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Thesis Title:

Assessing the Role of Protease-Activated Receptors in the Release of Inflammatory Mediators from Mast Cells

By

Hashem Alshurafa 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Master of Science.**

In

Experimental Medicine

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Assessing the Role of Protease-Activated Receptors in the Release of Inflammatory Mediators from Mast Cells** submitted by **Hashem Alshurafa** in partial fulfillment of the requirements for the degree of **Master of Science in Experimental Medicine.**

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To my mother, Diana, with my love

إهداء إلى أمي مع كل ألحب و ألإحترام و ألتقدير و ألإمتنان

ABSTRACT

Mast cells (MC) have been implicated in the development of inflammation mediated by serine proteases. Serine protease-induced cellular activation may be mediated through protease-activated receptors (PAR). We hypothesized that PAR1- and PAR2-activating peptides (PAR-AP) induce the release of inflammatory mediators by rat peritoneal MC (RPMC). We observed that the PAR2-AP, tc-LIGRL-NH_2 (tc-LIG), and the PAR1-AP, ApfFRChaCitY-NH_2 (Cit), mediated β-hexosaminidase (β-hex) and protease release by RPMC. Antigen (Ag) and the inactive control peptides, tc-OLRGIL-NH_2 (tc-OLR) and FSLLRY-NH_2, induced no significant release of β-hex or proteolytic activity. The ability of tc-LIG to induce the release of major rat MC proteases was investigated. We observed that unlike Ag, tc-LIG stimulated the release of MC protease (RMCP)-1, RMCP-5 and carboxypeptidase-A. Therefore, PAR-AP may stimulate mediator release by RPMC through mechanism(s) independent of the Ag-mediated pathways. This activation results in the release of proteases, which may activate PAR on other cells triggering further inflammatory cascades.

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Ab	Antibody
ACE	Angiotensin – Converting Enzyme
Ag	Antigen
β-hex	β-hexosaminidase
BMMC	Bone Marrow-Derived Mast Cells
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CBB	Coomassie Brilliant Blue
cGMP	Cyclic Guanosine Monophosphate
Cit	ApfFRChaCitY-NH_2 (PAR1-AP)
CNS	Central Nervous System
CP-A	Carboxypeptidase-A
CTMC	Connective Tissue Mast Cells
DAG	Diacylglycerol
DFP	Diisopropylfluorophosphate
DIP	diisopryopyl-phosphoryl
DMSO	dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DPPI	Dipeptidylaminopeptidase
ECL-2	2nd extracellular loop of protease-activated receptor
ECM	Extracellular Matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FGF	Fibrotic Growth Factor
FSL	FSLLRY-NH_2 (PAR1-CP)
GDP	Guanosine Diphosphate
GI	Gastrointestinal
GM-CSF	Granulocyte Monocyte-Colony Stimulating Factor
GTP	Guanosine Triphosphate
HEK	Human Embryonic Kidney Cells
HETEs	5-, 12-hydroxy-eicosatetraenoic acids

HRP	Horse Radish Peroxidase
HTB	Hepes-Tyrodes Buffer
ICAM-1	Intercellular Adhesion Molecule-1
IFN-γ	Interferon-Gamma
IgE, G	Immunoglobulin E, G
IL-6, 8	Interleukin-6, 8
IP3	Inositol 1,4,5-Trisphosphate
LIF	Leukemia Inhibitory Factor
L-NAME	N-nitro-L-arginine methyl ester
LPS	Bacterial Lipopolysaccharide
LSI	LSIGRL-NH_2 (PAR2-CP)
MC	Mast Cell
MMC	Mucosal Mast Cell
MMCP	Mouse Mast Cell Protease
MMCP-1-8	Mouse Mast Cell Protease-1-8
MC_T	Human Mast Cell (Tryptase-positive)
MC_{TC}	Human Mast Cell (Tryptase- and Chymase-positive)
mRNA	Messenger Ribonucleic Acid
MS	Multiple Sclerosis
mT-4	Mouse Tryptase-4
mTMT	Mouse Trans-Membrane Tryptase
Nb	*Nippostrongylus brasiliensis*
NGF	Nerve Growth Factor
NK	Neurokinin
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
PAF	Platelet Activating Factor
PAR	Protease-Activated Receptor
PAR-AP	Protease-Activated Receptor-Activating Peptide
PAR-CP	Protease-Activated Receptor-Control Peptide
PG	proteoglycan
PGE-2	Prostaglandin E2
PIP2	Phosphatidylinositol 4,5-Bisphosphate
PKC	Protein Kinase C
PLC	Phospholipase C

RBL	Rat Basophilic Leukemia Cells
RMCP-1,5	Rat Mast Cell Protease-1, 5
RPMC	Rat Peritoneal Mast Cells
rrTNF	Recombinant Rat Tumour Necrosis Factor
SBTI	Soybean Trypsin Inhibitor
SCF	Stem Cell Factor
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SLI	SLIGRL-NH_2 (PAR2-AP)
tc-LIG	*trans-cinnamoyl*-LIGRLO-NH_2
tc-OLR	*trans-cinnamoyl*-OLRGIL-NH_2
TFL	TFLLR-NH_2 (PAR1-AP)
TGF-β	Transforming Growth Factor-β
TNF	Tumour Necrosis Factor
We	Worm Equivalent

I. BACKGROUND

A. INTRODUCTION TO MAST CELL BIOLOGY

The mast cell (MC) was first discovered by Paul Ehrlich in 1879 using light microscopy. When stained with toluidine blue dye, this heavily granulated cell changed the color spectrum of the dye in a process called metachromasia (1). Since then, the MC has been of interest to cell biologists, pharmacologists, pathologists, immunologists and physicians mainly because of its central role in allergic disease. The initial experiments connecting the MC and allergic inflammation were reported by Ishizaka *et al.*, over 80 years after Ehrlich's discovery, where they showed that antigen (Ag) specific IgE can bind to high affinity receptors on MC and basophils (2). This was especially interesting when taken together with the discoveries that histamine, a bronchospasmic chemical involved in the anaphylactic reaction, is stored in the granules of MC and basophils (3, 4).

Since then, MC granules have been found to store many inflammatory mediators that can be released upon Ag challenge. These mediators can increase vascular permeability, bronchoconstriction, vasodilation and inflammatory cell chemotaxis both directly and indirectly by stimulating surrounding leukocytes and structural cells (5, 6). Thus, MC are recognized to be major effector cells of allergic inflammation. However, in addition to their prominent role in the immediate response of allergic inflammation, MC synthesize and secrete a plethora of cytokines, growth factors and other mediators that are implicated in chronic inflammation. These biologically active mediators have many regulatory functions on cells in their microenvironments. Indeed, MC-derived

mediators can stimulate nerves, vascular and epithelial changes, tissue remodeling and fibrosis that are implicated in many disease processes including: pulmonary fibrosis, angiogenesis in tumors, anaphylaxis, neuro-degenerative diseases, and arthritis (7-11). With these findings among others, MC are increasingly acknowledged for their immuno-regulatory roles beyond their classical involvement in acute allergic inflammation. Furthermore, MC are ubiquitously distributed and exhibit site-specific characteristics (12). These have important implications for their immunological roles and adaptation to their microenvironments.

B. PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL ROLES OF MAST CELLS *IN VIVO*

Inflammation is a response to living tissue injury and functions to limit and repair the damage. The causes of inflammation are diverse and include immunological reactions (allergy and autoimmunity), infection (bacteria, viruses and parasites), physical tissue damage (burns, trauma and radiation) and chemical agents (drugs and industrial pollutants). Histopathologically, inflammation consists of changes in vascular flow and alteration in vascular permeability leading to leukocyte migration (13). Acute inflammation is short term (seconds to hours) and is characterized by oedema (the exudation of fluid, plasma proteins) and extravasation of leukocytes, primarily neutrophils into the site of inflammation. In contrast, chronic inflammation is of longer duration (hours to years) and is characterized by the extravasation of lymphocytes, neutrophils, eosinophils and macrophages.

In addition to changes in vascular flow and extravasation of inflammatory cells, the inflammatory reaction leads to other processes. To limit the spread of the lesion and to start tissue repair and remodeling, substantial fibrosis occurs during inflammation. Furthermore, to allow for wound healing during chronic inflammation new capillaries have to be formed (angiogenesis). The ubiquitous distribution of MC and their ability to synthesize, store and release a wide range of inflammatory mediators, has allowed them to play potential key roles in many of these features of inflammation (Fig. 1). Understanding these associations and roles will foster a greater appreciation of the possible clinical significance of these cells in various inflammatory disorders.

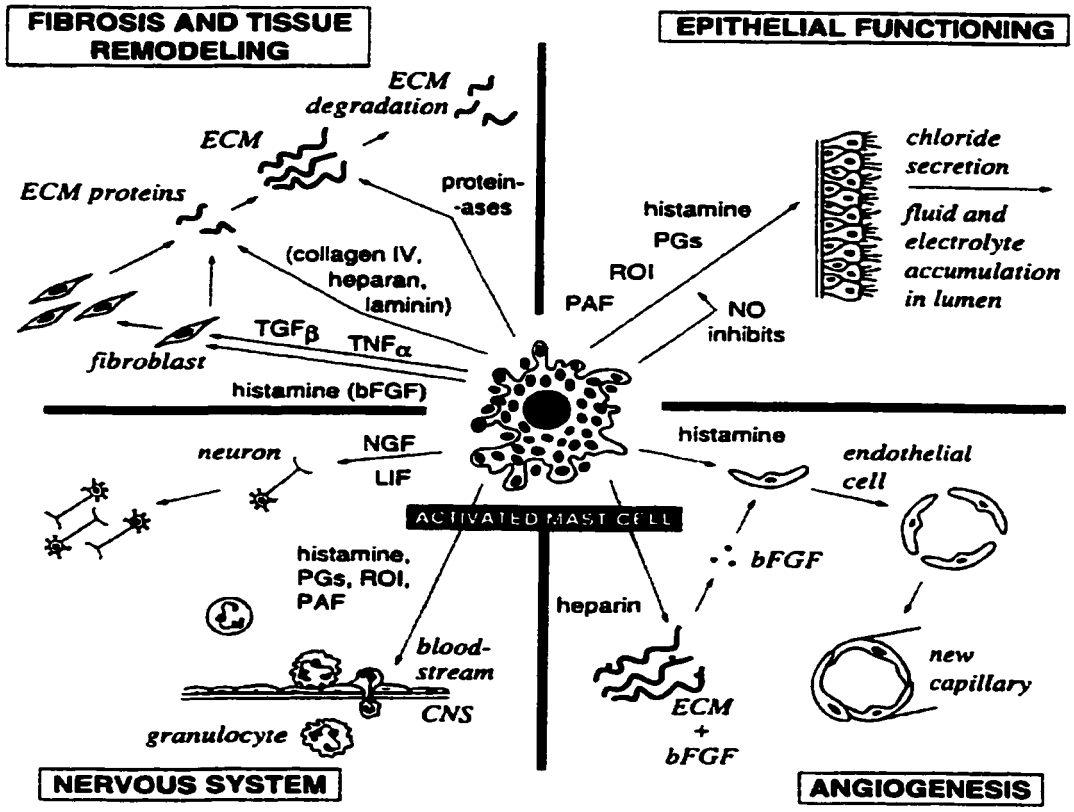

Figure 1: The effects of MC on their microenvironments. bFGF, basic fibroblast growth factor; CNS, central nervous system; ECM, extracellular matrix; LIF, leukemia inhibitory factor; NGF, nerve growth factor; PGs, prostaglandins; ROI, reactive oxygen intermediates; TGF-β, transforming growth factor-β, TNF, tumor necrosis factor. This figure was adapted from (19).

1. Allergic Inflammation

One of the major functions of MC *in vivo* is their response to allergens. The classical IgE-mediated allergic reaction is key in producing symptoms associated with food allergy, allergic asthma, conjunctivitis, and rhinitis (14-16). MC express high numbers (2 to 5 X 10^5 per rat MC) of high-affinity receptors (FcεR1) for immunoglobulin (Ig) E antibodies on their surfaces, and bind Ag-specific IgE (17). Upon re-exposure to sensitizing allergens, IgE antibodies on the MC surface are cross-linked and activate an intracellular signal transduction cascade that involves the phosphorylation and dephosphorylation of β and γ chains of FcεRI and numerous other proteins, activation of G proteins, adenyl cyclases, phospholipases and several ion channels (18). The outcome of this activation is the upregulation of inflammatory mediators and adhesion molecules which leads to the release of cytokines, membrane phospholipids such as leukotriene E4 and prostaglandins, proteases, and various vasoactive agents including histamine (19). Sensitized individuals can exhibit symptoms such as bronchospasm, rhinorrhea, and the cutaneous wheal and flare response following allergen exposure (20).

In addition to IgE-mediated activation, MC can be activated by a variety of other factors, including neuropeptides, bacterial products and proteases (21). For example, substance P activates ion channels in MC (22) and LPS induces the selective release of IL-6 from MC (23). Thus, MC are involved in inflammatory responses caused by a

variety of non-allergic mechanisms.

2. Fibrosis and Tissue Remodeling

There are increased numbers of MC and/or evidence of MC degranulation in many fibrotic conditions including, Crohn's disease (24), myocardial fibrosis in post-transplant hearts (25) and idiopathic pulmonary fibrosis (7), indicating that MC may be involved in fibrosis.

MC are assumed to be involved in tissue repair and/or overproduction of structural proteins in the basal lamina that takes place during fibrotic disease. For example, bone marrow-derived MC (BMMC) express mRNA and protein of collagen IV, laminin, and heparan sulfate proteoglycan (26). Although, unstimulated connective tissue MC (CTMC) are fibrogenic, treating CTMC with the activator compound 48/80 enhanced fibroblast proliferation and collagen secretion (7). As indicated earlier, MC produce many mediators that possess fibrogenic functions, including transforming growth factor-β (TGF-β), fibroblast growth factor (FGF) and tumour necrosis factor (TNF), shown to upregulate collagen gene expression in mouse skin fibroblasts (27).

During tissue remodeling process, cells migrate through tissues and cross the epithelial basal lamina. This involves the degradation of extracellular matrix (ECM) proteins. MC granule products, such as proteases, can cause damage to ECM. Indeed, there is an association between MC numbers and ECM proteolysis in lesions of rheumatoid arthritis (28), which are thought to be the result of degradation of structural proteins by MC-derived proteases. Human MC derived-chymase can degrade collagen IV and V, fibronectin, laminin and elastin, which are central basal lamina components

(29). Similarly, MC tryptase degrades fibronectin in the ECM of human fibroblasts.

3. Angiogenesis

Angiogenesis is a key feature of chronic inflammation, in which new capillaries are formed to allow for vascularization in wound healing. This process however, contributes to various pathologies including the growth of solid tumors, psoriasis and rheumatoid arthritis (8). The ubiquitous distribution of MC, their intimate association with newly formed vasculature, and their ability to produce histamine and TGF-β promotes a possible role in angiogenesis. *In vitro*, histamine is mitogenic for human microvascular endothelial cells (30), and *in vivo*-activated CTMC can induce histamine-mediated angiogenic responses in normal rats and mice (8). Furthermore, MC can produce structural proteins such as collagen VII (31) that are key in the construction of new blood vessels. These findings are especially important when considering that MC are known to accumulate around tumor sites and that tumor associated MC are often activated. Tumors >2mm in diameter depend on angiogenesis for growth. Therefore, it is not surprising that they release various angiogenic factors (8), including TGFβ-1, a potent MC chemotactic factor (32).

4. Epithelial Regulation

Epithelial cells line mucosal surfaces (including airways and the gastrointestinal tract), forming an essential barrier that controls the uptake of Ag, ions, solutes and water. Mucosal MC (MMC) are strategically located beneath the basal lamina providing the

potential for these cells to regulate epithelial functions. MC play a pivotal role in both IgE/Ag-dependent and IgE-independent regulation of the epithelium by releasing mediators that induce epithelial responses. For example, in isolated rat jejunum and human colon, IgE-mediated stimulation caused secretion of Cl^- via the action of histamine (33).

In additional, recent evidence suggests that the role of MC in the gastrointestinal mucosa is more than a reaction to Ag, but is to actively regulate the barrier and transport properties of the epithelium (34, 35). Chronic stress induces defects in the epithelial barrier that are associated with damage to mitochondria, and hyperplasia as well as activation of mucosal MC. MC deficient rats (Ws/Ws) do not exhibit the stress induced by epithelial abnormalities seen in normal rats (36). These and other recent studies in models of hypersensitivity and stress provide evidence that MC-mediated effects on mucosal function are caused either by the direct interaction of MC mediators with epithelial receptors and/or via an indirect pathway, which includes nerves and neurotransmitters (36, 37). Moreover, to emphasize the role of MC in controlling epithelial functions, these studies also highlight the ability of MC to interact with the nervous system.

5. The Nervous System

The communication between MC and nervous system has been implicated in a number of neurological diseases including multiple sclerosis (MS) and Alzheimer's disease (10, 38). MC and nerves can be found in close association throughout the body.

Both MC and cells of the nervous system produce many bioactive mediators that affect the function of the other. Stimulation of parasympathetic nerves in the ileum induces MC degranulation and histamine release (39). Similarly, MC stimulation alters electrical activity of enteric neurons partly through a histamine-mediated mechanism (40). Furthermore, MC-derived mediators such as nerve growth factor (NGF), IL-6, GM-CSF and LIF (a cholinergic neuronal differentiation factor) can potentially affect cells of the nervous system (19). Similarly, Schwann cells produce NGF and stem cell factor (SCF) that can alter MC function (41, 42). Thus, the bi-directional interaction between MC and nervous system cells may be important means for neuro-immune communication.

Supporting the concept that neuro-immune communication may be MC-mediated is an emerging body of *in vivo* evidence suggesting that intestinal effects of acute stress are MC-mediated (43). In rats colon, chronic stress significantly increases macromolecular flux (an epithelial barrier defect) and causes epithelial mitochondrial swelling. However, MC deficient rats (Ws/Ws) do not display these stress induced epithelial abnormalities (36), suggesting a central role for MC in stress induced colonic pathophysiology. Interestingly, these stress-induced epithelial changes in rats are similar to those that occur in hypersensitivity reactions to food Ag in allergic individuals, in which MC play a central role (43). The mechanism by which MC induce these epithelial abnormalities is still to be elucidated. However, it may occur through the release of MC mediators that directly interact with nerves to release neurotransmitters, which could act on epithelial receptors to loosen tight junctions in the epithelium allowing the passage of macromolecules between epithelial cells (44). Furthermore, in a model of acute

experimental allergic encephalomyelitis (a murine model of MS), MC-deficient mice display less disease incidence, delayed disease onset, and reduced mean clinical scores as compared with their wild-type littermates (45).

C. MAST CELL MEDIATORS

It is clear that the ability of MC to affect so many physiological conditions is caused by its secreted mediators and cell-to-cell contact, which have numerous effects on structural and immune cells, including neutrophils, eosinophils, monocytes/macrophages, lymphocytes, endothelial cells, epithelial cells and smooth muscle cells. We have previously discussed the ability of MC-derived mediators, such as proteases, to modulate the function of structural proteins that compose the ECM. In this section, MC-derived mediators will be examined in more detail with special emphasis on their mechanisms of release and functions.

MC synthesize a variety of mediators, including amines, neutral proteases, acid hydrolases, proteoglycans, free-radicals, lipid-derived mediators, and cytokines. For the MC to affect different physiological and pathophysiological processes, all these mediators must be carefully produced, tightly regulated, and selectively released upon appropriate stimulation.

1. Mechanism of Mast Cell Mediator Release

MC mediator production, storage and release are complex processes that are regulated by many factors. Traditionally MC mediators have been divided into two

groups based on the mechanism of production and release: a group of preformed mediators and a group of newly synthesized mediators. The former group includes biogenic amines and neutral proteinases, and the latter group includes lipid-derived mediators and many cytokines. However, it is important to recognize that this is an oversimplification. For example, it was believed that MC stored mediators are always released collectively upon MC activation by degranulation. This is inaccurate, as it has been shown that the release of histamine from MC can take place without the release of serotonin, another preformed mediator (46-49). This process, termed "piecemeal degranulation" has also been observed in basophils and eosinophils.

Nonetheless, in many cases, release of preformed mediators from MC occurs in the first 10 min post activation and does not require new protein synthesis. Alternatively, the MC can synthesize and release mediators that are either not stored in the granules or stored in small amounts. This process requires protein synthesis upon activation and usually occurs over a longer period. For example, Ag-mediated stimulation of rat peritoneal MC (RPMC) induces the release of IL-6. This release peaks at 6 to 9 hr post stimulation and is associated with mRNA upregulation and protein synthesis (23). However, not all newly-synthesized MC mediators are released over a long period of time. Lipid-derived mediators which depends on *de novo* protein synthesis can be synthesized and released by MC within 10 min post stimulation (50).

Given the diversity of MC mediators and considering that each mediator may have a broad range of functions, it is also misleading to categorize MC mediators into groups based on their assumed functions. In many reviews, MC mediators are categorized based

on their inflammatory function (proinflammatory vs. anti-inflammatory). Again, this is an oversimplification since many MC mediators possess both inflammatory and anti-inflammatory functions. For example, IFN-γ is commonly considered a proinflammatory cytokine (51), however, it has been shown to possess anti-inflammatory functions including the inhibition of TNF release by RPMC (52, 53).

Thus, each mediator has its own unique characteristics and functions. To study and understand more fully these characteristics and functions one should consider each mediator on its own as well as in the context of others released at the same time or sequentially.

2. Biogenic Amines

Histamine and serotonin are the two major biogenic amines present in MC granules in some species such as the rat. In human MC, histamine is the only biogenic amine expressed (19). The differential regulation of histamine and serotonin release by MC provided the first evidence for selective release of mediators from MC granules (46-49).

Histamine is commonly considered a potent proinflammatory and immunoregulatory mediator. It can cause bronchoconstriction, vasodilation, and increased vascular permeability (54-56). Histamine has four known receptors, which are present on many cell types including nerves, smooth muscle cells and MC (57). Histamine is synthesized in the Golgi apparatus by a process that includes the decarboxylation of histidine and is stored in MC granules at ~100 mM concentration

(58) RPMC store 10-30 pg/cell, whereas mucosal MC (MMC) contain much less (1-3pg/cell) (59). Release of MC histamine occurs immediately (within 10 min) upon activation by a variety of stimuli including Ag, calcium ionophores, compound 48/80 and others (19, 60).

3. Lipid-Derived Mediators

Lipid derived mediators are similar to MC amines in that they can be released rapidly (10 min) upon stimulation, but the former are not preformed and stored in the granules. After appropriate MC activation, arachidonic acid is released from phospholipids by enzymes including phospholipase (PL) A_2, PLC, and PLD, and metabolized by cyclooxygenase and lipoxygenase. This process occurs rapidly and produces prostaglandins, thromboxanes, leukotrienes and 5-, 12-hydroxy-eicosatetraenoic acids (HETEs). These lipid–derived mediators induce a broad spectrum of biological actions ranging from bronchorelaxation and bronchoconstriction to the recruitment and activation of inflammatory cells (61).

4. Acid Hydrolases

Acid hydrolases include β-hexosaminidase (β-hex), β-glucuronidase, β-D-galactosidase and arylsulfatase A. β-hex is found in the MC granules and its release occurs immediately and ends within 10 min of activation. β-hex is likely to work in concert with neutral proteases in the degradation of glycoproteins and proteoglycans (20).

Acid hydrolases are active optimally at an acidic pH (~ 4 pH, 269), such as at sites of inflammation. Thus, they may play a role in inflammatory processes. Although, the precise function of β-hex is unknown, its release is induced by most MC activators. Thus, β-hex is commonly measured as an indicator of MC activation and since it is always released in parallel with histamine it has been considered a marker for histamine release by enriched MC populations *in vitro*.

5. Cytokines

MC can express many of the known cytokines at least at an mRNA level. When activated, MC can release cytokines through various mechanisms. Some cytokines are constitutively produced and stored in MC granules and these can be released rapidly upon activation. Others are synthesized and released following selective activation. Thus, certain secretagogues can selectively stimulate the release of certain cytokines without the others. For example, LPS, thrombin and PAR1-AP, induce the selective release of IL-6, but not TNF from MC (21). Although MC have been shown to release many cytokines *in vitro*, which MC-derived cytokines are important in different *in vivo* settings remains to be clarified (23).

TNF was the first cytokine to be discovered in the MC (62) and is one of the most extensively studied MC cytokines. RPMC store approximately 400 pg/10^6 cells (63), whereas a much smaller amount is stored in rat intestinal MMC (~70 pg/10^6 cells) and human skin MC (64). Regardless, most MC are able to synthesize and release TNF upon appropriate stimulation. TNF is a typical example of multifunctional immunoregulatory

cytokines involved in hematopoiesis and inflammation. TNF induces expression of adhesion molecules (ICAM-1 and VCAM-1) on endothelial cells (65) and it is also a monocyte chemoattractant (66). High doses of TNF have major effects on intestinal inflammation, including the induction of shock, diarrhea, and necrosis of colonic epithelial and endothelial cells (67). TNF produced by skin MC may also mediate MC cytotoxicity against tumor cell targets (64).

Similarly, IL-6 has been well characterized in MC. It is both a stored mediator and can be released over a long period of time. IL-6 plays a key role in plasma cell development (68, 69), and is involved in the induction of the acute phase response (70, 71). IL-6 has been shown to mediate neurite growth *in vitro* (72). This cytokine has also been shown to inhibit TNF production in RPMC and cultured human monocytes (73, 74).

6. Neutral Proteases

Neutral proteases comprise a large proportion of the stored mediators in MC. They make up between 20 and 50% of human MC weight (75), and there is a wealth of different types, many of which appear to be MC specific in their distribution.

a. Types of Mast Cell Proteases

There is a clear pattern of heterogeneity among different MC populations based on their protease expression. One MC phenotype common in the human lung and intestinal mucosa has been named MC_T, since MC-specific tryptase is the only protease identified in the granules (10 pg/cell) (76). By contrast, most MC in skin are designated

MC_{TC} since they contain both tryptase (35 pg/cell) and a MC-specific chymase (4.5 pg/cell) in their granules (76). MC_{TC} also contain other proteases including carboxypeptidase A (CP-A, 34.5 kDa, 5-20 pg/cell) and cathepsin G-like protease (30 kDa) (77, 78).

Proteases in rodent MC can be divided into two groups: serine proteases and metalloproteinases. Serine proteases can be further divided into two groups; chymases and tryptases because their predicted substrate binding pockets are similar to those of pancreatic chymotrypsin and trypsin, respectively (79). Chymase is an endopeptidase that cleaves peptide bonds with hydrophobic side chains from carboxyl to amino direction. Tryptase also cleaves proteins from the carboxyl terminal to amino terminal, but this occurs at basic side chains such as Arg and Lys (80). Carboxypeptidase A (CP-A) is the only major metalloprotease stored in MC granules. This zinc metallocarboxypeptidase is an exopeptidase that cleaves proteins at aromatic and aliphatic amino acids from carboxyl to amino terminal (81).

RAT		MOUSE	
MMC	CTMC	MMC	CTMC
RMCP-2	RMCP-1	MMCP-1	MMCP-2
RMCP-3	RMCP-5	MMCP-2	MMCP-3
RMCP-4	RMCP-6	MMCP-L	MMCP-4
RMCP-8	RMCP-7	MMCP-9	MMCP-5
RMCP-9	CP-A		MMCP-6
RMCP-10			MMCP-7
			MMCP-8
			CP-A
			mTMT
			mT-4

Table 1: Neutral proteases in rodent MMC and CTMC subpopulations.

RPMC express RMCP-1 (24-30 pg/cell), RMCP-5 and carboxypeptidase A (20-25 pg/cell) as the three major MC proteases (Table 1). By contrast, rat mucosal MC express predominantly RMCP-2 (26 pg/cell) (82-84). Similarly, in the mouse, mRNA for MMCP-1 and 2 are exclusive to MMC and not CTMC. In rat, two tryptases have been identified RMCP-6 and 7. Small amounts of RMCP-6 and –7 may be expressed in mature RPMC (82). In the mouse, four tryptases have been identified, MMCP-6 is 32 kDa and has a known cDNA and seems to be stored in the granules (85). MMCP-7 is expressed early in MC development and can be lost with further maturation (86). Recently, two new mouse tryptases have been cloned; mouse tryptase-4 (mT-4) and mouse trans-membrane tryptase (mTMT) (87). These tryptases possess hydrophobic regions in their C-terminus that anchor them to the membrane (88). A human homolog for mTMT has been cloned (89).

The list of chymases in rodent MC is longer than that of tryptases. Recently, Lutzelshwab *et al.*, elucidated the structure and complexity of proteases expressed by different subpopulations of rat MC. Using cDNA libraries of RPMC and from the rat mucosal MC line RBL-1, 10 different serine proteases (RMCP-1-10), and the MC carboxypeptidase A were isolated and characterized (Table 1). Based on their protease content, three separate subpopulations of MC were found. Connective tissue MC (CTMC) from the ear and peritoneum that express the chymases RMCP-1 and -5, the tryptases RMCP-6, and -7 and the carboxypeptidase A. However, CTMC from these two organs may be regarded as separate subpopulations based on a large difference in the level of expression of RMCP-7. RMCP-2 and the three closely related proteases of the

RMCP-8, 9 and 10 subfamily were identified as major MC proteases in rat MMC. Unlike human MC, cathepsin G or cathepsin G-like proteases were not detected in any rat MC populations. Furthermore, by studying mRNA frequencies for the various proteases expressed by normal tissue MC, RPMC were found to have mRNA frequencies for major proteases in the range of 5 % of the total mRNA pool (90). These very high levels of proteases suggest a continuous production and release of proteases by MC. These levels are only found in terminally differentiated cells and are comparable to the expression of Ig (5-10% of total mRNA pool) in plasma cells.

Most of MC proteases range in size from 26 to 41 kDa (79, 90). Tryptases and chymases have different biochemical properties and thus, can be separated based on their pI. Tryptase has an acidic pI ranging between 5.95 and 6.5, while chymases and CP-A have a more basic pI of 9.4 to 10 (82). Using two-dimensional SDS-PAGE, our lab previously showed that RPMC contain six basic proteins of 26 to 38 kDa with pI $\geq$ 8.5. RMCP-1 was shown to be 26 to 28 kDa with pI > 9.0. By microsequencing the NH_2 terminal we identified two isoforms of RMCP-5 to be 29 and 30 kDa. We also identified 3 isoforms of CP-A to be 33, 35, and 36 kDa proteins with high homology to mouse MC carboxypeptidase A (82). Immunoblot studies with RMCP-1, RMCP-5 and CP-A showed at least three proteins which reacted with Ab to CP-A, whereas Ab to RMCP-5 detected three adjacent polypeptides, rather than just the two identified using microsequence analysis. Immunoblot analysis of proteins from intestinal MMC showed RMCP-2, but not RMCP-1, RMCP-5, or MC-CP-A, suggesting that these proteases are restricted in their distribution to selected MC populations (82).

b. Protease Structure and Production

MC proteases including CP-A are stored in MC granules in a fully active form. They are initially translated as preproenzymes and after directing them to the ER the signal peptide is cleaved. They become activated at an unknown point through the secretory pathway by the cleavage of the N-terminal propeptide. After removal of propeptide the protein undergoes refolding which forms the mature active site (79). In the case of tryptase, pro-tryptase may be cleaved by dipeptidylaminopeptidase (DPPI) (91). Similarly, CP-A requires cleavage of pro-enzyme to become activated (92).

After translation, neutral proteases undergo N-linked (asparagine) glycosylation in the ER and modification of carbohydrate moieties in Golgi apparatus (1 or 2 per protease) (93, 94). Multiple disulphide bonds are also created in the process. For example, tryptase contains four disulphide bonds (85). MC neutral proteases display the typical serine protease bilobed sandwich structure (95). CP-A possesses two globular domains and one large catalytic domain. A small pro-peptide domain covers the catalytic site.

In the granules, proteases are stored in macromolecular complexes (20, 000,000 Da) associated with proteoglycans (PG) (81, 96). Charge interactions play a role in PG-protease complexes. At the normal granule pH (5.5) most MC serine proteases have a net positive charge (97-100), whereas heparin and chondroitin sulfate E glycosaminoglycans are highly acidic. These charge differences are thought to play a role in maintaining the interaction. However, more than a simple charge mechanism is involved in PG-protease interaction, as even when tryptase is in high pH environments (after secretion), which

gives it a final net negative charge, it remains attached to PG (101, 102). In the granules, human tryptase forms tetramers that are stabilized by heparin and persistence of this association is required for activity (101). RMCP-2 may form disulfide bonded-homodimers (103). These granular interactions play key roles in the regulation of these proteases, as well as other mediators that are stored within the granules.

c. Regulation of Mast Cell Protease Function

Even though MC proteases are stored in an active form, the low pH of the granules helps prevent their protease activity and autolysis (97-99). Once the proteases are released into the ECM (pH 7.0), most of the proteases especially those with high pI, remain associated with negatively charged PG within their macromolecular complexes. In most cases these macromolecules remain in the tissue for over an hour. The large size of the exocytosed macromolecular complexes hinders the diffusion of the proteases (104, 105). However, low pI proteases such as the tryptase mMCP-7 loses its net positive charge once it is released into the 7 pH environment, allowing it to dissociate from the macromolecular complex. Nonetheless, heparin may provide sites for the accumulation of chymase on basement membranes and/or the surface of various cells near sites of MC activation (106).

A major mechanism of inactivating MC proteases *in vivo* is through endocytosis. A variety of cell types in connective tissue can endocytose and inactivate MC proteases or protease complexes, including macrophages, endothelial cells and fibroblasts (107-110). However, *in vitro* studies show that plasma inhibitors may also play a role in inactivating

some MC proteases. For example, α_1-antichymotrypsin inhibitor, α_1-protease inhibitor, secretory leukocyte protease inhibitor, lactoferrin, and α_2-macroglobulin can inhibit the activity of chymases (111-115, 115). Interestingly, serotonin, but not histamine, is competitive inhibitor of RMCP-I (116). Human MC tryptases purified from lung are resistant to inactivation by protease inhibitors of plasma (117-119). The role of heparin in regulating the function of MC protease is complex. MC heparin can protect RMCP-1 from inactivation by numerous plasma inhibitors (113). However, it can also enhance the inactivation by some, such as secretory leukocyte protease inhibitor (120).

In vitro, many non-physiological protease inhibitors have been used in the study of MC protease function. Among the most prominent is soybean trypsin inhibitor (SBTI), which is used as a broad spectrum MC protease inhibitor. SBTI has been shown to strongly inhibit human lung tryptase, human pituitary tryptase, human skin chymase, RMCP-1 ($K_i = 8.3 \times 10^{-8}$) and RMCP-2 (106).

In summary, the ubiquitous distribution of MC and their ability to synthesize, store and release a wide range of mediators, allows them to play key roles in many diseases, including inflammatory diseases, fibrotic diseases, tumors, and neuro-degenerative diseases (7). Among the most potent of MC inflammatory mediators are proteases, which possess a broad spectrum of regulatory effects on structural and immune cells, as well as the ECM. The mechanism by which proteases influence their microenvironment is not well understood, but there is little doubt that understanding these mechanisms could lead to identification of novel therapeutic targets.

D. SERINE PROTEASES: MECHANISM OF CELLULAR ACTIVATION

Serine proteases are members of a large family that includes thrombin, trypsin, granzymes, and MC chymases and tryptases. They are produced and released by several cell types. Their ability to proteolytically modify a variety of substrates makes them important players in various processes, such as homeostasis, inflammation and tissue remodeling. The role of serine proteases in inflammation has been well documented. They are directly involved in the activation and deactivation of many inflammatory proteins, in degradation of the extracellular matrix and in cellular activation. (121). Indeed, serine proteases have direct effects on a number of cells including monocytes, smooth muscle cells, and lymphocytes (122). However, the mechanism by which serine proteases activated cells is not understood.

Recently, protease-activated receptors (PAR) were identified (123). The first one recognized was a G-protein coupled receptor specifically activated by thrombin. Since its discovery, three other PAR subtypes have been cloned based on a conserved DNA sequence and activity (124). PAR are expressed by a variety of cell types including, endothelial cells, epithelial cells, fibroblasts, monocytes, platelets, smooth muscle cells and neurons. To begin to define the main physiologic activator of each PAR subtype, appropriate consideration has to be given to the physiological location of the cells expressing it, the level of expression of PAR protein on the cell surface, the concentration

of proteases surrounding PAR-expressing cells and the presence of protease inhibitors in the microenvironment. Thus, the identification of primary PAR activators is complicated and difficult. Nonetheless, thrombin is generally considered the main physiological activator of PAR-1 and –3, whereas, PAR-2 and –4 are primarily activated by trypsin-like proteases, such as MC tryptase (122).

PAR-1 and –2 are the most extensively studied of all PAR and many aspects of their mechanism of activation are well characterized. They have been implicated in many physiological functions including, protease-induced inflammation, chemotaxis, mitogenesis, embryogenesis, and brain function and repair (124). In this section, an overview of the characteristics of PAR-1 and -2 will be reviewed with emphasis on their mechanism of action, signaling and pharmacology.

PAR-3 and PAR-4 can be activated by thrombin and trypsin, respectively. However, their functions are not as well understood. Given that there are no known PAR3-AP available for investigation, it is difficult to study their function. PAR-4 has been very recently identified and few analytical reagents are available. Thus, our study was limited to PAR-1 and PAR-2.

1. Protease-Activated Receptors

a. Receptor Structure and Mechanism of Activation

PAR possess three extracellular loops, three intracellular loops, an extracellular NH_2 terminus involved in activation and an intracellular COOH terminus involved in deactivation and G-protein mediated signaling (Fig. 2). Thus, PAR belong to the seven

transmembrane domain G-coupled receptor superfamily. The extracellular NH_2 terminus of PAR contains a cleavage site for certain serine proteases. In the case of PAR-1, the thrombin cleavage site is followed by a series of charged residues (125). The charged domain can interact with the anion-binding site on thrombin, allowing the initial binding of thrombin to the receptor. This is thought to cause a conformational change in the receptor, which exposes the cleavage site on PAR to the catalytic site of thrombin (126). This anion-biding domain is considered essential since its deletion leads to the loss of PAR-1 activation by thrombin.

Serine protease cleavage of PAR (Fig. 3a) exposes a new NH_2-terminus (tethered ligand) and changes conformation of the receptor allowing the tethered ligand to interact with the activation site on the 2^{nd} extracellular loop (ECL-2) of the receptor. Peptides corresponding to the tethered ligand domains of PAR-1, PAR-2 and –4 are able to interact directly with the activation site and act as agonists on their respective receptors (Fig. 3b) (125). These peptides are useful tools in studying specific receptor function, since they are generally more selective in their activation of PAR subtypes than serine proteases, which can activate multiple PAR.

PAR STRUCTURE

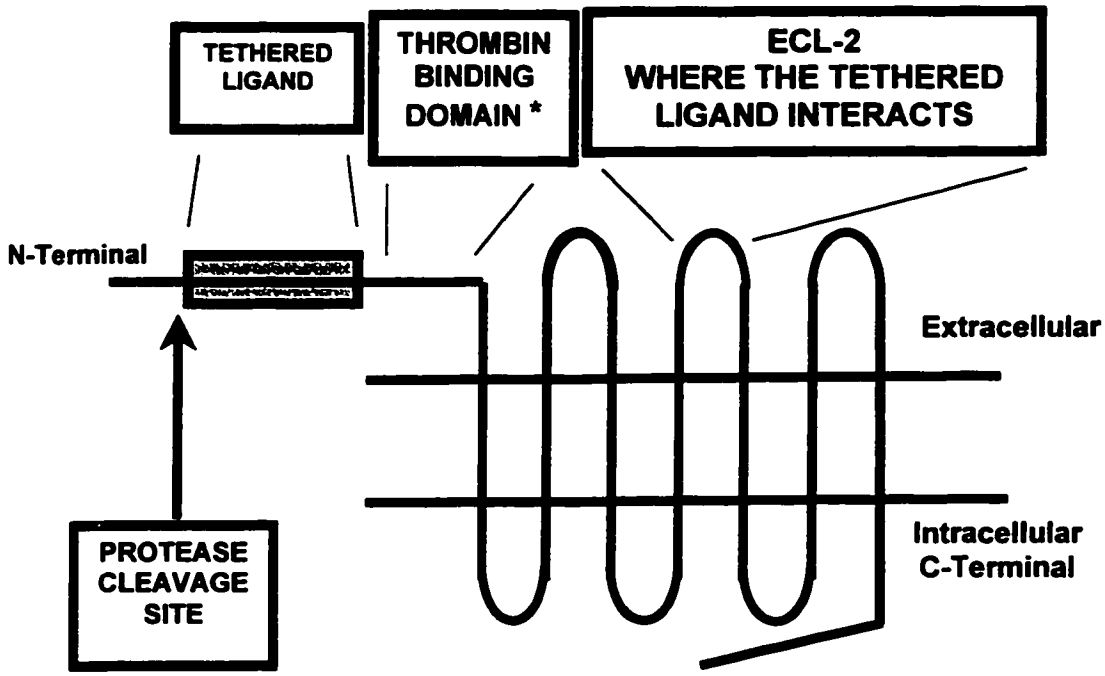

Figure 2: Structural features of PAR. The areas involved in PAR activation are highlighted. The N-terminal protease cleavage site, the anion bind region to which thrombin binds, the 2nd extracellular loop and the C-terminal tail involved in deactivation and signaling. * indicates a region found only in PAR-1.

b. Signaling Mechanism

When agonists interact with G protein-coupled receptors they induce conformational changes that allow G protein activation (127). The α subunit of heterotrimeric G proteins catalyzes the exchange of GDP by GTP. Both the α and βγ heterodimer will then activate different effector enzymes and ion channels. The signal is terminated when GTP is hydrolyzed. Of the PAR, the signaling mechanisms of PAR-1 that is activated by thrombin have been most extensively studied. PAR-1 couples to several different G proteins (Fig. 4) including $G_{12/13}$, G_i and $G_{q/11}$. The signal transduction pathway via Gq protein activates phospholipase C-β (PLCβ) that in turn hydrolyses phosphoinositide (PIP_2) to inositol triphosphate (IP_3) and diacylglycerol (DAG). DAG activates protein kinase C (PKC) and IP_3 interacts with the endoplasmic reticulum to release Ca^{2+} stores (128). In CCL-39 cells activation of PAR-1 inhibits cAMP generation in a pertussis toxin (PT)-sensitive manner, suggesting that G_i-like proteins may be involved (128) in PAR-1-mediated signaling. Also, antibodies to $G_0\alpha$ inhibit PAR-1 activation in Chinese hamster lung fibroblasts (CCL-39) (129) suggesting the involvement of G_0 in PAR-1 mediated signaling in these cells. Thrombin stimulates incorporation of photoreactive GTP analog [α-^{32}P]GTP azidoanilide into G_{12} and G_{13} in platelets (130), suggesting their involvement as well. It has been shown that multiple G proteins can mediate similar PAR-1-derived mitogenic signals in a single cell type. This can be seen in Balb/c 3T3 cells (Gq and Gi_2)(131) and in Chinese hamster CCL-39 cells (G_q and G_o) (132).

Like PAR1-AP, PAR2-AP and trypsin can activate G_i. The effects of PAR2-AP on adhesion of human gastric carcinoma cell line, MKN-1, to vitronectin can be almost completely blocked by the G_i inhibitor PT, whereas cell adhesion to fibronectin was PT insensitive. Thus, the activation of integrin $\alpha_5\beta_1$ and integrin $\alpha_v\beta_3$ by PAR-2-stimulated signaling are mediated by different G proteins in the same cell. Furthermore, Src kinase was shown to play a role in the regulation of integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ by the trypsin/PAR-2 signaling (133). Cleavage and activation of PAR-2 by trypsin results in activation of phospholipase C-β, formation of IP_3, mobilization of Ca^{2+}, and release of arachidonic acid metabolites and prostaglandins (126), suggesting that PAR-2 couples with $G_{\alpha q}$ or $G_{\alpha 0}$.

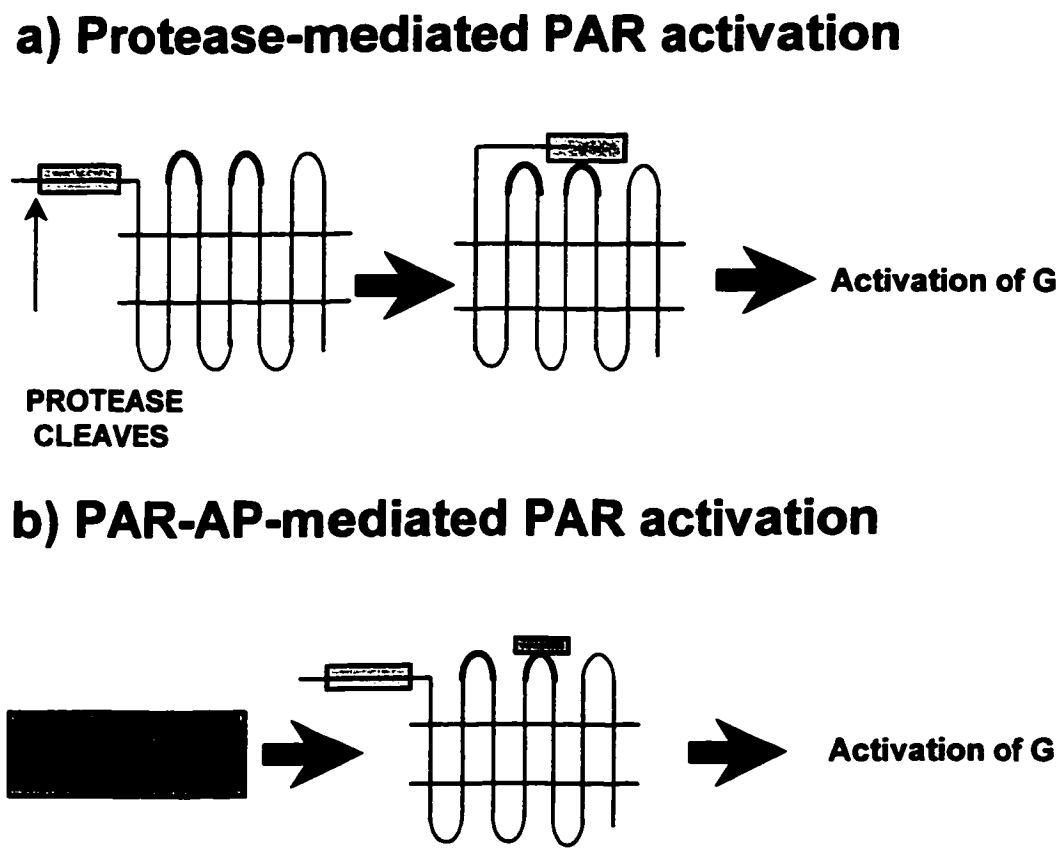

Figure 3: Mechanisms of PAR activation. a)Proteases cleave at the NH_2 terminus of PAR causing a conformational change that allows the tethere ligand to interact with the ECL-2. This activates a G-protein mediate signal. b) PAR can be activated directly by synthetic peptides (PAR-AP) mimicking the tethered ligand sequence.

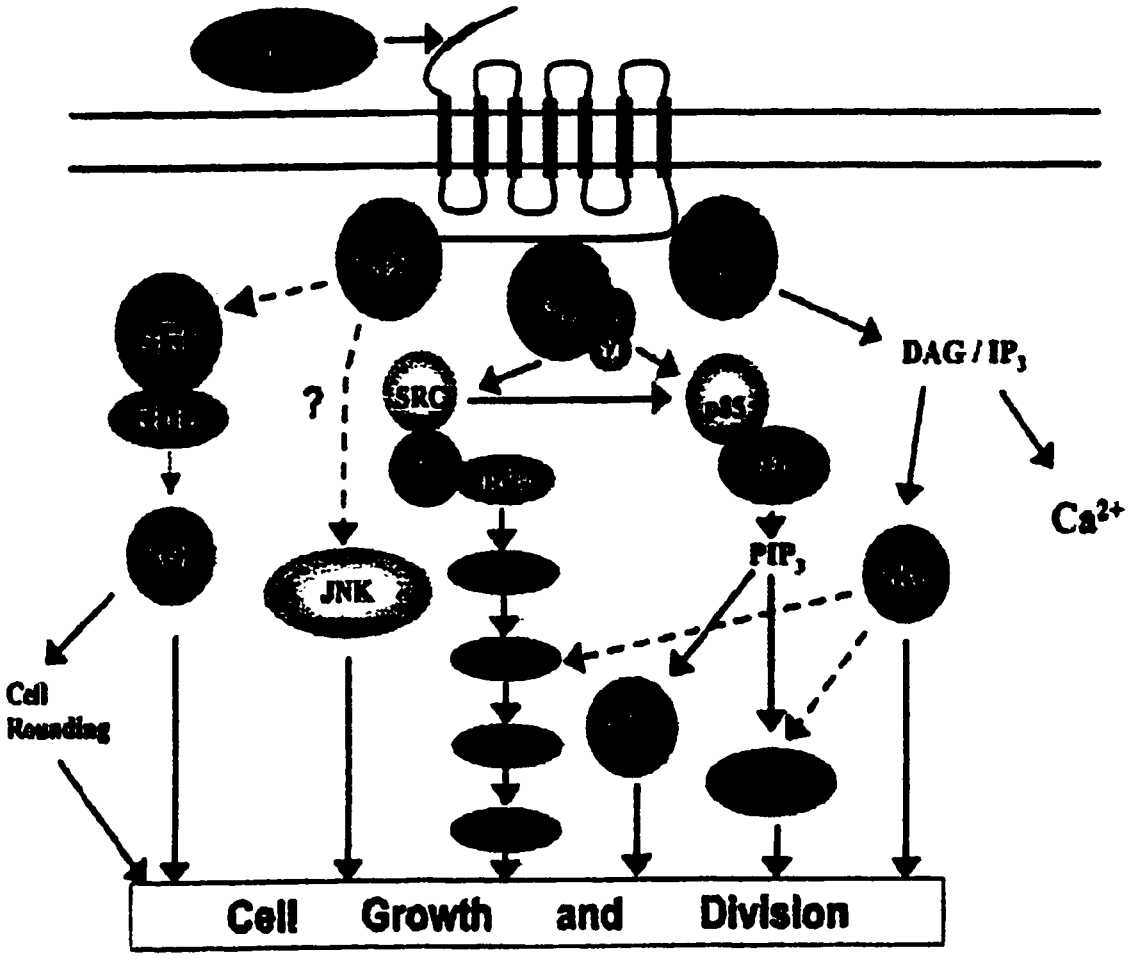

Figure 4: G-protein-dependent signaling pathways regulated through activation of PAR-1. The major signaling events regulated by PAR-1. Dashed lines represent putative pathways where the intermediates have not been identified or fully demonstrated for PAR-1, but are a feature of other G-protein-coupled receptors. Some well identified intermediates and precursors have been omitted for sake of clarity. DAG, diacylglycerol; SRC, $pp60^{src}$ and related kinases; MAPK, p42/44 mitogen-activated protein kinase; PKB, protein kinase B; GER, Rho GTP exchange factor; PIP3, phophatidylinositol 3,4,5-trisphosphate. This figure was adapted from (122).

c. Deactivation and Desensitization

Desensitization of PAR-1 has been studied extensively. As for most G-protein-coupled receptors, PAR-1 desensitization may be mediated by the uncoupling of the receptor from the G-protein, followed by internalization (134). This requires phosphorylation of distinct residues in the C-terminal, which is mediated by G-protein receptor kinases (135). However, other mechanisms can cause PAR receptor shutoff (122) (Fig. 5).

Receptor deactivation can also occur through proteolytic cleavage and destruction of the tethered ligand of the receptor. For example, plasmin can cleave sites in the N-terminus domain and desensitize PAR-1 to thrombin-mediated Ca^{2+} signalling (136). In this case, the receptor remains responsive to PAR1-AP-activation. PAR shutoff can also be mediated by ligand sequestration, which involves the removal of the tethered ligand from within the binding pocket of ECL-2 (137).

Once the receptor is activated, PAR-1 is internalized into endosomes within the 60 sec. This process does not require proteolytic cleavage of the receptor since it can be mediated by PAR1-AP. A large percentage of the receptors are then transported to lysosomes for degradation in a process mediated by the C-terminal domain of PAR-1. However, a portion of PAR-1 are recycled to the cell surface.

In most cell types, PAR-1 are found both expressed on the surface and intracellularly. The receptors are constantly cycling between the intracellular pool and

the surface, by a mechanism independent of agonist activation, trafficking and C-terminus phosphorylation (138). This is thought to allow rapid receptor replacement for reactivation, independent of new receptor synthesis. However, this intracellular pool is not available for reactivation in every cell type, since in some cells activation recovery is slow and requires protein synthesis (139).

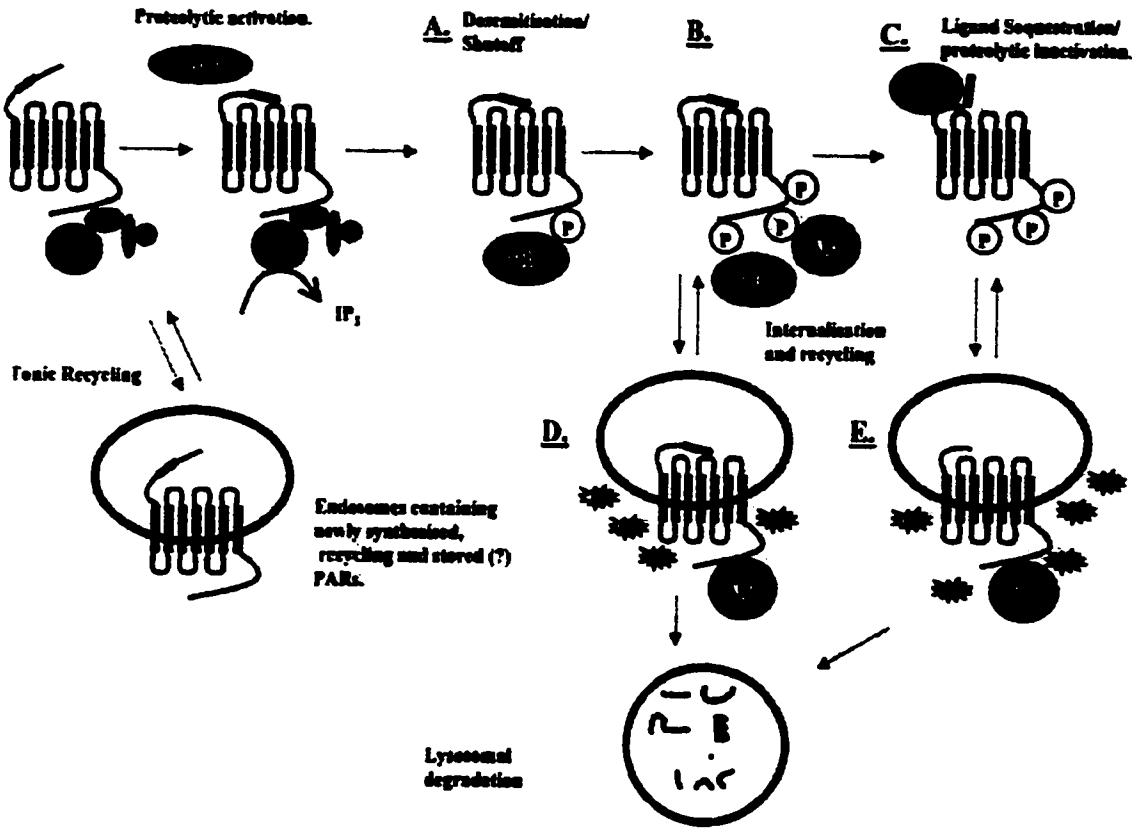

Figure 5: Modes of PAR-1 desensitization. C-terminal phosphorylation at distinc residues can mediate rapid receptor shutoff (A), followed by further phosphorylatio and receptor internalization (B). Additional modes of receptor desensitization ma involve either sequestration and or proteolytic degradation of the tethered ligand (C). It is unclear whether receptor internalization predominantly occurs immediately following phosphorylation (D), and a portion of this population recycled to the plasma membrane before sequestration, or whether the ligand sequestration event occurs prior to any internalization (E). Indeed, if the latter scenario is the case, then this pool of internalized receptors must itself recycle to some degree and presumabl remain sensitive to soluble agonist. AR, b-arrestin; C, clathrin; α, β, and γ, G protein subunits; THR, thrombin. This figure was adapted from (122).

E. PATHOPHYSIOLOGICAL ROLES OF PROTEASES

The cellular functions of PAR in many cases are similar to the cellular functions of the serine proteases that activate them. Thus, to understand the physiological roles of thrombin and trypsin/tryptase, the physiological roles of PAR-1 and PAR-2 should be considered. The focus of this section will be on two proteases, thrombin and tryptase and their respective PAR. However, it is important to note that trypsin-like proteases can activate PAR-1 and thus some of the PAR-1 mediated effects might be caused by trypsin-like proteases *in vivo* (126). Futhermore, in this section we will review some of the physiological and pathophysiological functions of chymase and CP-A, which are the primary proteases produced by RPMC.

1. Thrombin and PAR-1

During vascular injury, the initiation of the coagulation cascade leads to generation of thrombin. The blood clot is formed after thrombin cleaves fibrinogen to form fibrin, which activates clotting factors V, VIII, XIII and protein C (140). Besides its fundamental role in the coagulation cascade, thrombin has many cellular functions. It has been implicated in many disease states including inflammatory disorders, CNS-diseases, and cancer.

a. Thrombosis and Vascular Remodeling

Thrombin's pivotal role in the regulation of platelet aggregation and clot formation makes it an important therapeutic target for cardiovascular diseases. The developments of antithrombotic agents were initially focused on the selective inhibition of proteolytic activity by thrombin. However, complete enzymatic inhibition of thrombin *in vivo*, has major side effects, such as prolonged bleeding (141). Thus, the identification of PAR opened new research opportunities to develop selective PAR antagonists. Indeed, inhibiting PAR-1 activity with PAR-1-specific antibodies reduced platelet-dependent cyclic flow in a monkey model of experimental arterial thrombosis without significantly altering hemostatic parameters. The PAR-1 antibodies were also shown to inhibit platelet aggregation *ex vivo* (142). Thus, highly selective PAR-1 antagonists are being developed and clinically tested.

PAR-1 has also been implicated in other cardiovascular disease conditions such as balloon catheter injury (143) and vascular remodeling (144). This has been shown by many *in vitro* and *ex vivo* studies in which thrombin and PAR1-AP stimulated mitogenesis in smooth muscle cells, endothelial cells and fibroblasts, either directly or by the release of growth factors (145-147).

b. Inflammation

Thrombin and PAR-1 have many proinflammatory functions. These include vasomotor functions, effects on vascular permeability and the induction of inflammatory cell chemotaxis. Thrombin affects vasculature either directly or by inducing the release

of vasoactive mediators by other cells. Thrombin and PAR1-AP relax dog isolated coronary arteries (85), rat aorta (104) and constrict human umbilical arteries (148). The direct endothelial cell-mediated vasodilatory effects of thrombin and PAR-1 are NO dependent while the vasoconstrictory effects require extracellular Ca^{2+} (149, 150). In human umbilical veins, thrombin-mediated contraction of endothelial cells is rapid and results in gap formation that increases vascular permeability (151). The indirect effects of thrombin and PAR-1 may be mediated via inflammatory cells. For example, thrombin and PAR1-AP activation may induce the release of histamine from certain MC types (152), which is a potent vasoconstrictory agent (153). The effects of thrombin on MC will be discussed in considerable detail later in this chapter.

Inflammatory cell infiltration is a characteristic of chronic and acute inflammation. Thrombin and PAR-1 may cause leukocyte chemotaxis by mediating the release of chemotactic mediators. Direct activation of endothelial cells by thrombin and PAR-1 induces the release of monocyte chemotactic protein-1 (154). Furthermore, thrombin was shown to induce neutrophil adhesion and extravasation (155, 156) by a mechanism that involved the upregulation of P-selectin by endothelial cells (146). PAR-1 has also been implicated in crescentric glomerulonephritis (157), in which PAR-1 deficient mice display decreased crescent formation and a reduction in the infiltration of inflammatory cells (158).

c. Cancer

Thrombin has been shown to induce adhesion in many tumor cells (159), through upregulation of adhesion molecule expression such as P-selectin. Thrombin also

promotes invasion of breast tumor cells (160) and enhances experimental pulmonary metastasis (161). Although, thrombin's involvement in tumor cell invasion and metastases is established, the role of PAR in this process is under investigation. However, recent studies show that PAR-1 antisense cDNA inhibits the invasion of metastatic breast carcinoma cells in experimental models (162).

d. Neurological Disorders

During and after brain injury, thrombin is one of the blood borne factors that may interact with brain tissue. Thrombin can enter the interstitial fluid of the CNS and initiate neuronal cell cytotoxicity (163). In rat brain, thrombin infusion causes inflammatory cell chemotaxis, proliferation of mesenchymal cells, and stimulation of angiogenesis. In addition, thrombin has been implicated in posttraumatic hyperexcitability and seizure (164, 165).

In the CNS, PAR1-AP induce neurite retraction (166), and protect neurons and astrocytes from death induced by hypoglycaemia and oxidative stress (167). PAR1-AP have many other functions on brain astrocytes, including the induction of morphological changes (168), and proliferation (169). PAR-1 can also activate astrocytes to release endothelin-1 and NGF (170, 171).

2. Trypsin-Like Proteases and PAR-2

Trypsin-like proteases are serine proteases that possess a substrate-binding pocket similar to pancreatic trypsin. Although pancreatic trypsin and MC-derived tryptase have

different biological localizations they are known to bind and cleave similar substrates. Tryptase makes up to 20% of total protein within human MC and is responsible for at least 95% of the trypsin-like activity (172). Tryptase is found in almost all MC and is the major secretory protease of human MC (173). Tryptase can degrade many proteases including neuropeptides and basement membrane proteins *in vitro* (29, 174). In addition to these degradative functions, tryptase has many cellular functions. One of the most prominent functions of tryptase and its PAR-2 is their role in inflammation.

Because tryptase is stored in MC granules and can be released upon MC degranulation, it has been considered as a marker for MC activation. This protease appears to be important clinically because of its association with many inflammatory diseases. High levels of tryptase have been detected in the serum of patients with systemic anaphylaxis and in bronchoalveolar lavage fluid (BAL) of patients with bronchial asthma and interstitial lung disease (175-177). High levels of tryptase can be found fluids from nasal lavage of patients of allergic rhinitis and in synovial in fluid from arthritis patients (178, 179). Even skin blister fluid from patients with allergic contact dermatitis has increased tryptase concentrations (180).

Because tryptase is released upon MC degranulation, PAR-2 may be involved in the pathogenesis of a variety of acute inflammatory disorders. Indeed, studies in PAR-2 knock out mice provide direct evidence for the involvement of PAR-2 in immediate inflammation, as the lack of PAR-2 in mice results in a delayed onset of inflammation (181). Furthermore, in cultured endothelial cells PAR-2 mRNA is upregulated after the addition of IL-1α, TNF-α, or LPS supporting a role for PAR-2 during inflammation (182).

In vivo, PAR2-AP stimulation of rat mesenteric postcapillary venules contributes to several early inflammatory events, including leukocyte rolling, adherence, and recruitment, by a mechanism that is platelet-activating factor (PAF)-dependent (183). In addition, PAR-2 has been implicated in a variety of inflammatory conditions, including pain and intestinal and airway diseases (184-186).

a. Neurological Regulation and Inflammatory Pain

There are multiple lines of evidence suggesting a role for tryptase and PAR-2 in the regulation of neurological function. PAR-2 agonists can activate neuronal cells. PAR-2 stimulation is toxic to hippocampal neurons (187) and may contribute to motility disturbances during intestinal trauma (188). Tryptase is elevated in the cerebrospinal fluid of MS patients (189). PAR-2 is expressed by a variety of CNS cells, including afferent sensory nerves (187, 190), rat astrocytes and C6 glioma cells (191, 192). Many of these cells can respond to trypsin-like proteases and PAR2-AP activation. For example, in rat glial cells, tryptase and PAR2-AP activate a transient rise in Ca^{2+} (191). Moreover, a novel rat trypsin-like protease expressed in rat brain activates A172 glioblastoma cells by a PAR-2-mediated mechanism. MC tryptase as well as PAR1 and PAR2-AP regulate morphology, growth and survival of guinea-pig myenteric neurons and astrocytes (188).

Neurons may be notably involved in the trypsin, tryptase and PAR-2-mediated inflammation. This is supported by a recent study in which trypsin, tryptase and PAR2-

AP were shown to directly signal to neurons to mobilize calcium and stimulate release of neuropeptides that mediate inflammatory edema (190).

Given that MC are in close proximity with sensory nerve endings in both animals and humans, activation of MC can release tryptase, which may act on PAR-2 on nerves to release inflammatory neuropeptides (190). Indeed, trypsin-induces contraction of guinea pig bronchus in a mechanism that is neurokinin (NK)-mediated (193). In intestinal studies, PAR-2 has been implicated in hyperalgesia of conscious rats (186) and can mediate long-lasting thermal hyperalgesia (185), suggesting a role for trypsin-like proteases in pain-associated inflammatory conditions, such as inflammatory bowel disease, where there is evidence that PAR-2 may be activated (122).

b. Airway Inflammation

The role of tryptase in pulmonary diseases has been repeatedly demonstrated especially in allergic pulmonary disorders such as asthma. *In vivo,* tryptase inhibitors significantly reduce allergen-induced airway and cutaneous responses suggesting a role for tryptase in airway inflammation (194, 195). Furthermore, high levels of tryptase have been found in bronchoalveolar lavage fluid from patients with allergic alveolitis, bronchial carcinoma (177) and asthma (176). Tryptase has potent effects on the epithelium as for example, stimulation of human H292 epithelial cells with tryptase induced mitogenesis, IL-8 release and upregulation of intracellular adhesion molecule-1 (ICAM1) (196, 197). The size and number of epithelial cells lining the airway mucosa is

increased in asthmatic subjects who have high levels of tryptase in the airways (198, 199).

Although tryptase has been strongly shown to have proinflammatory roles in the airways, the role of PAR-2 in airway inflammation is not well understood. Cocks *et al.* (61) showed that in the mouse, PAR-2 activation mediated bronchorelaxation of isolated bronchi by a mechanism that is PGE-2 and epithelial dependent. Similarly in guinea pigs, both PAR-2 and trypsin mediated relaxation of isolated bronchi, and in the rat PAR2-AP inhibited serotonin-induced bronchoconstriction *in vivo* (61, 200). Another study in guinea pigs showed that PAR2-AP inhibits histamine-mediated bronchoconstriction (54), through a prostaglandin-independent mechanism. However, in another study, contrasting results were shown. Like trypsin, the PAR2-AP, SLIGRL-NH_2 (SLI), caused bronchoconstriction in guinea pig intrapulmonary bronchi, which was inhibited by NK-receptor antagonists and was potentiated by NO. However, in the same study trypsin and SLI relaxed isolated trachea and main bronchi suggesting differential PAR-2 functions in different locations in the airways (201). Thus, whether one role of PAR-2 in the airways is to cause bronchorelaxation or bronchoconstriction requires more investigation.

There are other *in vivo* studies that support a strong proinflammatory role for PAR-2 in the airways. For example, PAR-2 activation of airway epithelial cells mediates the release of the eosinophil survival-promoting factor GM-CSF and matrix metalloproteinases (202, 203). In addition, there is a growing body of evidence suggesting that PAR-2-induced proinflammatory functions are MC-mediated. These

will be discussed in detail later in this chapter. Finally, both tryptase and PAR2-AP stimulate human airway smooth muscle cells to proliferate, suggesting a role of PAR-AP in airway remodeling. Tryptase may also enhance proliferation of fibroblasts.

Given this wide profile of disease association, tryptase has been investigated as a potential target for therapeutic intervention (204). Thus, understanding the mechanisms that regulate release of MC tryptase and elucidating the mechanisms by which tryptase functions may lead to new discoveries in the management of inflammatory disorders.

3. Chymases

MC chymases can degrade neuropeptides, generate angiotensin II, stimulate fibroblast proliferation, destroy high-molecular-weight kininogen and degrade basal lamina *in vitro*. Chymase can also cleave pro-collagenase, many cytokines and bioactive peptides. Thus, chymase has important functions in inflammatory tissues through its proteolytic activities (205). Chymase effects *in vivo* are not limited to tissue and protein degradation. Chymase may also have important effects on chemotaxis and cellular activation. Chymase can cleave IgG1 to produce a fragment that has chemotactic activity for neutrophils *in vitro* and *in vivo* (206). In humans, MC chymase is also a neutrophil chemotactic factor for macrophages, eosinophils and other inflammatory cells *in vivo* (207, 208).

Being a major MC protease, chymase functions have been implicated in several inflammatory diseases. However, chymase is prominent for its angiogenic functions. It generates angiotensin II from angiotensin I in human, primate and dog and thus can

replace angiotensin–converting enzyme (ACE) that causes hypertrophy and remodelling of cardiovascular tissue (205).

Chymase may also have important regulatory functions on PAR. Studies showed that chymotrypsin cleaves PAR-1 tethered ligand at site that would deactivate the receptor (209, 210). Indeed, treatment of fibroblasts with chymase renders them insusceptible to thrombin activation. This effect depends on chymase proteolytic activity (111). Similarly, treatment of platelets with thrombin and PAR1-AP causes aggregation. However, when pre-treated with chymotrypsin, platelets only respond to PAR1-AP and not thrombin (211). Interestingly, thrombin is also sensitive to rapid inactivation by RMCP-1 (212). This inactivation is increased significantly when heparin is bound to RMCP-1 (213). Thus, chymotrypsin-like proteases may regulate thrombin function by two mechanisms; the inactivation of thrombin and its receptor (211).

4. Carboxypeptidase A

The biological functions of carboxypeptidase (CP)-A are poorly known, as are its physiological and pathophysiological substrates. However, many neuropeptide and vasoactive peptides can be degraded by CP-A *in vitro* including: angiotensin, kinetensin, enkephalin, neuromedin N, neurotensin, and xenopsin (214, 215). Since CP-A is an exopeptidase and rat MC protease (RMCP)-1 (see below) is an endopeptidase the cleavage of substrates by RMCP-1 exposes new sites for CP-A degradation. For example, apolipoprotein B can be degraded sequentially by RMCP-1 and then CP-A (216). However, some of the above substrates, such as enkephalin, are cleaved by CP-A and not

the other MC proteases. Neurotensin, however, is cleaved only by chymase and CP-A (217). Thus, CP-A can work alone or sequentially with other MC proteases to degrade substrates.

In summary, proteases are involved in various pathophysiological conditions. The effect of proteases on their microenvironment can be mediated directly by the degradation of ECM protein or activation of structural cells. However, proteases can also affect pathophysiological processes through the activation of effector cells to release their mediators. MC are implicated in protease-induced inflammation *iv vivo*. Thus, understanding the effects of proteases on MC are key to understanding protease-mediated inflammatory processes.

F. EFFECTS OF SERINE PROTEASES ON MAST CELL FUNCTION

In this section, the current knowledge of the effects of serine proteases and PAR-AP on MC will be reviewed. Considering that MC produce, store and constitutively release numerous proteases both *in vivo* and *in vitro* (19), studying the effects of proteases on MC involves assessment of autocrine and paracrine regulation of MC. Moreover, since the release of serine proteases is increased in response to some stimuli, proteases may constitute means by which MC communicate with other cells. Numerous serine proteases are also released by other cells including neutrophils, and thus proteases may also be means by which other cells communicate with MC and influence their functions.

1. Effects of Thrombin on Mast Cells

The effect of thrombin on inflammatory responses and in several diseases, were described in the previous section. In this section, we provide evidence that many of these effects of thrombin are MC-mediated. The effect of thrombin on BMMC has been well characterized. Thrombin binds directly to the surface of MC and activates MC (218). Thrombin-stimulation of murine BMMC *in vitro* results in rapid formation of inositol phosphates and β-hex release (218). This activation may be physiologically relevant because near physiologic levels of thrombin can trigger degranulation of BMMC and a transient rise in intracellular cAMP(219). The activation is dose dependent and is reliant on the proteolytic activity of thrombin since pretreatment of thrombin with 0.2 mM diisopropylfluorophosphate (DFP), a potent serine protease inhibitor, abolishes 90% of the degranulation activity of thrombin (219). This may involve a PAR. The thrombin effect on murine MC is not limited to release of stored mediator, as it can also regulate cytokine release. A recent study in mouse PMC and C1.MC/C57.1 MC showed that both thrombin and PAR1-AP mediate the selective release of IL-6, but not TNF (21). Investigating the mechanism of activation showed that both PI_3 and sphingosin-kinase are involved in this process. The fact that both thrombin and PAR1-AP can activate murine MC to release stored mediators and cytokines provides good evidence that at least some of the pathophysiological roles of thrombin are MC-mediated.

There is a marked heterogeneity in the response of MC to thrombin, as although thrombin activates mouse MC degranulation and cytokine release, the response of RPMC

to thrombin seems to be different. In an early study that compared the effect of thrombin on the release of stored mediators from mouse BMMC and RPMC, both cells released stored mediators upon exposure to IgE-Ag, whereas only BMMC and not RPMC were stimulated by thrombin (220). This is consistent with another study in which thrombin was shown to stimulate heparin secretion by RPMC, independent of histamine release. This activation was dependent on the proteolytic activity of thrombin, since DFP-thrombin was unable to activate MC (221).

In contrast, Strukova *et al.*, who investigated thrombin-mediated events implicated in MC activation, showed that thrombin can activate histamine release by RPMC (152). In this study, the responsiveness of RPMC to different forms of thrombin was investigated by studying ion conductance, intracellular pH, cyclic guanosine monophosphate (cGMP) concentration, and release of histamine. By measuring Na/H^+ exchange in reactions involving protein kinase C, α-thrombin was able to activate MC. Also, it gave a simultaneous elevation in cell conductance and capacitance. Low concentrations of α-thrombin stimulated an increase in cGMP and simultaneous decrease of histamine release. However, they also showed that higher concentrations of thrombin (> 1 μmol/L) induced an increase of histamine release. They also reported other thrombin-mediated RPMC events that were dependent on the proteolytic activity of thrombin. PAR1-AP was shown to induce the release of NO by RPMC (222). This release was inhibited by L-NAME or the constitutive NO-synthase inhibitor, calmidazolium. PAR1-AP also inhibits PAF release from A23187-treated MC in an NO-dependent mechanism.

The second line of evidence supporting MC involvement in thrombin-mediated inflammation is in rat studies. Injection of thrombin or PAR1-AP into the hind paw induces edema, as demonstrated by increased vascular permeability by extravasation of Evans blue and ^{125}I-labeled serum albumin (153). Interestingly, pretreatment of rats with compound 48/80, which depletes MC of their storesof mediator, completely abolishes the PAR1-AP induced edema. The release of bioactive amines, serotonin and histamine, are key in mediating the PAR-1-induced edema, as the serotonin/histamine antagonist cryproheptadine and the histamine H2 receptor antagonist cimetidine reduced significantly the edema. This suggests that PAR1-AP induced edema is MC-dependent, and at least partially mediated by histamine and/or serotonin. Indeed, histochemical studies demonstrated PAR1-AP-mediated MC degranulation in this system (153).

Thus, both *in vivo* and *in vitro* studies suggest a role for MC in thrombin and PAR-1-mediated inflammation. These effects may be mediated by the release of prestored mediators, including histamine and proteases or newly-synthesised mediators such as IL-6.

2. Effects of Trypsin and Tryptase on Mast Cells

MC may be involved in microvascular leakage (223) induced by administration of MC tryptase (194). *In vitro*, tryptase can stimulate histamine release by human tonsillar (224), guinea pig and rodent (223) MC. The response of MC to tryptase activation seems to be different from one MC type to another, since unlike tonsillar MC, foreskin MC showed no histamine response to tryptase activation (207). The tryptase inhibitor

APC366 inhibits IgE-dependent MC activation. Tryptase inhibitor also inhibits calcium ionophore-induced histamine release (225). Taken together these results suggest a role for tryptase in an autocrine and/or paracrine amplification mechanism of MC activation. There remains a possibility that the inhibitors used could have effects on MC activity through mechanisms other than those caused by the inhibition of tryptase. However, considered together these reports strongly suggest a role for tryptase in MC activation.

The *in vivo* evidence of MC involvement in tryptase and PAR2-AP-mediated inflammation is contradictory. In sheep airways, Abraham *et al.* showed that aerosolized PAR2-AP and trypsin caused bronchoconstriction, which can be blocked by the histamine antagonist diphenydramine, suggesting the involvement of MC histamine. Indomethacin had no effect on the bronchoconstriction (226).

Kawabata *et al.* examined the effect of the administration of trypsin and the PAR2-AP, SLI, on vascular permeability in rat hind paw, and showed that the administeration of SLI (10-100 nmol per paw) or trypsin enhanced vascular permeability and caused oedema formation. By contrast, the administration of an inactive control peptide at an equivalent dose did not induce the inflammatory changes. SLI-induced increase in vascular permeability can be abolished by repeated pre-treatment with compound 48/80, suggesting that activation by SLI induces acute inflammation, at least partially, via MC degranulation in rat hind paw (227).

In contrast, Vergnolle *et al.* showed potentially conflicting results in the same rat hind paw system with PAR2-AP. They followed the development of oedema and monitored granulocyte infiltration in the rat hind paw following the injection of two

PAR2-AP, tc-LIG and SLI. Both peptides induced significant oedema, and after the PAR2-AP injection, paw tissues showed disruption of tissue architecture along with an inflammatory cell infiltrate. Furthermore, in the inflamed paw, PAR-2-immunoreactivity was expressed on endothelial cells as well as on the infiltrating inflammatory cells. Oedema induced by injection of either of the two PAR2-AP was slightly reduced in rats pre-treated with compound 48/80, suggesting the involvement of MC. However, it was confusing that pre-treatment of rats with cromolyn, a MC stabilizer, had no effects on inflammation (228).

Similarly, in isolated segments of rat upper airways PAR2-AP (SLI) and PAR1-AP (TFL and SFL) caused contractions that were augmented by indomethacin. Compound 48/80 caused a weaker contraction and pretreatment with compound 48/80 did not cross-desensitize the SLI-mediated contraction (229). Taken together these studies demonstrate that the administration of PAR2-AP induces an acute inflammatory response characterized by a persistent oedema and granulocyte infiltration. However, MC involvement in this system requires more investigation.

In summary, *in vitro* studies show that MC tryptase has marked effects on MC. Furthermore, tryptase inhibition seems to augment IgE- and Ca^{2+} ionophore-induced MC activation. This suggests a role for tryptase in MC activation through autocrine and/or paracrine amplification of MC activation. These roles may be mediated through PAR-2.

3. Effects of Chymotrypsin and Chymase on Mast Cells

The effects of chymotryptic proteases on MC have been studied for many years. Chymase is thought to directly activate certain types of MC. However, marked heterogeneity is evident in the effects of chymase and chymase inhibitors on MC. In humans, the effects of chymase inhibitors on MC are selective to specific types of MC and not others. IgE-mediated histamine release by skin and lung MC is inhibited by chymostatin and soybean trypsin inhibitor (0.5 μM), whereas tonsil MC is unresponsive to the effects of the inhibitors (230). Even though the effects of chymase inhibitors are different in different human MC types, chymase has not been shown to activate any human MC directly (230).

On the other hand, the response is quite different when the effects of chymotrypsin on RPMC are investigated. MC activation with this enzyme induces a dose dependent release of histamine and PGD_2 (231). As with human MC, IgE-mediated histamine release by rat MC is significantly inhibited by chymase inhibitors. Multiple studies show that RPMC activation can be inhibited by broad spectrum and highly specific inhibitors of chymase, including, L-tosylamide-2-phenylethyl chloromethyl ketone, chymostatin, certain peptide boronic acid inhibitors, a Bowman-Birk soybean protease inhibitor, as well as anti-RMCP-1 antibodies. (231-233). Interestingly, chymase inhibitors do not inhibit histamine released by calcium ionophore-stimulated MC, suggesting that the chymotryptic dependency is specific to IgE-mediated activation.

The mechanism by which chymase activates MC is not known. However, some studies demonstrated that chymase-mediated MC activation is inhibited by pre-treatment

of MC with trypsin (234, 235). This suggests that a trypsin-responsive element (receptor or substrate) is involved in the functional interaction of chymase with MC, perhaps involving PAR.

In summary, chymase is stored in MC secretory granules in a form that is catalytically active, and can be released upon MC activation. The response of MC to chymase and effects of chymase inhibitors on MC provides evidence that chymases may act on MC by a feedback regulatory mechanism that amplifies MC activation by triggering further MC mediator release. This may be mediated by PAR.

G. EXPRESSION OF PROTEASE-ACTIVATED RECEPTORS ON MAST CELLS

1. PAR mRNA in Mast Cells

The *in vivo, ex vivo* and *in vitro* studies linking MC to thrombin, tryptase, PAR1- and PAR2-AP-mediated inflammation suggested that PAR-1 and PAR-2 are expressed on MC. Recent studies in our laboratory showed that RPMC express PAR-1 and PAR-2 mRNA (unpublished data). This was shown by using both solution phase RT-PCR as well as *in situ* RT-PCR. Incubation of RPMC with PAR1- and PAR2-AP and IFN-γ modulated mRNA expression. Both, PAR1-AP, Cit (40 μM), and PAR1-CP, FSL (40 μM), upregulated the expression of PAR-1 mRNA, while PAR2-AP, tc-LIG and SLI, and PAR2-CP, LSI had no effect on PAR-1 mRNA. Treatment with Cit, FSL (40 μM) and

tc-LIG (10 μM) but not SLI (10 μM), upregulated PAR-2 mRNA expression. Furthermore, PAR-2 mRNA expression was downregulated by IFN-γ treatment.

These results suggest that RPMC express PAR-1 and –2 mRNA and that the expression can be modulated by PAR1- and PAR2-AP. However, because the "partially inactive" control peptide FSL was able to upregulate PAR-1 and PAR-2 expression, questions remain about the relevance of this and the appropriateness of FSL as an inactive control peptide for PAR-1 in the rat MC. Our findings differ from findings reported by another group (236) who reported mRNA for PAR-1 but not PAR-2 in RPMC.

2. PAR-1 and PAR-2 Receptor Expression on Mast Cells

PAR-1 and PAR-2 have been localized to human MC and rat MC. Using immunohistochemistry and double immunofluorescence techniques D'Andrea *et al.* demonstrated PAR-1 and 2 on a variety of human cells, including MC (192, 237). The only other report of MC expression of PAR is in the rat. Immunofluorescent staining of rat bronchi showed that both epithelial cells and MC express PAR-1 (229). Direct evidence of PAR-2 expression on rat MC is not available.

H. RATIONALE, CONCEPTUAL MODEL AND SPECIFIC OBJECTIVES

1. Rationale

a. PAR-2

Tryptase has multiple potent pathophysiological functions and has been implicated in many diseases. Some *in vivo* studies suggested that tryptase-induced inflammatory functions may be MC-mediated (227). However, other similar studies contradicted these findings. Thus, whether or not MC play a role in tryptase/trypsin and PAR2-AP-mediated inflammation remains unresolved. *In vitro,* tryptase/trypsin induce histamine release by MC and tryptase/trypsin inhibitors decrease IgE- and calcium ionophore-mediated histamine release. However, there are no studies showing the effects of PAR2-AP on MC function. Thus, we set out to study the function of PAR2-AP on MC activation.

b. PAR-1

Thrombin plays a fundamental role in various pathophysiologic diseases and can activate many cell types through the cleavage of PAR-1. The functions of thrombin allow it to contribute to many inflammatory diseases including, neurological disorders, and cancer (122). *In vivo* studies suggest that MC are involved in thrombin- and PAR1-AP-induced edema. Indeed, evidence of MC degranulation was evident in the rat paw model of edema (153). Some of thrombin's effects on MC may be mediated through

PAR-1 since, both thrombin and PAR1-AP activate MC to release NO (immediate) and IL-6 (24 hr) *in vitro*. However, even though thrombin has been shown to induce histamine release *in vitro*, there is no evidence showing direct PAR-1-mediated MC release of stored mediators. Thus, we set out to investigate the effects of PAR1-AP on the release of stored mediators by RPMC.

MC store a variety of inflammatory mediators that can be released selectively and collectively upon activation. A large proportion of MC stored mediators are proteases. The functions of tryptases and chymases on MC include further activation and release of stored mediators from MC. Given that chymase has been shown to inactivate PAR-1 in keratinocytes and render the receptor unresponsive to thrombin (111), and that chymases can also directly inactivate thrombin, we set out to investigate whether PAR1-AP induce the immediate release of MC proteases.

2. Conceptual Model

We set out to investigate the expression of PAR-1 and PAR-2 on the surface of MC and to study the ability of PAR1-AP and PAR2-AP to induce mediator release from MC. Our model (Fig. 6) was that PAR-AP interact with their respective PAR on MC surface and induce a G-protein-mediated release of inflammatory mediators, including cytokines (IL-6 and/or TNF), histamine/β-hex and proteases (RMCP-1, RMCP-5 and CP-A).

To understand the effects of PAR-AP on MC, studies in the lab were divided into two aims: (1) determining the mechanism(s) by which PAR-AP activates MC; and (2)

investigating the effects of the PAR-AP on release of various MC-mediators. These projects were conducted simultaneously and while other members of our research group were investigating Aim 1, my research and thesis were mainly focused on the second objective (Aim 2). Considering that the two projects are interdependent, some results from the former study will be discussed below.

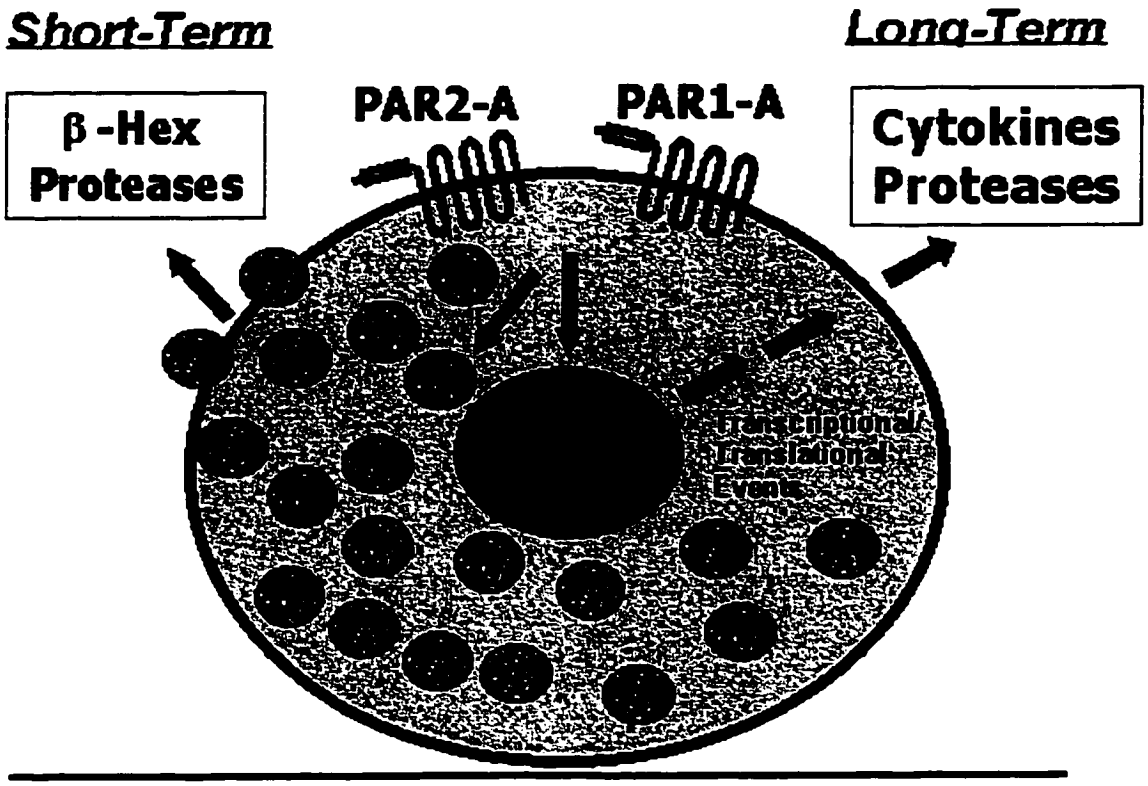

Figure 6: Conceptual Model

Effects of PAR-AP-activation on MC function. Our model was that PAR1-A and PAR2-AP interact with their respective PAR on MC surface and induce a G-protein-mediated release of inflammatory mediators, including cytokines, histamine/β-hex and proteases.

3. Specific Objectives:

1. To investigate the effect of PAR2-AP on MC stored mediator release.
2. To investigate the effect of PAR2-AP on the release of proteases from MC and to characterize exact proteases released by MC upon PAR2-AP-stimulation.
3. To investigate the ability of PAR2-AP to modulate cytokine release from PMC.
4. To investigate the ability of PAR1-AP to induce the release of preformed mediators by MC.

4. Hypotheses:

To investigate the above objectives we hypothesized that:

1. **PAR-2 stimulation induces immediate β-hex release from RPMC, which is associated with morphological changes.**
2. **PAR-2 stimulation induces the release of proteolytic activity from MC, which can be inhibited by SBTI.**
3. **PAR-2 stimulation induces the release of RMCP-1, RMCP-5 and CP-A from RPMC.**
4. **PAR-2 stimulation induces the release of TNF and/or IL-6 by RPMC.**

These hypotheses where tested by the use of PAR2-AP, tc-LIG, and appropriate negative controls.

5. **PAR-1 induces the release of β-hex from RPMC.**
6. **PAR1-AP induces immediate release of proteolytic activity by RPMC.**

These hypotheses were tested by the use of the PAR1-AP, Cit, and its appropriate negative controls.

II. MATERIALS AND METHODS

A. REAGENTS

Compound 48/80 and 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (β-hexosaminidase substrate) were purchased from Sigma Chemical Co. (St. Louis, MO). PAR-1 and PAR-2 activating and control peptides (Table 2) were synthesized by the Peptide Synthesis Facility, Faculty of Medicine, University of Calgary. These peptides were determined to be 95 % pure by mass spectrometry and HPLC. SBTI, was purchased from Sigma Chemical Co. (St. Louis, MO). Polyclonal RMCP-5 and CP-A antibodies were produced and characterized as described previously (82). Briefly, RMCP-5 (15 amino acids) and CP-A (12 amino acids) NH_2-terminal sequences were synthesized at Zymogenetics Inc, Seattle, WA, and then used to immunize rabbits to develop specific polyclonal anti-protease antibodies. Professor H. Miller, Edinburgh, Scotland, kindly provided rabbit antibody to RMCP-1.

B. ANIMALS AND THEIR SENSITIZATION

Outbred male Sprague-Dawley rats (weight 250-500 g) were purchased from Charles River Canada Inc., (St. Constant, Quebec) and used as the source of RPMC. Rats were maintained in an isolation room with filter-topped cages to minimize unwanted infections. For the experiments where MC were activated through their IgE receptor, rats were sensitized by infection with (3000 third-stage larvae of) *Nippostrongylus brasiliensis* (*Nb*), by a single subcutaneous injection of 0.5 mL, as described previously

(238). This experimental protocol was approved by the University of Alberta Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care (protocol numbers: 041/02/02 (2001), 025/02/01 (2000), 025/02/00 (1999)).

C. HARVESTING AND ENRICHMENT OF PERITONEAL MAST CELLS

Rats were sacrificed by exposure to high concentrations of CO_2 (dry ice) in a filtered cage. Ethanol (70%) was sprayed on the ventral abdominal surface. Hair and skin were removed from the ventral peritoneal region using ethanol sterilized-scissors. Fifteen ml of ice-cold Hepes-Tyrodes buffer (HTB) was injected into the peritoneal cavity using a 20 ml syringe and an 18G needle. HTB consisted of 4 g NaCl, 0.5 g glucose, 0.1 g KCl, 73.5 mg $CaCl_2$, 0.5 g bovine serum albumin (BSA, fraction V, ICN Biomedicals., Aurora, Ohio), 1.43 g HEPES and 31.2 mg NaH_2PO_4-$2H_2O$ in 500 ml sterile water, adjusted to pH 7.3 and filter sterilized. The abdomen was massaged for 30 sec to increase cell recovery, opened and the liquid was gently aspirated into ice-cold polypropylene tubes using a bulb plastic pipette. The peritoneal cells were washed by centrifugation (5 min, 150 g, 4 °C) and resuspended in 5 ml of HTB.

MC were enriched by centrifugation through a discontinuous density gradient of Percoll (Pharmacia, Uppsala, Sweden,), as described previously (53). Briefly, 30% isotonic Percoll (20ml) was layered on top of 80 % isotonic Percoll (15 ml). Five ml of total cell suspension in HTB was layered on top of the 30 % Percoll layer and centrifuged through the Percoll density gradient (20 min, 4 °C). The pellet, containing the RPMC

(>95 % purity, contaminating cells were mostly red blood cells and a very small number of lymphocytes), was collected following aspiration of the supernatant. The purity and viability of the RPMC recovered from the pellet was determined using morphological characteristics, by light microscopy and trypan blue staining. Cell viability was >95 %.

D. MAST CELL ACTIVATION

After isolation and enrichment, RPMC were rested in complete RPMI (5 % fetal bovine serum (FBS), pH 7.2) in a 50 ml cylindrical tube for 2 hr at 37°C and 5.0 % CO_2. After incubation, the cells were washed twice by centrifugation (150 g) and resuspended in HTB or RPMI at 1 x 10^6 cells/ml. Cells were placed in 1.5 ml eppendorf tubes or in 48 well plates, incubated at 37°C in the incubator for 10 min, and then the same volume of prewarmed (37°C) PAR-AP or controls in complete RPMI or HTB were added, to give a final cell concentration of 0.5 x 10^6 cells/ml. The cells were incubated for different times (10 min to 24 hr) depending on the experiment. Experiment requiring incubation of cells for >20 min were conducted in complete RPMI.

In the initial experiments, different concentrations of the PAR-AP and controls were used to establish the optimal concentrations. To measure spontaneous release of mediators by RPMC, cells were mixed with media alone. As positive controls, either compound 48/80 (0.5 μg/ml) and *Nb* Ag (1 to 100 We/ml, isolated as shown in (238)) were mixed with cells under the same conditions. After incubation, tubes were placed on ice for 10 min before cells were centrifuged (150 g) to separate supernatant from cells. The supernatants were collected in eppendorf tubes and the same volume of fresh media

was added to the pellets, which were then resuspended. Cell viability was assessed at different times. Cell pellets and supernatants were stored at -70°C until assayed for its content of β-hex, cytokines or proteolytic activity.

For experiments requiring visualization of morphological changes in MC, the same procedure was used as outlined above, except that cells were incubated and activated in 48 well plates. Pictures were taken immediately after the incubation period using an inverted light microscope (400X) connected to a microscope photometer (D104, Photon Technology International) and 35mm camera (Nikon). Kodak 100 film was used.

E. INDUCTION AND QUANTIFICATION OF β-HEXOSAMINIDASE SECRETION

Purified MC were suspended at 3.5 x 10^5 to 5 x 10^5 cells/ml. One hundred µl of cells were prewarmed to 37°C and stimulated for 10 or 20 min with 100 µl of various concentrations of the PAR-AP (SLI, LSI, FSL, TFL, Cit, tc-LIG, tc-OLR, see table 2 and 3), to induce β-hex release. Compound 48/80 (0.75 µg/ml), and the secretory/excretory *Nb* worm Ag (5 and 10 worm equivalents/ml) were also used as positive controls. β-hex was measured in the supernatants and cell pellets, as described by Schwartz and Austen (239). Briefly, 50 µl of sample was incubated with 50 µl of β-hex substrate (1 mM 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide dissolved in DMSO and 0.2 M sodium citrate) for 2 hr at 37 °C. To stop the reaction 100 µl 0.2 M Tris Base was added. Fluorescence was measured using a CytoFluor 2350 Fluorescent Spectrophotometer at

450nm (excitation 356 nm). Results are expressed as β-hex released as a percent of total β-hex (pellet + supernatant). In some figures, values shown have been corrected for the spontaneous β-hex release as indicated in the figure legends.

F. CYTOKINE ASSAYS

Supernatants from activated RPMC were thawed and analysed for TNF and IL-6 content using an Endogen ELISA kit (TNF) and a Biosource International ELISA kit (IL-6). The sensitivity of the TNF assay was < 10 pg/ml. The recovery of rTNF (400 pg/ml) added to serum averaged 94 %, n= 7. This ELISA does not cross react with IL-1α, IL-1β, or rat IL-6. The sensitivity of the IL-6 ELISA was < 8 pg/ml. The recovery of rIL-6 added to tissue culture medium with 10 % FBS averaged 102%. This assay does not cross-react with rat IL-1β, IL-4, IL-10, MIP-2, or TNF.

G. PROTEOLYTIC ACTIVITY ASSAY AND PROTEASE INHIBITION ASSAY

To measure PAR-AP, Ag and compound 48/80-mediated release of proteoltyic activity, supernatants (55 µl) from stimulated RPMC (containing secreted TNF) were placed in wells of a 96-well plate. Seven µL of medium, SBTI, inhibitory antibody to RMCP-1 (1/10) or isotype control (1/10) was added to the supernatants and mixed by tapping. After 2 min incubation, 7 µl of exogenous TNF (1.5 ng/ml) or medium was added to the supernatants and mixed by tapping on the plate to give a final concentration

of 150 pg/ml. The plate was incubated at 37°C / 5% CO_2 for 8 hr. Then TNF content was measured by ELISA. Percent TNF proteolysis was measured by the following formula:

% TNF degraded = 1 – (TNF recovered / (rrTNF seeded + TNF released) X 100)

H. SDS-PAGE, COOMASSIE BLUE STAINING AND WESTERN BLOT ANALYSIS

For these experiments RPMC were activated in serum free HTB and supernatants were concentrated using Centricon (YM-10) centrifugal filter devices as described in the user guide (Millipore, Bedford, MA). Briefly, samples were placed into Centricon tubes and centrifuged for 2 hr at 500 x g. Filtrate was discarded and concentrated supernatants were mixed with 2X sample buffer (0.5 mL1M Tris-Cl, 1 ml DTT, 2 mL 10% SDS, 1 mL glycerol, 0.5 mL 0.12 bromophenol blue) supplemented with 2% β-mercaptoethanol and separated on a 12% SDS-polyacrylamide gel.

For Coomassie Brilliant Blue (CBB) staining, gels were placed in plastic dishes on a tilt table and CBB stain was added (0.25% CBB, 50% methanol, 10% acetic acid) for 30 min. For destaining, the gel was washed repeatedly with prewarmed destain (40% methanol, 10% acetic acid) until the desired intensity was achieved. For Western blot analysis, gels were transferred electrophoretically (25 V, 35 min) using the Semi-Dry Trans Blot System, to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membranes were either incubated overnight at 4°C or for 1 hr at 37°C while rocking in 5% milk (Bio-Rad) in tris buffered saline containing 0.02% Tween and then blocked in

the same buffer containing 5 % goat serum for 1 hr. The membranes were probed with 1/1000 dilution of anti-RMCP-1, 1/600 anti-CP-A, 1/5000 anti-RMCP-5 and then incubated with donkey anti-rabbit HRP-conjugated antibody (1:5000). Protein bands were detected by enhanced chemiluminescence using ECL Western blotting detection system (Amersham Pharmacia Biotech, Quebec, Canada).

I. STATISTICS

All values are given as mean ± standard error of mean (SEM) for the numbers of experiments noted and statistical analyses were performed using the Student's *t*-test and ANOVA.

III. RESULTS

A. THE SELECTION OF PAR-ACTIVATING PEPTIDES USED

A serine protease can usually activate more than one type of PAR. For example, trypsin has the ability to activate PAR-1, -2 and –4 (122). To study the function of distinct PAR subtypes, various PAR-AP have been synthesized based on the conserved sequence of the tethered ligand. These peptides have been tested in many systems to define their activity, specificity and potential usage (240). PAR-AP have been useful in characterizing the functions of specific PAR, as well as their mechanism of activation, signaling and expression.

Although these peptides may be useful in investigating the function of PAR on several cell types, one has to be careful in the selection of which PAR-AP and PAR-control peptides (PAR-CP) to use. There have been studies showing differential potency, and selectivity of PAR-peptides (241). Furthermore, RPMC synthesize many potent proteases, some of these proteases are spontaneously released from RPMC *in vitro* and *in vivo* (242), and could degrade PAR-AP before they would be able to activate MC. Thus, the stability of the PAR-AP in an *in vitro* RPMC system should be considered before the peptides can be used.

In this project, two PAR1-AP, one PAR1-CP, two PAR2-AP and two PAR2-CP were initially considered to study the effects of PAR-1 and –2 on MC. Before selecting which peptides to use in our system we considered three factors: 1) relative selectivity of PAR1-AP and PAR2-AP for their respective receptors; 2) relative potency of the PAR-

AP used; 3) stability and half-life of PAR-AP in our system. The PAR-AP and –CP were selected based on the recommendations of Dr. Morley Hollenberg from the University of Calgary, who has studied these PAR-AP and published extensively on their structural requirements for activity, receptor cross-reactivity and receptor selectivity (240, 241, 243-245). In this section the characteristics of the PAR-peptides used in our study will be reviewed with an emphasis on the three factors outlined above.

1. PAR1-AP

Two PAR1-AP were considered for this study, Cit and TFL; a PAR1-CP (FSL) was used for comparison.

Agonist	Abbreviation	Target
ApfFChaCitY-NH_2 (Ala-*p*-fluoroPhe-Arg-3-cyclohexylAla-Citrulline-Tyr-NH_2)	**Cit**	PAR1-Activating Peptide (PAR1-AP)
TFLLR-NH_2	**TFL**	PAR1-Activating Peptide (PAR1-AP)
FSLLRY-NH_2	**FSL**	PAR-1 Relatively Inactive Reverse Sequence Control (PAR1-CP)

Table 2. Peptides used for studying PAR-1 activation. The reverse sequence control peptide is relatively inactive, although it possesses some biological activity in other systems (228, 245-247) However, its activity is significantly less than the corresponding PAR-AP.

a. Selectivity:

Kawabata *et al.*, tested various PAR1-AP for their PAR-2 cross-reactivity in a calcium signaling-based assay utilizing human embryonic kidney cells (HEK) (245). The study showed that most PAR1-AP are not completely selective to PAR-1 but can also activate/desensitize PAR-2. Of the tested peptides, Cit and TFL were found to have the best selectivity for PAR-1. In the HEK cell assay, the relative selectivity of Cit for PAR-1, as compared to PAR-2 was about 280:1, whereas selectivity of TFL was 220:1. However, at concentrations over 50 μM for Cit and over 70 μM for TFL, the peptides cross-desensitized PAR-2. Thus, at least with HEK cell system, Cit and TFL are non-reactive with PAR-2 if used at concentrations under 50 μM. The ability of PAR1-AP to desensitize PAR-2 was also studied using a radioligand-binding assay in KNRK cells transfected with rat PAR-2 (240). In this study, the relative potency of a series of PAR1-AP to desensitize PAR-2 was compared to a potent radiolabelled PAR2-AP. Again, most agonists used showed some cross-reactivity with PAR-2. However, out of all the PAR1-AP studied, TFL was the least reactive with PAR-2, with no Ca^{2+} inducing activity at concentrations lower than 70 μM. Therefore, initially both Cit and TFL were used in our investigations.

b. Potency:

The relative potencies of the PAR1-AP Cit and TFL in the PAR-1 desensitization assays as well as a platelet aggregation assay showed that Cit is a more potent activator of

PAR-1 than TFL (245). The IC_{50} values for Cit and TFL are 1 and 2.5 μM respectively, meaning less Cit is needed than TFL to cause the same PAR-1 desensitization effect.

c. Stability and Mast Cell-Compatibility:

Preliminary studies with PAR-AP from our laboratory showed that Cit (10-40 μM) activated RPMC Ca^{2+} flux and β-hex release. However, TFL (0.01-40 μM) was unable to activate MC β-hex release. This may have been due to proteolytic inactivation of TFL by MC proteases that had been spontaneously released. Therefore, TFL was eliminated and Cit was chosen as the primary PAR1-AP for our functional studies.

d. PAR1-Control Peptides:

FSL were used as an inactive control peptide. FSL represents scrambled sequences for TFL and Cit.. This PAR1-CP has been shown to have reduced PAR-1 activity as compared to its active counterparts.

Multiple studies suggested that proteases such as cathepsin G and tryptase can cleave inside the tethered ligand of PAR-1 and PAR-2 respectively (248). Therefore, without modifications to the PAR1-AP (such as in Cit), we would expect that the PAR-AP, which are identical to the PAR-1 tethered ligand to also be cleaved by MC proteases.

2. PAR2-AP

Two PAR2-AP (tc-LIG and SLI) and 2 PAR2-CP (tc-OLR and LSI) were considered for this study.

Agonist	Abbreviation	Target
trans-cinnamoyl-LIGRLO-NH_2	**tc-LIG**	PAR2-Activating Peptide (PAR2-AP)
trans-cinnamoyl-OLRGIL-NH_2	**tc-OLR**	PAR2-Relatively Inactive Reverse Sequence Control (PAR2-CP)
LRGILS-NH_2	**LRG**	PAR2-Relatively Inactive Reverse Sequence Control (PAR2-CP)
SLIGRL-NH_2	**SLI**	PAR2-Activating Peptide (PAR2-AP)
LSIGRL-NH_2	**LSI**	PAR2-Relatively Inactive Reverse Sequence Control (PAR2-CP)

Table 3. The selectivity of PAR2-AP, as determined in biological systems other than MC. The reverse sequence control peptides are relatively inactive, but they possess some biological activity in other systems (228, 245).(246, 247) However, their activity is significantly less than their corresponding agonists.

a. Selectivity:

Hollenberg *et al.*, studied the effects of PAR2-AP on a PAR-1 expressing HEK cell calcium assay. None of the PAR2-AP activated PAR-1. Therefore, unlike some of the PAR1-AP, these PAR2-AP are highly selective at least with respect to PAR-1 (241, 246, 249, 249, 250).

b. Potency:

The relative potency of these PAR2-AP has been studied in three different systems; (1) by measuring contraction of endothelium-denuded and intact human umbilical vein ring preparations; (2) by reading intracellular calcium signals in cultured rat kidney KNRK cells expressing a cloned human PAR-2; and (3) by monitoring ion transport across rat jejunum preparations mounted in Ussing chambers. In these systems, SLI was found to be more potent than tc-LIG (246, 251).

c. Stability and Compatibility in the Mast Cell System:

Preliminary studies with Ca^{2+} influx and β-hex release by MC showed tc-LIG to be a potent activator of MC. However, the PAR2-AP SLI did not cause Ca^{2+} influx or β-hex release. tc-LIG contains the *trans-cinnamoyl* modification. This modification stabilizes the peptide by making it less sensitive to aminopeptidase degradation. Thus, SLI inactivity may be due to aminopeptidase-mediated proteolysis. Indeed, in the presence of amastatin, an aminopeptidase inhibitor, SLI (1 μM) stimulated significant β-hex response (10.4 ± 5.2 %) from RPMC. However, even in the presence of amastatin (1 μM), the SLI response was weak relative to tc-LIG. Since protease inhibitors are known to have direct effects on MC (See chapter 1), we eliminated the use of SLI and used tc-LIG as the primary activating peptide for our functional studies.

d. PAR2-Control Peptides:

tc-OLR, LSI and LRG were used as inactive control peptides. LSI and tc-OLR are the reverse sequences for SLI and tc-LIG, respectively. These PAR2-CP have been shown to have reduced PAR-2 activity their active counterparts (228, 245-247). LRG is the reverse sequence of tc-LIG without the *trans*-cinnamoyl group. This was used initially as a control for tc-LIG before tc-OLR was available.

B. EFFECTS OF PAR2-AP, tc-LIG, ON MAST CELL MORPHOLOGY

Release of stored mediators from MC causes morphological changes that can be observed by light microscopy. Purified RPMC were incubated with tc-LIG (PAR2-AP 10μM), tc-OLR (PAR2-CP, 10μM), Ag (5-10, We/ml) or media alone for 8 hr. MC were examined at 10 min, 1h, 4h, and 8h under light microscopy (400X). At all time points, non-treated RPMC appeared round, heavily granulated and displayed a clear dark refractile edge around their surface (Fig. 7a). tc-LIG-treated MC showed rapid changes in morphology upon stimulation. At 10 min post-stimulation, tc-LIG-treated cells lost their roundness and showed evidence of granule release. MC granules were visible in the media of tc-LIG-stimulated MC but not of sham treated cells. After 4 hr of incubation, cells started to exhibit loss of the refractile edge and began to flatten and spread. This phenomenon was more apparent at 8 hr post stimulation (Fig. 7c). Throughout the 8 hr incubation, MC granules were visible in the surrounding media of the tc-LIG-stimulated MC. tc-OLR (10μM)-treated MC exhibited similar morphological changes to tc-LIG-treated cells, however, to a much lesser extent (Fig. 7d). Ag-treated cells (Fig. 7b) were morphologically indistinguishable from non-treated cells.

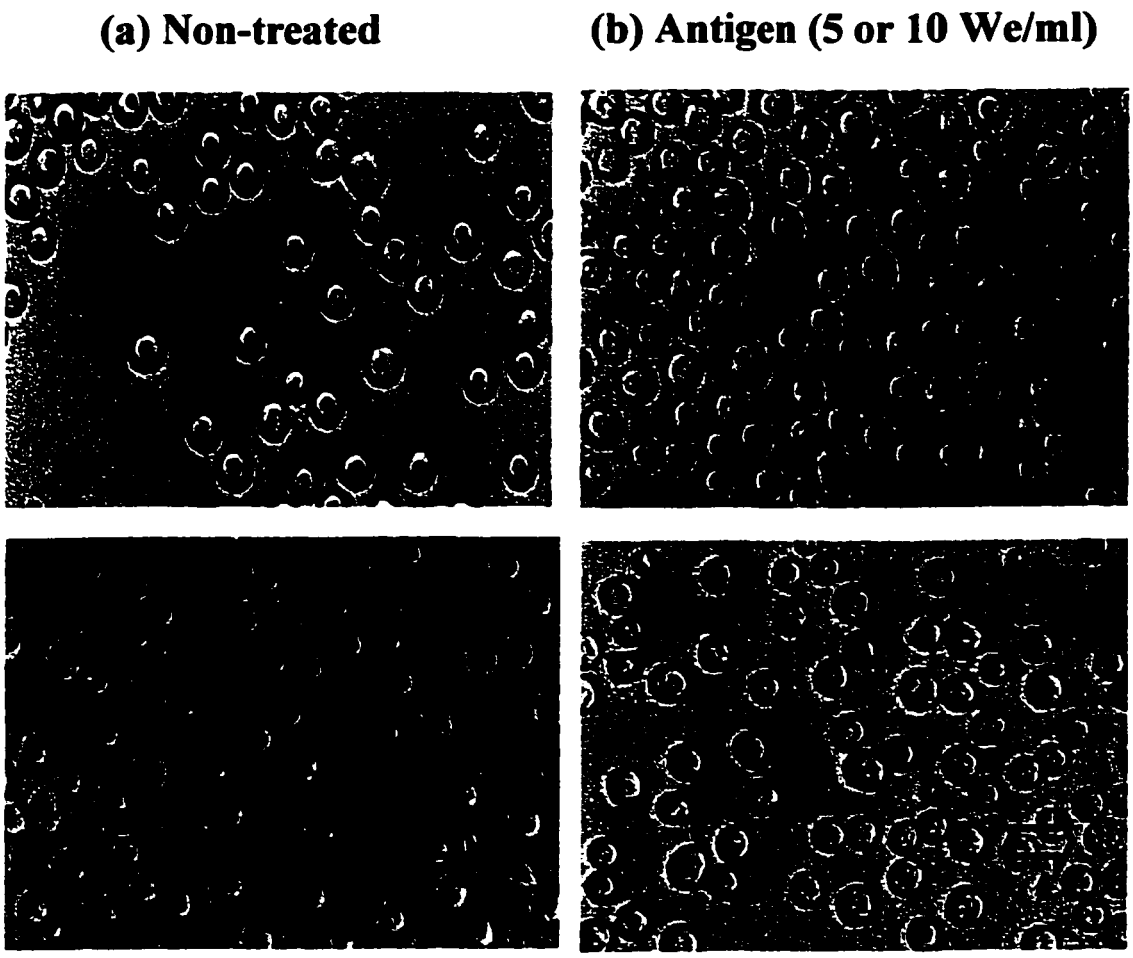

Figure 7: Comparison between tc-LIG and antigen-induced morphological changes in mast cells. Non-treated (a) antigen-treated (b), tc-LIG (PAR2-AP)-treated (c) and tc-OLR (PAR2-CP)-treated (d) RPMC after 8 hr incubation. Arrows point out evidence of degranulation, flattening, spreading and adhesion. Original magnification 400X. These photographs are representative of 4 independent experiments (n=4).

C. EFFECTS OF PAR2-AP, tc-LIG AND SLI, ON β-HEXOSAMINIDASE RELEASE FROM MAST CELLS

To confirm that the rapid morphological changes induced by tc-LIG were associated with release of stored mediators by RPMC, we examined the effects of PAR2-AP, SLI and tc-LIG, PAR2-CP, tc-OLR and LSI, and Ag on β-hex release from highly enriched (95 %) RPMC. MC were incubated with Ag (10 We/ml) as a positive control, tc-LIG or SLI (10 μM) as putative activating agents, and LSI or tc-OLR (10 μM) as putative, inactive control peptides. tc-LIG at concentrations from 0.05 to 1 μM significantly ($p<0.05$) induced β-hex release as compared to media alone (<3 %) (Fig. 8 and 9). Ag produced a significant increase in β-hex release (10 % ± 2), while tc-OLR showed no effect on β-hex release at doses (0.05-1 μM). (Fig. 9). However, higher concentrations of tc-OLR (eg, 10 μM) induced a significant release of β-hex (32 ± 2 %). Interestingly, neither the second PAR2-AP, SLI (40 μM) nor its PAR2-CP, LSI (40 μM), induced significant β-hex release (Fig. 10).

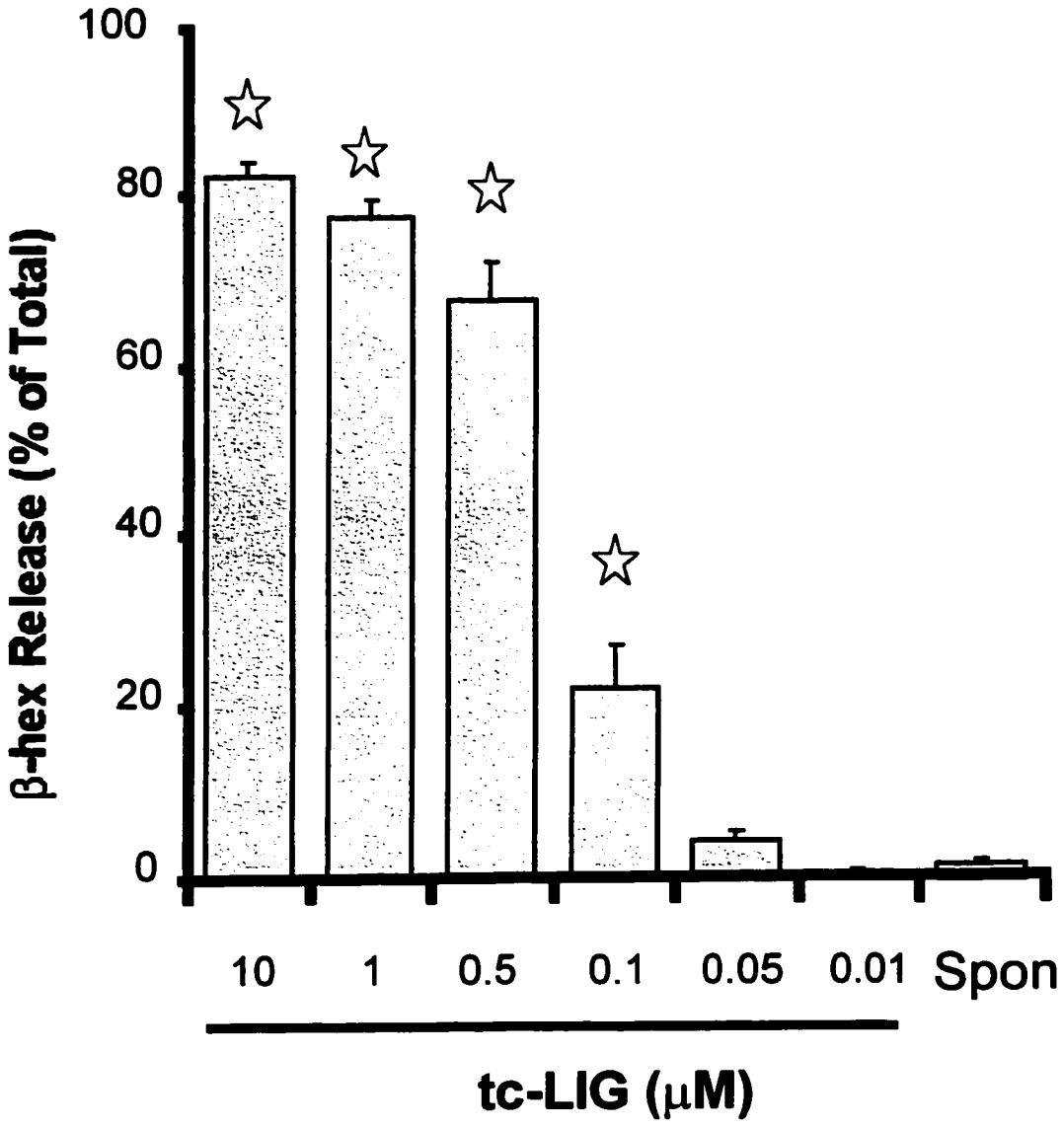

Figure 8. Dose response of tc-LIG (PAR2-AP)-mediated β-hex release from mast cells. β-hex release from RPMC after a 10 min incubation with tc-LIG (10-0.01 μM). β-hex release is represented as % of total (mean ± SEM). Statistical analysis was performed using ANOVA (F=286 and F_{crit}=2.59898059). Star indicates statistically significant difference from spontaneous ($p<0.05$, n=3 to 7 independent experiments).

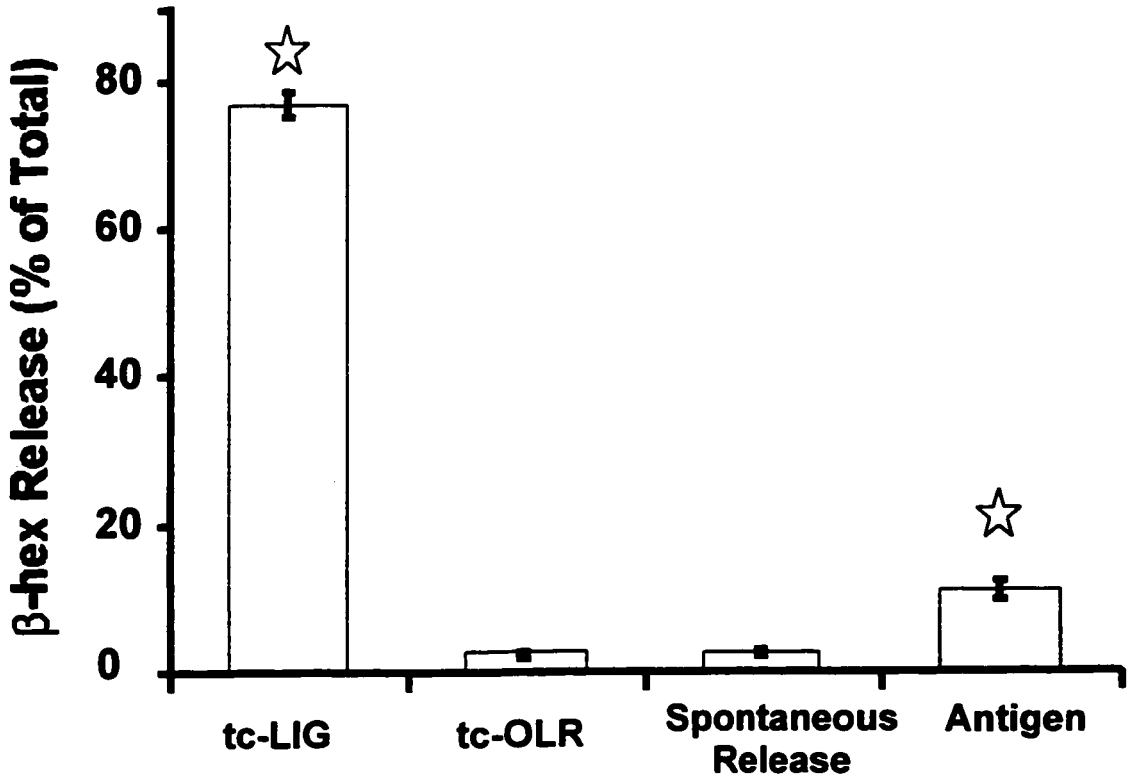

Figure 9. Comparison among antigen, tc-LIG (PAR2-AP) and tc-OLR (PAR2-CP)-mediated β-hex release from mast cells. β-hex release from RPMC after 10 min incubation with tc-LIG (1μM), tc-OLR (1μM) and antigen (5 or 10 We/ml). β-hex release is represented as % of total (mean ± SEM). Star indicates statistically significant difference from spontaneous ($p<0.05$ n=5 independent experiments).

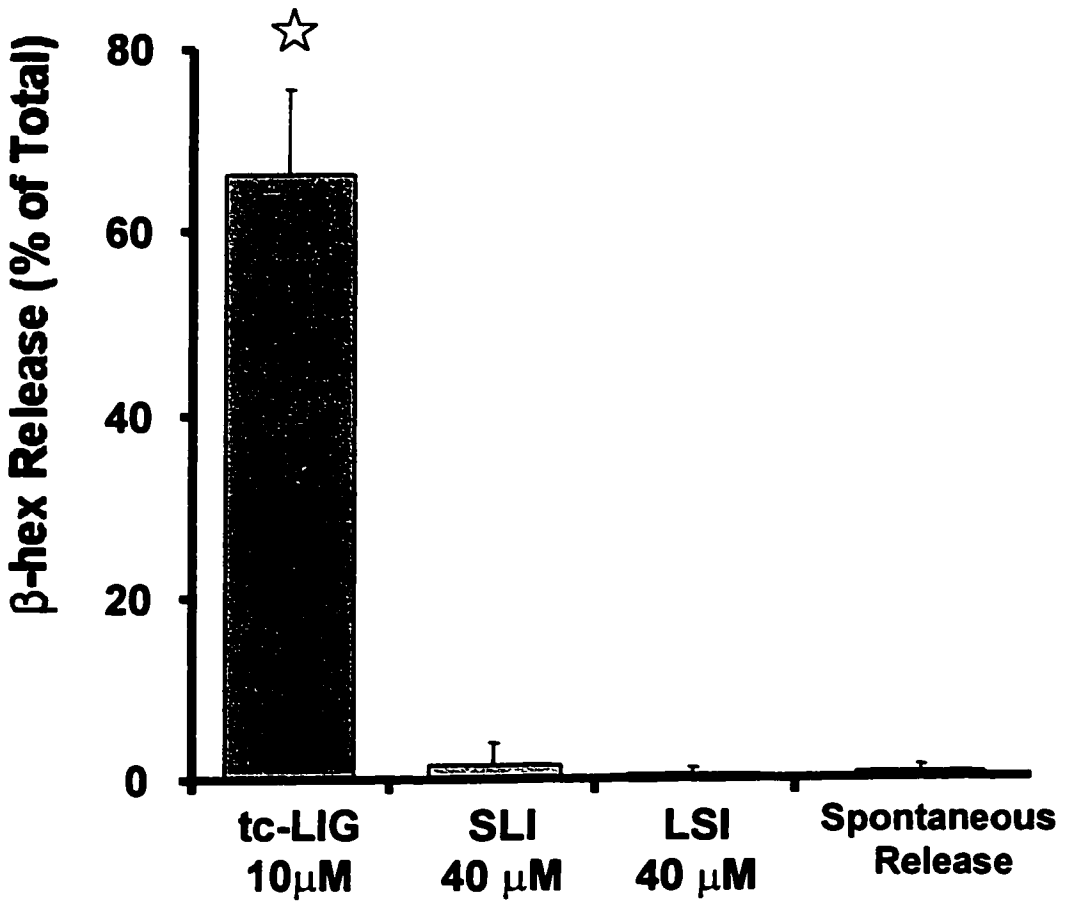

Figure 10. Comparison among tc-LIG (PAR2-AP), SLI (PAR2-AP) and LSI (PAR2-CP)-mediated β-hex release from mast cells. β-hex release from RPMC after a 10 min incubation with tc-LIG (10 μM), SLI (40 μM) and LSI (40 μM). β-hex release is represented as % of total (mean ± SEM). Star indicates statistically significant difference from spontaneous ($p<0.05$ n=3 independent experiments).

D. EFFECTS OF PAR2-AP, tc-LIG, ON TNF AND IL-6 SECRETION FROM MAST CELLS

Considering that RPMC store TNF and IL-6 in their granules and given that thrombin and PAR1-AP can induce the release of IL-6 release by BMMC, we examined the effects of PAR2-AP on IL-6 and TNF production from highly enriched (95%) RPMC. RPMC were incubated with Ag (10 We/ml), tc-LIG (0.01-10 μM) and tc-OLR (10 μM) for 20 min or 8 hr and TNF release was measured by ELISA. MC released low levels of TNF (21 ± 3 pg/ml, n=4) spontaneously, after 20 min. Neither Ag, tc-LIG or tc-OLR had a significant effect on short-term (20 min) TNF release above what was released spontaneously (data not shown). However, after 8 hr incubation, Ag (106 ± 33.6 pg/10^6 cells/ml, $p< 0.05$) but not tc-LIG or tc-OLR induced a significant release of TNF (Fig. 11 and 12). tc-OLR appeared to increase TNF secretion over baseline, but this did not reach statistical significance.

Given that release of TNF and IL-6 by RPMC is mediated through different mechanisms (73), we then examined the effect of Ag, tc-LIG and LRG (PAR2-CP) on production of IL-6 by RPMC over 8 hr. Again, we observed that Ag but not tc-LIG (0.1-10 μM) nor LRG (10 μM) induced production of IL-6 (Fig. 12). Ag-mediated IL-6 production did not reach statistical significance.

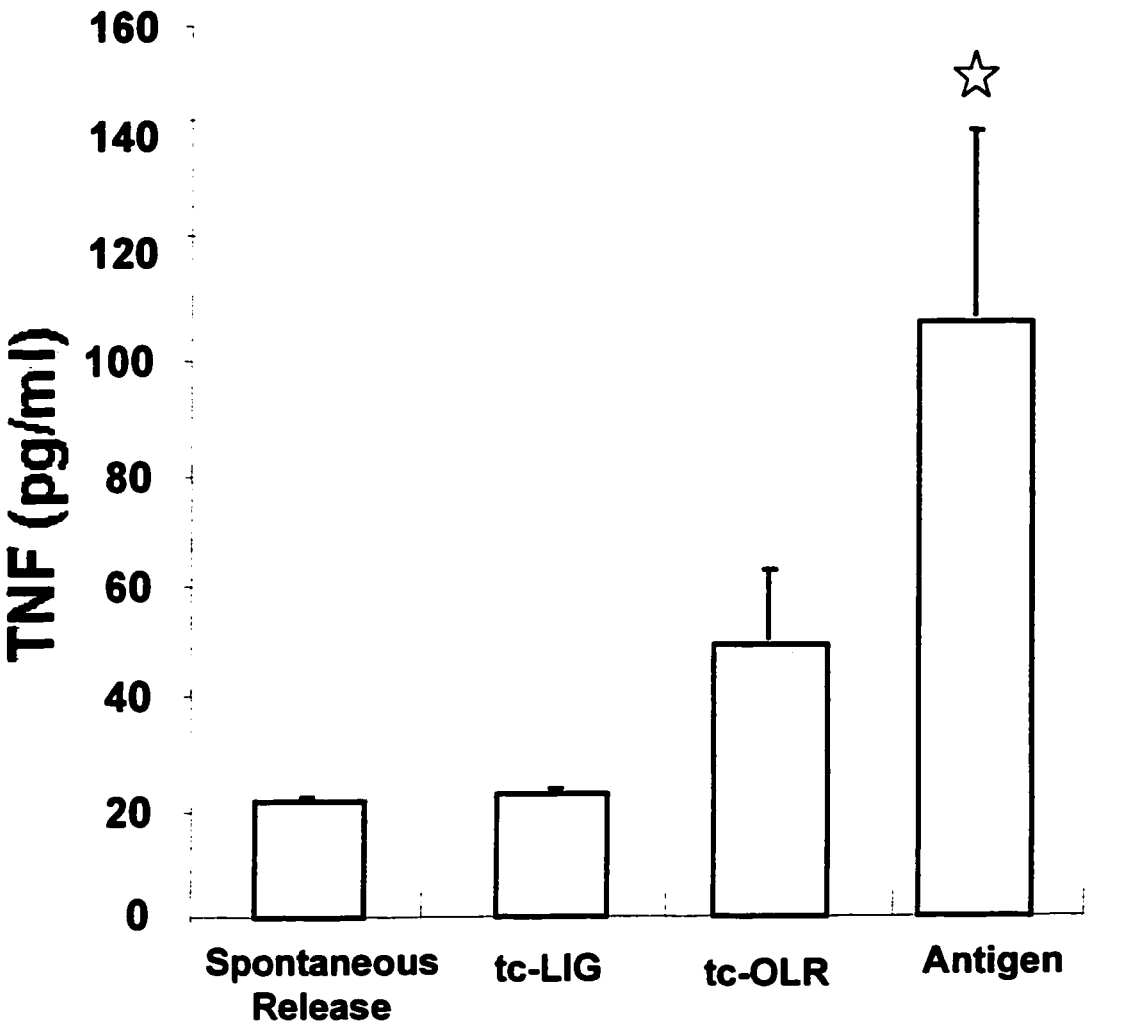

Figure 11. Comparison among tc-LIG (PAR2-AP), tc-OLR (PAR2-CP) and antigen-mediated TNF release from mast cells. TNF release from RPMC (1 X 10^6 cells) after 8 hr incubation with tc-LIG (10μM), tc-OLR (10μM) and antigen (5 or 10 We/ml). (Mean ± SEM). Star indicates statistically significant difference from spontaneous ($p<0.05$, n=4 independent experiments).

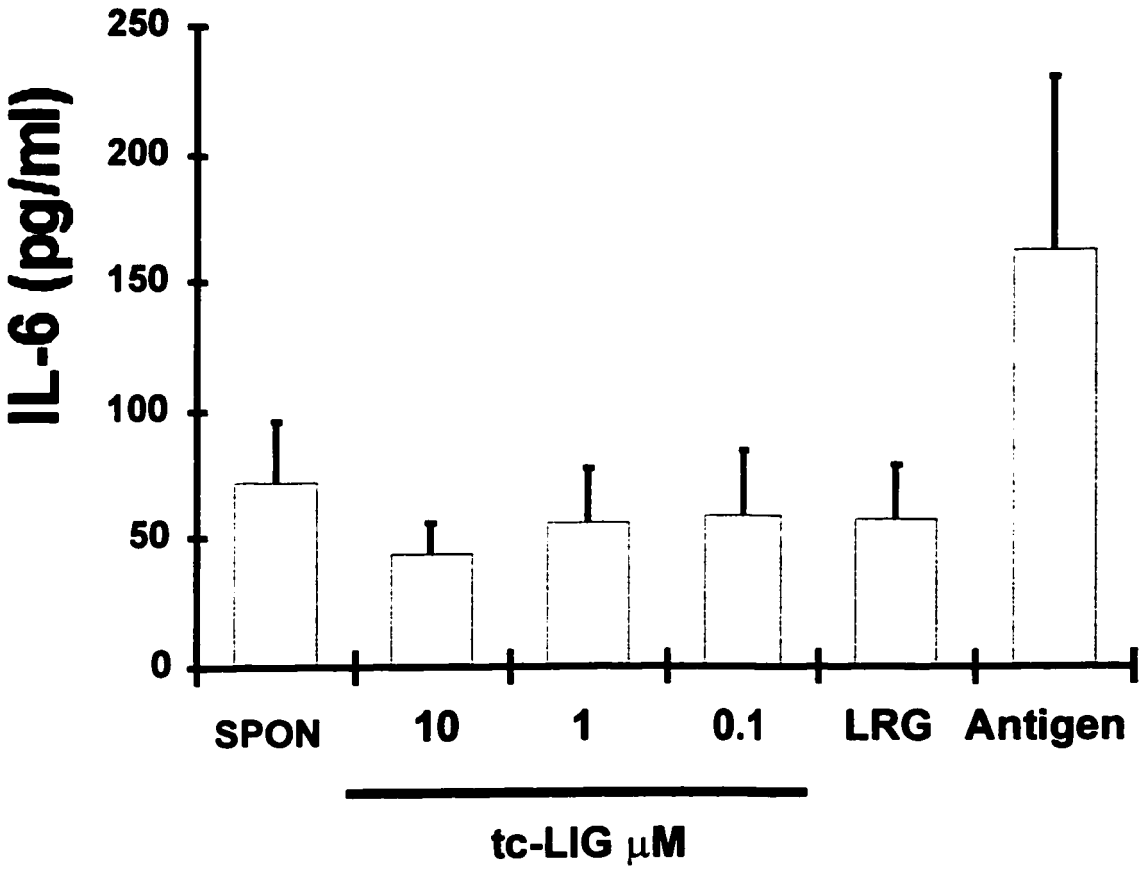

Figure 12. Comparison among tc-LIG (PAR2-AP), LRG (PAR2-CP) and antigen-mediated IL-6 release from mast cells. IL-6 release from RPMC (1 X 10^6 cells) after 8 hr incubation with tc-LIG (10- 0.01 μM), LRG (10 μM) and antigen (5 or 10 We/ml). (Mean ± SEM, n=3 independent experiments).

E. EFFECTS OF PAR2-AP, tc-LIG, ON RELEASE OF PROTEOLYTIC ACTIVITY FROM MAST CELLS

Given that both TNF and β-hex are stored in MC and that only β-hex is released upon tc-LIG stimulation, we examined the ability of tc-LIG to induce the release of protease(s) that would potentially degrade TNF. Sensitized RPMC were incubated with tc-LIG (0.01–10 μM), Ag (10 We/ml), compound 48/80 (0.5 μg/ml) or tc-OLR (10 μM) for 8 hr at 37°C. Supernatants or media were seeded with 150 pg/ml of recombinant rat TNF (rrTNF) and incubated for an additional 8 hr. TNF remaining in the supernatants or media was measured by ELISA. Supernatants from sham-treated MC showed significant loss of seeded TNF (17 ± 7 %) as compared to media. Supernatants from tc-LIG-treated MC showed a higher loss of the seeded TNF (44 ± 5 %) above spontaneous ($p<0.05$), suggesting tc-LIG-mediated release of proteolytic activity. Supernatants from tc-OLR- and Ag-stimulated cells showed no significant loss of TNF over that which occurred in sham-treated cells (Fig. 13).

The experiment was also conducted at 20 min after stimulation. Similar results were found, except supernatants form sham-treated cells showed 23 ± 7 % proteolytic activity, while supernatants from the tc-LIG-treated MC showed 30 ± 4 % proteolytic activity above that which occurred in sham-treated cells. The tc-LIG-induced release of proteolytic activity was dose-dependent and vanished at 0.05 μM (Fig. 14).

Based on the β-hex and cytokine results, tc-LIG seems to be functionally similar to the well characterized MC secretagogue, 48/80. Thus, we compared tc-LIG-mediated

release of proteolytic activity with that induced by 48/80. Similar to tc-LIG, 48/80 (0.75 μg/ml) induced the release of proteolytic activity (30 ± 2 %) from RPMC (Fig. 14), suggesting that tc-LIG and 48/80 may be similar at least in terms of their release of stored mediator from RPMC.

The observation that SLI did not stimulate RPMC to release β-hex suggested that SLI does not activate RPMC. However, it may be that SLI activates MC through a different mechanism than tc-LIG and thus may activate pathways other than those involved in the release of β-hex. Thus, to investigate this possibility we examined the ability of SLI and its PAR2-CP, LSI, to release proteolytic activity from RPMC (Fig. 15). A small but significant increase in proteolytic activity over spontaneous release was induced by SLI (40 μM, 7 ± 1 %, $p<0.05$). However, SLI-mediated proteolytic activity was not significantly higher than the inactive control peptide (40 μM, 7 ± 5 %). It is important to note that this experiment was only repeated 3 times. Therefore, one has to be cautious not to rely on the small differences between SLI, LSI and sham-mediated release of proteolytic activity.

Given that Ag and tc-LIG activation of RPMC induced different cytokine responses, we compared tc-LIG and Ag-induced release of proteolytic activity. Ag (10 We/ml) induced an increase in the release of proteolytic activity over spontaneous. However, it did not reach significance.

To confirm that tc-LIG-mediated TNF proteolytic activity is a result of serine protease activity, before seeding the supernatants with TNF, they were mixed with the broad spectrum serine protease inhibitor, SBTI (1 mg/ml). SBTI inhibited (82 %) of TNF

loss from the supernatants of tc-LIG (10 μM) stimulated MC (Fig. 16), confirming that tc-LIG- induced loss of TNF is by serine proteolytic activity. tc-OLR and Ag induced no significant proteolytic activity.

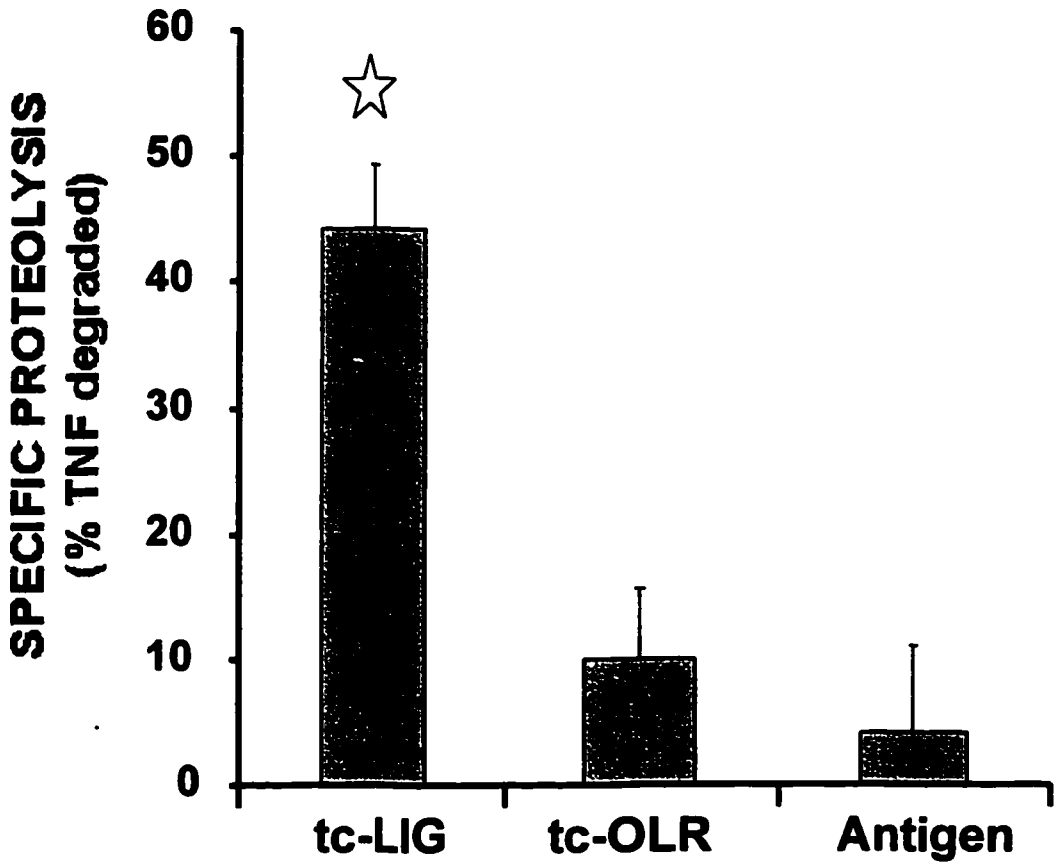

Figure 13. Comparison among tc-LIG (PAR2-AP), tc-OLR (PAR2-CP) and antigen-mediated release of proteolytic activity from mast cells. Supernatants from tc-LIG (10 μM), tc-OLR (10 μM), or antigen (5 or 10 We/ml)-treated (8 hr) RPMC were incubated with 150 pg/ml of TNF. Specific proteolytic activity was calculated as % TNF degraded from expected (seeded + released). Values indicate proteolytic activity after the subtraction of release that occurred from sham-treated cells (spontaneous 17 ± 7 %) (Mean ± SEM, n=4-5 independent experiments). Star indicates statistically significant difference from spontaneous ($p<0.05$).

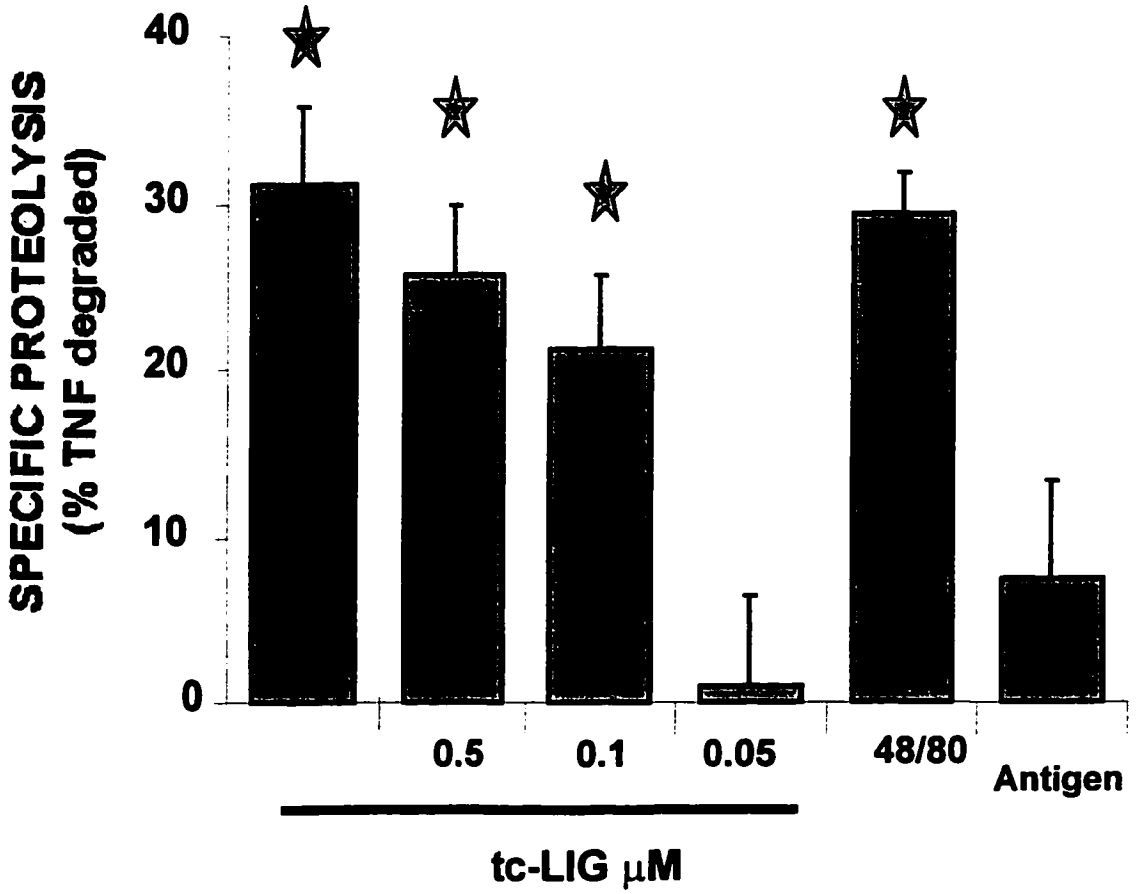

Figure 14. Dose response of the release of TNF-degrading proteolytic activity by tc-LIG (PAR2-AP)-stimulated mast cells. Supernatants from tc-LIG (10 μM), antigen (5 or 10 We/ml) or 48/80 (0.75 μg/ml)-treated (20 min) RPMC were incubated with 150 pg/ml of TNF. Specific proteolytic activity was calculated as % TNF degraded from expected (seeded + released). Values indicate proteolytic activity after the subtraction of spontaneous release (22 ± 5%) (Mean ± SEM). Star indicates statistically significant difference from spontaneous ($p<0.05$, n=4 to 5 independent experiments).

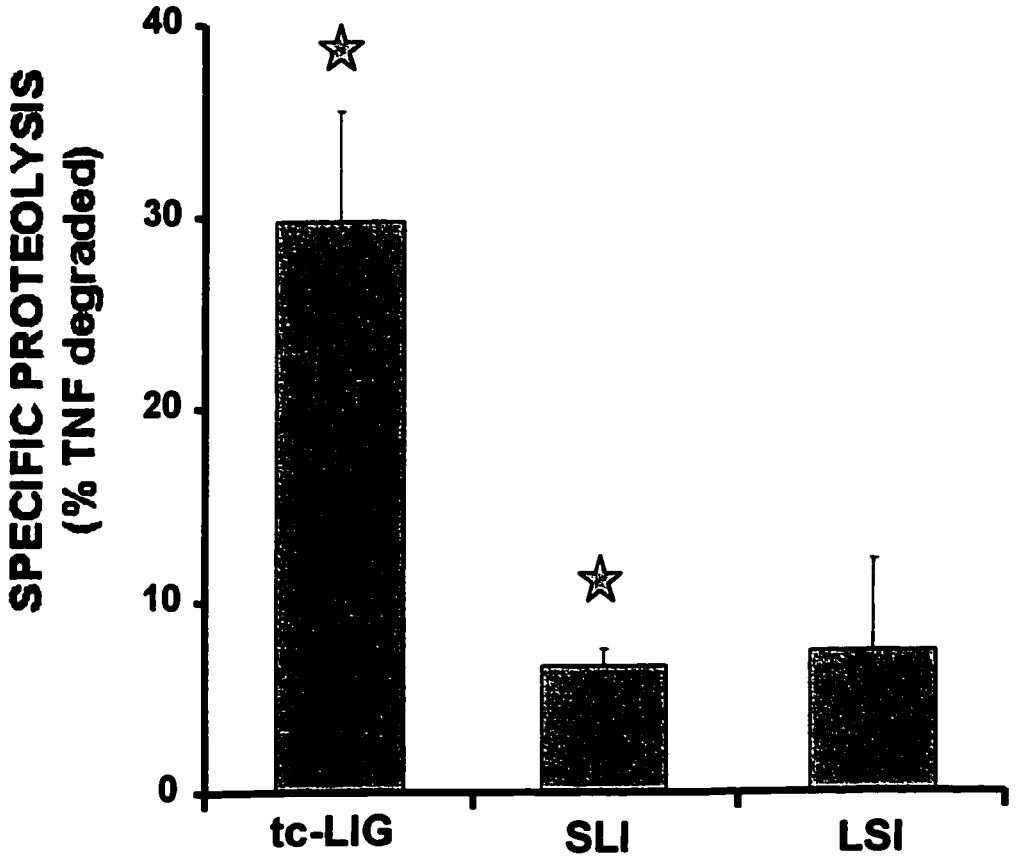

Figure 15. Release of proteolytic activity by SLI (PAR2-AP) and LSI (PAR2-CP)-stimulated mast cells. Supernatants from tc-LIG (10 μM), SLI (40 μM), and LSI (40 μM)-treated (20 min) PMC were incubated with 150 pg/ml of TNF. Spontaneous release was 23 % ± 7. Specific proteolytic activity was calculated as % TNF degraded from expected (seeded + released) Values indicate proteolytic activity after the subtraction of spontaneous release (23 % ± 7). (Mean ± SEM). Star indicates statistically significant difference from spontaneous ($p<0.05$, n=3 independent experiments).

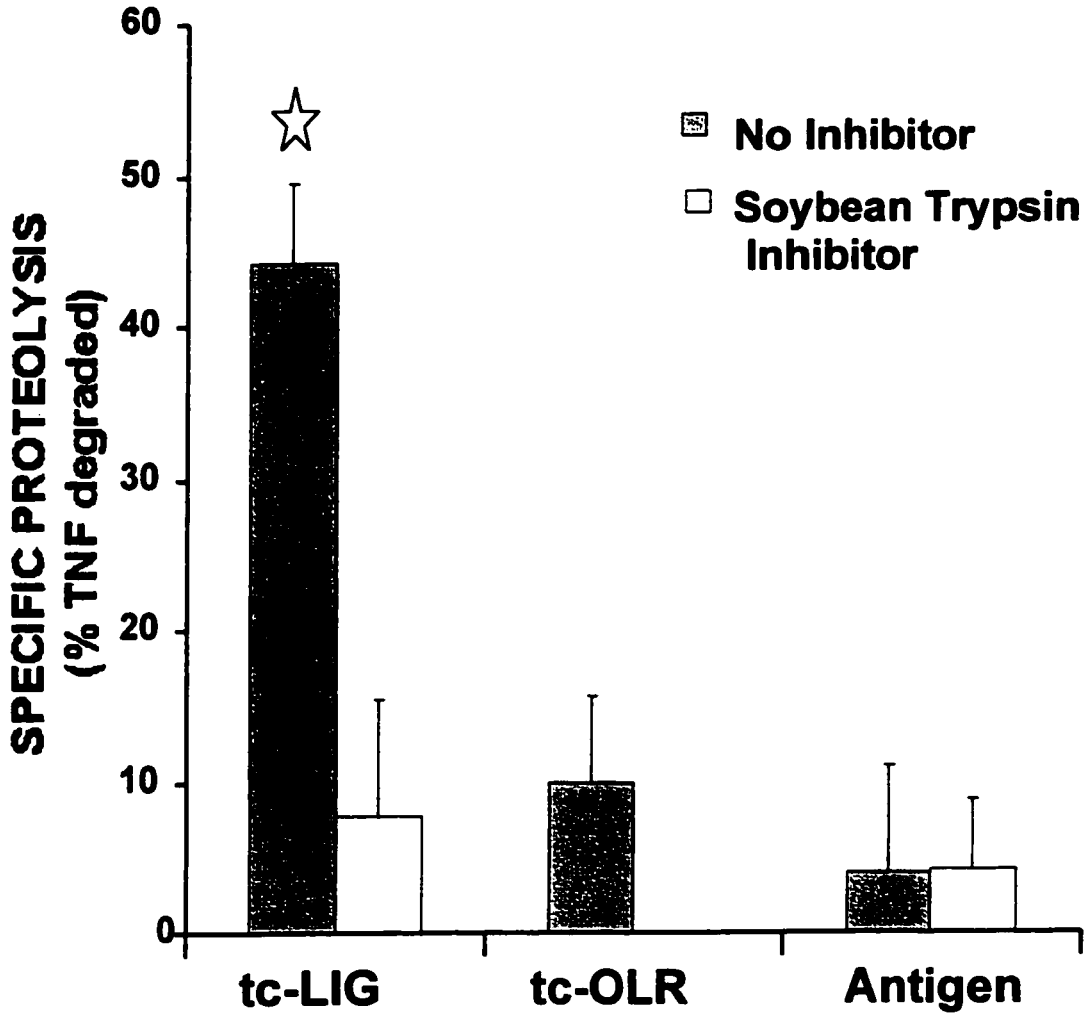

Figure 16. Effect of Soybean Trypsin Inhibitor on proteolytic activity in supernatants of tc-LIG (PAR2-AP)-stimulated mast cells. Supernatants from tc-LIG (10 μM), tc-OLR, and antigen (5 or 10 We/ml)-treated (8 hr) RPMC were incubated with or without SBTI (1mg/ml) before 150 pg/ml of TNF was added. Specific proteolytic activity was calculated as % TNF degraded from expected (seeded + released). Values indicate proteolytic activity after the subtraction of spontaneous release (17 % ± 7) Star indicates statistically significant difference from spontaneous ($p<0.05$, n=4 to 5 independent experiments). (Mean ± SEM).

F. EFFECTS OF PAR2-AP, tc-LIG, ON THE RELEASE OF RMCP-1, RMCP-5 AND CP-A

Proteases comprise up to 50% of RPMC by weight (75). To identify some of the proteases that are released by tc-LIG (PAR2-AP) stimulation of MC, supernatants from tc-LIG- and Ag-stimulated RPMC were collected and concentrated (10X). Then, SDS-PAGE and CBB staining was preformed. Large bands of Mr ranging from 27-30 kDa and 39-45 kDa were visible in the tc-LIG stimulated supernatants but not in the sham-treated or Ag treated supernatants (Fig. 17). This suggested the possibility of tc-LIG-mediated release of the proteases RMCP-1 (~26-28 kDa), RMCP-5 (~29-30 kDa) and CP-A (~33-36 kDa) (82).

To confirm the release of these proteases, Western blot analyses were conducted on supernatants from tc-LIG and Ag-activated cells using antisera developed against peptides corresponding to NH_2 terminal sequences of RMCP-5 and MC-CP-A and antiserum against RMCP-1 protein. In supernatants from tc-LIG-treated MC one band for RMCP-1 (30 kDa), two bands for RMCP-5 (34 and 35 kDa), and three bands for CP-A were detected (40, 41 and 42 kDa) (Fig. 18). The release of all three proteases was dose-dependent and was not detectable in supernatants stimulated with tc-LIG concentrations lower than 0.05 μM (Fig 18). Ag (10 We/ml) induced no detectable amount of the three proteases (Fig. 17).

Interestingly, 48/80 (0.5 mg/ml) induced the release of all three proteases in similar levels as compared to tc-LIG (0.5 μM), supporting the hypothesis that tc-LIG and 48/80 have functional similarities (Fig. 18).

To determine whether the release of RMCP-1, 5 and CP-A is an immediate release of stored mediators or a long-term production of the proteases, we examined the release of the RMCP-1, 5 and CP-A at two different time points, 20 min and 8 hr by Western Blot analysis. At 20 min tc-LIG induced a release of RMCP-1, RMCP-5 and CP-A (Fig. 19).

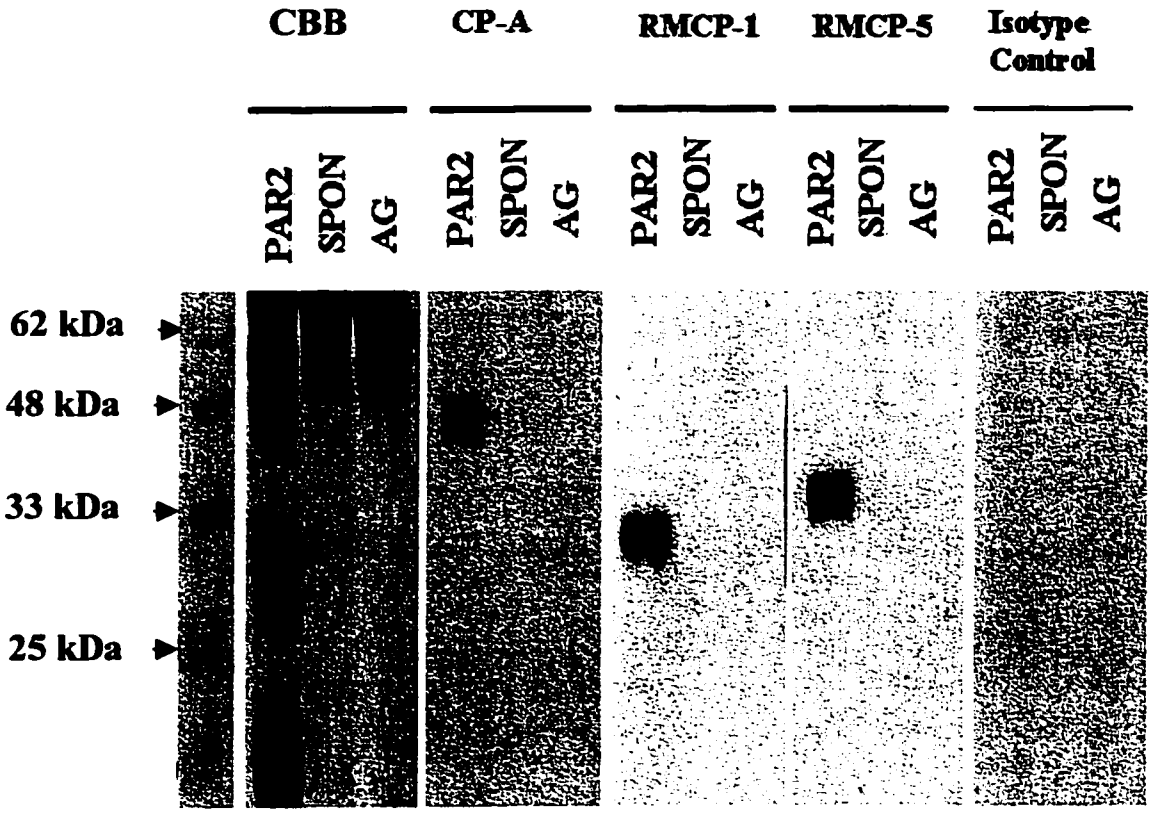

Figure 17. The release of RMCP-1, -5 and CP-A by tc-LIG (PAR2-AP) and antigen-stimulated mast cells. Supernatants from tc-LIG (10 μM) and antigen (10 We/ml) and sham-treated (spon) mast cells were concentrated (10 X) and Western blot analysis preformed for CP-A, RMCP-1 and RMCP-5 on the same membrane. Western blots shown are representative of 3 independent experiments. Membranes were stripped and reblotted in the following order (CP-A, RMCP-5, and RMCP-1).

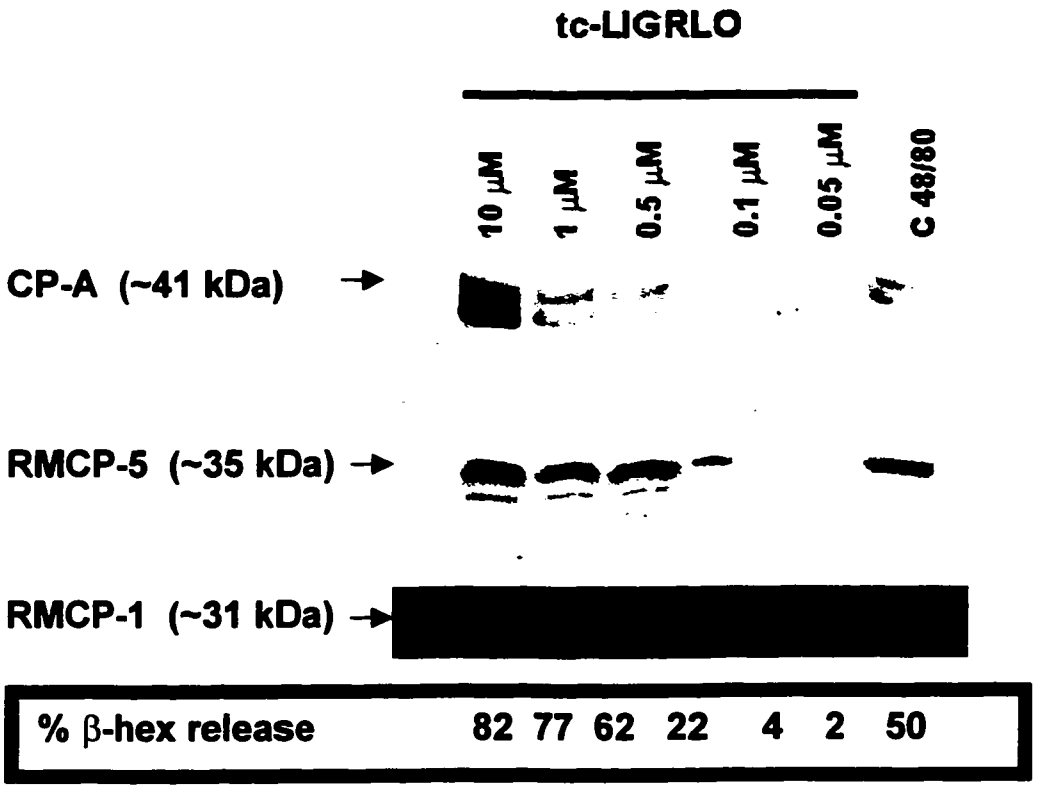

Figure 18. Dose response for the release of RMCP-1, -5 and CP-A by tc-LIG-stimulated mast cells as compared to average β-hex release. Supernatants from tc-LIG (10-0.5 μM) and 48/80 (0.75 μg/ml)-stimulated mast cells were concentrated and Western blot analysis performed for CP-A, RMCP-1, and RMCP-5. Western blots shown are representative of 2 independent experiments. β-hex release (n=3 independent experiments).

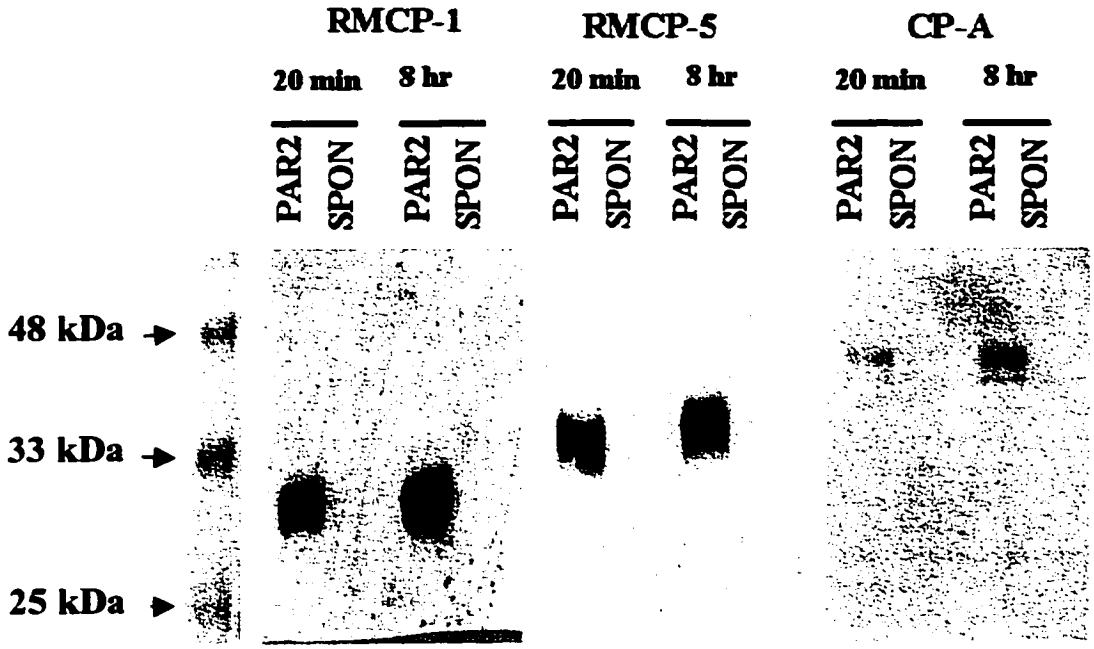

Figure 19. Short-term (20 min) versus long-term (8 hr) release of RMCP-1, -5 and CP-A by tc-LIG-stimulated mast cells . Supernatants from RPMC incubated with tc-LIG (10 μM) were concentrated (10 X) and Western blot analysis performed for CP-A, RMCP-1 and RMCP-5 (n=1).

G. EFFECTS OF RMCP-1 ANTISERUM ON TNF PROTEOLYSIS

To test if RMCP-1 is involved in tc-LIG-mediated release of proteolytic activity, we examined the ability of RMCP-1 antiserum to inhibit the TNF degradation in the supernatant of non-treated and tc-LIG-activated MC. Normal rabbit serum (1:10) and RMCP-1 (1:10) antiserum were mixed with the supernatants of sham-treated and tc-LIG-stimulated MC before TNF was seeded. After incubation for 8 hr at 37°C, TNF was measured by ELISA. Sham-treated RPMC released 25 ± 7 % of proteolytic activity which was consistent with our other studies. RMCP-1 antiserum (1:10) completely abolished the proteolytic activity (96 %) in the supernatants from sham-treated MC (Fig. 20). Normal rabbit serum had no effect on the proteolytic activity, suggesting that RMCP-1 is the main TNF degrading protease released from sham-treated RPMC. RMCP-1 antiserum (1:10) partially but significantly ($p<0.05$) inhibited the proteolysis of TNF in the supernatant from the tc-LIG-activated MC by 36 %. When the release of proteolytic activity from sham-treated cells was subtracted from the tc-LIG mediated release of proteolytic activity both in the presense of RMCP-1 antiserum or normal serum, no significant inhibition was found to be mediated by the Ab (Fig. 20b). This however, does not necessarily mean that the RMCP-1 is not involved in the degradation of TNF because only one dose of the antiserum was used. It is likely that the dilution of the antiserum used is enough to inhibit the small release of proteolytic activity from sham-treated cells but not sufficient to inhibit the large release of RMCP-1 induced by tc-LIG.

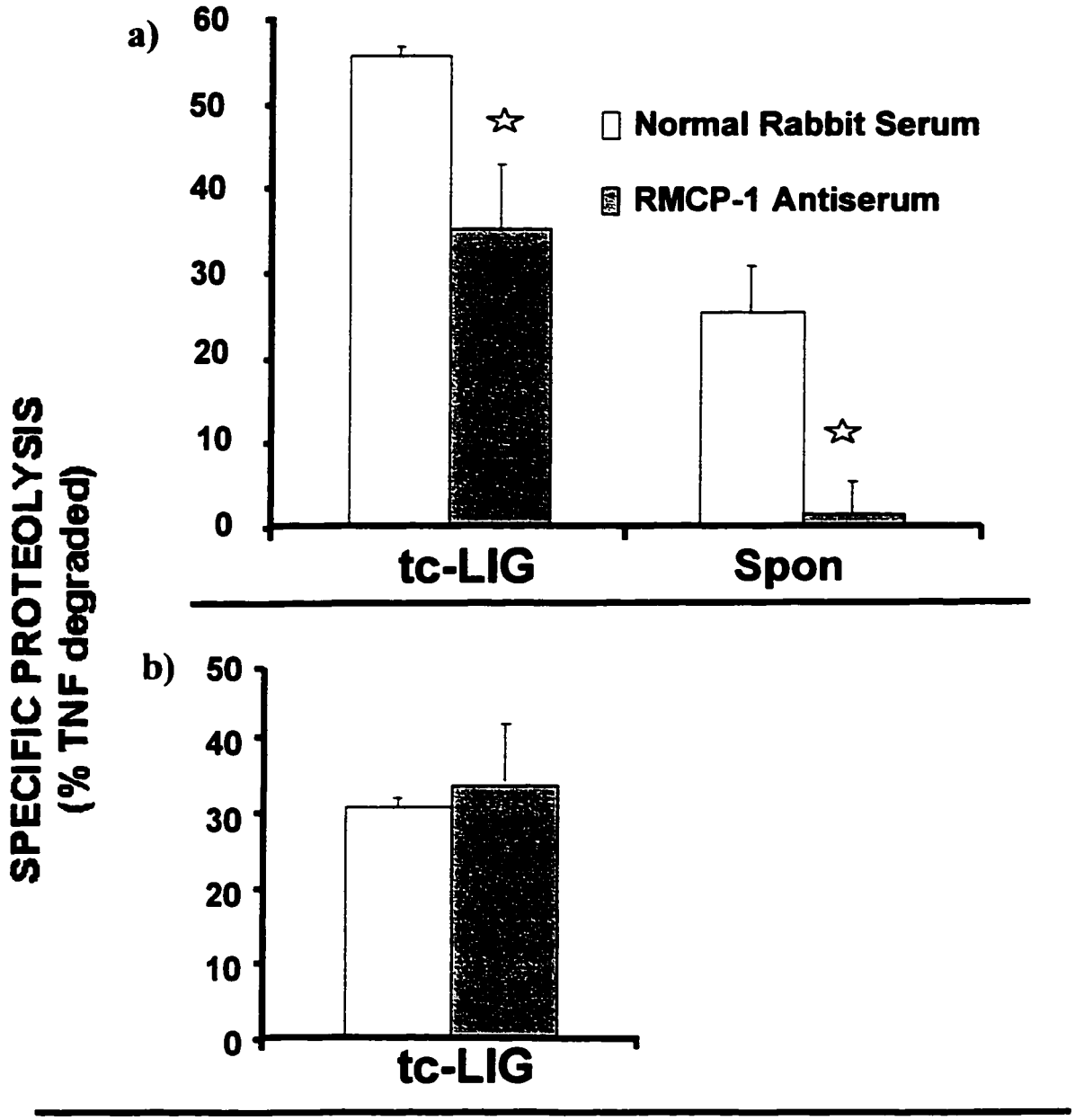

Figure 20. Effect of RMCP-1 antiserum on proteolytic activity in the supernatants of sham and tc-LIG-stimulated mast cells. Supernatants from tc-LIG-treated (20 min) or non-treated mast cells were incubated with 150 pg/ml of TNF in the presence of normal rabbit serum or RMCP-1 antiserum (1:10). Proteolytic activity was calculated as % TNF degraded from expected (seeded + released). a) Effect of RMCP-1 antiserum on the proteolytic activity in the supernatants of sham and tc-LIG(10μM)-treated mast cells. b) Effect of RMCP-1 antiserum on the proteolytic activity in the supernatant of tc-LIG-stimulated MC after the subtraction of proteolytic activity from sham-treated MC. (Mean ± SEM, n=4 to 5 independent experiments).

H. EFFECTS OF THE PAR1-AP ON RELEASE OF β-HEXOSAMINIDASE FROM MAST CELLS

To study the effects of PAR1-AP on release of stored mediators from MC, we examined the effects of Cit, TFL, and FSL (Table 2) on β-hex release from highly enriched RPMC. MC were incubated with Cit and TFL (PAR1-AP, 10 μM) as putative activating agents, and FSL (PAR1-CP, 10 μM) as an inactive control peptide. Cit significantly induced β-hex release (9% ± 3, $p<0.05$, Fig. 21) as compared to media alone (1% ± 1). Neither TFL nor FSL induced significant β-hex release.

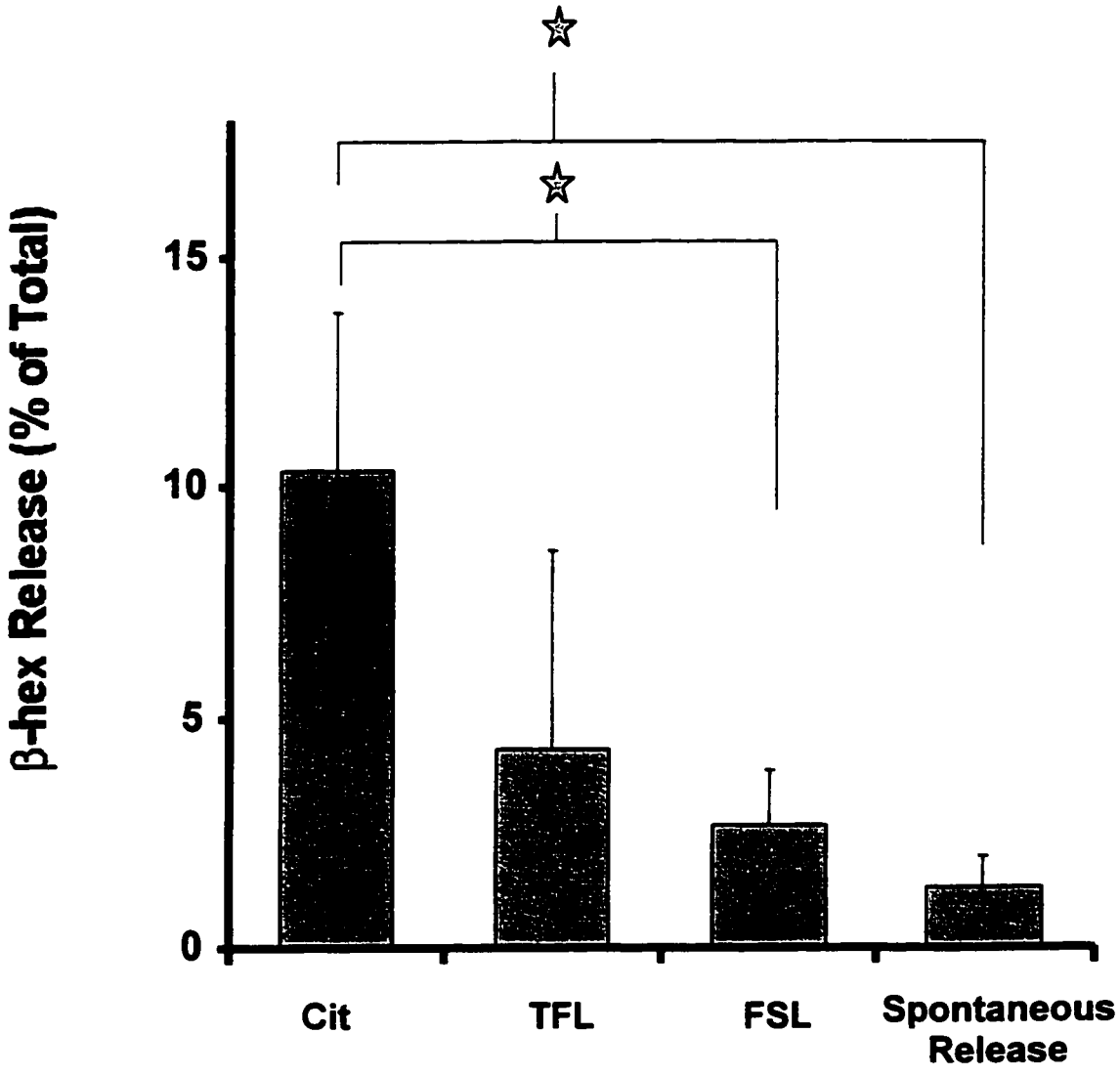

Figure 21. Comparison among Cit (PAR1-AP), TFL (PAR1-AP)- and FSL (PAR1-CP)-mediated β-hex release from mast cells. β-hex release from RPMC after a 10 min incubation with Cit (10 μM), TFL (10 μM) and FSL (10 μM). β-hex release is represented as % of total (mean ± SEM, n=3). Star indicates statistically significant difference from spontaneous or FSL ($p<0.05$ n=3 independent experiments) induced release.

I. EFFECTS OF THE PAR1-AP ON RELEASE OF PROTEOLYTIC ACTIVITY FROM MAST CELLS

We examined the ability of Cit and TFL to induce the release of proteolytic activity that would degrade TNF. Sensitized RPMC were incubated (20 min) with Cit and TFL (10 μM) as activating agents and FSL (10 μM) as a partially inactive control peptide. tc-LIG (10 μM) was used as a positive control. Supernatants were collected and seeded with rrTNF (150 pg/ml) for 8 hr. TNF remaining in the supernatants was measured by ELISA.

Supernatants from sham-treated MC showed significant proteolytic activity (23 ± 9 %). Supernatants from the Cit-treated MC showed a significant increase in the proteolytic activity (25 ± 8 %) after subtraction of spontaneous (Fig. 22). Supernatants form TFL showed a significant increase in proteolytic over spontaneous (7 ± 2 %), although the magnitude of TFL release was much smaller than Cit. FSL-treated RPMC did not release a significant amount of proteolytic activity after subtracting spontaneous.

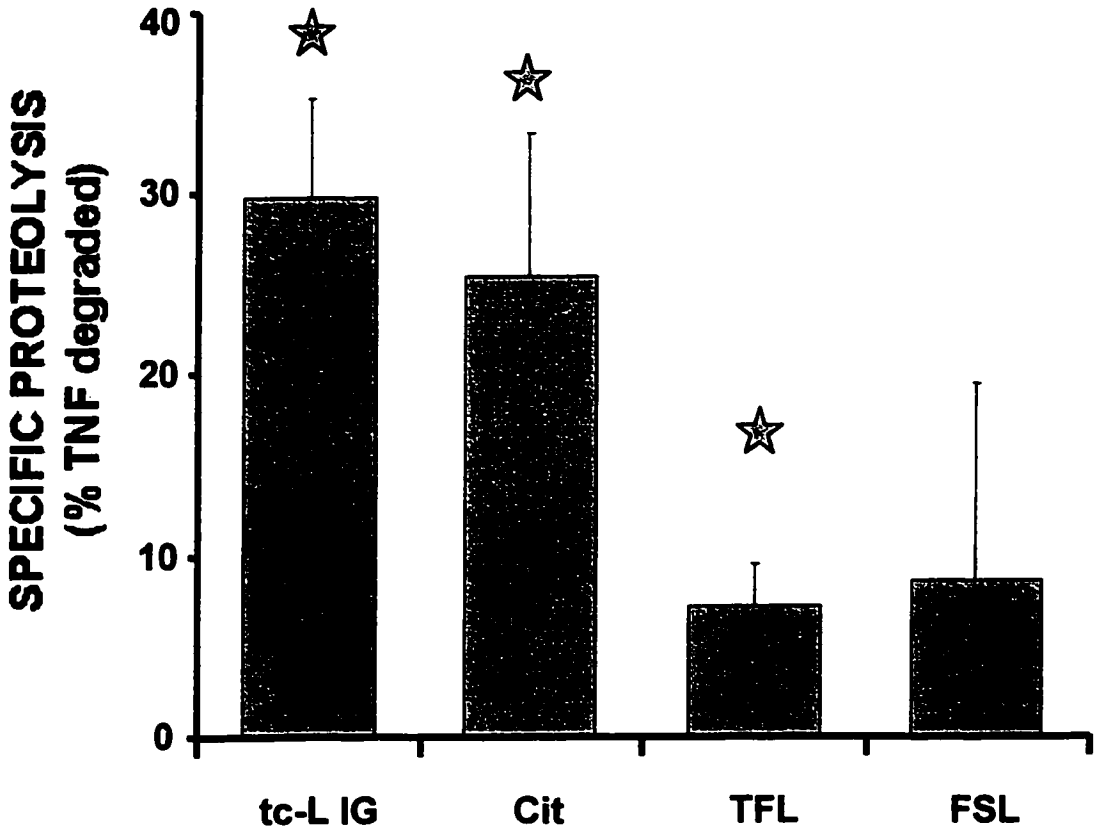

Figure 22. Release of proteolytic activity by Cit, TFL, (PAR1-AP) and FSL (PAR2-CP)-stimulated mast cells. Supernatants from tc-LIG (10 μM), Cit (10 μM), TFL (10 μM), and FSL (10 μM)-treated (20 min) RPMC were incubated with 150 pg/ml of TNF. Specific proteolytic activity was calculated as % TNF degraded from expected (seeded + released). Values indicate proteolytic activity after the subtraction of spontaneous release (23 ± 7%). (Mean ± SEM). Star indicates statistically significant difference from spontaneous ($p<0.05$, n=3 independent experiments).

IV. DISCUSSION, FUTURE DIRECTION AND CONCLUSIONS

A. DISCUSSION

We have previously shown that RPMC express PAR-1 and PAR-2 mRNA, which can be regulated by cytokines and PAR1-AP and PAR2-AP. We have also shown that RMCP-1, RMCP-5 and CP-A are stored in MC and are the primary proteases produced by RPMC. In the present study we demonstrate that tc-LIG, a PAR2-AP, can activate MC and that activation induces the release of RMCP-1, -5 and CP-A. The PAR2-AP mediated protease release is accompanied by the release of β-hex, but not by release of stored or newly synthesized IL-6 or TNF. This is different than IgE-mediated MC activation, which releases β-hex stores along with IL-6 and TNF. Furthermore, we show that Cit (PAR1-AP) can activate MC to release TNF-degrading proteolytic activity, which is accompanied by the release of β-hex.

1. The Effects of PAR2-AP on Mast Cells

The involvement of MC in PAR2-AP-mediated inflammation *in vivo* has been uncertain and somewhat controversial. In this study we provide the first direct evidence of effects of PAR2-AP on MC *in vitro*. Our observations are consistent with those of Abraham *et al.*, which demonstrated that histamine is involved in PAR2-AP and tryptase-mediated bronchoconstriction in sheep (226). Our observations are also consistent with

observations by Kawabata *et al.* (227), who suggested the involvement of MC in increased oedema caused by PAR2-AP or trypsin administration in the rat paw.

The effects of trypsin-like proteases on MC have been documented (223). However, the mechanisms by which trypsin-like proteases activate MC are unknown. Given that activation of MC by trypsin-like proteases induces release of stored mediators from MC, and given that trypsin/tryptase are the primary activators of PAR-2, our observations suggest that effects of trypsin/tryptase on MC may be mediated through PAR-2. However, tryptase can also cleave PAR-1 and PAR-4, and thus these receptors may also be involved in the trypsin/tryptase-mediated activation of MC. Our results also suggest that the PAR2-AP-mediated inflammation may involve the release of MC-specific chymases. Chymase has been shown to be a potent chemoattractant for inflammatory cells (207). Thus, it may contribute to MC-mediated inflammation induced by PAR-2 activation.

The fact that supernatants from MC activated by tc-LIG could degrade TNF and that this was completely inhibited by SBTI indicates that TNF-degrading proteases released by tc-LIG are predominantly serine proteases. SBTI is a broad-spectrum serine protease inhibitor that inhibits activity of both tryptases and chymases. Given that RMCP-1 and RMCP-5 are present in the supernatants of tc-LIG-stimulated MC, it is likely that these proteases are involved in the TNF degradation. However, we cannot rule out PAR2-AP release of other RMCP including tryptases (RMCP-6 and RMCP-7), which may contribute to the TNF-degradation.

It is interesting that chymases are released in parallel with CP-A. Previous reports show that endopeptidase function of serine proteases may expose sequential CP-A-specific termini allowing its exopeptidase activity to contribute to degradation (216).

Previous studies have shown that activation of MC to release protease activity may be induced by a variety of agents both *in vivo* and *in vitro*. However, in previous studies release of proteases from RPMC was determined by measurement of proteolytic activity. Since the proteolytic characteristics of many of the MC proteases are similar and since antibodies for many MC proteases were not commercially available, it has been difficult to identify the exact proteases released upon MC activation. This is the first study that shows RMCP-1 and RMCP-5 release from RPMC by Western blot analysis of the supernatants in addition to proteolytic assay. The size of released RMCP-1 (~30 kDa), RMCP-5 (2 close bands, ~34 kDa) and CP-A (3 close bands, ~41 kDa) are similar to the sizes of the stored forms of these proteases that we published previously (82). The different bands for RMCP-5 and CP-A are likely due to different glycosylation, as it was previously shown that treatment of MC lysate with PNGase F markedly reduced the heterogeneity of the protein bands from stored proteases (82). Taken together, our data supports the hypothesis that proteases are stored in their active form and are not modified before or during release. However, this does not rule out partial autoproteolysis, which has been previously suggested for RMCP-1 (90).

Other studies have shown the release of proteases from MMC. The release of RMCP-II by rat MMC have been reported to be induced by Ag challenge, in parasitic infections, and during anaphylaxis (252-254). The release of RMCP-II mouse

counterpart, MMCP-I, can be increased during parasitic infections (255). Furthermore, rat CP-A can be released by 48/80, Ca^{2+} ionophore and Ag activation of RPMC (256).

RMCP-1, RMCP–5 and CP-A were not detected in the supernatants of sham-treated MC using Western blot analysis. This may be because tc-LIG (10 μM) induced release of a large amount of the proteases as compared to spontaneous release. The spontaneous release was lower than the sensitivity of the Western blot at the conditions used. However, we show that MC spontaneously release TNF-degrading proteolytic activity that can be inhibited by SBTI and RMCP-1 antiserum. This is consistent with other studies that showed spontaneous release of chymases by MC (242). Similarly, Ag may also have induced release of a small amount of RMCP-1 that was lower than the sensitivity of the Western blot procedure. However, the increase of proteolytic activity mediated by Ag did not reach statistical significance.

The spontaneous release of RMCP-1 is especially interesting because other studies reported that human MC chymase could rapidly cleave thrombin and inactivate PAR-1, rendering the receptor unresponsive to thrombin activation. At the same time, the responsiveness of the receptor to PAR1-AP is maintained (211). Indeed, our studies as well as others showed that RPMC can be activated by Cit but not thrombin (Project (1), unpublished data), suggesting that PAR-1 on freshly isolated RPMC may have been modified in this manner.

RMCP-1 antiserum did not inhibit the tc-LIG-mediated proteoltyic activity while completely inhibiting the spontaneous proteolytic activity. The inability of the serum to totally inhibit the tc-LIG-mediated protease may be because the dose of RMCP-1

antiserum used (1:10) was too low. To better undertand the involvement of RMCP-1 in tc-LIG mediated activation of MC a full dose response must be performed. However, the possibility that the RMCP-1 antibodies do not recognize the RMCP-1 released by tc-LIG-mediated activation of RPMC can not be ruled out. Le Trong et al., (104) showed that polypeptides smaller than ~17 kDa were hydrolyzed by heparin-bound RMCP-1, whereas, larger proteins were not hydrolyzed. In contrast, non heparin-bound RMCP-1 hydrolysed larger proteins. Given that TNF is larger than 17 kDa it may not be a good substrate for heparin-bound RMCP-1.

We showed that Ag does not release detectable TNF-degrading proteolytic activity. The lack of proteolytic activity does not necessarily indicate that proteases are not released, since it may be due to an Ag-mediated simultaneous release of protease inhibitors stored in the MC. Indeed, MC produce and release Secretory Leukocyte Protease Inhibitor (a chymase inhibitor) and Latexin (a CP-A inhibitor) (256-258).

We also chose to investigate the possibility that PAR-AP release IL-6 and TNF because of the important biologic activities of these cytokines, their association with inflammatory disorders such as asthma and inflammatory bowel disease (259), and evidence that PAR1-AP induces cytokine release in mouse BMMC (12).

Our data suggests that MC may regulate TNF function by directly degrading the protein. Given that both TNF and RMCP-1 are stored and released from MC, our *in vitro* finding may suggest an important mechanism by which MC regulate TNF function *in vivo*. This is the first study to show RMCP-1 can degrade TNF. It may be that such

proteolytic activity directed against TNF and possibly other cytokines is an important anti-inflammatory function for RMCP-1.

Our studies demonstrate differences between tc-LIG and Ag-mediated activation of MC. Both Ag and tc-LIG induced the release of β-hex, but there were clear differences between Ag and tc-LIG in cytokine release. Unlike PAR2-AP, Ag stimulated the release of both TNF and IL-6 suggesting that PAR2-AP and Ag may work through different mechanisms. However, in other studies in our lab, Dr. Grant Stenton showed that both PAR1-AP and PAR2-AP appear to work at least partially through Gαi protein. We have previoulsy shown that G_i is implicated in Ag-induced histamine secretion by MC (260). Thus, while Ag, PAR1-AP and PAR2-AP may be acting on the MC through different mechanisms, they may also share some components of their activation pathways which is G_i mediated. This is consistent with studies by Gordon *et al.* who showed there are fundamental differences in MC signaling mechanisms associated with thrombin and Ag challenge (21). Unlike Ag, thrombin and PAR-1 induce the selective release of cytokine from MC. Furthermore, an additive IL-6 response was observed with a high-level costimulation of thrombin and Ag suggesting that their pathways contain at least some degree of independence. However, they also showed that threshold thrombin receptor and low levels of FcεRI signaling synergized for IL-6 secretion, suggesting that they may also signal through an overlapping pathway. Indeed, both PI_3 and sphingosine kinases are involved in thrombin and PAR-1 signaling for IL-6 release in MC. In addition, both PI_3 (250, 261) and sphingosine kinases (262) have been shown to be key in Ag-mediated Ca^{2+} signaling in MC. Gordon *et al.* also showed that, like Ag-induced

TNF release, thrombin-induced IL-6 release is downregulated by the PI_3 kinase inhibitor wortmannin (263). However, Ag-induced IL-6 release is not affected by wortmannin.

Recent evidence suggests that 48/80 along with other cationic compounds can activate MC by directly interacting with a PT sensitive component (260). This interaction can be blocked by BAK and neuraminidase (NA). To compare the tc-LIG mechanism of activation with 48/80 mechanism, Dr. Grant Stenton from our lab performed desensitization studies with different PAR-AP. His studies suggested that PAR1-AP (Cit) and PAR2-AP (tc-LIG) may activate MC through a 48/80-like mechanism of action. However, PAR2-AP may also possess a second mechanism of signalling that is distinct from that of 48/80, since 48/80 only partially desensitized Ca^{2+} response to tc-LIG. We investigated the ability of BAK, a Gαi inhibitor, to suppress tc-LIG-mediated β-hex release. BAK inhibited both compound 48/80 and of tc-LIG-mediated (0.1 μM, 62%) β-hex release. Furthermore, the removal of sialic acid from PMC, using NA (2U/ml), suppressed Cit (10 mM, 52%) and tc-LIG (0.5 mM, 29%)-induced β-hex release, suggesting that the Cit and tc-LIG are at least partially dependent on cell surface sialic acid residues. In summary, the desensitization experiments showed that PAR1-AP, Cit, may work through a mechanism similar to 48/80, while PAR2-AP pathway may overlap partially with 48/80 mechanism of MC activation. The tc-LIG-mediated release of proteolytic activity may be mediated through a 48/80-like mechanism, since 48/80 induced similar levels of proteolytic activity and protease release.

tc-LIG induced immediate (within 10 min) and long-term (8 hr) morphological changes in RPMC that were detectable by light microscopy. Evidence of early activation was revealed by the loss of cell roundness, granularity and appearance of aggregates (granules) surrounding the cells (Fig. 3). At later time points (4 to 8 hr), the morphological changes induced by tc-LIG changed to loss of refractile edge, flattening and spreading. These morphological observations suggested that tc-LIG (PAR2-AP)-activated MC to release some granular contents at early time points (10 min) and to induce adhesion at later time points (4-8 hr). This phenomenon is consistent with other studies of PAR-2. Trypsin and PAR2-AP stimulate integrin $\alpha_5\beta_1$-dependent adhesion to fibronectin by human gastric carcinoma cells (133). *In vivo,* intraperitoneal injections of PAR2-AP (SLI and tc-LIG) caused significant increase in leukocyte migration into the peritoneal cavity (183), a process that requires adhesion of leukocytes to the endothelium. It has been shown previously that cytokines such as TNF can induce ICAM-1 expression on MC (264). Other cytokines, such as IL-4 have been shown to induce adhesion molecule expression and enhance MC survival in an autocrine fashion (264). Thus, the long-term (8 hr) adhesion-like morphological changes of MC may be a direct cause of tc-LIG stimulation or an indirect effect caused by the upregulation and release of cytokines, such as IL-4 (265).

2. The Effects of PAR1-AP on Mast Cells

Previous studies from our laboratory showed that MC express mRNA for PAR-1 and that PAR-1 mRNA expression on RPMC can be regulated by PAR1-AP and

cytokines. Others have shown immunochemically that MC express PAR-1 on their surfaces (229, 266). We describe here the ability of a PAR1-AP, Cit, to activate RPMC inducing the release of β-hex and proteolytic activity. This is the first study demonstrating the release of stored mediators by RPMC in response to PAR1-AP activation. Strukova *et al.* showed a PAR1-AP-mediated release of NO by RPMC (222). This release was inhibited by L-NAME or the constitutive NO-synthase inhibitor, calmidazolium. PAR1-AP also inhibits PAF release from A23187-treated MC in an NO-dependent mechanism (222). PAR1-AP has also been shown to induce selective IL-6 release by BMMC (21).

The PAR1-AP, Cit, induces the release of stored proteases by RPMC. Interestingly, a second PAR1-AP TFL and a putatively inactive PAR1-CP, FSL, also caused a small release of proteolytic activity. However, FSL-induced proteolytic activity did not reach statistical significance. This suggests that PAR1-AP can mediate release of proteases in a manner consistent with PAR-1-mediated mechanism.

Thrombin, a PAR-1 activating protease, has been implicated in inflammatory diseases (149). However, the mechanism by which thrombin interacts with the immune system to induce inflammation is not well understood. Our study provides evidence that thrombin-induced inflammation may be working through the activation of PAR-1 on MC, in turn inducing the release of MC-proteases and amplifying the PAR inflammatory cascade.

It is not surprising that the TFL has decreased activity in our system considering that it has unprotected chymase-cleaving sites. Given that MC chymase can cleave the

tethered ligand of PAR-1, it is possible that it would cleave within TFL, thus destroying or reducing its activity. By contrast, Cit has been modified to increase stability and potency.

Finally, the two chymases (RMCP-1 and RMCP-5) released by RPMC in response to tc-LIG activation may have many functions *in vivo* relevant to PAR-1. Chymase has been suggested to cleave PAR-1 rendering it unresponsive to proteolytic activation (209). Chymase has also been shown to be involved in the chemotaxis of inflammatory cells (207, 208). Chymases are also able to degrade multiple structural proteins of the extracellular matrix (205). In addition, chymases have been implicated in a variety of diseases including cardiovascular disease (205). Furthermore, the release of chymase by PAR2-AP suggests interdependent regulation between PAR-1 and PAR-2. Chymase has been shown to inhibit the ability of tryptase to activated cells possibly through deactivating PAR-1 (209, 210). Thus, PAR2-AP mediated release of chymase may act to shutoff PAR-1.

3. The Mechanism of PAR-AP Activation of Mast Cells

Only one of the two PAR2-AP (tc-LIG) and one of the PAR1-AP (Cit) were able to activate β-hex release from RPMC. The other peptides, SLI and TFL, although active in other cells, were unable to induce a β-hex or proteolytic activity response above sham in RPMC unless in the case of SLI, the protease inhibitor AM was present. This may be due to simple degradation of the inactive peptides by spontaneously released proteases. However, this also raised the possibility that the active peptides activated MC through a

PAR-independent mechanism. In this section, evidence for and against a classical PAR-mediated mechanism will be discussed.

In contrast to SLI, tc-LIG possesses a *trans-cinnamoyl* group, which acts to stabilize the peptide and prevent its degradation by aminopeptidases. Thus, the difference between these two PAR2-AP peptides in their ability to activate RPMC may be due to differential susceptibility to aminopeptidase degradation. Indeed, in the presence of AM, an aminopeptidase inhibitor, SLI did induce β-hex release. However, the levels of activation by SLI were much lower than those of tc-LIG which is inconsistent with the differential effects of these peptides in other systems, where SLI is either similar or more potent than tc-LIG.

It is unlikely that the *trans-cinnamoyl* modification on tc-LIG is solely responsible for tc-LIG activation of the MC because it is present on the reverse sequence peptide (tc-OLR). tc-OLR induced no β-hex release except when a high dose was used (10μM). For example, tc-LIG at 1 μM induced the release of 67% of β-hex stores in RPMC, whereas tc-OLR induced no significant release (< 5 %) at this dose.

The fact that SLI is less active on RPMC *in vitro* may suggest that tc-LIG activation of RPMC is mediated through a non-classical PAR-2 receptor that responds differentially to PAR2-AP. This is consistent with a recent study in which a polymorphic form of human PAR-2 (PAR2F240S) was found to display reduced sensitivity to trypsin and differential responses to different PAR2-AP (267). In this PAR-2 form, the single mutation in the secondary extracellular loop rendered the receptor 4-fold more sensitive to tc-LIG and 2.8 fold less sensitive to SLI. Constructing this same mutation in the rat

PAR-2 led to comparable results, suggesting that polymorphism may play a role in responsiveness to PAR2-AP (267). Thus, our results may suggest the expression of a non-classical allelic form of PAR-2 on the surface of Sprague-Dawley RPMC that responds to different PAR2-AP in a differential manner.

Similarly, the relatively low activity of TFL (putative PAR1-AP) in our system may be due to proteolytic degradation of the peptide. Alternatively, Cit activation of RPMC may be mediated through a non-classical PAR-1 receptor that responds differentially to PAR1-AP. This is difficult to confirm considering the lack of available antagonists for PAR-1. Pharmacological studies in our laboratory are currently investigating the mechanism of activation.

Finally, TFL and Cit have been used in many *in vivo*, *ex vivo* and *in vitro* studies to investigate PAR-1 functions in the rat. Our results provide evidence that one of the reasons why Cit and TFL show differential responses in *in vivo* and *ex vivo* studies may be that MC are differentially responsive to these two agonists of PAR-1.

Other studies in our lab provided evidence both for and against classical PAR-1 and PAR-2 mediated effects for PAR1-AP and PAR2-AP, respectively. The following section describes the latest pharmacological evidence for and against a classical PAR-1 and PAR–2 activation mechanism induced by PAR1-AP and PAR2-AP in RPMC.

There are at least two lines of evidence suggesting that PAR1-AP work through a classical PAR-1 mechanism: (1) RPMC express a classical PAR-1 mRNA; PAR1-AP but not PAR-2 upregulated the mRNA expression of PAR-1 and IFN-γ downregulated the mRNA expression. Cloning and sequencing of the RT-PCR amplified products that

confirmed they were PAR-1 and PAR-2. (2) Cit activates Ca^{2+} and β-hex released by RPMC. The activation can be blocked by G_i inhibitor, BAK. This is consistent with other studies that showed PAR-1 may work through G_i.

On the other hand we have evidence suggesting that PAR1-AP work through a mechanism unlike classical PAR-1: (1) One PAR1-AP (TFL) has no effect on MC β-hex release. This may be due to degradation of TFL or may suggest a non-classical PAR-1 on RMPC that responds differently to different PAR1-AP. (2) Interestingly, human thrombin had no effect on RPMC activation. This is consistent with other studies that showed no effects of thrombin on RPMC (220). This finding could be due to chymase degradation of thrombin or chymase-dependent inactivation of PAR-1 tethered ligand. However, it may also suggest the lack of PAR-1 on RPMC, and thus some other mechanism of activation by Cit. Finally, (3) neuraminidase (NA) inhibited the Ca^{2+} signalling in Cit-activated RPMC but not in HEK293 cells that express PAR-1 and PAR-2, suggesting that Cit-mediated activation of RPMC does not involve classical PAR-1.

There are at least three lines of evidence suggesting that PAR2-AP work through a classical PAR-2 mechanism in RMPC: (1) RPMC express PAR-2 mRNA and, PAR1-AP and PAR2-AP can upregulate mRNA expression of PAR-2. IFN-γ downregulated the mRNA expression. This is consistent with other studies that showed that PAR mRNA could be regulated by cytokines (182). Furthermore, both active peptides, tc-LIG and SLI, released β-hex and both inactive peptides did not, although the effect of SLI requires the presence of protease inhibitor AM. (2) Trypsin desensitizes tc-LIG-induced calcium signal. (3) tc-LIG-mediated activation is inhibited by $G_{\alpha i}$ inhibitors.

The evidence for a mechanism of activation or RPMC by PAR2-AP that is unlike a classical PAR-2 mechanism is that NA inhibited the tc-LIG-mediated Ca^{2+} flux in RPMC, but did not inhibit it in HEK293 cells that naturally express PAR-2. This suggests that tc-LIG-mediated activation of MC is different from tc-LIG-mediated activation of HEK293 cells in that tc-LIG may work through a sialic acid-dependent mechanism in the MC.

To summarize, there are still several questions left unanswered with regards to whether the PAR1-AP and PAR2-AP used in this study activate the MC through PAR-1 and PAR-2, respectively. Do RPMC express PAR proteins and do the agonists activate MC through classical PAR receptor mechanisms? Due to a limitation in reagents (namely the lack of a specific antibody for PAR, or PAR antagonists) we were unable to obtain sufficient data to unequivocally answer these questions. However, regardless of the precise mechanism involved, our results shed light on the effects of PAR-AP on MC. These effects are important to the field since many investigators are currently using these peptides in attempts to understand PAR-induced inflammation *in vivo*. Our results provide strong evidence that caution must be used in interpretation of the mechanisms involved when PAR-AP are employed.

B. FUTURE DIRECTIONS

While these investigations provide evidence for PAR1-AP and PAR2-AP-mediated activation of MC, many questions remain unanswered.

1) **Do MC synthesize and express classical PAR?**
   a. Clearly, this question will be easier to answer once antagonists are produced and a broader spectrum of antibodies and molecular tools are available. Then, PAR protein could be defined, isolated and characterized using Ab. The activation of MC with PAR-AP in the presence of anti-PAR inhibitory Ab and/or antagonists could be assessed.

2) **Are the effects of PAR-AP on MC mediated through PAR?**
   a. This question could be answered by comparing the effects of PAR-AP on MC from normal and PAR knockout mice.
   b. We have suggested that the low level of activity of SLI and TFL in the absence of protease inhibitors is a result of proteolytic degradation of the peptides and may not be due to differential response to the PAR-AP. This hypothesis must be tested.
      i. This could be accomplished in collaboration with Altrogge *et al.*, who developed galactosidase PAR-AP as a recombinant protein obtained by fusion of beta-galactosidase (209). Using these peptides the degradation of SLI or TFL by supernatants from non-treated cells could be quantified.
   c. In the presense of AM, SLI induces the release of β-hex. However, the levels of β-hex release are much lower than those induced by tc-LIG suggesting that

they may work through different mechanisms. Thus, comparing signaling proteins involved in SLI (in the presense of AM) and tc-LIG activation of RMPC may help to confirm whether or not these peptides work through the same mechanism; eg. does SLI in the presence of AM signal through Gαi?

3) **Do PAR play a role in inflammatory diseases in which MC are implicated?**
   a. One way to answer this question is by studying the effects of blocking PAR-2 and PAR-1 in animal models of inflammatory disease, in which MC and PAR have been implicated (e.g. asthma). PAR activity may be blocked by antisense cDNA, PAR antagonists or inhibitory antibodies.
   b. PAR2-AP-activated MC displayed some characteristics of adhesion and activation. More work needs to be done to understand this adhesion and its significance. These studies should include assessing adhesion molecule upregulation, expression and/or activation by tc-LIG-activated MC. This can be accomplished using Western blot analysis and flow cytometry.
4) **Does the release of proteases regulate MC function in an autocrine and/or paracrine manner?**
   a. To answer this question we could assess the effects of PAR-1-4 antagonists or inhibitory Ab on MC activation through different mechanisms (eg, Ag and proteases).
   b. Assessing tc-LIG-mediated release of proteases at early (20 min) and late (8 hr) time points showed that the release is not limited to a rapid secretion of stored mediators such as histamine and β-hex but may also involve new synthesis of

protease. Alternatively, release of stored proteases may occur over an extended period of time. Experiments must be conducted to confirm these results and to show whether steady state levels of mRNA or $t_{1/2}$ of mRNA for MC proteases are upregulated by tc-LIG activation.

5) **Can the difference in the effect of proteases on different populations of MC be explained?**

   a. Both thrombin and PAR1-AP activate IL-6 release by mouse BMMC. However, in our lab only PAR1-AP and not thrombin activated MC to release β–hex. Are the differences between MC from different species and between subtypes of MC limited to thrombin or do PAR1-AP also function differently on MC from various sources. To better understand the similarities between that mouse and rat system it would be interesting to compare the PAR1-AP mediated IL-6 release in both systems.

   b. Whether thrombin can activate RPMC is uncertain. Our lab as well as others have previously shown that thrombin cannot activate secretion of stored mediators by RPMC. This is interesting when considering that RPMC spontaneously released RMCP-1. Given that RMCP-1 has the ability to rapidly degrade thrombin and its receptor (212), it would be interesting to investigate if thrombin would activate RPMC in the presence of RMCP-1 inhibitory Ab.

C. CONCLUSIONS

This study describes new findings that have important implications on our understanding of the roles of MC in inflammation and homeostasis. Firstly, we showed that RPMC releases serine protease activity as well as RMCP-1, -5 and CP-A in response to tc-LIG (putative PAR2-AP) activation. The release is dose and time-dependent. Secondly, we presented evidence that RPMC spontaneously release TNF-degrading proteolytic activity that can be completely abolished by RMCP-1 inhibitory antibodies, suggesting that RPMC spontaneously release RMCP-1. This suggest a new function for RMCP-1; the ability to degrade TNF. We also showed that RPMC releases proteolytic activity and β-hex in response to PAR1-AP activation.

Our study does not confirm that PAR-AP act through classical PAR. Ongoing research in our laboratory is focusing on characterizing the mechanism by which PAR-AP activate MC. However, our study provides evidence that tc-LIG-mediated activation of MC is different from Ag-mediated activation in that the latter induces release of cytokines (TNF and IL-6), while tc-LIG does not. Whether PAR1-AP induces cytokine release by RPMC remains to be investigated. However, given that PAR1-AP induces IL-6 release by mouse PMC, it is likely that this occurs in RPMC. The peptides studied in this thesis have been used in many *in vivo*, *ex vivo* and *in vitro* studies to investigate PAR-2 functions in the rat (268). Our results provide evidence that may explain differential effects of the PAR-AP *in vivo*.

PAR may have multiple effects on mast cell mediated inflammation (Fig. 23). PAR activation may cause the release of MC proteases including chymases, which have vasomotor and bronchomotor effects. Chymases may degrade surrounding cytokines, inactivate PAR-1 on surrounding cells and degrade extracellular matrix (ECM) proteins. Chymases may also be chemotactic to monocytes and neutrophils. The release of β-hex and histamine may also contribute to the inflammation. Our observation that PAR-AP activate the release of proteases from MC suggest that PAR are involved in the autocrine regulation of MC. Moreover, the release of proteases from MC may stimulate secretion from neighbouring MC and thus provide an amplification signal in inflammatory responses.

Finally, investigating the role of PAR2-AP on MC may lead to the elucidation of important mechanisms by which PAR-2 is involved in inflammatory processes and would help understand the mechanism by which MC regulates mediator release. Understanding these mechanisms may lead to new therapeutic avenues for inflammatory diseases, such as asthma in which MC and PAR-2 have been implicated.

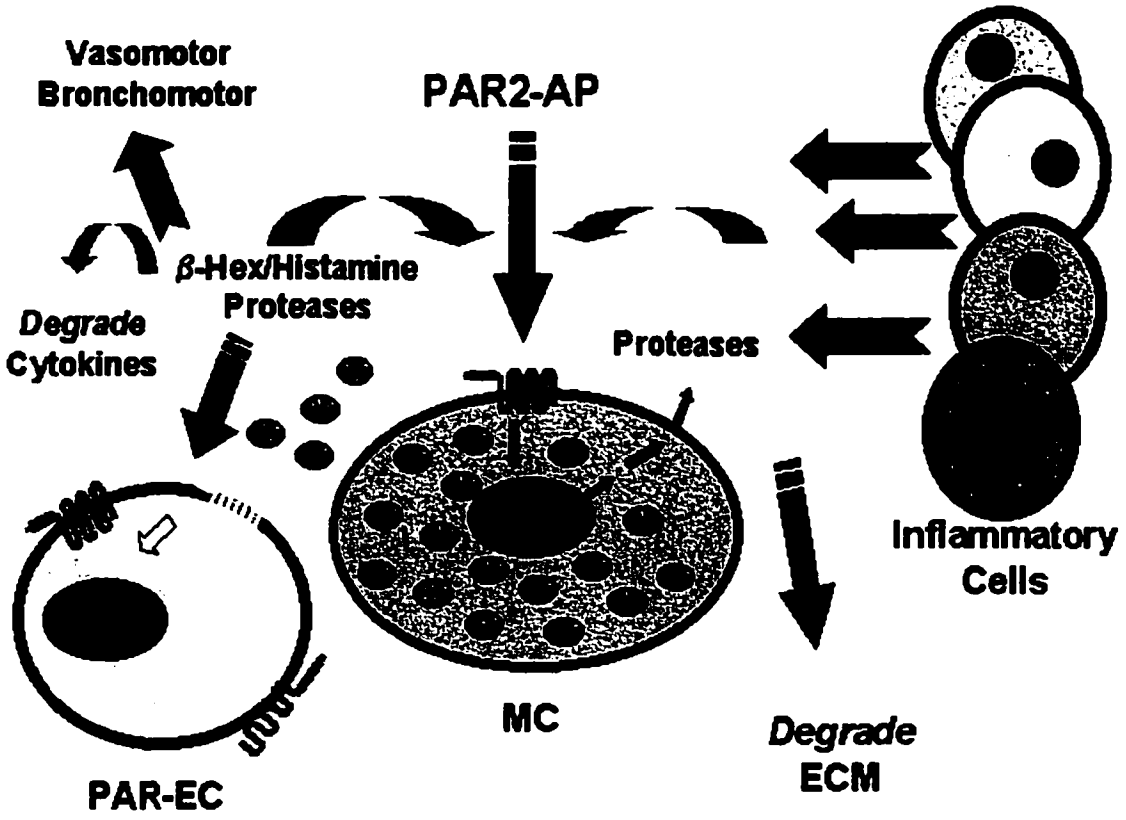

Figure 23: PAR-2-induced effects on mast cell mediated inflammation. PAR-2 activation may cause the release of mast cell chymases which have vasomotor and bronchomotor effects. Chymases may degrade surrounding cytokines, inactivate PAR-1 on surrounding cells and degrade extracellular matrix (ECM) proteins. Chymases may also be chemotactic to monocytes and neutrophils. The release of β-hex and histamine may contribute to the inflammation. PAR-EC, PAR-expressing cells;

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