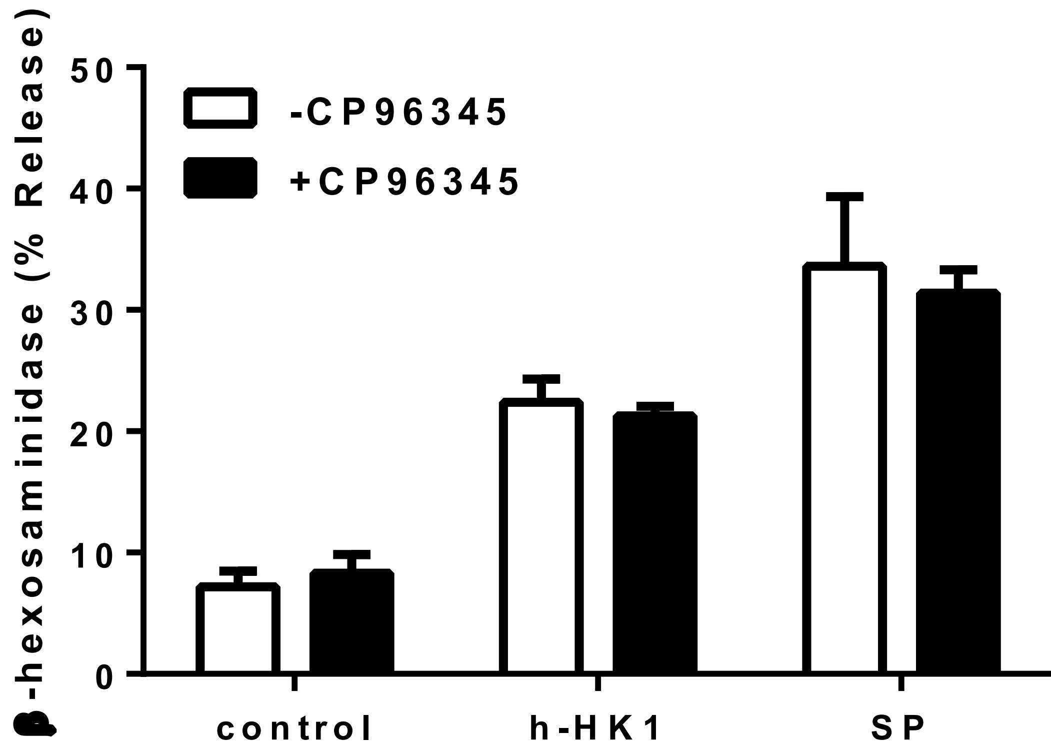


Figure 3. Receptor expression level in RBL-2H3 cells transiently expressing NK-1R.

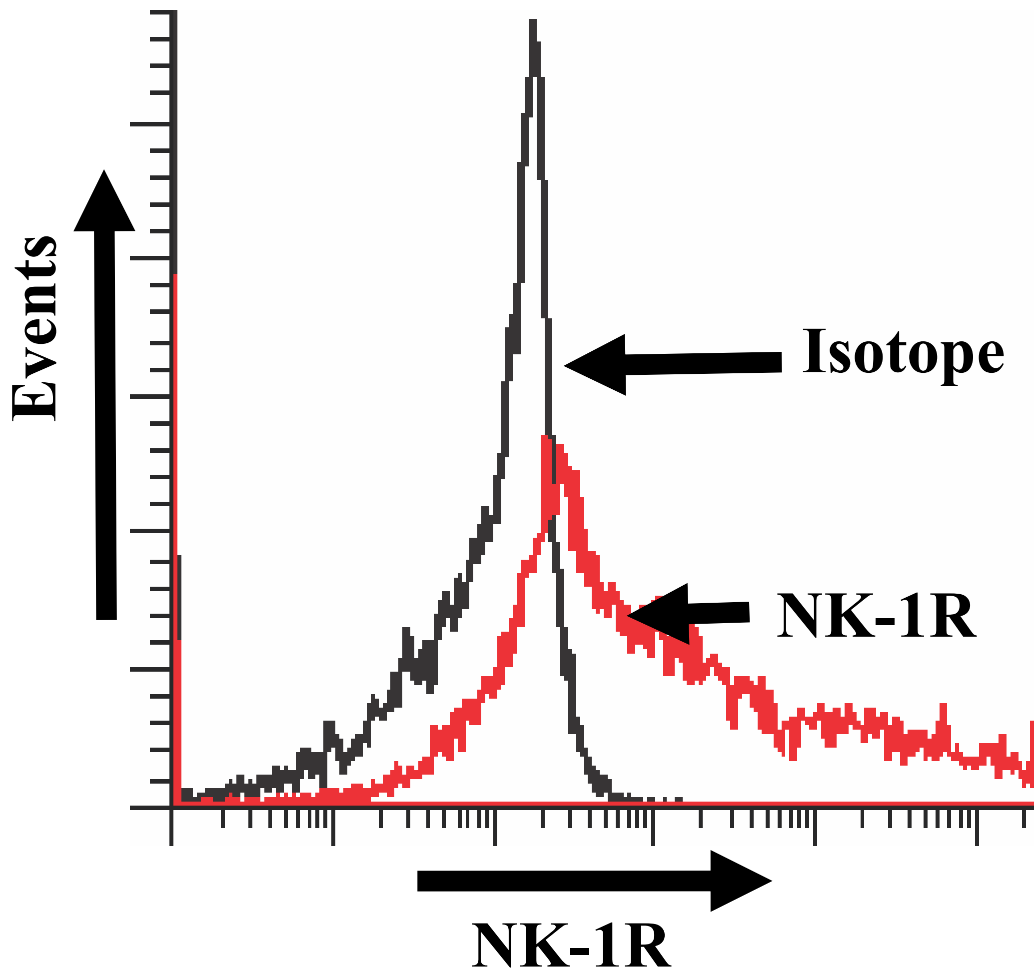


Figure 4. NPs induced degranulation in RBL-2H3 cells transiently expressing NK-1R.

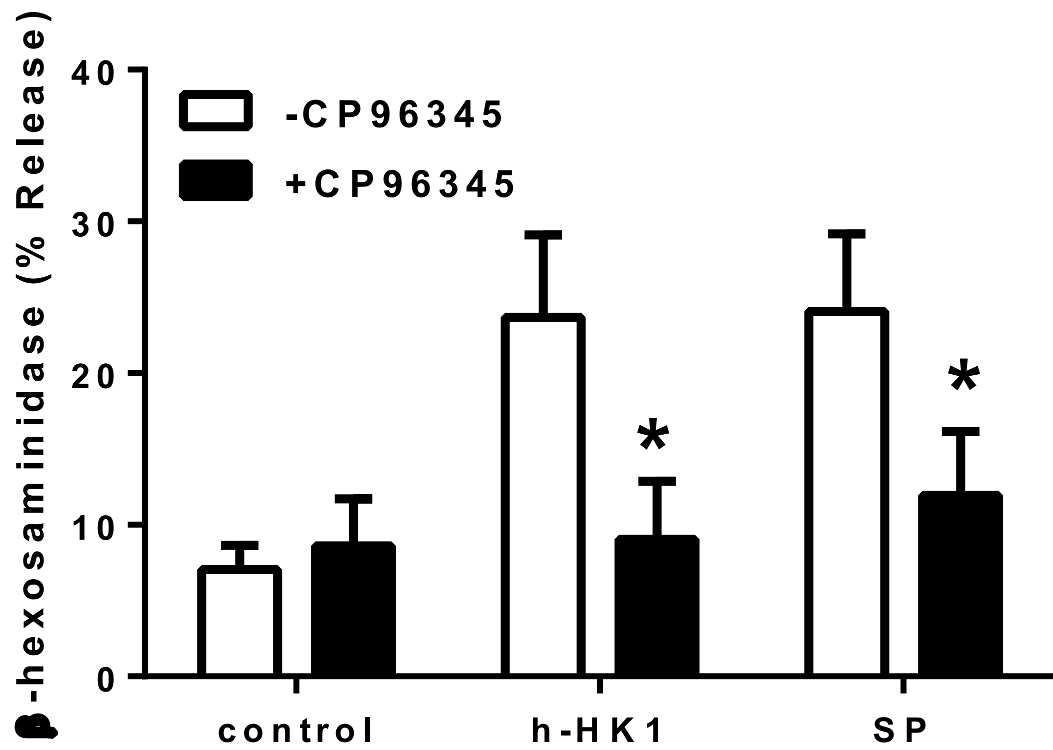


Figure 5. Receptor expression level in RBL-2H3 cells stably expressing MRGPRX2.

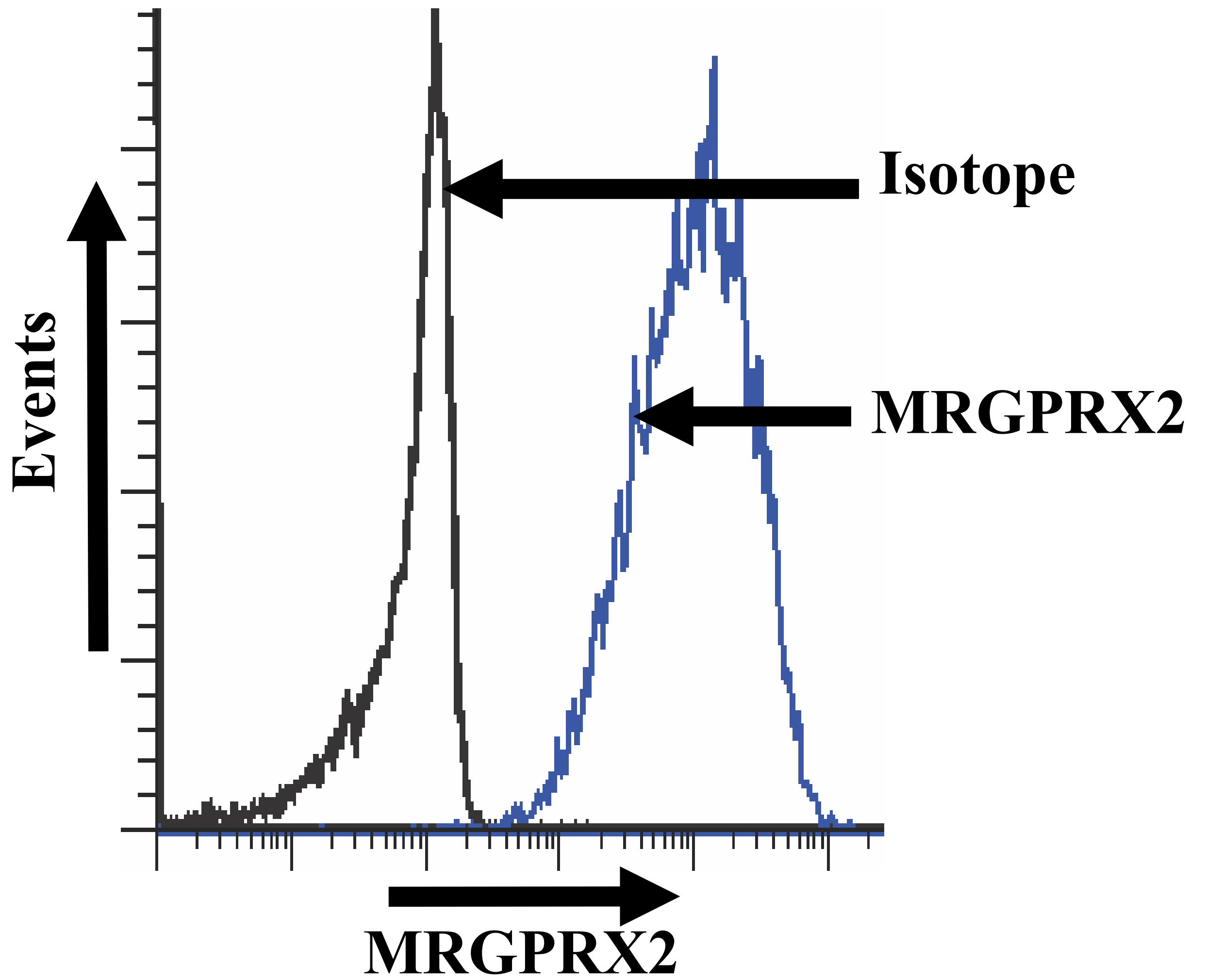


Figure 6. NPs induced degranulation in RBL-2H3 cells stably expressing MRGPRX2.

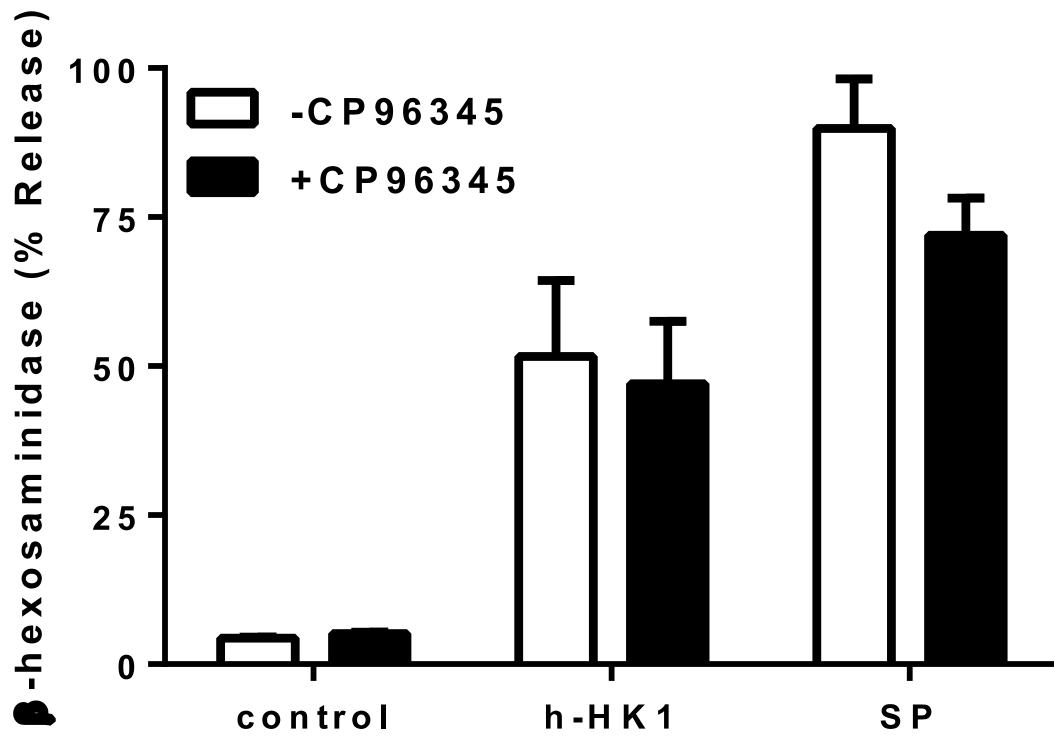


Figure 7. Cholesterol depletion in RBL-2H3 cells stably expressing MRGPRX2.

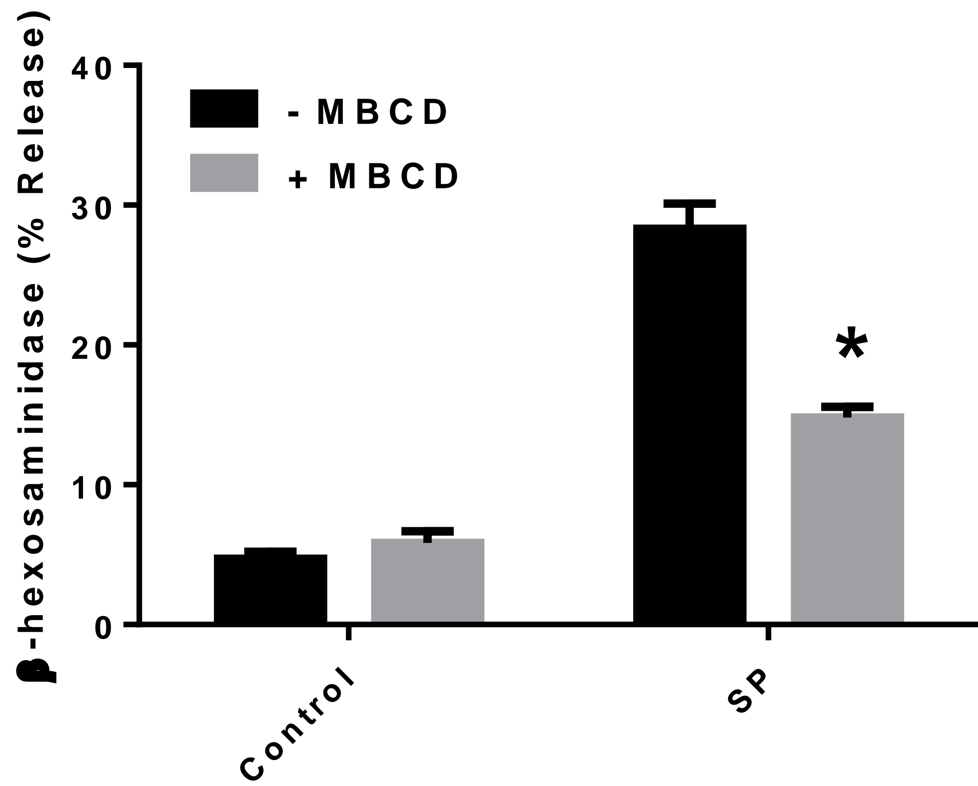


Figure 8. Receptor expression level in RBL-2H3 cells stably expressing CRAC mutant of MRGPRX2 (M8).

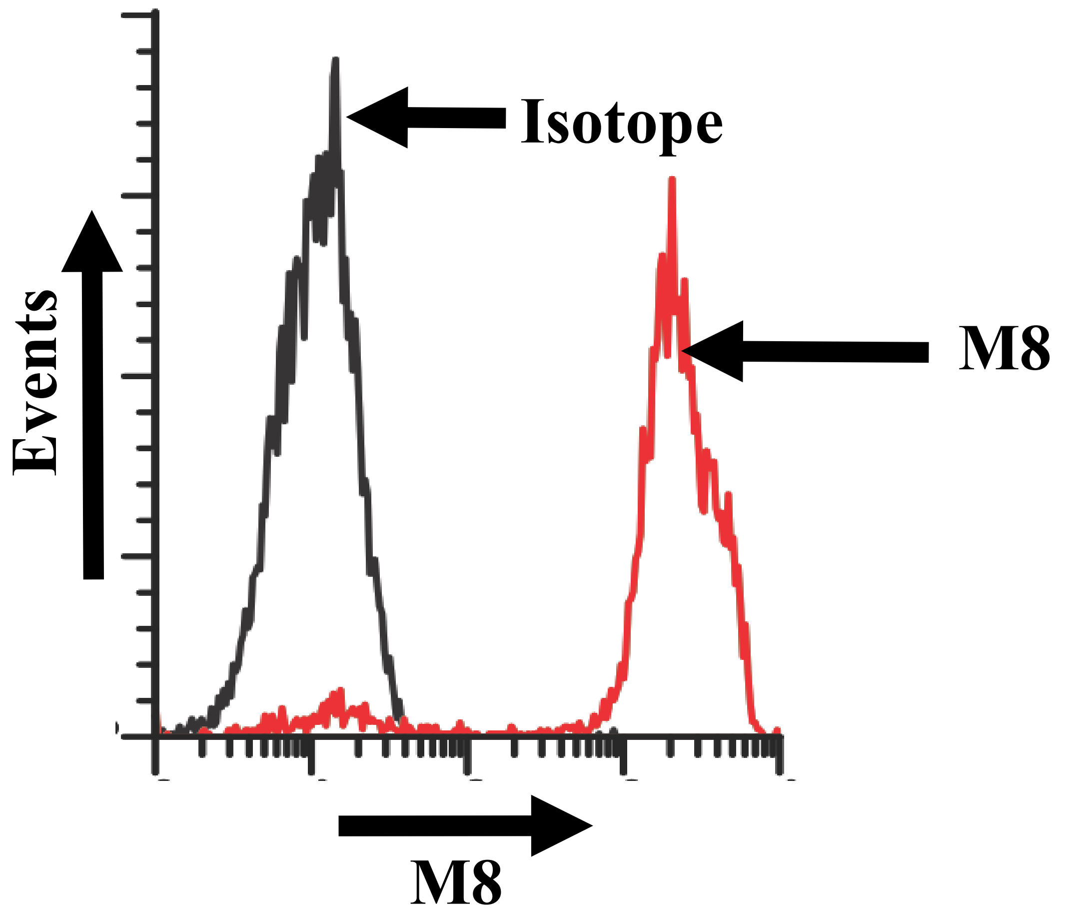


Figure 9. Receptor expression level in RBL-2H3 cells stably expressing CRAC mutant of MRGPRX2 (M10).

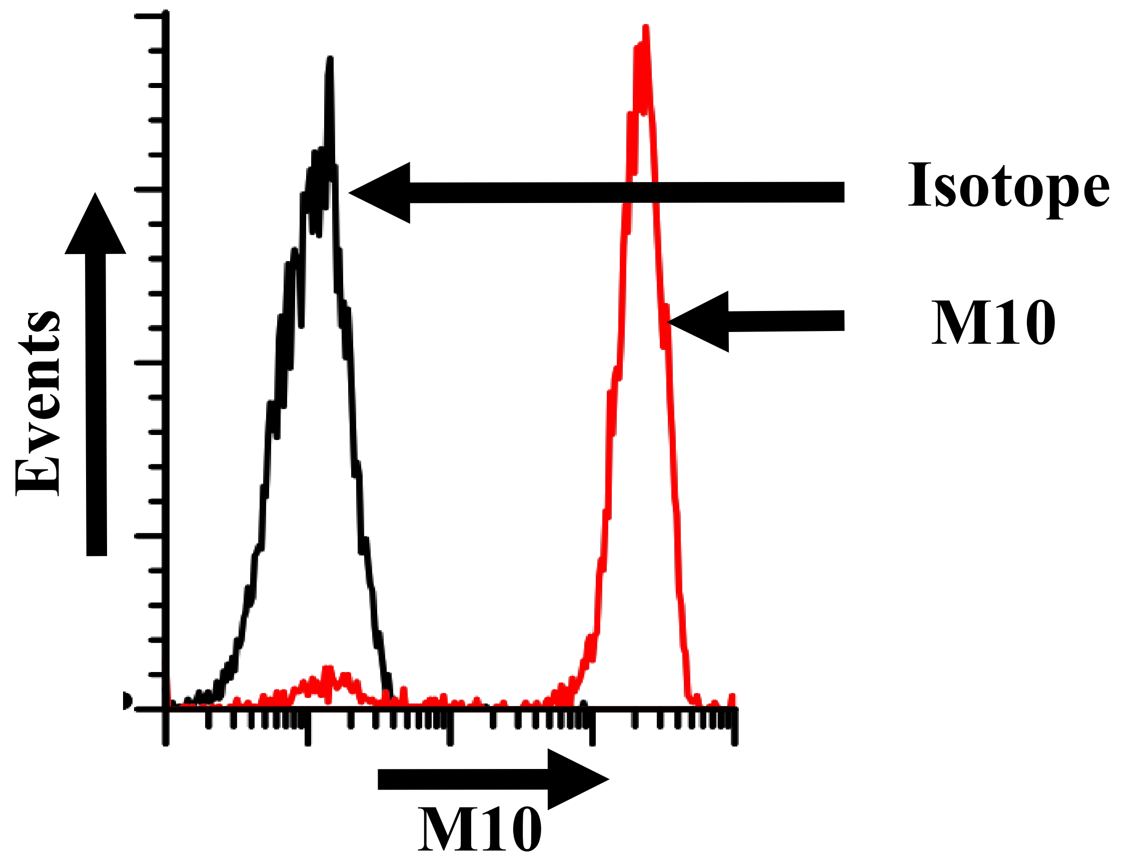


Figure 10. NPs induced degranulation in RBL-2H3 cells stably expressing MRGPRX2 and CRAC mutants of MRGPRX2 (M8 and M10).

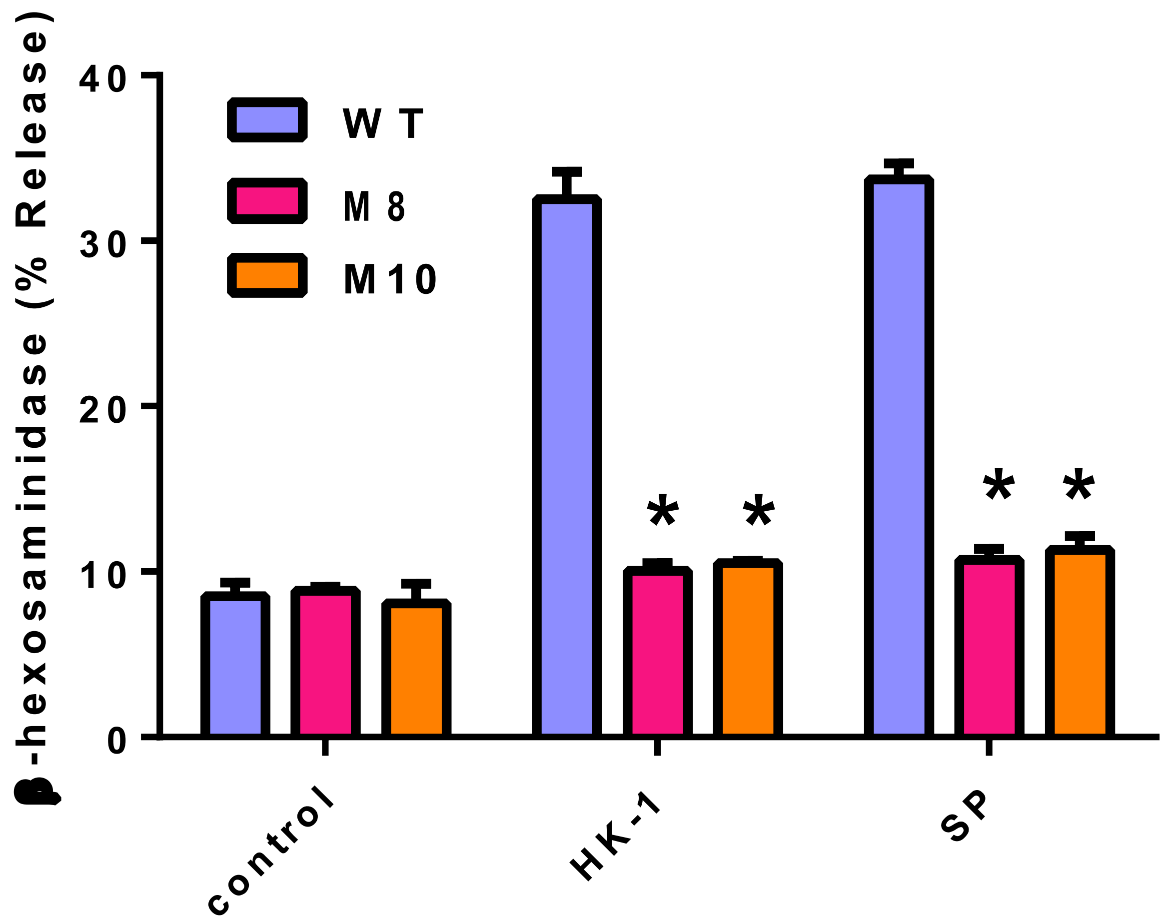


Figure 11. Other compounds induced degranulation in RBL-2H3 cells stably expressing MRGPRX2 and CRAC mutants of MRGPRX2 (M8 and M10).

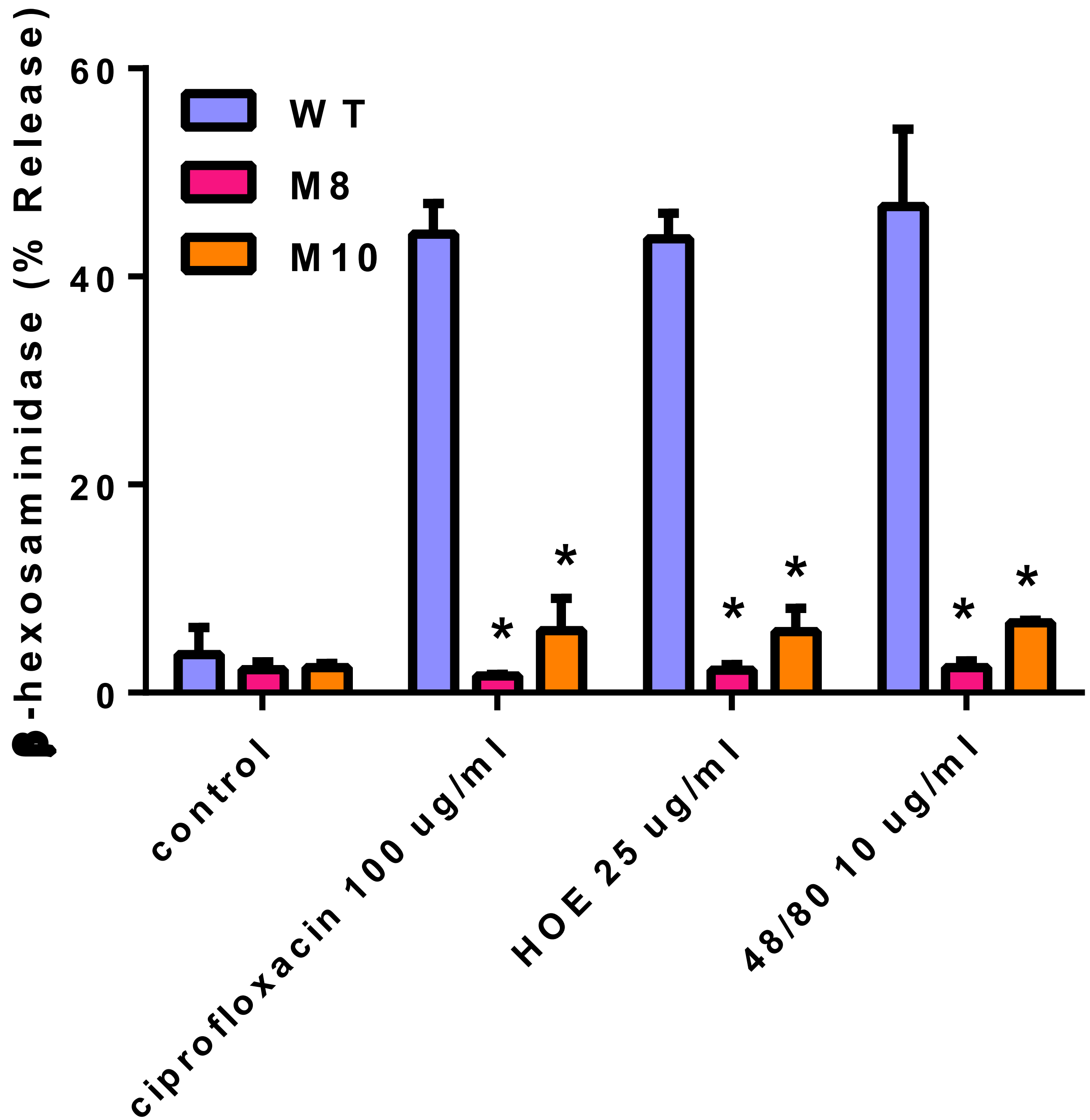


Figure 12. Lipid raft compartment and MRGPRX2 by confocal microscopy.

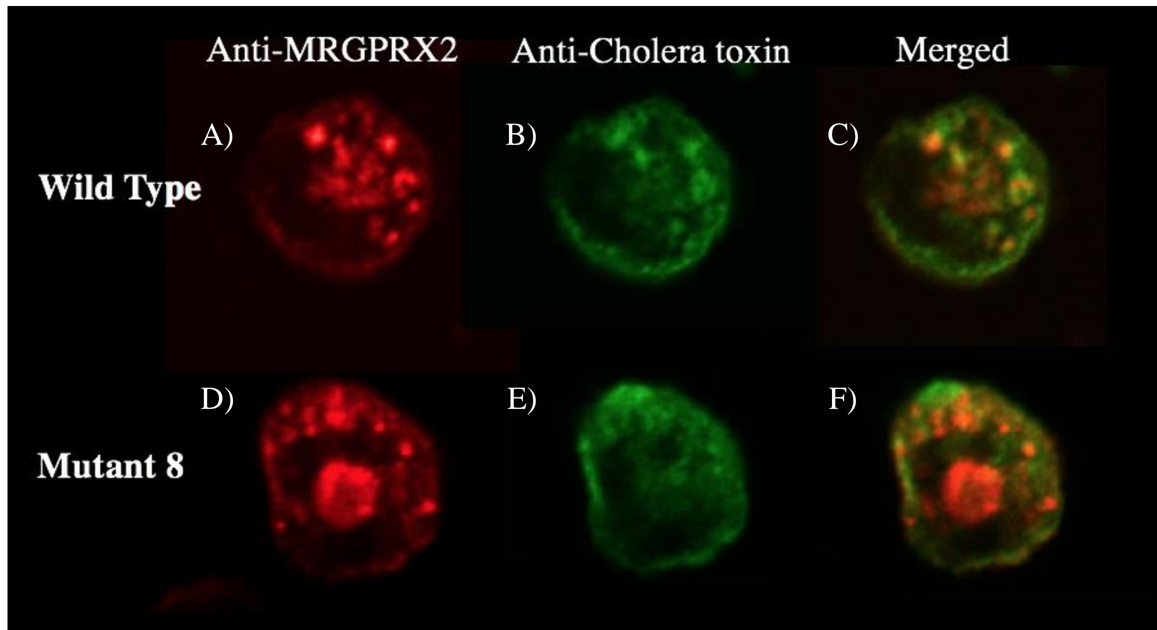
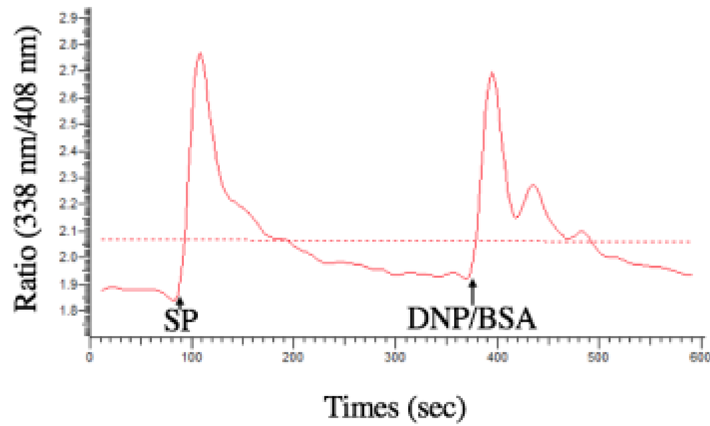
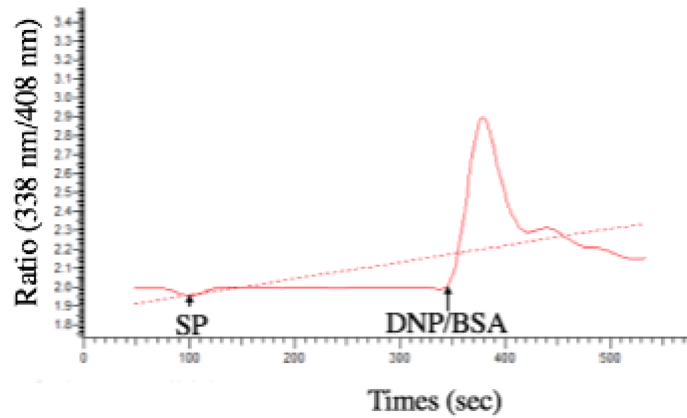


Figure 13. NPs induces Ca^{2+} mobilization in RBL-2H3 cells stably expressing MRGPRX2 (A) and CRAC mutant of MRGPRX2 (M8) (B).

A)



B)



CHARPTER 8

Figures legend

Figure 1 Receptor expression level in LAD2 (human MCs). LAD2 cells were incubated with anti-Tac1r followed by goat anti-rabbit IgG-FITC and PE anti-human MRGX2. Representative histogram of expression level of MRGPRX2 (blue line) and NK-1R (red line) as analyzed by flow cytometry is shown.

Figure 2 NPs induced degranulation in LAD2. LAD2 cells were incubated with CP93645 (10 µM) and stimulated with SP (1 µM) and HK-1 (10 µM). Percent degranulation was determined. Data are mean ± S.E. of three experiments. Statistical significance was determined by unpaired t-Test (2 groups) and Anova-Williams test (multiple comparisons).

Figure 3 Receptor expression level in RBL-2H3 cells stably expressing MRGPRX2. Cells were incubated with PE anti-human MRGX2. Representative histogram of expression level of MRGPRX2 (blue line) as analyzed by flow cytometry is shown.

Figure 4 NPs induced degranulation in RBL-2H3 cells stably expressing MRGPRX2. Cells were incubated with CP93645 (10 µM) and stimulated with SP (1 µM) and HK-1 (10 µM). Percent degranulation was determined. Data are mean ± S.E. of three experiments. Statistical significance was determined by unpaired t-Test (2 groups) and Anova-Williams test (multiple comparisons).

Figure 5 Receptor expression level in RBL-2H3 cells transiently expressing NK-1R. Cells were incubated with anti-Tac1r followed by goat anti-rabbit IgG-FITC.

Representative histogram of expression level of NK-1R (red line) as analyzed by flow cytometry is shown.

Figure 6 NPs induced degranulation in RBL-2H3 cells transiently expressing NK-1R. Cells were incubated with CP93645 (10 μM) and stimulated with SP (1 μM) and HK-1 (10 μM). Percent degranulation was determined. Data are mean ± S.E. of three experiments. Statistical significance was determined by unpaired t-Test (2 groups) and Anova-Williams test (multiple comparisons).

Figure 7 Cholesterol depletion in RBL-2H3 cells stably expressing MRGPRX2. Cells were incubated with MβCD (10 mM) for cholesterol depletion and stimulated with SP (1 μM). Percent degranulation was determined. Data are mean ± S.E. of three experiments. Statistical significance was determined by unpaired t-Test (2 groups) and Anova-Williams test (multiple comparisons).

Figure 8 Receptor expression level in RBL-2H3 cells stably expressing CRAC mutant of MRGPRX2 (M8). Cells were incubated with PE anti-human MRGX2. Representative histogram of expression level of CRAC mutant of MRGPRX2 (M8) (red line) as analyzed by flow cytometry is shown.

Figure 9 Receptor expression level in RBL-2H3 cells stably expressing CRAC mutant of MRGPRX2 (M10). Cells were incubated with PE anti-human MRGX2. Representative histogram of expression level of CRAC mutant of MRGPRX2 (M10) (red line) as analyzed by flow cytometry is shown.

Figure 10 NPs induced degranulation in RBL-2H3 cells stably expressing MRGPRX2 and CRAC mutants of MRGPRX2 (M8 and M10). Cells were stimulated with HK-1 (10 μM) and SP (1 μM). Percent degranulation was determined. Data are

mean $\pm$ S.E. of three experiments. Statistical significance was determined by unpaired t-Test (2 groups) and Anova-Williams test (multiple comparisons).

Figure 11 Other compounds induced degranulation in RBL-2H3 cells stably expressing MRGPRX2 and CRAC mutants of MRGPRX2 (M8 and M10). Cells were stimulated with Ciprofloxacin (100 µg/ml), HOE 140 (25 µg/ml), and 48/80 compound (10 µg/ml). Percent degranulation was determined. Data are mean $\pm$ S.E. of three experiments. Statistical significance was determined by unpaired t-Test (2 groups) and Anova-Williams test (multiple comparisons).

Figure 12 Lipid raft compartment and MRGPRX2 by confocal microscopy. Cells were incubated with PE anti-human MRGX2 (red) (A) and cholera toxin subunit B, Alexa Fluor 488 conjugate antibody (green) (B). Overlay of MRGPRX2 (red) and cholera toxin (green) showed that there is colocalization (orange) (C). The same as CRAC mutant presents the MRGPRX2 (red) (D), and lipid raft part (green) (E). Overlay of MRGPRX2 (red) (D) and cholera toxin (green) (E) showed that there are colocalization (orange) (F).

Figure 13 NPs induces Ca^{2+} mobilization in RBL-2H3 cells stably expressing MRGPRX2 (A) and CRAC mutant of MRGPRX2 (M8) (B). Cells were incubated with Indo-1AM and IgE, washed in Ca^{2+}-free buffer, and stimulated with SP (1 µM). Extracellular Ca^{2+} was determined. After 2 mins, cells were exposed to DNP/BSA (100 ng/ml) and extracellular Ca^{2+} was again determined. Traces represent results from three similar experiments.

CHARPTER 9

Bibliography

1. Melissa Krystel-Whittemore, K.N.D., and John G. Wood, Mast Cell: A Multi-Functional Master Cell. Front Immunol, 2015. 6: p. 620.
2. Hariharan Subramanian, K.G., Qiang Guo, Ryan Price, Hydar Ali, Mas-related Gene X2 (MrgX2) Is a Novel G Protein-coupled Receptor for the Antimicrobial Peptide LL-37 in Human Mast Cells. J Biol Chem, 2011. 286(Dec 30): p. 44739-44749.
3. Mirjam Urb, D.C.S., The Role of Mast Cells in the Defence against Pathogens. PLOS Pathogens, 2012. 8(4).
4. Michael F. Gurish, K.F.A., Developmental Origin and Functional Specialization of Mast Cell Subsets. Immunity, 2012. 37(1): p. 25-33.
5. Joakim S. Dahlin, J.H., Mast cell progenitors : Origin, development and migration to tissues. Molecular Immunology, 2015. 63.
6. Laurent L Reber, R.S., Kaori Mukai, and Stephen J Galli, Potential effector and immunoregulatory functions of mast cells in mucosal immunity. Mucosal Immunol, 2015. 8: p. 444-463.
7. A. A. Irani, N.M.S., S. S. Craig, G. Deblois, and L. B. Schwartz, Two types of human mast cells that have distinct neutral protesae compositions Proc. Natl. Acad. Sci. USA, 1986. 83: p. 4464-4468.
8. Stephen J. Galli, J.K., Michele A. Grimbaldeston, Adrian M. Piliponsky, Cara M.M. Williams, and Mindy Tsai, MAST CELLS AS *"TUNABLE"* EFFECTOR AND IMMUNOREGULATORY CELLS: Recent Advances. Annual Review of Immunology 2005. 23: p. 749-786.
9. Hariharan Subramanian, K.G., Narayanan Parameswaran, and Hydar Ali, Regulation of Fc RI Signaling in Mast Cells by G Protein-coupled Receptor Kinase 2 and Its RH Domain. The Journal of Biological Chemistry 2014. 289(30): p. 20917-20927.
10. Krishnaswamy G, A.O., Chi DS, THe human mast cell : an overview. Methods in Molecular Biology, 2006. 315.
11. TC Moon, C.S.L., KE Morris, C Marcet, T Yoshimura, Y Sekar and AD Befus, Advances in mast cell biology: new understanding of heterogeneity and function. Mucosal Immunology, 2010. 3.
12. Frandji P, O.C., Cacaraci F, Lapeyre J, Peronet R, David B, et al., Antigen-dependent stimulation by bone marrow-derived mast cells of MHC class II-restricted T cell hybridoma. J Immunol, 1993. 11(Dec 1): p. 6318-28.
13. Frandji P, T.C., Oskeritzian C, David B, Desaymard C, Mecheri S, Exogenous and endogenous antigens are differentially presented by mast cells to CD4+ T lymphocytes. Eur J Immunol, 1996. Oct;26(10): p. 2517-28.
14. Malaviya R, T.N., Ross EA, Abraham SN, Pfeifer JD. , Mast cells process bacterial Ags through a phagocytic route for class I MHC presentation to T cells. J Immunol, 1996. 156(4)(Feb 15): p. 1490-6.
15. Broide, D.H., Molecular and cellular mechanisms of allergic disease. The Journal of Allergy and Clinical Immunology, 2001. 108(Number 2): p. S65.
16. Cruse G, B.P., Mast cells in airway diseases and interstitial lung disease. Eur J Pharmacol, 2015. May 8.
17. Pundir P, K.M., The role of G protein-coupled receptors in mast cell activation by antimicrobial peptides: is there a connection? Immunol Cell Biol, 2010. Aug(88(6)): p. 632-40.
18. Gilfillan AM, B.M., Regulation of mast cell responses in health and disease. Crit Rev Immunol, 2011. 31(6): p. 475-529.
19. Okayama Y, O.Y., Nakazawa T, Church MK, Mori M, Human skin mast cells produce TNF- alpha by substance P. Int Arch Allergy Immunol, 1998. Sep(117 suppl): p. 48-51.
20. Kulka M, S.C., Tancowny BP, Grammer LC, Schleimer RP, Neuropeptides activate human mast cell degranulation and chemokine production. Immunology, 2008. Mar(123(3)): p. 398-410.
21. Erjavec F, L.F., Florjanc-Irman T, Skofitsch G, Donnerer J, Saria A, et al, Release of histamine by substance P. Naunyn Schmiedebergs Arch Pharmacol, 1981. Aug(317(1)): p. 67-70.
22. Lowman MA, B.R., Church MK, Characterization of neuropeptide-induced histamine release from human dispersed skin mast cells. Br J Pharmacol, 1988. Sep(95(1)): p. 121-30.
23. Grundemar L, H.R.N.Y., peptide YY and C-terminal fragments release histamine from rat peritoneal mast cells. Br J Pharmacol, 1991. Dec(104(4)): p. 776-8.
24. Maurer M, T.T., Granstein RD, Bischoff SC, Bienenstock J, Henz B, et al, What is the physiological function of mast cells? Exp Dermatol, 2003. Dec(12(6)): p. 886-910.

25. Nicola Robas, E.M., and Mark Fidock, Mrgx2 is a high potency cortistatin receptor expressed in dorsol root ganglion The Journal of Biological Chemistry, 2003. 278(November 7, 2003).
26. Yoshimichi Okayama, H.S.a.C.R., Targeting Human Mast Cells Expressing G-Protein-Coupled Receptors in Allergic Diseases. Allergology International 2008. 57(No3): p. 197-203.
27. Haihui Wu, M.Z., Eric Y.P Cho, Wenqi Jiang,Ou Sha, The Origin, Expression, Function and Future Research Focus of a G Protein-coupled Receptor, Mas-related Gene X2 (MrgX2). Progress in HIstochemistry and Cytochemistry, 2015. 50(1-2): p. 11-17.
28. Tatemoto K, N.Y., Tsuda R, Konno S, Tomura K, Furuno M, et al, Immunoglobulin E- independent activation of mast cell is mediated by Mrg receptors. Biochem Biophys Res Commun, 2006. Nov 3(349(4)): p. 1322-8.
29. Subramanian H, G.K., Lee D, Bayir AK, Ahn H, Ali H, beta-Defensins activate human mast cells via Mas-related gene X2. J Immunol, 2013. Jul 1(191(1)): p. 345-52.
30. Subramanian H, K.S., Collington SJ, Qu H, Lambris JD, Ali H, PMX-53 as a dual CD88 antagonist and an agonist for Mas-related gene 2 (MrgX2) in human mast cells. Mol Pharmacol, 2011. Dec 30(286(52)): p. 44739-49.
31. Okayama Y, S.H., Ra C, Targeting human mast cells expressing g-protein-coupled receptors in allergic diseases. Allergol Int, 2008. Sep(57(3)): p. 197-203.
32. Takeda Y, C.K., Takeda J, Sachais BS, Krause JE, Molecular cloning, structural characterization and functional expression of the human substance P receptor. Biochemical and Biophysical Research Communications, 1991. 179(3): p. 1232-40.
33. Ehsan Azimi, V.B.R., Kai-Ting C Shade, Robert M. Anthony, Sebastien Talbot, Paula Juliana Seadi Pereira and Ethan A. Lerner, Dual action of neurokinin-1 antagonist on Mas-related GPCRs. JCI insight, 2016. October.
34. Chiu IM, v.H.C., Woolf CJ, Neurogenic inflammation and the peripheral nervous system in host defense and immunopathology. Nat Neurosci, 2012. 15: p. 1063-7.
35. Douglas SD, L.S., Neurokinin-1 receptor: functional significance in the immune system in reference to selected infections and inflammation. Ann N Y Acad Sci 2011. 1217: p. 83-95.
36. Zhang Y, L.L., Furlonger C, Wu GE, Paige CJ Hemokinin is a hematopoietic-specific tachykinin that regulates B lymphopoiesis. Nat Immunol, 2000. 1: p. 392-397.
37. Sumpter TL, H.C., Pleet AR, Tkacheva OA, Shufesky WJ, Rojas-Canales DM, Morelli AE, Autocrine hemokinin-1 functions as an endogenous adjuvant for IgE-mediated mast cell inflammatory responses. J Allergy Clin Immunol, 2015. 135: p. 1019-1030.
38. Nakae S, H.L., Yu M, Monteforte R, Iikura M, Suto H, Galli SJ, Mast cell-derived TNF contributes to airway hyperreactivity, inflammation, and TH2 cytokine production in an asthma model in mice. J Allergy Clin Immunol, 2007. 120: p. 48-55.
39. Fujisawa, D., et al., Expression of Mas-related gene X2 on mast cells is upregulated in the skin of patients with severe chronic urticaria. J Allergy Clin Immunol, 2014. 134(3): p. 622-633 e9.
40. Idahosa, C.N., The mast cell receptor Mas-Related Gene X2 (MrgX2) in health and disease Dental theses, 2015.
41. Roast, K., F. Fleischer and K. Nieber, Neurokinin 1 receptor antagonists-between hope and disappointment Med Monatsschr Pharm, 2006. 29: p. 200-205.
42. Boot, J.D., S. de Haas, S. Tarasevych, C. Roy, L. Wang, D. Amin, J. Cohen, P. J. Sterk, B. iller, A. Paccaly, J. Burggraaf, A.F. Cohen and Z. Diamant, Effect of an NK1/NK2 receptor antagonist on airway responses and inflmmation to allergen in asthma. Am J Respir Crit Care Med, 2007. 175: p. 450-457.
43. Quartara, L., M. Altamura, S. Evangelista and C. A. Maggi, Tachykinin receptor antagonists in clinical trials. Expert Opin Investig Drugs, 2009. 18: p. 1843-1864.
44. Hens, G., U. Raap, J. Vanoirbeek, I. Meyts, I. Callebaut, B. Verbinnen, B. M. Vanaudenaerde, P. Cadot, B. Nemery, D. M. Bullens, J. L. Ceuppens and P. W. Hellings, Seletive nasal allergen provocation induces substance P-mediated bronchial hyperresponsiveness. Am J Respir Cell Mol Biol, 2011. 44(4): p. 517-523.
45. Gerl, K.S.a.M.J., Revitalizing membrane rafts: new tools and insights. Naure Reviews, 2010. 11(October): p. 688-699.
46. Hancock, J.F., Lipid rafts: contentious only from simplistic standpoints Nature Reviews Molecular Cells Biology, 2006. 7: p. 456-462.
47. Pike, L.J., Rafts defined: a report on the keystone symposium on lipid rafts and cell function. Journal of Lipid Research, 2006. 47: p. 1597-1598.
48. Adriana Maria Mariano Silveira e Souza, V.M.M., Matia Celia Jamur, and Constance Oliver, Lipid Rafts in Mast Cell Biology. Journal of Lipids, 2010. 2011(1-11).
49. Colbeau, A., Nachbaur, J. Vignais, P.M., Enzymic characterization and lipid composition of rat liver subcellular membranes Biochim. Biophys. Acta, 1971. 249: p. 462-492.

50. Li, H.P., V., Peripheral-type benzodiazepine receptor function in cholesterol transport. Identification of a putative cholesterol recognition/interaction amino acid sequence and consensus pattern. Endocrinology, 1998. 139: p. 4991-4997.
51. Li, H.Y., Z.; Degenhardt, B,; Teper, G.; Papadopoulos, V. , Cholesterol binding at the cholesterol recognition/interaction amino acid consensus (CRAC) of the peripheral-type benzodiazepine receptor and inhibition of steroidogenesis by an HIV TAT-CRAC peptide. Proc. Natl. Acad. Sci. USA, 2001. 98: p. 1267-1272.
52. Jamin, N.N., J.M.; Ostuni, M.A.; Vu, T.K.; Yao, Z.X.; Murail, S.; Robert, J.C.; Giatzakis, C., Papadopoulos, V.; Lacapere, J.J., Characterization of the cholesterol recognition amino acid consensus sequence of the peripheral-type benzodiazepine receptor. Mol . Endocrinol., 2005. 98: p. 1267-1272.
53. Tamara Azarashvili, O.K., Yulia Baburina, Irina Odinokova, Vladimir Akatov, Igor Beletsky, John Lemasters and Vassilios Papadopoulos Effect of the CRAC Peptide, VLNYYVW, on mPTP Opening in Rat Brain and Liver Mitochondria. International Journal of Molecular Science, 2016.
54. Epand, R.M.S., B.G.; Epand, R.F., Peptide-induced formation of cholesterol-rich domains. Biochemistry, 2003. 42: p. 14677-14689.
55. Miller, C.M.B., A.C.; Mittal, J. , Disorder in cholesterol-binding functionality of CRAC peptides: A molecular dynamics study. J. Phys. Chem., 2014. 118: p. 12169-13174.
56. Md. Jafurulla, S.T., Amitabha Chattopadhyay, Identification of cholesterol recognition amino acid consensus (CRAC) motif in G-protein coupled receptors. Biochemical and Biophysical Research Communications, 2010. 404: p. 569-573.
57. K. Simons, D.T., Lipid rafts and signal transduction. Nat. Rev. Mol. Cell Biol, 2000: p. 31-39.
58. D.M. Rosenbaum, S.G.F.R., B.K. Kobilka, The structure and function of G-protein-coupled receptors. Nature, 2009. 459: p. 356-363.
59. K. Burger, G.G., F. Fahrenholz, Regulation of receptor function by cholesterol. Cell. Mol. Life sci., 2000. 57: p. 1566-1592.
60. T.J. Pucadyil, A.C., Role of cholesterol in the function and organization of G-protein coupled receptors. Prog. Lipid Res, 2006. 45.
61. Y.D. Paila, A.C., Membrane cholesterol in the function and organization of G-protein coupled receptors. Subcell. Biochem, 2010. 51: p. 439-466.
62. Cong JIn, C.P.S., Guojie Li, Erin N. Potts, Kristina J. Riebe, Gregory D. Sempowski, W. Michael Foster, and Soman N. Abraham., Particulate allergens potentiate allergic asthma in mice through sustained IgE-mediated mast cell activation The journal of Clinical Investigation, 2011. 121: p. 941-955.
63. Oskeritzian, C.A., et al., Surface CD88 functionally distinguishes the MCTC from the MCT type of human lung mast cell. J Allergy Clin Immunol, 2005. 115(6): p. 1162-8.
64. Fukuoka Y, X.H., Sanchez-Munoz LB, Dellinger AL, Escribano L, Generation of anyaphylaxins by human beta-tryptase from C3, C4 and C5. J Immunol, 2008. 180: p. 6307-6316.
65. Shin JS, S.C., Jin C, LeFurgey EA, Abrahab SN, Harboring of particulate allergens within secretory compartments by mast cells following IgE/FcepsilonRI-lipid raft-mediated phagocytosis. J Immunol. 177(9): p. 5791-5800.
66. Brown DA, L.E., Functions of lipid rafts in biological membranes. Annu rev Cell Dev Biol, 1998. 14: p. 111-136.
67. Anderson RG, J.K., A role for lipid raft shells in targeting proteins to caveolae, rafts, and other lipid domains. Science, 2002. 296(5574): p. 1821-1825.