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Interferon-gamma modulates chloride flux and chloride channels in mast cells

by

Marianna Kulka ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

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Dedication

This thesis is dedicated to my parents who have given me unwavering support and have always believed in my ability to succeed. Thank you for everything.

This thesis is also dedicated to my husband who has given me his advise and help throughout this project. Thank you for being unwavering in your optimism and support.

Abstract

Mast cells (MC) are important effector cells in innate immunity and inflammatory responses. Their activation and subsequent degranulation releases several pro-inflammatory mediators that have a variety of targets including smooth muscle, lymphocytes, eosinophils and submucosal glands. A number of biologically active molecules including cytokines downregulate MC mediator release and understanding their mechanism of action may open new avenues to therapeutic interventions in diseases such as asthma where MC play a major role. Interferon-γ (IFN-γ), a pleotropic cytokine produced by T lymphocytes and several other cell types, is an inhibitor of MC proliferation, differentiation, mediator release and adhesion yet the mechanism of IFN-γ-mediated inhibition of MC functions is poorly understood. The central hypothesis of this project is that Cl^- flux in MC is one target that is altered by IFN-γ and that changes in Cl^- channel expression might be one mechanism that mediates the effect of IFN-γ. Studies using [Cl^-] sensitive fluorochromes and ^{36}Cl show that IFN-γ decreases Cl^- influx in resting MC. Light scatter measurements and colloid titration analysis show that IFN-γ changes in Cl^- flux are independent of changes in cell volume but accompanied by a decrease in cell surface charge. Screening for expression of γ-aminobutyric acid type A receptor ($GABA_AR$) subunits, ClC channel members and the cystic fibrosis transmembrane conductance regulator (CFTR) reveals that MC expressed mRNA for all of these channels. Expression of at least one of these channels, CFTR, is upregulated by IFN-γ. Furthermore, blocking CFTR using pharmacologic inhibitors decreases mediator release from MC. Antisense oligonucleotides to CFTR mRNA and MC derived from

CFTR knock-out mouse bone marrow methodologies were developed and open new avenues for future studies of CFTR expression in MC. In conclusion, IFN-γ decreases Cl^- flux in MC perhaps by regulating the expression of several Cl^- channels.

Preface

This thesis has been written in paper format according to the guidelines of the University of Alberta. Each chapter stands alone as a separate document and is written in the style of the Journal of Immunology. With no exception, the experimental data in this thesis was generated by Marianna Kulka.

Acknowledgements

This book is the "history and emblem of [my] ardent, ambitious, imaginative, yet practical and laborious life" for the past five years and much like Hawthorne's Aylmer I have placed herein an incomplete accounting of my studies during my PhD program. The journey has by no means been an easy one and I have many people to thank for their support and guidance. Certainly, my supervisor, Dr. Dean Befus, has taught me invaluable scientific principles and challenged me to rigorously apply hypothesis driven method to experimentation. I will be forever grateful for his mentorship.

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I had the great privilege to work with many outstanding scientists throughout the past five years and I thank those individuals who have inspired me or challenged me to achieve a high level of scientific integrity. Dr. Angus MacDonald began some of the experiments that lead to my project and I thank him for helping teach me even the most basic science skills. Mark Gilchrist's and Rene Dery's help with lab techniques was also invaluable and I thank them for their patience and time. I thank Dr. Harrisios Vliagoftis and Dr. Paige Lacy because they were always willing to help and offer advice whenever experiments hit an unexpected snag.

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Lastly, and perhaps most importantly, I must thank Iwona Kuklicz for keeping me "sane with sushi" and the rest of the gang for making sure that my imagination got a workout....too much at times!

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Abbreviations

2,5 OAS	2,5 oligoadenylate synthetase
48/80	polybasic compound used to activate mast cells
5-HT	5-hydroxytryptamine, serotonin
ADP	adenosine diphosphate
Ag	antigen
AMPK	adenosine 5'-monophosphate-activated protein kinase
ANOVA	analysis of variance
ATP	adenosine triphosphate
BDZ	benzodiazepine
β-hex	β-hexosaminidase
BMMC	bone marrow cultured mast cells
BM	bone marrow
Ca^{2+}	calcium
cAMP	adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CD34	cluster of differentiation 34
CD45	cluster of differentiation 45
Cl^-	chloride
ClC	voltage-gated chloride channel family
ClC-Kb	kidney specific ClC chloride channel member
CFC	poly(diallyldimethyl-ammonium chloride) (Cat-floc)
CFTR	cystic fibrosis transmembrane conductance regulator
c-kit	receptor for stem cell factor
c-kit ligand	stem cell factor (SCF)
cGKII	cGMP-dependent protein kinase type II
CNS	central nervous system
CTMC	connective tissue mast cells
DADS	4,4'-diaminostilbene-2,2'-disulfonic acid
DAG	diacylglycerol
DEPC	diethyl pyrocarbonate
DIDS	4,4'-diisothiocyano-2,2'-stilbenedisulphonate
DNP	dinitrophenol
DPC	diphenylamine-2-carboxylate
DTT	dithiothreitol
EC_{50}	excitatory concentration (that gives 50% of response)
EDTA	ethylenediaminetetraacetic acid
E_{Cl}	equilibrium potential for chloride ions
ECP	eosinophil cationic protein

ERK	extra cellular receptor activated kinase
EPO	eosinophil peroxidase
ER	endoplasmic reticulum
ERAD	ER-associated protein degradation
FBS	fetal bovine serum
FcεRI	high affinity Fc-epsilon receptor-I
FcγRII	Fc-gamma receptor II
FcγRIIB	Fc-gamma receptor II B – an inhibitory isoform
FFA	flufenamic acid
F	fluorescence intensity
fMLP	formyl-methionyl-leucyl-phenylalanine
GABA	γ-aminobutyric acid
$GABA_AR$	γ-aminobutyric acid-type A receptor (chloride channel)
$GABA_BR$	γ-aminobutyric acid-type B receptor
GAS	IFN-gamma activated site
GM-CSF	granulocyte-macrophage colony-stimulating factor
GSNO	s-nitrosoglutathione
GTP	guanosine triphosphate
HCO_3^-	bicarbonate ion
HMC-1	human mast cell line
HTB	Hepes Tyrode's buffer
IC_{50}	concentration that inhibits effect by 50%
$I_{Clswell}$	volume-activated chloride current
IFN-γ	interferon-gamma
IgE	immunoglobulin E
IκB	inhibitory factor kappa-B
IL-3	interleukin-3
iNOS	inducible nitric oxide synthase
IP3	inositol triphosphate
IRF	IFN regulatory factor
IRS	inhibitory receptor superfamily
I_{SK}	*Shaker* potassium channel current
ISRE	interferon-stimulated response elements
ITAM	immunoreceptor tyrosine activation motifs
ITIM	immunoreceptor tyrosine-based inhibition motifs
JAK	Janus kinase
K_{ATP}	ATP-regulated potassium channels
K_{Ca}	Ca^{2+}-dependent potassium channels
K_d	dissociation constant
$[K]_i$	intracellular potassium concentration

$[K]_e$	extracellular potassium concentration
KIR	killer cell inhibitory receptor
K_{SV}	Stern-Volmer constant
LPS	lipopolysaccharide
L-NMMA	N-mono-methyl-L-arginine
LTC_4	leukotriene C_4
mAb	monoclonal antibody
MAFA	mast cell function-associated antigen
MAP	mitogen-activated protein
MBP	myelin basic protein
MCP-1	monocyte chemotactic protein-1
MC	mast cell
MCCP	mast cell committed precursors
MHC II	major histocompatibility complex type II
MIP1 α/β	monocyte inhibitory protein-1
MMC	mucosal mast cells
M-MLV	Moloney-murine leukemia virus
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MQAE	N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide
NCS	nitrogen carbon sulfide group
NED	nedocromil sodium
NF-AT	nuclear factor of activated T cells
NF-κB	nuclear factor kappa-B
NGF	nerve growth factor
NHERF or EBP50	Na^+/H^+ exchange regulatory factor
NK	natural killer cells
NMDA	N-methyl –D-aspartate
NMG	N-methyl-D-glucamine
NO	nitric oxide
NPPB	5-nitro-2-(3-phenylpropylamino) benzoic acid
OD	optical density
ODN	oligodeoxynucleotides
ORCC	outwardly rectifying chloride channel
P1, P2	purinergic receptor-1 and 2
PAF	platelet activating factor
PBA	sodium 4-phenylbutyrate
PC	phosphatidylcholine
PCR	polymerase chain reaction
PDZ	protein-protein binding motif
PGD_2	prostaglandin D_2

pH_i	intracellular pH
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PMA	phorbol myrisate acetate
PMC	peritoneal mast cells
PMSF	phenylmethylsulfonyl fluoride
RANTES	regulated upon activation normal T-cell expressed and secreted protein
RBL-2H3	rat basophilic leukemia
RCMC	rat cultured mast cells
ROMK	renal outer medullary K^+ channel
RPMI	Rosweli Park Memorial Institute
RT	reverse transcriptase
$S1/S1^d$	mast cell-deficient mice lacking stem cell factor
SCF	stem cell factor (c-kit ligand)
SCG	sodium cromoglycate
SH2,3	protein-protein binding motif
SITS	4-acetamido-4-isothiocyano-2,2-disulfonic acid
SOC	store-operated channels
SPQ	6-methoxy-N-(3-sulfopropyl)quinolinium
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNP	sodium nitroferricyanide
STAT	signal transducers and activators of transcription
TB	toluidine blue
TGFβ1	transforming growth factor-β1
TNF	tumor necrosis factor
TNP	trinitrophenol
VAMP	vesicle-associated membrane protein
VRAC	volume regulator anion channels
WE	worm equivalent
W/W^v	mast cell-deficient mice lacking mast cell committed precursors

Chapter 1

General Introduction

Mast cells (MC) are important effector cells in innate immunity and inflammatory responses. Their activation and subsequent degranulation releases several pro-inflammatory mediators that have a variety of targets including smooth muscle, lymphocytes, eosinophils and submucosal glands (1). A number of biologically active molecules including cytokines downregulate MC mediator release and understanding their mechanism of action may open new avenues to therapeutic interventions in diseases such as asthma where MC play a major role. IFN-γ, a pleotropic cytokine produced by T lymphocytes and several other cell types, is an inhibitor of MC proliferation, differentiation, mediator release and adhesion (2-4) yet the mechanism of IFN-γ-mediated inhibition of MC functions is poorly understood. Previous studies in our lab used differential display to analyze the mRNA expression of IFN-γ-treated and untreated MC. Preliminary data suggested that IFN-γ upregulated a 218 bp fragment. A 51 bp portion of this fragment had 72% homology to ClC3, a Cl^- channel, and suggested that IFN-γ regulated Cl^- channel expression and possibly Cl^- channel function. **Therefore, the central hypothesis of this PhD project is that Cl^- flux in MC is one target that is altered by IFN-γ and that changes in chloride channel expression might be one mechanism that mediates the effect of IFN-γ.** Although IFN-γ modulates the expression of more than 200 genes (5) its effect on ion movements and ion channels has received little attention. Furthermore, expression of specific chloride channels and their role in MC function is poorly understood. Therefore, the four major aims of this PhD project were as follows:

A. **To characterize the effect of IFN-γ on Cl^- flux in MC.** Cl^- fluxes in resting peritoneal mast cells (PMC) and a rat cultured mast cell line (RCMC) were tested using [Cl^-] sensitive fluorochromes and ^{36}Cl. Cl^- flux of IFN-γ-treated and untreated MC was compared. Since changes in ion flux are dependent upon changes in cell volume and surface charge, light scatter measurements and colloid titration techniques were used to measure differences in cell volume and surface charge between IFN-γ-treated and untreated MC.

B. **To characterize chloride channel expression in MC.** There are a large number of ion channels and co-transporters that could facilitate the flux of Cl^- ions in MC.

For this study, the three main classes of Cl^- channels were investigated. MC were screened for mRNA expression of γ-aminobutyric acid type A receptor ($GABA_AR$) subunits, ClC channel members and the cystic fibrosis transmembrane conductance regulator (CFTR). Although MC expressed mRNA for all of these channels, the lack of reagents (e.g. antibodies, specific pharmacologic inhibitors, etc) to ClC prompted us to focus on CFTR. CFTR is a complex and multi-faceted chloride channel, mutations in which are responsible for cystic fibrosis. CFTR function in MC was characterized using cAMP analogues and pharmacologic inhibitors.

C. **To determine the effect of IFN-γ on the expression of these chloride channels.** The effect of IFN-γ on $GABA_AR$ subunits and ClC mRNA in MC was assessed. IFN-γ effects on CFTR mRNA and protein regulation were also studied and the role of nitric oxide (NO)-dependent pathways in CFTR expression was examined.

D. **To determine the role of chloride channels in MC function.** To determine the possible role of CFTR in MC function, antisense to CFTR mRNA was designed and used to block CFTR expression in MC. The effect of antisense on MC mediator release was measured.

These aims are based upon a foundation of literature that suggested that MC activation is partly chloride dependent and that chloride flux might be important in MC function.

I. Introduction to Mast Cells

A. Mast cell biology

1. Localization and morphology

Mature MC are found throughout the body, predominantly located near blood vessels and nerves and beneath epithelia (1,6). MC were first described by Paul Ehrlich in the 1870's who described them as "*mast zellen*" or "fat cells" because they were abundant in the tissue of well-fed animals (7). He identified them by their metachromatic properties, which depend on the presence and degree of sulfation of proteoglycans such as heparin

stored in cytoplasmic granules. Basic dyes such as toluidine blue bind the negatively charged proteoglycans and change color from blue to red.

MC are relatively large cells (approximately 20 μm in diameter; (8)) with round nuclei, membrane-bound granules and lipid bodies (Fig 1.1). MC granules contain stored mediators such as histamine, β-hexosaminidase (β-hex) and proteases in a proteoglycan matrix. MC circulate in the blood as committed precursors expressing CD34, CD45, FcεRI, FcγRII and c-kit (1,9). These precursors migrate to the tissues where it is believed MC undergo the last stages of tissue-specific differentiation. MC differentiate from MC committed precursors (MCCP) in the presence of stem cell factor (SCF or c-kit ligand) that binds MC c-kit receptors. Knowledge of MC differentiation has largely come from studies of MC-deficient mice such as the W/W^v MC-deficient mouse (on a WBB6F1 background) that is deficient in MCCP (10,11) and the Sl/Sl^d MC-deficient mouse (WCB6F1 background) that lacks functional SCF (12).

2. Heterogeneity and localization

MC phenotype and profile of mediator secretion in response to different secretogogues depend largely on their environment. Indeed, MC from different tissues can be heterogeneous (13). MC heterogeneity involves serine protease content, response to secretogogues, response to chemotactic factors and production of lipid mediators. In rodents, there at least two recognized subsets of MC, connective-tissue type (CTMC) and mucosal type (MMC). CTMC are present in the skin, peritoneal cavity, lymphoid organs and the lungs of rodents and are particularly abundant around large and small blood vessels and nerve endings (1). In rodents, MMC are present in the mucosa of the gastrointestinal tract but human MMC are found predominantly in intestinal mucosa and alveolar spaces in the lung (1).

CTMC store large amounts of histamine in their cytoplasmic granules, express CTMC-specific proteases, are activated via FcεRI stimulation, contain heparin proteoglycan and preferentially make prostaglandin D_2 (PGD_2). Unlike MMC, CTMC show little T cell dependence. MMC contain less histamine, express MMC-specific proteases, contain chondroitin sulfate proteoglycan and preferentially make leukotriene

C_4 (LTC_4). The presence of MMC *in vivo* depends on T cells since athymic mice and humans with T cell immunodeficiency are deficient in MMC (14,15). MC cultured from rat bone marrow in the presence of IL-3 (BMMC) resemble MMC based on the high level of granule-associated chondroitin sulfate, low histamine concentration and proteinase content (16,17).

MMC and CTMC differ in their response to stimuli. For example, a rat basophilic cell line (RBL) that bears close resemblance to MMC does not respond to P2 agonists (purinergic activation), but rat CTMC express P2 receptors, respond to P2 agonists and migrate toward extracellular ADP, which binds the P2 receptor (18,19). CTMC and MMC respond to secretogogues differently, reflecting their phenotypic distinctiveness (see next section).

MC also reside in the brain and belong to either CTMC or MMC phenotypes (20). Brain MC are found intracranially, in the dura mater, leptomeninges, choroid plexus and the parenchyma of the brain (particularly the thalamus) (21-24). In the rat brain, there is a correlation between MC numbers and content of histamine (25) and isolated rat brain MC release 5-hydroxytryptamine (5-HT; serotonin) and histamine in response to neuropeptides and neurotransmitters released from nearby neurons (26). In birds and rodents, MC numbers in the brain are increased in response to courting, taking care of young, aggressive behaviour, long photoperiods and hibernation (27,28), but stress and isolation decrease the number of MC in the brain. Behavioural control of migration of MC suggests that this trafficking is hormone dependent (29,30).

B. Mast cell activation

1. FcεR-dependent activation

MC are highly specialized effector cells that can respond to a number of stimuli. One of the best characterized mechanisms of activation is antigen-mediated crosslinking of surface FcεRI. FcεRI aggregation stimulates the release of preformed cytoplasmic granules (containing histamine, proteoglycans, and MC-specific proteases), newly synthesized mediators such as tumor necrosis factor (TNF) and lipid-derived mediators,

including platelet activating factor (PAF) and the arachidonic acid metabolites PGD_2 and LTC_4 (Fig 1.2)(1).

FcεRI is composed of one α chain, one β chain and two γ chains (Fig 1.3). Both the α and γ chains must be present to have cell surface expression (31). IgE binds the α chain causing conformational changes and activation of the β and γ chains which initiate signal transduction via their immunoreceptor tyrosine activation motifs (ITAM). The Lyn tyrosine kinase is constitutively associated with the carboxy terminal cytoplasmic tail of the FcεRI β chain (32). When the FcεRI is crosslinked by antigen, Lyn phosphorylates the ITAM in the cytoplasmic domains of FcεRI β and γ chains and recruits Syk tyrosine kinase to the ITAM of the γ chain (33). Syk becomes activated and phosphorylates other kinases, activating a signalling cascade.

Some of these proteins include phospholipase C (34) which hydrolyzes phosphatidylcholine (PC) to produce inositol triphosphate (IP3) and diacylglycerol (DAG). DAG phosphorylates and activates protein kinase C (PKC) (35) that can initiate granule mobilization to the plasma membrane. The other PC product, IP3, activates intracellular Ca^{2+} channels, releasing intracellular Ca^{2+} stores from the sarcoplasmic reticulum. High intracellular Ca^{2+} concentrations (or perhaps the depletion of intracellular Ca^{2+} stores) activate store-operated channels (SOC) to initiate influx of extracellular Ca^{2+} (Fig 1.4). Other ions fluxes such as Cl^- are probably also important in facilitating influx of Ca^{2+}. Upon FcεRI crosslinking, for example, MC uptake Cl^- and only in the presence of extracellular Cl^- is maximal mediator release achieved (36). Threshold levels of intracellular Ca^{2+} in combination with DAG activate PKC, which phosphorylates the myosin light chains and leads to disassembly of actin-myosin complexes beneath the plasma membrane, thereby allowing granules to come into contact with the plasma membrane (37,38). Fusion of the granule membrane and the plasma membrane is mediated by interactions between soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE), SNARE vesicle-associated membrane protein (VAMP) and SNARE syntaxin (39). Granule fusion with the plasma membrane is regulated by the guanosine triphosphate (GTP)-binding form of Ras-related Rab proteins (40). Activated adenylate cyclase elevates cyclic adenosine monophosphate (cAMP)

levels, and cAMP activates protein kinase A (PKA) which may participate in a negative feedback loop to inhibit degranulation (41).

2. FcεR-independent activation

MC can also be activated by FcεRI-independent mechanisms by substances such as polybasic compounds, peptides, cytokines and complement-derived anaphylatoxins. The polybasic compound, 48/80, contains a cationic region adjacent to a hydrophobic moiety, which penetrates MC membranes and activates G proteins (Fig 1.4) (42-44). Interestingly, polybasic compounds only activate rodent CTMC, not MMC (13,45,46). The bee venom peptide, mellitin, and adrenocorticotropic hormone share sequence homology to the C_H4 domain of IgE, which allows them to bind to FcεRI and activate MC via the Lyn/Syk pathway (47,48). Neuropeptides such as substance P induce MC activation in an FcεRI-independent mechanism through activation of G proteins (42). Complement peptides such as C5a bind to specific receptors on mast cells and stimulate degranulation and potentiate anaphylactic reactions (49).

3. Mast cell mediators

FcεRI crosslinking initiates degranulation and the release of histamine, proteases and enzymes such as β-hex. These biologically active molecules have a variety of functions and several can bind to receptors on different cell types.

Upon activation, MC also synthesize and secrete a wide range of cytokines such as IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-13, IL-16, TNF and granulocyte-macrophage colony-stimulating factor (GM-CSF) and chemokines such as monocyte chemotactic protein-1 (MCP-1), monocyte inhibitory protein-1 (MIP-1 α/β) and regulated upon activation normal T-cell expressed and secreted protein (RANTES) (1,13). Cytokine production by activated MC is a consequence of newly induced cytokine gene transcription. Activation of various adapter molecules by Syk leads to nuclear translocation of nuclear factor of activated T cells (NF-AT) and NF-κB, as well as activation of AP-1 by stress-activated kinases such as c-Jun N-terminal kinase. The

presence of cytokines in the tissues can in turn modulate MC functions. TNF, IL-6, GM-CSF, MIP-1α and SCF cause MC to release monoamines such as serotonin (13).

Another set of newly synthesized products released by IgE-dependent activation of MC are the lipid mediators such as leukotrienes and prostaglandins. Synthesis of lipid mediators is initiated by the cytosolic enzyme phospholipase A_2 ($cPLA_2$) that is activated by increases in cytoplasmic Ca^{2+} and phosphorylation by the extra cellular receptor activated kinase (ERK; a member of the mitogen-activated protein (MAP) kinases). ERK kinase is activated by the kinase cascade that is initiated by the receptor ITAM. Once activated, $cPLA_2$ hydrolyzes membrane phospholipids into arachidonic acid, which is modified by cyclooxygenase or lipooxygenase enzymes to produce prostaglandins and leukotrienes.

4. Mast cell chemotaxis

Another consequence of MC activation is chemotaxis. MC chemoattractants include antigen, transforming growth factor-β1 (TGFβ1), angiogenic factors, complement components (C1q, C3a and C5a), hematopoietic growth factors including SCF (50) and adenine nucleotides which are neuronal secretory products that bind P1 and P2 purinoceptors (19). MC chemotaxis is dependent on tyrosine kinase activity and G_i activation since both genistein and pertussis toxin inhibit MC migration (50-52). As in neutrophils, that chemotax over vitronectin or fibronectin, MC chemotaxis is marked by influx of extracellular Ca^{2+} ions (19).

C. Mast cells are important cells in innate immunity

A principal protective role of IgE and MC-mediated immune reactions is the eradication of parasites. Mice genetically deficient in MC show increased susceptibility to infection by tick larvae and immunity can be restored by adoptive transfer of serum containing IgE to tick larvae (53). MC also play an important protective role as part of the innate immune response to bacterial infections. MC-deficient mice are less capable of clearing bacterial infections of the peritoneum than normal mice (54). This protective

effect can be restored by introduction of MC expressing toll-like receptors (TLR) which can bind lipopolysaccharide (55). The protective role of MC in innate immunity is mediated by MC production of IL-1, IL-6 and TNF and depends on TNF-dependent influx of neutrophils to the peritoneum (55). In antigen-challenged mice, local production of C5a activates local MC to produce 5HT and TNF, which induces ICAM-1 and VCAM-1 expression on endothelial cells leading to T cell recruitment (56). MC are also capable of phagocytosis of bacteria and presentation of bacterial antigens via MHC II to T cells (57).

D. Mast cells in disease

1. Mast cells in allergy

MC degranulation is a central component of allergic diseases and pathologic manifestations depend on the tissues in which MC mediators have effects, as well as duration of the inflammatory process. Allergic individuals typically have elevated levels of serum IgE and express more FcεRI receptors on MC, a high proportion of which are occupied by IgE (58). Inhalation or ingestion of the allergen activates MC in the lung or gut respectively and initiates the allergic response. In systemic immediate hypersensitivity, the allergen is present systemically as with an insect bite. When activated by allergen systemically, it is believed that MC and basophil mediators contribute to vasodilation and exudation of plasma in the vascular beds throughout the body resulting in fall in blood pressure, constriction of upper and lower airways, hypersensitivity of the gut, over-production of mucus in the gut and lung, and urticarial lesions (hives) on the skin (59).

2. Mast cells in asthma

MC activation and degranulation is also important in asthma. Asthma is an inflammatory disease caused by repeated immediate hypersensitivity reactions in the lung leading to the pathologic triad of intermittent and reversible airway obstruction, chronic bronchial inflammation with eosinophils, and bronchial smooth muscle cell hypertrophy and hyperreactivity to bronchoconstrictors (60). The majority of asthmatics show

positive skin test responses to the injection of one or more common allergens such as house dust mite allergens and in these patients, airway obstruction can be triggered by inhalation of these allergens (61). The allergens activate MC via surface FcεR releasing pro-inflammatory mediators such as LTC_4 and PAF, which bind to receptors on smooth muscle and induce constriction. One of the hallmarks of MC function in asthma is their ability to recruit other cells such as lymphocytes and eosinophils. Allergen-activated MC release tryptase, IL-5 and RANTES, which recruit neutrophils and eosinophils to the lung (62-65). Eosinophils in the lung become activated and release several mediators such as PAF, LTC_4, major basic protein, eosinophil cationic protein (ECP) and eosinophil peroxidase (EPO), which contribute to airway hyperresponsiveness and tissue damage (63,66,67). Activated MC also release IL-16 and a newly described chemokine, lymphotactin, which recruit lymphocytes to the lung (68,69). However, a smaller group of patients are non-atopic (58) and their asthma reactions may be triggered by non-immune stimuli such as drugs, cold and exercise – suggesting that alternative mechanisms of MC degranulation may underlie the disease.

3. Mast cells in other diseases

Abnormally high numbers of MC can also cause disease. Recent genetic findings indicate that mastocytosis, a rare disease characterized by a primary pathological increase in mast cells in a variety of tissues, is caused by mutations in the c-kit receptor (70). MC-mediated diseases display a sexual selectivity, suggesting that MC behave differently in males and females. MC express estrogen receptors and can be activated by estrogen and estrogen receptor antagonists and have been implicated in recruiting inflammatory cells in female-related bladder disease (71-73). Recent studies have shown that MC are found in the vicinity of multiple sclerosis lesions and may be important in brain disease (74). Myelin basic protein (MBP), a major suspected immunogen in multiple sclerosis, activates MC to produce tumor necrosis factor (TNF), which activates astrocytes to produce NO and kill surrounding neurons (75). Additionally, proteases from brain MC are capable of producing the Alzheimer amyloid β-protein implicated in this neurodegenerative disease (76).

E. Mast cell inhibitors

There are several inhibitory compounds/receptors that attenuate FcεRI-induced MC activation. These compounds bind inhibitory receptors that are structurally and functionally related. The inhibitory receptor superfamily (IRS) are single-pass transmembrane receptors that contain either C-type lectin (i.e. CD22, Ly49, NKG2 and mast cell function-associated antigen [MAFA]) or Ig-like (i.e. FcγRIIB, killer cell inhibitory receptor [KIR], and gp49B1) extracellular domains (77). All IRS members contain immunoreceptor tyrosine-based inhibition motifs (ITIM). ITIM-containing inhibitory receptors differentially recruit SHP-1, SHP-2 and/or SHIP *in vivo* in a manner dependent on phosphorylation of ITIM tyrosines (78-80). The first identified and most extensively studied member of the IRS is FcγRIIB, a low-affinity IgG receptor that functions in regulation of capping, endocytosis and phagocytosis in response to binding multivalent antigen (81). Two forms of FcγRIIB are expressed in murine mast cells (81,82). Coaggregation of FcγRIIB1 and FcγRIIB2 inhibits FcεRI-mediated release of serotonin and TNF (82).

Drugs that inhibit MC function include theophylline, prostaglandin analogues (83), corticosteroids, β-agonists, and cromolyn compounds such as nedocromil sodium (NED) and sodium cromoglycate (SCG). The precise mechanisms by which some of these drugs inhibit MC activation are poorly understood. However, their effects on MC physiology are diverse. Theophylline is a phosphodiesterase inhibitor (84) and increases intracellular cAMP concentration (85). β_2-agonists such as salbutamol and salmeterol inhibit the release of preformed and newly synthesized MC mediators (86). NED and SCG downregulate TNF release in PMC by up to 40% (87-89). PMC pretreated with NED or SCG for 20 min and then stimulated with antigen (Ag) for 6 hr show a decrease in both TNF mRNA and TNF protein, an effect that is cycloheximide dependent. Interestingly, NED and SCG block chloride current in MC (90-92). Cromolyn inhibits Cl^- channel activity with an IC_{50} of 15 ± 2 μM (92). Cl^- channel activity is completely blocked when 1 mM cromolyn is applied to the intracellular side of an inside-out patch but this inhibition

can be reversed upon washing out cromolyn (92). The fact that drugs that inhibit MC degranulation also inhibit Cl^- channel function initiated the central hypothesis of this thesis that suggests chloride is an important ion in MC activation cascades.

The third group of MC "inhibitors" include cytokines, which have a variety of affects on MC physiology some of which are inhibitory. These cytokines include IL-10 (93), TGF-β (94) and interferon-gamma (IFN-γ) (95).

II. Introduction to Interferon-gamma

IFN-γ is secreted by activated T cells (cytotoxic and Th1), macrophages and natural killer (NK) cells in response to viral infection or T cell activators such as ConA or anti-CD3 (5). IFN-γ is an acid labile homodimer composed of two heavily N-glycosylated proteins that bind two IFN-γ receptor α chains (IFN-γRα; Fig 1.5) (96). Interferons (α, β and γ) were discovered based on their antiviral activity but exert several biological effects including modulation of immune function and inhibition of cell growth (96). As an immunomodulator, IFN-γ influences the migration of thoracic duct B and T lymphocytes from blood to lymph (97), inhibits mast cell-mediated cytotoxicity (4,89), and stimulates macrophage phagocytosis (98). IFN-γ modulates immune responses involved in host defenses against infectious agents and tumors by up-regulating MHC class I and class II, affecting IgG heavy chain switching and stimulating the production of immunomodulatory cytokines such as IL-12 and TNF and of antiviral proteins such as 2,5 oligoadenylate synthetase (2,5 OAS) and RNAse L (99,100). IFN-γ also influences basic physiological functions of non-immune cells such as epithelial cells (101), fibroblasts (102), and keratinocytes (103,104).

In immediate type hypersensitivity diseases, where MC play a significant effector role, IFN-γ production is abnormal. For example, in patients with allergic asthma, the percentage of IFN-γ-producing T lymphocytes is considerably lower (5.7% versus 23.5 % in normal subjects, $P<0.001$) but the level of IL-4-producing T lymphocytes is the same (105,106). Studies of atopic asthmatics revealed reduced expression of IFN-γ mRNA in bronchoalveolar lavage (BAL), 24 hr post endobronchial allergen challenge (107). In

adoptive transfer experiments, IFN-γ has been shown to have a suppressive effect on airway eosinophilia (108). Therefore, it was postulated that individuals with asthma may possess genetic differences in IFN-γ receptor (IFN-γR) genes and a study revealed that some common polymorphisms were associated with increased total serum IgE levels, although these were not correlated with severity of disease (109). Peripheral blood mononuclear cells isolated from children with food allergy, however, show normal levels of IFN-γ when stimulated with phytohemagglutinin (110) suggesting that decreased IFN-γ production may be antigen-specific.

A. IFN-γ receptor (IFN-γR)

The IFN-γR (Fig 1.5) was initially characterized in the early 1980s in radioligand binding studies conducted in several laboratories on a variety of cell types (96). These experiments showed that most primary and cultured cells expressed a moderate level of high affinity binding sites for IFN-γ. The interaction of IFN-γ with its receptor was not inhibited by other interferon classes, suggesting that IFN-γ bound a receptor distinct from the one bound by IFN-α and IFN-β.

Subsequent experiments showed that the IFN-γR was actually composed of two subunits. The ligand-binding component is referred to as the α chain IFN-γR1 or CDw119. The second subunit is called the β chain, accessory factor-1 (AF-1) or IFNγR2. For purposes of clarity the two subunits of the IFN-γR will be referred to as the α and β chains. Each IFN-γR is composed of two α and two β chains (Fig 1.5). The human α chain is encoded by a 30 kb gene located on the long arm of chromosome 6 and the murine homologue is a 22 kb gene present on chromosome 10 (96). Although expression of the fully mature α chain protein varies widely among tissues (200 to 25,000 sites/cell), there does not appear to be a direct correlation between the extent of α chain expression and the magnitude of the IFN-γ-induced response (96). The human β chain is encoded to chromosome 21 and the murine homologue resides on chromosome 16 (111). Whereas transcription of the α chain does not appear to be externally regulated, the gene encoding the β chain contains several potential binding sites for a variety of externally regulated

activated transcription factors (111). The α chains exhibit very little overall sequence homology between species (52.5% between human and murine), thus the activity of IFN-γ is species specific (96).

IFN-γ binding to its receptor induces the dimerization of IFN-γR subunits and allows association of JAK1 and JAK2, two members of the Janus tyrosine kinase (JAK) family, resulting in JAK activation by tyrosine phosphorylation (Fig 1.5). Activated JAK phosphoylates tyrosine at position 440 on the β chain of IFN-γR, creating a docking site for signal transducer and activator of transcription-1 (STAT1) molecules (112). STAT1 is then phosphorylated on tyrosine 701, leading to its dimerization and translocation to the nucleus, where it binds to the IFN-gamma-activated site (GAS) sequences of promoters to regulate expression of downstream genes (113). Following IFN-γR ligation, the IFN-γR/IFN-γ complex is internalized and enters an acidified compartment wherein the complex dissociates and free IFN-γ trafficks to the lysosome where it is degraded (114).

B. IFN-γ regulated gene transcription

Treatment of cells with IFN-γ causes the transcription of genes that are otherwise expressed at low levels or are quiescent (5). There are more than 200 genes that are regulated by IFN-γ either via changes in gene transcription or mRNA stability (5,115,116). Transcription of IFNγ-inducible genes is initiated by STAT1 translocation through the nuclear membrane and binding to specific sequences of promoter regions (Fig 1.6). There are two isoforms of STAT1 termed α and β, however only STAT1α is capable of translocating to the nucleus and initiating gene transcription (117). STAT1α contains a conserved amino-terminal dimerization domain, an IFN regulatory factor (IRF)-binding domain, a DNA-binding domain, SH2 and SH3 domains and a carboxyl-terminal transcription activation domain (118). Phosphorylated STAT1α specifically binds to unique elements called GAS with a consensus sequence of AANNNNNTT (119) and initiates transcription of genes such as the proteasome subunit LMP2 (120,121), major histocompatibility complex type II (MHC II; (121)), CIITA (122) and components

of the respiratory burst oxidase such as gp91, p47 and p67 phox (123,124) (Fig 1.6). STAT1α also activates the transcription of IRF family of transcription factors and one of these, IRF1, binds to interferon-stimulated response elements (ISRE) sequences, initiating gene transcription of inducible NO synthase (iNOS) and MHC I (121,125). These alterations in gene transcription ultimately change the phenotype and response of the cell.

The essential role of STAT1 in IFN-γ-dependent signalling has been demonstrated in cells that do not express this factor (126). However, there is also evidence for STAT1-independent responses to IFN-γ. Expression of kinase-negative JAK1 mutants in wild-type cells inhibits the development of the antiviral response without affecting the activation of STAT1 or STAT1-dependent gene expression in response to IFN-γ (127). Although STAT1 is important for the antiviral response, STAT1-null mice are 100 times more resistant to murine cytomegalovirus and Sindbis virus than are mice lacking expression of IFN-γ (128). Furthermore, IFN-γ inhibits the proliferation of wild-type but not STAT1-null fibroblasts (117). JAK1 and JAK2 are required for the induction of STAT1-independent gene expression by IFN-γ (127,128). It is possible that JAK phosphorylates other STAT family members, possibly STAT3 which can induce the transcription of some of the same immediate-early genes (129-131). Most of the STAT1-independent genes are induced rapidly (0.5-1 h) suggesting that they may have similar promoters and that *de novo* synthesis of transcription factors (such as IRF1) is not required (131).

Many IFN-γ-mediated effects are induced indirectly and are the result of pathways that are initiated as a result of IFN-γ regulation of gene transcription. One of those indirect messengers is NO. Nitric oxide is a radical synthesized from L-arginine and molecular oxygen by many cell types, including MC. IFN-γ initiates production of NOS in MC as well as some of the second messengers involved with NO production (132,133). NO exerts a range of physiological, toxicological and immunoregulatory effects and may be important in immunity by influencing the balance between functional subsets of T helper cells and in IgE mediated allergic reactions by inhibiting mast cell activation and histamine release (134,135). In fact, NO appears to be one of the principle intermediaries of IFN-γ-mediated downregulation of MC functions (133).

C. IFN-γ effects on MC

IFN-γ has suppressive actions on several MC functions such as clonal proliferation, differentiation, TNF-mediated cytotoxicity, adhesion and mediator release (2,3,95,136,137). These effects appear to be transcription/translation dependent and are probably the consequence of several changes in MC gene expression. However, several questions remain. Exactly which genes does IFN-γ modulate and how? How does the expression of these genes effect MC functions? Currently, studies of IFN-γ effects on MC have created more questions than answers. For example, IFN-γ induces the expression of MHC class I and class II, yet downregulates the presentation of soluble antigens in MHC class II (138,139). IFN-γ may mediate some of its effects by upregulating FcγRI and FcγRIII expression (140). The ligation of FcγRIIB receptor subsets initiates major inhibitory pathways in MC (see section I.D. above). However, this does not explain IFN-γ inhibition of MC degranulation in response to antigen. The exact mechanism of IFN-γ-mediated inhibition of MC mediator release is unknown. The central hypothesis of this thesis is that IFN-γ inhibits MC functions in part by modulating chloride channel function and/or expression.

III. Introduction to chloride channels

A. Chloride channels perform a variety of important cell functions

Chloride is the most abundant extracellular anion in nature (141). Plant, yeast, bacterial and mammalian cells express chloride channels to move chloride ions and power several important cellular functions and maintain homeostasis. Chloride ion is important in functions such as maintaining cell volume, resting membrane potential, and intracellular pH (141). Ion channels are classified according to their electrochemical properties (e.g. conductance, ion selectivity, voltage-dependence), their mode of regulation (e.g. by ligands, calcium, G-proteins) and the tissues in which they have been

found (142). Electrochemical properties are often confusing and mechanisms of activation often overlap, but there are at least three distinct classes of Cl^- channels classified according to their mechanism of activation; the volume and voltage-gated ClC, the ligand-gated γ-aminobutyric acid-type A receptor ($GABA_AR$) and the phosphorylation and nucleotide-gated cystic fibrosis transmembrane conductance regulator (CFTR). All three classes contain transmembrane regions that are composed of hydrophobic amino acids (Fig 1.7; (142). The transmembrane regions form a pore in the plasma membrane through which Cl^- ions pass. Although all three classes of chloride channels facilitate the flux of Cl^- ions, they share no homology and have diverse roles.

B. The γ-aminobutyric acid receptor ($GABA_AR$):

The γ-aminobutyric acid receptor ($GABA_AR$) is a highly specialized chloride channel that is assembled as a pentamer and is mainly expressed in the brain and CNS (143). The $GABA_AR$ was cloned a decade ago and belongs to the family of multi-subunit proteins that form ligand-gated ion channels (143). The receptor/channel is a heteroligomer composed of five subunits, usually a combination of α, β, γ, and δ (143). There have also been reports of a sixth subunit (ρ) but its expression is rare (144). However, all $GABA_AR$ studied in the brain and CNS contain at least one α (53 kDa) and one β (56 kDa) subunit (143). Each subunit is encoded by a separate gene and has four membrane-spanning domains, which contribute to the wall of the ion channel (Fig 1.8). Sequence analysis of the α, β and γ subunit mRNA has revealed amino acid variability and has created 16 subunit subtypes (6α, 4β, 3γ, 1δ and 2ρ (143)). The pharmacological characteristics of the $GABA_AR$ are determined by the composition of particular subunits (144) and variations in subunit composition confers alterations in GABA and benzodiazepine (BDZ) ligand binding (143). Precisely how these amino acid differences account for changes in ligand binding are unknown, but there is evidence that glycosylation sites might play a role. For example, small variations among α subtypes (α1-4) involve putative glycosylation sites (143) in a region that may contribute significantly to pharmacological subtype differences as well as variable cellular

regulatory differences (143). Many of the studies characterizing the contribution of different subunits to $GABA_AR$ function have come from injection of cDNA encoding these subunits into oocytes. For example, *Xenopus* oocytes injected with cDNA for α_1 and β_1 produced GABA-dependent inward currents and responded to GABA (EC_{50} = 2 μM) with a reversal potential of 27 mV (145). Therefore, studies of $GABA_AR$ must first define receptor composition.

Phosphorylation plays an important role in the regulation of ligand-gated channels. The $GABA_AR$ complex is phosphorylated by several protein kinases *in vitro* and the addition of Mg^{2+}-ATP to the internal pipette solution prevents the time-dependent decrease of GABA-activated currents suggesting that phosphorylation confers an activated (open) conformation to the channel (146). However, recent studies on rat neurons have shown that PKA and PKC negatively regulate the activity of $GABA_AR$, probably through phosphorylation of the receptor (147). PKC phosphorylation sites are located in the intracellular domains of the β and γ2 subunits, and increased PKC phosphorylation causes a reduction of GABA-activated currents in hippocampal neurons and those expressed in oocytes injected with mRNA (148). Also, increases in cAMP-dependent protein kinase activity decreases $GABA_AR$ mediated $^{36}Cl^-$ uptake by brain microsacs (149) showing that $GABA_AR$ are no longer conducting a Cl^- current when phosphorylated. These findings show that, unlike CFTR and ClC, $GABA_AR$ are inhibited by phosphorylation.

$GABA_AR$ is activated by γ-aminobutyric acid (GABA), the principal inhibitory neurotransmitter in the central nervous system (CNS). GABA binding to $GABA_AR$ allosterically opens the channel pore allowing chloride ions to flow through the channel and into the cell (150). The $GABA_AR$ also has several binding sites for other compounds that alters its chloride channel pore by allosteric mechanisms. These compounds include ethanol and BDZ, which likely bind all of the subunits with different affinities (150). MC express BDZ binding sites and BDZ has been shown to inhibit bone marrow-derived MC proliferation and mediator release (151-153).

GABA also binds $GABA_BR$. The $GABA_BR$ receptor has only recently been cloned and shows similarities to the metabotropic glutamate receptor (143). $GABA_BR$ is

not an ion channel like $GABA_AR$ but linked to G-proteins that produce several divergent effects (143).

C. The ClC chloride channels

A second class of Cl^- channels is the ClC whose first known member was cloned in 1990 from *Torpedo marmorata* electric organ (154). ClC is the largest gene family of Cl^- channels and, although members have been highly conserved during evolution, they have less than 30% homology amongst them (155). At least nine mammalian isoforms exist and there are homologues in a wide range of other species including bacteria, *Drosophila* (fly), *Saccharomyces* (yeast), *Neurospora* (mold), *Arabidopsis* (plant) *and Caenorhabditis* (worm) suggesting that ClC channels mediate cellular events that are essential for life (156-158).

The function and general structure of most ClC channels is incompletely understood due to lack of specific inhibitors and an inability to express some channels in oocytes. ClC protein monomers have predicted molecular masses ranging from 69 to 110 kDa and hydropathy analyses indicate that they possess 13 hydrophobic domains of which 12 may span the cell membrane (Fig 1.7). The COOH and NH_2 terminals of the protein are located in the cytoplasm (159).

Mutations in some of these ClC can cause severe diseases (160,161). Mutations causing loss of function of ClC-5 result in Dent's disease characterized by low molecular weight proteinuria, hypercalciuria, nephrolithiasis and renal failure (162). Mutations of a kidney-specific ClC (ClC-Kb) result in Bartter's Syndrome characterized by hypokalemic metabolic alkalosis and renal salt wasting (163). Generalized congenic myotonia (Becker's) and dominant myotonia (Thomsen) are muscle disorders transmitted as autosomal recessive traits and caused by allelic mutation of ClC-1 (164). Mutation in ClC-7 can result in infantile malignant osteoporosis (165).

ClC-1 is specifically expressed in skeletal muscle and accounts for approximately 75 % of the ionic conductance of the resting sarcoplasmic membrane (166). In contrast, ClC-2 is widely expressed with strong expression in brain neurons and lung epithelia

(167). ClC-2 can be activated by cell swelling and acidic extracellular pH and is involved in limiting intracellular Cl^- concentration (168). ClC-3 is Ca^{2+}-sensitive, volume regulated Cl^- channel that maintains a variety of cellular functions during osmotic perturbation (169). ClC-3 channels are opened by changes in cell volume and are expressed in epithelial cells, hepatocytes and neuronal cells (170-172). ClC-3, ClC-4 and ClC-5 belong to the same branch in the ClC family and are highly homologous (173). ClC-4 is expressed in human skeletal and vascular smooth muscle and is activated by external pH changes, inhibited by DIDS and has consensus sequences for protein kinase A (PKA) phosphorylation (174,175). ClC-5 is mainly expressed in endocytic vesicles of proximal tubule cells of the kidney and mutation results in Dent's disease (161,162,176). ClC-6-mediated currents are inhibited by extracellular cAMP or the Cl^- channel inhibitor 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) (177) and there is some evidence that ClC-6 is expressed in vascular smooth muscle (174). ClC-7 resides in late endosomal and lysosomal compartments (178) and is important in bone resorption by osteoclasts (165). The ClC-K channels are kidney-specific and a recent ClC-K1 knock-out mouse model has implicated malfunctions of this channel in diabetes insipidus (159).

Patch clamp studies have shown that MC exhibit Cl^- channel activity (36,92,179). These Cl^- channels are activated by antigen in sensitized MC, and blocked by 4,4'-diisothiocyano-2,2'-stilbenedisulphonate (DIDS) and NPPB, Cl^- channel inhibitors (180,181). Inhibition of the Cl^- current by extracellular DIDS is voltage and time dependent and also inhibits antigen and 48/80-stimulated β-hexosaminidase release (182). In a human mast cell line (HMC-1), patch-clamp studies have identified the presence of Ca^{2+}–activated Cl channels and RT-PCR has confirmed that HMC-1 express mRNA for ClC3 and ClC5 (183). However, the identity of specific ClC members in rat MC is unknown.

D. The cystic fibrosis transmembrane conductance regulator (CFTR)

1. CFTR structure and activation

A third class of Cl^- channels currently has one member, the cystic fibrosis transmembrane conductance regulator (CFTR), cloned in 1989 (184). CFTR is a cAMP-activated Cl^- channel that belongs to the gene family of ATP-binding cassette (ABC) transporters which generally function as active transporters driven by ATP hydrolysis. Yet, CFTR is unique among the ABC transporters since it is the only member that can conduct Cl^- currents and inhibit EnaC activity (185). CFTR has 12 transmembrane spans arranged in two domains joined by a large, negatively charged regulatory (R) region, attached to two nucleotide binding folds (NBF) (Fig 1.7). The R domain contains consensus sequences for phosphorylation by protein kinase A (PKA) and by protein kinase C (PKC) (186). PKC phosphorylation of CFTR is diacylglycerol-dependent but Ca^{2+}-independent since PKC inhibitors or antisense oligonucleotides to PKC-ε block cAMP-activated CFTR Cl^- current (187). In intestinal epithelium, studies have shown that membrane targeting of cGMP-dependent protein kinase (cGK) type II induces CFTR Cl^- channel activity (186). Therefore, CFTR activity is controlled by phosphorylation events but, unlike $GABA_AR$, phosphorylation opens the CFTR Cl^- channel. CFTR activity is also regulated by ATP hydrolysis at the NBF. Evidence suggests that hydrolysis of one ATP molecule at NBF1 opens the channel and hydrolysis of one ATP molecule at NBF2 closes the channel (186).

2. CFTR is a chloride channel and conductance regulator

In normal secretory cells such as lung epithelial cells, CFTR plays a predominant role in both cAMP and Ca^{2+}-activated secretion of electrolytes (Fig 1.9). Cl^- ions are taken up from the blood (basolateral side) by the Na^+-K^+-$2Cl^-$ cotransporter which also takes up K^+. Cl^- leaves the cell via apically expressed CFTR, Na^+ is secreted via the paracellular shunt and K^+ is secreted via K^+ channels. Increases in intracellular cAMP or prostaglandin E_2 (PGE_2) activates CFTR-mediated Cl^- transport (Fig 1.9). In absorptive epithelial cells, in the sweat glands for example, Na^+ is absorbed by the luminal Na^+ channels (ENaC) and

Cl^- is absorbed via the basolateral shunt and probably via CFTR Cl^- channels. Na^+ is pumped out of the cell by the basolateral Na^+-K^+-ATPase, whereas Cl^- and K^+ leave the cell via Cl^- channels, respectively. In absorptive epithelial cells that coexpress CFTR and ENaC, stimulation of CFTR leads to inhibition of ENaC (185).

The structure/function of most ion channels is characterized by using pharmacologic agents to block the channel pore. Unfortunately, there are no known peptide blockers of CFTR and no known organic blockers that exhibit a high affinity and exclusive selectivity for CFTR. However, some Cl^- channels can be blocked by three classes of compounds; the disulfonic stilbenes, the arylaminobenzoates and sulfonylurea compounds. The disulfonic stilbenes, such as DIDS, block a wide variety of Cl^- channels (188) but DIDS does not block CFTR-mediated whole cell Cl^- currents (189). The arylaminobenzoates such as diphenylamine-2-carboxylate (DPC), flufenamic acid (FFA), and NPPB, are the most heavily studied blockers of the CFTR Cl^- channel. Sulfonylureas such as glibenclamide and tolbutamide reduce the open probability of CFTR in a structure-dependent and concentration-dependent manner by direct interaction with the channel as recorded in cell-free membrane patches (188). In each case, glibenclamide inhibition was reversible and had an inhibitory concentration (IC_{50}) of approximately 2 to 30 μM (188). Both DPC and glibenclamide block CFTR by accessing intracellular binding sites and inhibit single channels at hyperpolarizing membrane potentials with simple kinetics (188).

In addition to its role as a Cl^- channel, CFTR acts as a "conductance regulator," coordinating an ensemble of transmembrane ion fluxes in polarized epithelia. Like at least two other members of the ABC transporter family, CFTR modulates ion channel activity of ENaC, an outwardly rectifying Cl^- channel, Ca^{2+}-activated and volume-activated Cl^- channels, the ROMK K^+ channel; anion exchangers and the aquaporin 3 water channel (190,191). In fact, CFTR expression (independent of Cl^- channel activity) is sufficient to modify other ion channels. For example, volume-activated chloride current ($I_{Clswell}$) via volume regulator anion channels (VRAC) is significantly (66.5 $\pm$ 8.8 %) reduced in cells transfected with wild-type CFTR and is independent of activation of CFTR (192). Activation of the Cl^-/HCO_3^- anion exchanger requires CFTR expression

but not Cl^- channel activity (193). CFTR's conductance regulation may be mediated via protein-protein interactions via the NBF2 since mutations in the NBF2 (see Fig 1.7) alters regulation of exchanger activity independent of their effect on Cl^- channel activity (193). NBF1, however, may also be involved in conductance regulation since cAMP-dependent activation of CFTR inhibits ENaC activity via NBF1 (185). Other mechanisms of CFTR-mediated conductance regulation may involve ATP release and autocrine activation of signalling pathways that modulate permeation pathways (194). The COOH terminus of CFTR interacts with adenosine 5'-monophosphate-activated protein kinase (AMPK) and inhibits CFTR whole-cell Cl^- conductances (195).

Recent evidence shows that CFTR also interacts with numerous intracellular signalling and scaffolding proteins. The COOH terminus of CFTR contains a PDZ domain-binding motif that is capable of binding the Na^+/H^+ exchange regulatory factor (NHERF or EBP50), which also contains two PDZ domains (196). It has been speculated that NHERF functions as a scaffold in a macromolecular complex that facilitates the trafficking and localization of CFTR (196). These observations have re-defined CFTR as a regulatory molecule, modulating the activity of a multi-protein complex colocalized at the plasma membrane.

3. CFTR expression in non-epithelial cells

While CFTR expression was generally considered to be epithelial cell specific, evidence for CFTR expression and/or function has also been observed in other cell types, including lymphocytes, Sertoli cells, heart muscle cells, tracheal submucosal gland cells and hypothalmic neurons (197-201). CFTR-dependent Cl^- transport is responsible for modulation of membrane potential in several cell types, lymphocyte activation, $CD8^+$ T cell-mediated cytotoxicity and volume regulation. However, the physiological relevance of CFTR expression in non-epithelial cells is unknown (198,202,203).

4. Regulators of CFTR expression

There are several substances that modulate CFTR expression and function. Interestingly some substances increase CFTR expression while inhibiting CFTR-

dependent Cl^- flux. Sodium 4-phenylbutyrate (PBA), a short-chain fatty acid that functions as an ammonia scavenger enhances CFTR protein expression 6 to 10 fold and increases the intensity of CFTR staining in the apical membrane but reduces CFTR Cl^- current across the apical membrane of epithelial cells (204). Paradoxically, butyrate restores cAMP-activated Cl^- current in ΔF508 CF epithelial cells by increasing the expression of ΔF508 CFTR in the plasma membrane (205).

5. CFTR mutations and disease

Mutations in CFTR result in the common genetic disease cystic fibrosis (CF) characterized by defective fluid transport and excessive mucus production in a variety of epithelial cells. In normal airways, ion transport is switched from net absorption under resting conditions to a net secretion when exposed to secretogogues (Fig 1.10A). However, CF airways are unable to enhance their secretory transport upon stimulation (Fig 1.10B). The CF abnormality is due in part to the impaired secretion of Cl^- located preferentially in the submucosal glands and the enhanced absorption of Na^+ and consecutive hyperabsorption of electrolytes, which takes place in the surface epithelium (Fig 1.10). The reduced airway surface liquid layer results in improper clearing of mucus from the submucosal glands and impaired mucociliary clearance in CF airways. Impaired secretion in pancreatic cells is dependent upon CFTR-mediated abnormalities in gap junction coupling (206).

Although lung disease is the major cause of mortality, gastrointestinal disease is the first hallmark of CF in affected newborns and remains a major cause of morbidity throughout life (207). Intestinal disease in CF is primarily targeted to the small intestine and is characterized by defective alkalinization of secretions in the proximal small intestine, luminal obstruction by thick mucoid secretions, and malabsorption (208). The hyperactivation of CFTR Cl^- channels in intestinal epithelium by several enterotoxins causes a massive secretory diarrhea that is responsible for a large part of the infant death in developing countries.

The most common abnormality in CF epithelial cells arises from an inability to synthesize and translocate CFTR to the surface of the cell (209). CFTR proteins are

synthesized and properly folded in the endoplasmic reticulum (ER). CFTR folding and maturation in the ER is an inefficient, temperature-sensitive process, and between 70-80% of wild-type CFTR is degraded via ER-associated protein degradation (ERAD) pathway (210). The ERAD involves the recognition, dislocation and proteolysis of misfolded polypeptides by the 26S proteasome, a multicatalytic enzyme complex localized to the cytoplasm and nucleus. However, inhibition of proteasomes has failed to promote the processing of core-glycosylated wild-type as well as ΔF508 mutated CFTR (210). Only about 20-30% of newly synthesized CFTR enters the secretory pathway after its ATP-dependent conformational maturation assisted by cytosolic and ER-resident chaperones such as heat shock proteins 70 and 90 (Hsp 70 and Hsp 90) and the ER lumenal chaperone calnexin (211-214). These chaperones are thought to facilitate CFTR maturation because they transiently associate with CFTR in the ER and their dissociation coincides with CFTR maturation. Processing of the high mannose N-linked glycan to complex oligosaccharides in the cis/medial Golgi region decreases the electrophoretic mobility of CFTR (approximately 180 kDa), providing a convenient method to monitor trafficking of CFTR (210). Once expressed on the cell membrane, CFTR has a relatively slow turnover *in vivo* of more than 12 hr (215). Defective CFTR-mediated Cl^- conductance of CF epithelia is principally caused by abnormal CFTR trafficking. The most common ΔF508 point mutation impairs biosynthetic processing of CFTR by disrupting posttranslational folding at the ER (209).

Abnormalities associated with CF are thought to occur because of inadequate CFTR expression in epithelial cells. However, some findings suggest that defective CFTR expression in immune cells may also contribute to the pathophysiology of CF. Impaired CFTR function in T cells, for example, results in abnormal IL-10 production (216). Neutrophils isolated from CF patients show deficient PKC-mediated mediator release and chemotaxis (217).

6. Animal models of CF

A number of mouse models of CF have been developed by targeted disruption of the CFTR gene. This approach has had mixed success in providing a valid model of human

disease because most CFTR (-/-) mice develop lethal intestinal obstruction, uncommon in human CF. It is not surprising that CF mice do not have the same disease as humans since there are few similarities in anatomy and physiology between mouse and human that are pertinent to lung and pancreatic function. For example, CFTR is not expressed at high levels in the mouse pancreas, but in humans, the most abundant CFTR expression is in the pancreas (218). Further, the mouse pancreatic ducts express high levels of a calcium-activated chloride channel that might compensate for the presence of a mutant CFTR channel (219). Lastly, there are significant differences in villus distribution for CFTR in the mouse proximal small intestine compared to those reported for human (220).

The rat model of CFTR dysfunction may be more relevant to human disease since the cellular distribution of CFTR in the rat intestine resembles that of human (221). The recent generation of cloned sheep has focused attention on an ovine model of CF. Ovine CFTR cDNA shows a high degree of conservation at the DNA coding and predicted polypeptide levels with human CFTR, much greater than the similarity between mouse and human CFTR (222). The development of CFTR expression in sheep is equivalent to that observed in humans and the sheep and human lungs show anantomical and electrophysiological similarities (222). Therefore, CF studies may eventually move to other, more physiologically relevant species such as the rat or sheep.

E. Chloride channels in mast cells

Electrophysiological measurements show that MC in the resting state express currents indicative of inwardly rectifying and outwardly rectifying K^+ channels, several types of Ca^{2+} channels, GTP-activated Na^+ channels and Cl^- channels. (36,179,223). The best described currents in the MC are K^+ currents recognized in resting cells, during differentiation and during response to stimuli. MMC grown from bone-marrow, express two K^+ conductances that are absent in progenitor cells, and an outwardly rectifying K^+ conductance initiated by ligation of the P2 receptor (224). Ligation of the P2 receptor on RBL cells also activates an outwardly rectifying K^+ channel (225). The P2 receptor is coupled to G proteins and can stimulate mediator (225,226) release, suggesting that K^+

conductances may be involved in mediator release. FcεRI crosslinking increases the open probability of an inwardly rectifying K^+ channel that affects Ca^{2+} uptake in RBL cells and may potentiate mediator release (227).

Although the expression of Na^+ channels in non-excitable cells is rare, MC express a voltage and GTP -dependent Na^+ current (228). The role of this Na^+ current is unknown, although studies of other cell types suggest that increases in intracellular Na^+ can regulate cell volume (229), modulate excitability through changes in activities of the Na^+/Ca^{2+} exchanger, and ATPases (230) and regulate Na^+-activated K^+channels (231). Studies using $^{86}Rb^+$, an analogue of Na^+, show that FcεRI crosslinking increases $^{86}Rb^+$ uptake by a Ca^{2+}-independent mechanism, possibly via the Na^+/K^+ dependent ATPase (232). The absence of extracellular Na^+ ions increases MC sensitivity to Ca^{2+} such that addition of extracellular Ca^{2+} can act as a stimulus (233). These data suggest that Na^+ uptake may be important for FcεRI-mediated signalling and possibly mediator release.

There is evidence to suggest that Cl^- may also be important in MC functions. For example, resting MC display a Cl^- current. Whole cell patch clamp has revealed small reversible, outwardly rectifying currents suggesting a Cl^- current (234). In addition, resting PMC uptake ^{36}Cl ion and presumably reach an equilibrium distribution of Cl^- ions (36). Stimulation of PMC with anti-IgE induces a significant and rapid uptake of extracellular $^{36}Cl^-$ compared to unstimulated cells and in the absence of extracellular $^{36}Cl^-$, anti-IgE-induced histamine release is decreased by at least 30% (235). In contrast, A23187 (0.1 μM), substance P (20 μM) or 48/80 (0.1 μg/mL) induced histamine release is unaffected by the removal of extracellular Cl^-, even though the release of histamine is comparable to that of anti-IgE (235). A23187, substance P and 48/80 also had no effect on $^{36}Cl^-$ uptake suggesting that Cl^- uptake is associated with FcεRI aggregation and not with these secretogogues. 48/80 and substance P are thought to trigger MC secretion by activating G proteins, while A23187 initiates MC secretion by directly elevating intracellular Ca^{2+} (42,236) (see section I.A above).

Friis et al. demonstrated that antigen-mediated histamine release from rat PMC is inhibited when extracellular chloride is replaced with either isethionate or gluconate anions (36). This study also showed that resting MC absorb ^{36}Cl at a constant rate,

reaching a steady state after 1 hr. Following antigen stimulation, the rate of ^{36}Cl uptake increases 25 fold (36). Uptake of chloride ions following anti-IgE stimulation has been correlated to histamine secretion, suggesting that chloride ion influx is involved in histamine secretion (235).

The role of Cl^- flux is most likely a complex process, possibly involving several other ion channels. Release of histamine from antigen-activated PMC is blocked by the putative Cl^- channel blockers, DIDS and NPPB (36,235,237). NPPB also blocks increases in ^{36}Cl following IgE-mediated stimulation (235). However, DIDS does not have any effect on ^{36}Cl uptake following stimulation suggesting that DIDS-sensitive chloride channels are not involved in MC mediator release (36). Preincubation of Cl^- channel inhibitors with polycationic secretogogues has a profound effect on their potency. DIDS and a related compound, SITS, inhibit histamine release from 48/80 stimulated PMC only when 48/80 and DIDS are added separately to PMC (238) (see discussion of DIDS specificity in Chap 8). Mixing of 48/80 with DIDS prior to addition to PMC showed very little inhibition of histamine release suggesting that the polycationic 48/80 and the inhibitor molecules may interact electrochemically and less free 48/80 may be available for MC stimulation. PMC preincubated with DIDS for 10 min and stimulated with anti-IgE potently inhibited histamine release (238). PMC preincubated with SCG (another inhibitor of Cl^- current) for 10 min and stimulated with either 48/80 or anti-IgE also showed decreased histamine release (238).

Cotransporters of chloride or other ion channels may also play a role in chloride movement in MC. However, ouabain which inhibits the Na^+/K^+ ATPase, has no effect on antigen-stimulated chloride uptake in PMC (36). Furosemide, an inhibitor of $Na^+/K^+/2Cl^-$ significantly reduces anti-IgE stimulated chloride uptake (235).

F. Nitric oxide regulates ion channel function

Recent studies have shown that MC produce NO in response to stimuli such as IFN-γ (132) (Fig 1.11). Production of NO could have profound effects on MC ion channels since NO is an important messenger that allows channels to communicate with remote

sensors (physically separate molecules such as receptors) via cAMP, cGMP and G proteins. In fact, neuronal NOS type-1 coclusters with several ion channels such as *Shaker*-type K^+ channels and N-methyl-D-aspartic acid (NMDA)-receptors which may reflect site-specific NO signalling pathways (239,240). The study of NO modulation of ion channels in MC is very immature yet MC express several ions channels all of which may be regulated by NO. For example, exogenous NO can activate CFTR Cl^- currents and MC express CFTR (182). Patch clamp recordings show that in epithelial cells and normal T cell clones, NO can activate a cGMP-dependent CFTR-mediated Cl^- current which is absent in cells derived from CF patients (198). Loss of CFTR activity by over-expressing its regulatory domain, reduces NOS mRNA expression and decreases overall NO production in epithelial cells (241).

In endothelial cells, NO production is triggered by an influx of Ca^{2+}, but is independent of intracellular Ca^{2+} concentration (242). Release of Ca^{2+} from intracellular stores by histamine in the presence of an inhibitor of receptor-activated Ca^{2+} influx does not induce the production of NO (242). Thus, NO production appears to require influx of Ca^{2+} through Ca^{2+} channels and may be dependent upon close proximity between Ca^{2+} channels and NOS. It has been shown that eNOS resides in calveoli, which also contain calmodulin and various ion channels including Ca^{2+} and K^+ channels (239) (Fig 1.11). When activated by an agonist, Ca^{2+} channels can open, facilitating the entry of extracellular Ca^{2+} and increasing the local concentration of Ca^{2+} on the internal side of the plasma membrane. Ca^{2+} ions bind calmodulin and induce the formation of the NOS-Ca-calmodulin complex, thereby dissociating the enzyme from the plasma membrane and activating its catalytic activity.

NO modulates smooth muscle contraction via cGMP-dependent activation of K^+ channels (243). In some instances, such as hypercholesterolemia, the reduced contribution of NO in resting vasomotor tone is accompanied by an increase contribution of NO-dependent Ca^{2+}-dependent K^+ (K_{Ca}) channels (244). This data suggests that there exists a tightly controlled balance between NO-mediated and NO-independent K_{Ca} channel contributions to resting vasomotor tone and that the NO-independent group of K^+ channels is activated whenever NO regulation is absent and vice versa.

V. Conceptual Model and Central Hypothesis

Since MC mediators cause deleterious effects on surrounding tissue in diseases such as asthma and anaphylactic shock, understanding the mechanisms of MC mediator release and MC function has become increasingly important. The observation that cytokines can alter MC phenotype and inhibit many of their functions has provided a useful tool for dissecting these activation signals. IFN-γ is a potent downregulator of several MC functions including proliferation, adhesion and mediator release but the mechanism of its effect is unknown. As reviewed above, IFN-γ signals through the JAK/STAT1 pathway and regulates the expression of genes in MC such as MHC class II and FcγRI (245). Aggregation of FcγR after IFN-γ treatment induces mediator release and IFN-γ-mediated MHC class II upregulation turns MC into potent antigen-presenting cells when activated via FcεRI (139,246). However, these observations do not explain the nature of IFN-γ-mediated changes in MC phenotype resulting in inhibition of activation.

One important facet of MC activation (both in mediator release and chemotaxis) may be Cl^- flux, which is required for maximal MC mediator release induced by at least some secretogogues. Chloride currents and one particular chloride channel, CFTR are regulated by IFN-γ in epithelial cells, suggesting that a similar effect may occur in MC (Fig 1.12). **Based on these observations and the data reviewed above, we hypothesize that IFN-γ has an effect on Cl^- flux and/or chloride channel expression in MC.** Specifically, IFN-γ binding to its receptor regulates transcription of several genes, some of which may be chloride channels, resulting in changes in MC Cl^- flux (Fig 1.10). Although direct effects of IFN-γ on ion channel function appear to be rare (e.g. Na^+-K^+-$2Cl^-$ cotransporter data, (247)), IFN-γ may also modulate chloride channel function via STAT-independent pathways. These chloride channels may involve ClC, $GABA_AR$ or CFTR any of which may modulate Cl^- flux during MC activation. Therefore, this project has three aims as mentioned in section I; (1) to determine the influence of IFN-γ on Cl^- flux in MC; (2) to characterize the expression of three major chloride channel types (ClC,

GABA$_A$R and CFTR); and (3) assess the effect of IFN-γ on expression of these chloride channels.

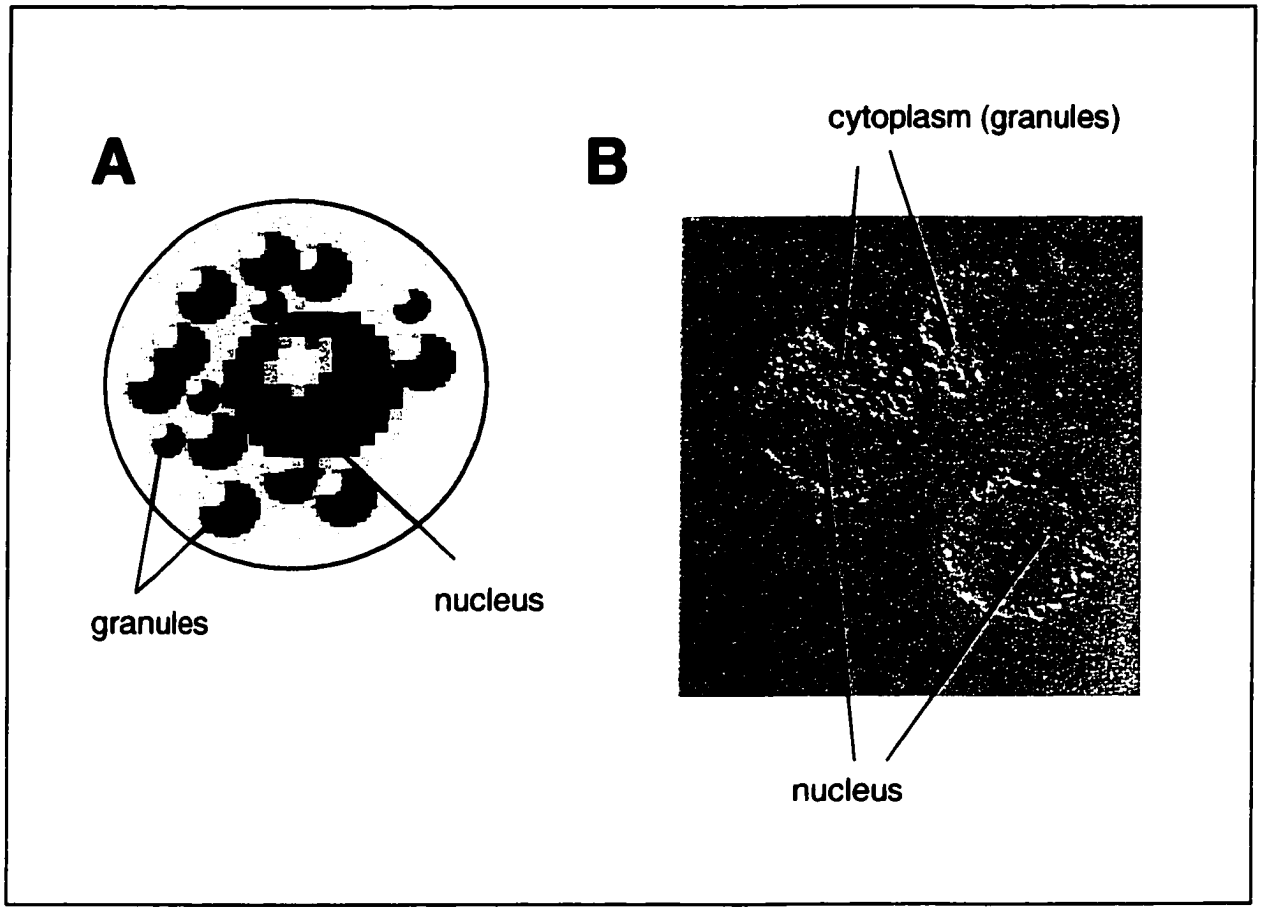


Figure 1.1. Morphology of a mast cell. (A) Schematic diagram of a mast cell showing granules, nucleus and ruffled membrane. (B) Phase contrast photograph showing three rat cultured mast cells with their large nucleus and granular cytoplasm (625 X).

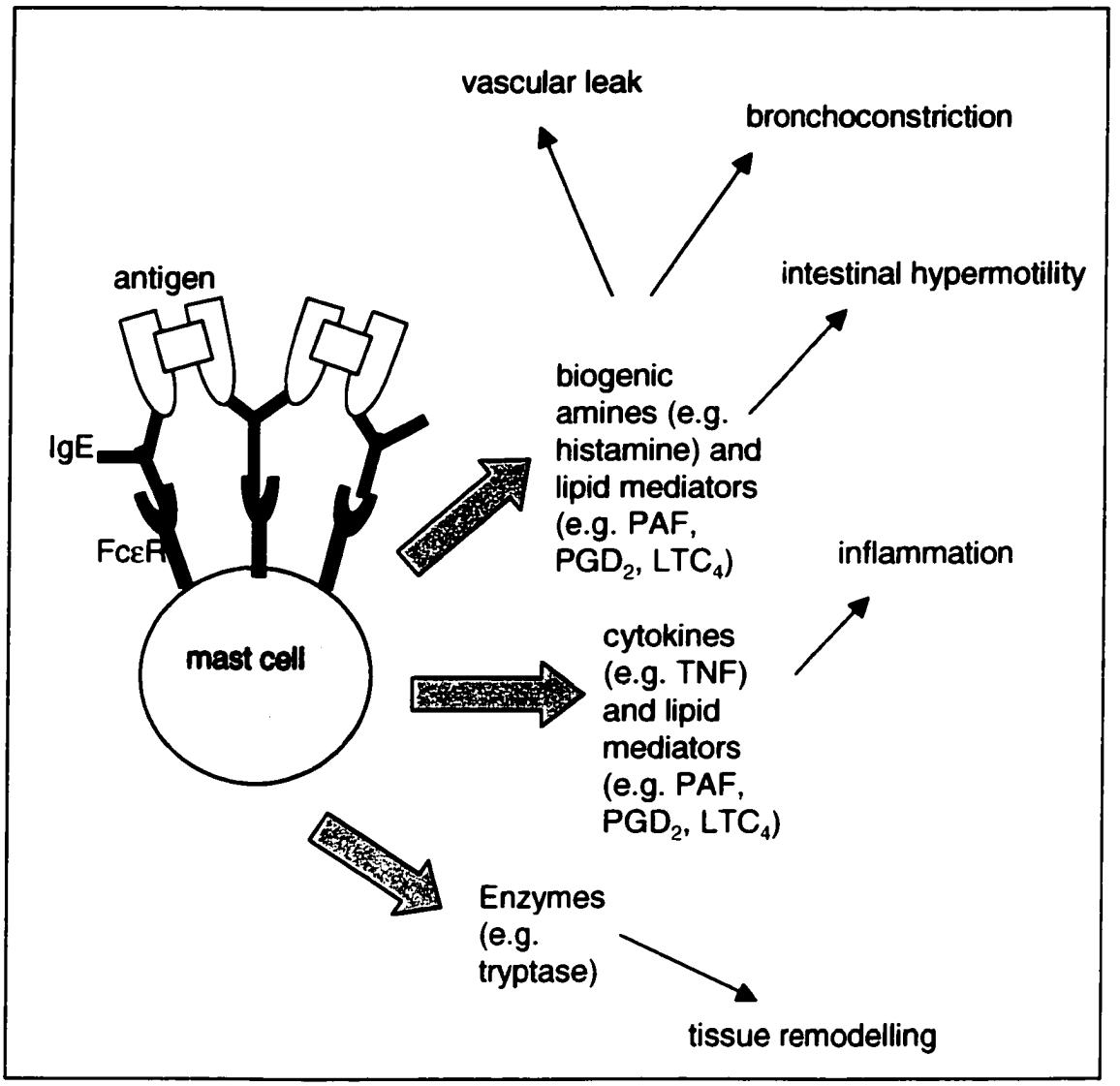


Figure 1.2. The role of mast cells in immediate hypersensitivity disease. Mast cells store biogenic amines such as histamine and enzymes in their granules that are released by stimuli. Mast cells also release cytokines and lipid mediators which are largely synthesized upon cell activation. The biogenic amines and lipid mediators both contribute to inflammation, which is part of the late phase reaction. Enzymes probably contribute to tissue remodeling. Activated mast cells also release cationic proteins such as enzymes that are toxic to parasites and host cells.

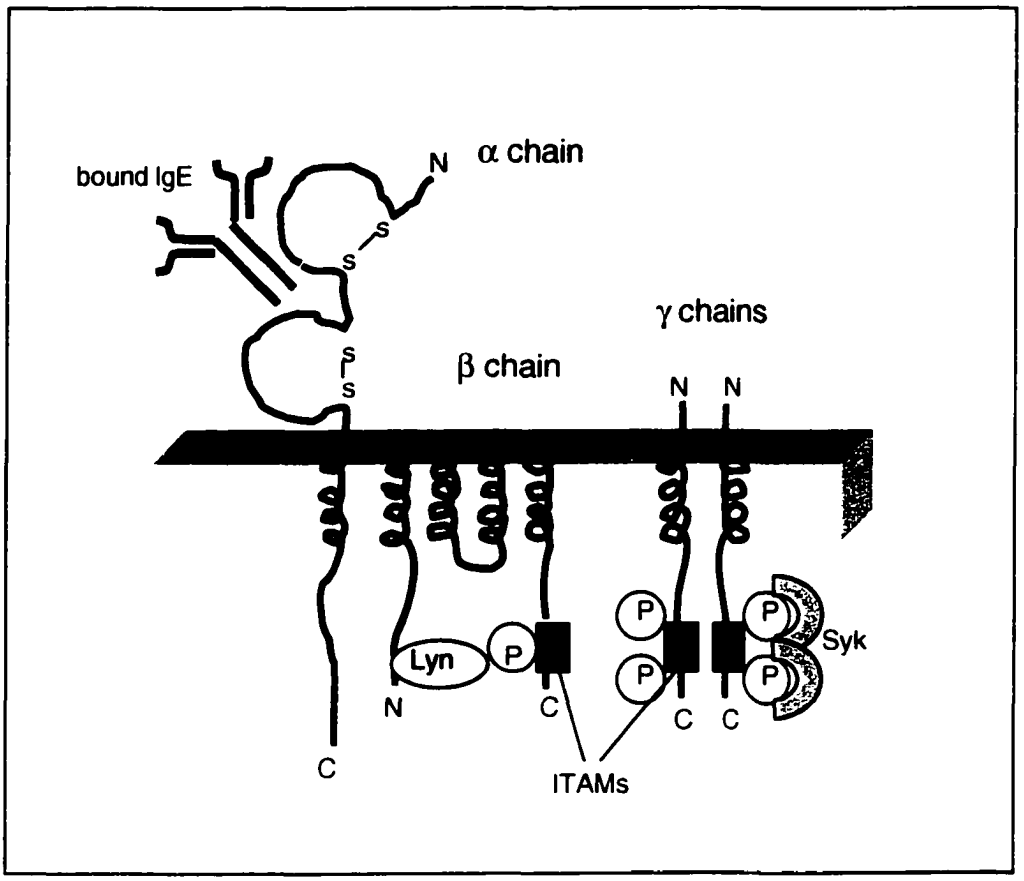


Figure 1.3. Model of the FcεR. IgE binds to the Ig-like domains of the α chain. The β and γ chains mediate signal transduction via their immunoreceptor tyrosine activation motifs (ITAM), similar to those found in the T cell receptor complex, which recruit Lyn and Syk kinases.

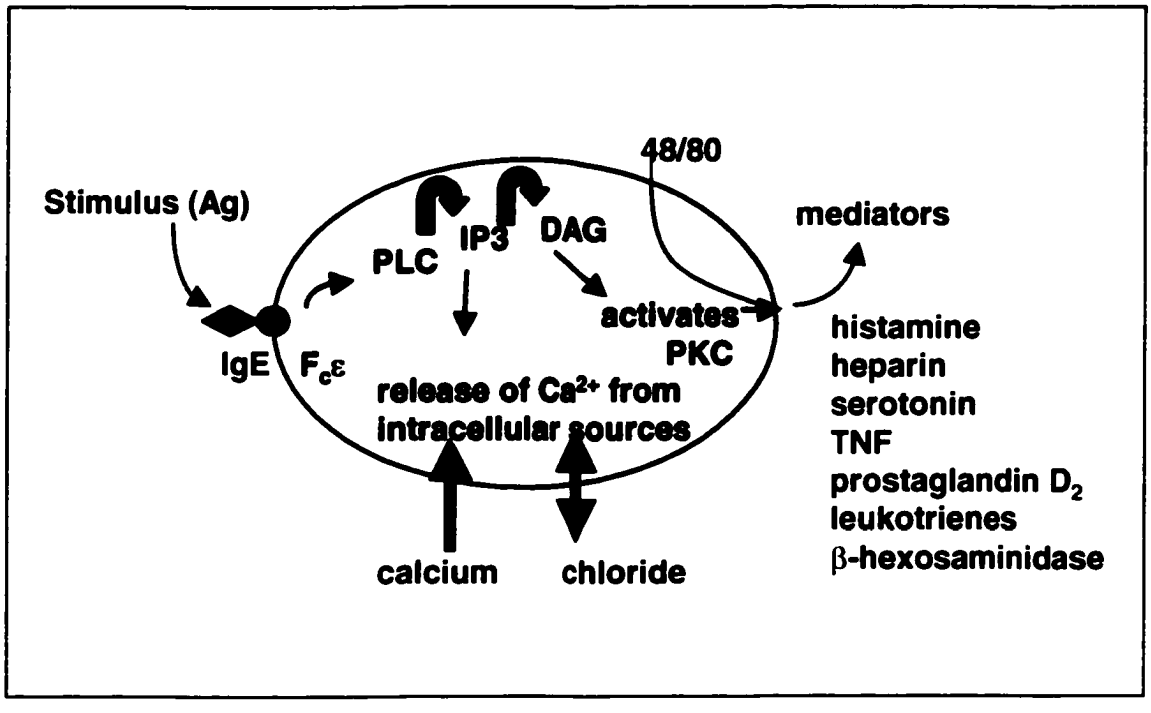


Figure 1.4. Model of IgE/FcεR-mediated or 48/80-mediated activation of mast cells.

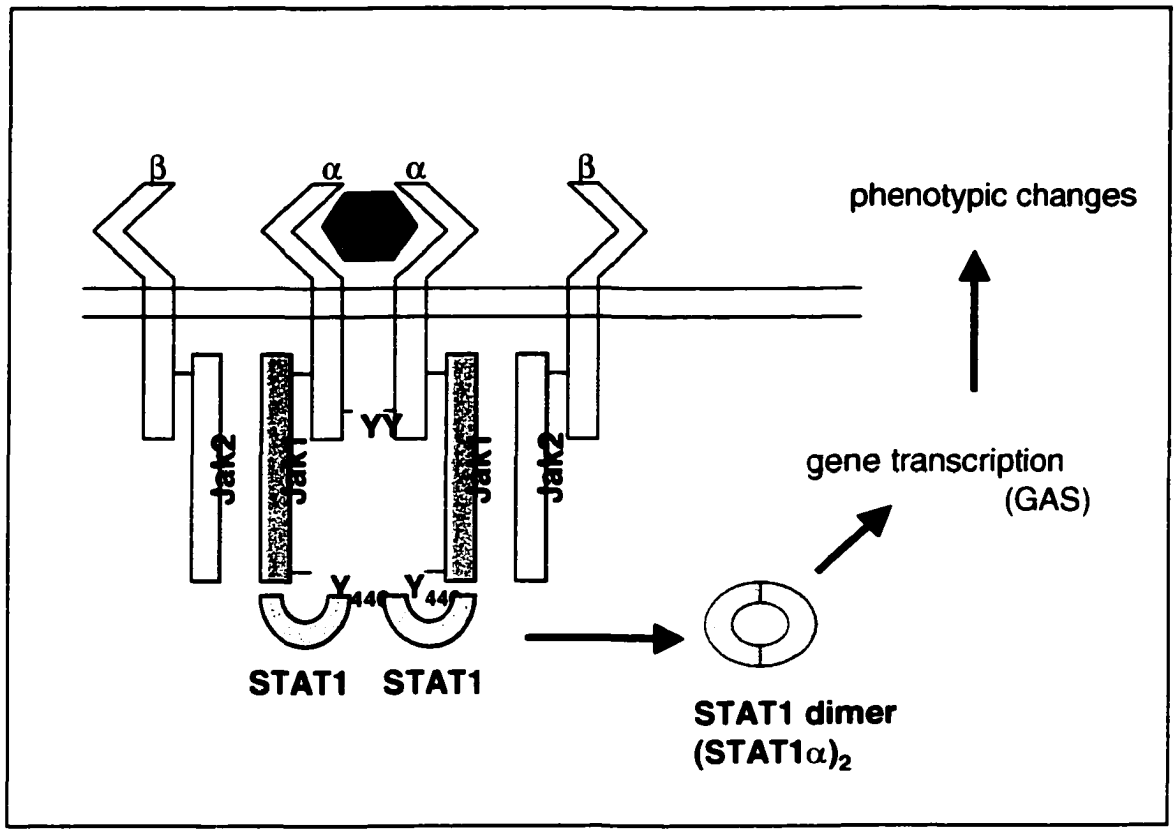


Figure 1.5. IFN-γ induced signal transduction via the IFN-γR. Homodimeric IFN-γ binds two IFN-γR α chains carrying the JAK1, leading to receptor α chain dimerization, association with the β chains carrying JAK2. JAK1 and JAK2 transphosphorylate one another, and the β chains of the IFN-γR, creating a binding site for two cytosolic STAT1α molecules. STAT1α bind to the β chain and are phosphorylated by the JAKs which allows them to dimerize and translocate to the nucleus and initiate transcription of genes possessing the IFN-γ activated site (GAS) in their promoter.

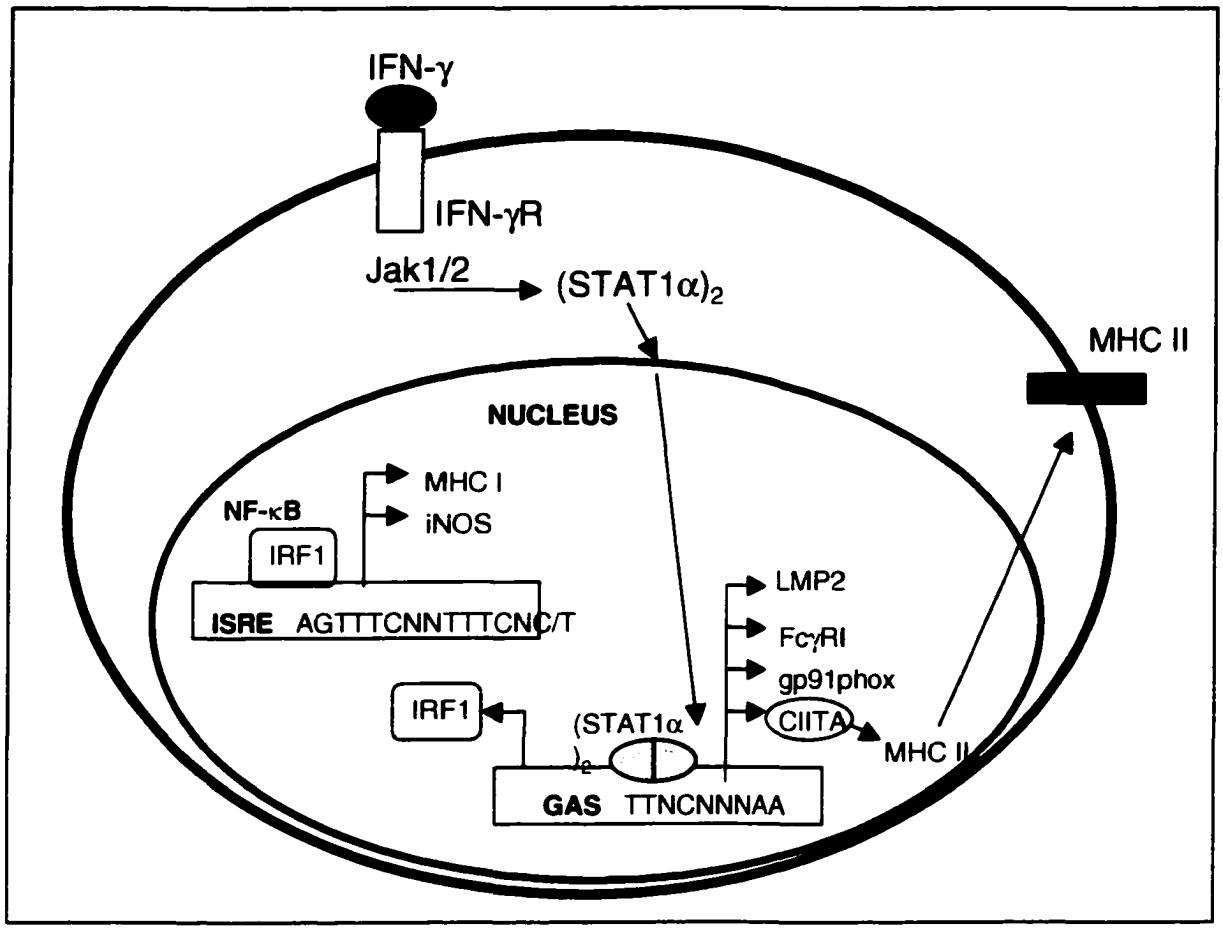


Figure 1.6. IFN-γ mediated changes in gene transcription and expression. IFN-γ binding the IFN-γR leads to α chain dimerization, association with β chains, transphosphorylation by the JAKs and ultimately phosphorylation of preformed cytosolic STAT1α subunits which dimerize ($(STAT1\alpha)_2$) and translocate to the nucleus. In the nucleus, $(STAT1\alpha)_2$ binds to IFN-γ activated site (GAS) sequences and initiates transcription of genes such as LMP2, FcγRI, gp91 phox, CIITA and interferon response factor (IRF). CIITA is a transcription cofactor and initiates transcription of major histocompatibility complex type II (MHC II) genes. IRF is also a transcription factor and interacts with NF-κB to bind the interferon-stimulated response elements (ISRE) and initiate transcription of genes such as MHC I and inducible nitric oxide synthase (iNOS).

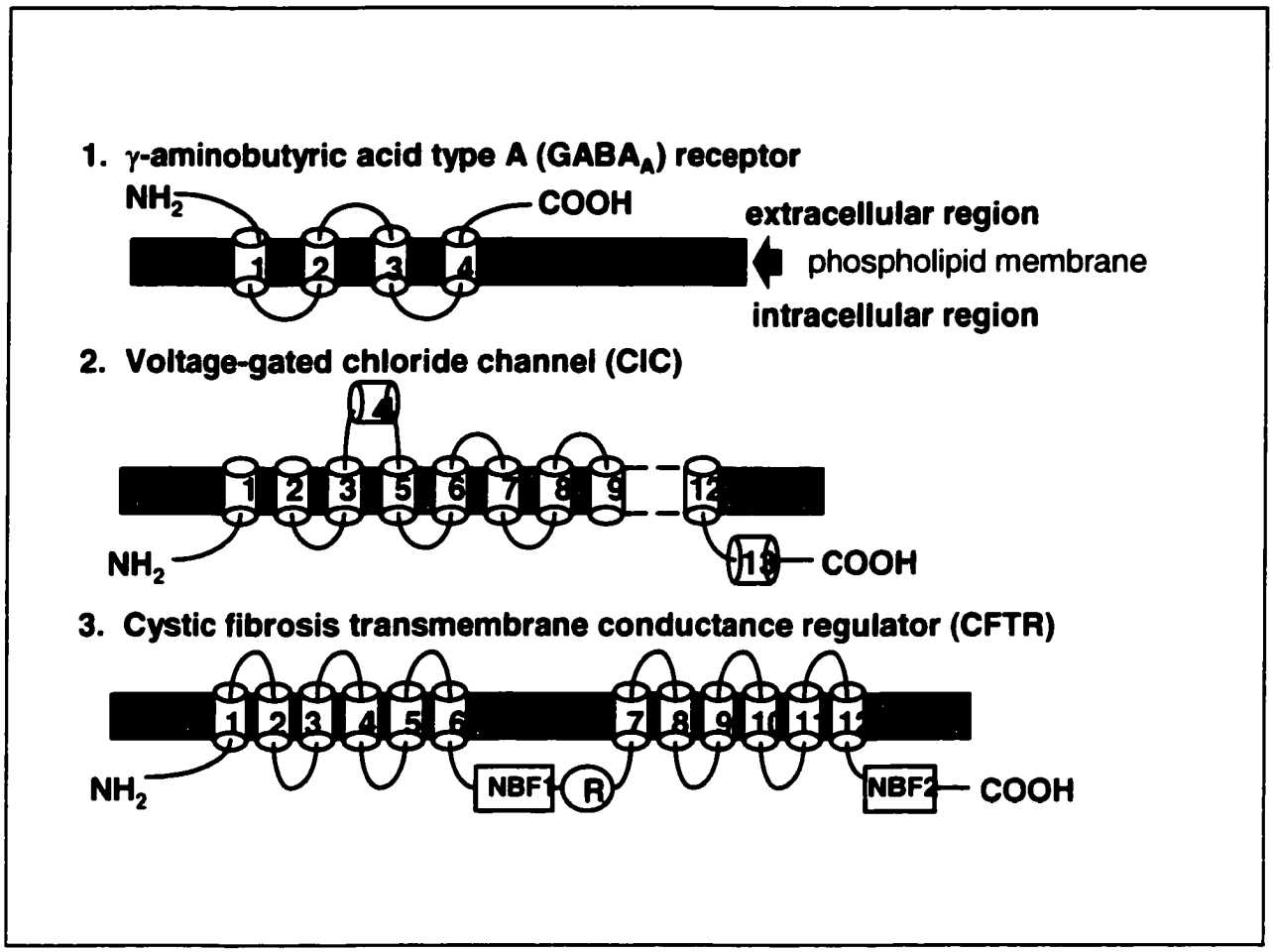


Figure 1.7. Model of the of the three major classes of mammalian chloride channels: the voltage gated ClC, the ligand gated $GABA_AR$ and the cAMP-gated CFTR. Each class of chloride channels may be composed of several subunits, all of which have several transmembrane regions that form the pore of the channel. $GABA_AR$ is commonly composed of up to five subunits. ClC structures are largely unknown, although they are postulated to have at least 13 hydrophobic domains, some of which may form transmembrane regions. The CFTR is the most complex, possessing a negatively charged regulatory domain (R) and two nucleotide binding folds (NBF).

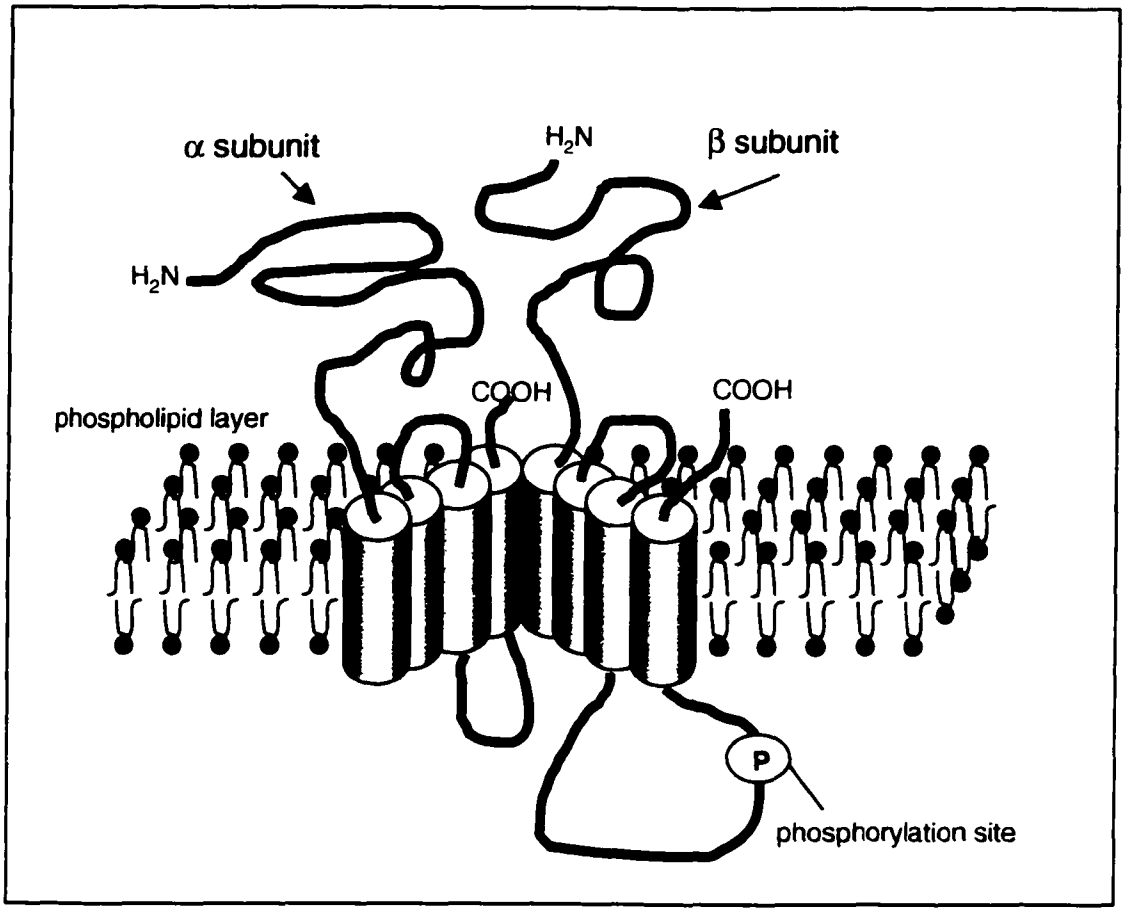


Figure 1.8. Model of the $GABA_AR$ chloride channel protein complex. This ligand-gated channel is a hetero-oligomer composed of up to five subunits. Each subunit has four membrane-spanning domains that contribute to the wall of the channel. Receptors in the brain and CNS contain at least one α and one β subunit, shown here.

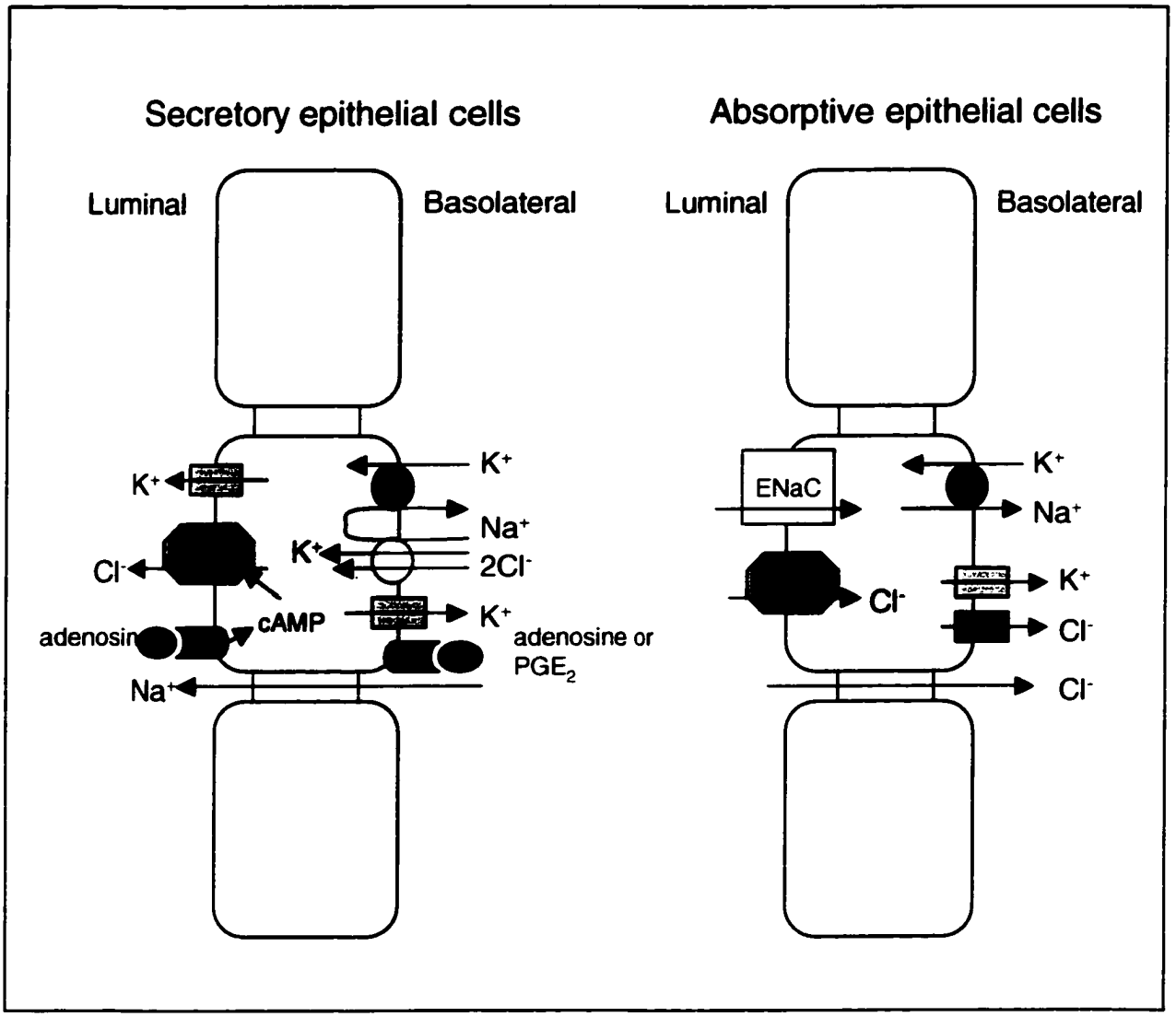


Figure 1.9. Models of electrolyte secretion and absorption in the airways and intestinal epithelium. In secretory cells, Cl^- is taken up from the basolateral side by the Na^+-K^+-2 Cl^- cotransporter. K^+ recycles via basolateral K^+ channels, and Na^+ is pumped out of the cell by the Na^+-K^+-ATPase (dark green). Cl^- leaves the cell via luminal (apical) cystic fibrosis transmembrane conductance regulator (CFTR; light green) Cl^- channels, and Na^+ is secreted via the paracellular shunt. K^+ is also secreted to the luminal side via luminal K^+ channels. Depending on the tissue, intracellular cAMP is increased and secretion is activated by adenosine or prostaglandin E_2 (PGE_2). In absorptive epithelial cells, Na^+ is taken up by luminal epithelial Na^+ channels (ENaC; white). Cl^- is transported via the basolateral shunt and probably via CFTR Cl^- channels. Na^+ is pumped out of the cell by the basolateral Na^+-K^+-ATPase, whereas Cl^- and K^+ leave the cell via Cl^- and K^+ channels, respectively.

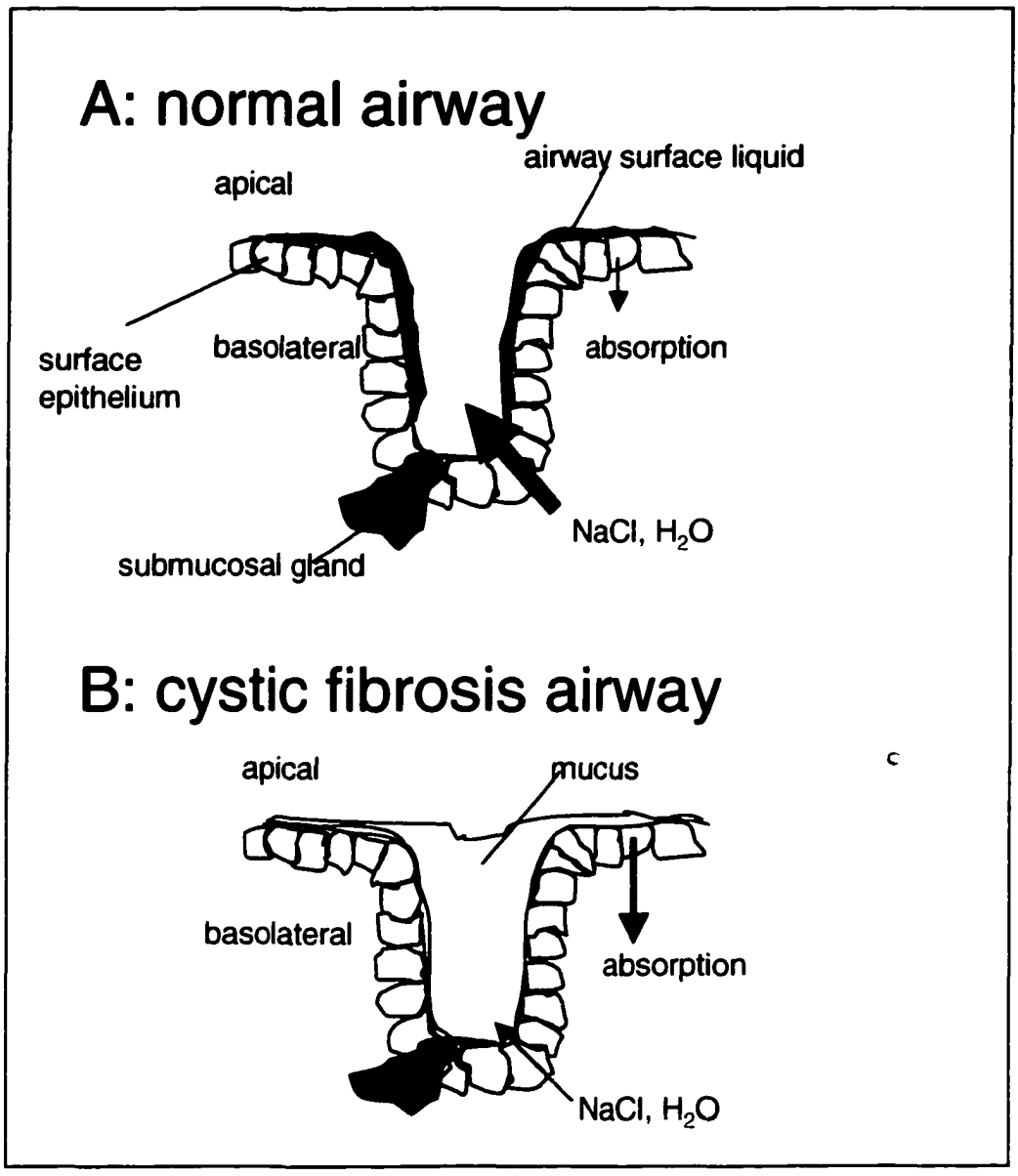


Figure 1.10. Model of the airway epithelium consisting of an absorptive surface epithelium and secretory submucosal glands. The airway epithelium is covered with a thin layer of surface liquid which composed of electrolytes secreted from the epithelial cells. The surface liquid is essential in clearing the mucus from the submucosal glands and in proper muciliary clearance. In normal airways (A), epithelium is able to upregulate its net secretion of electrolytes but in cystic fibrosis (B) the epithelium is unable to increase secretion and displays net absorption.

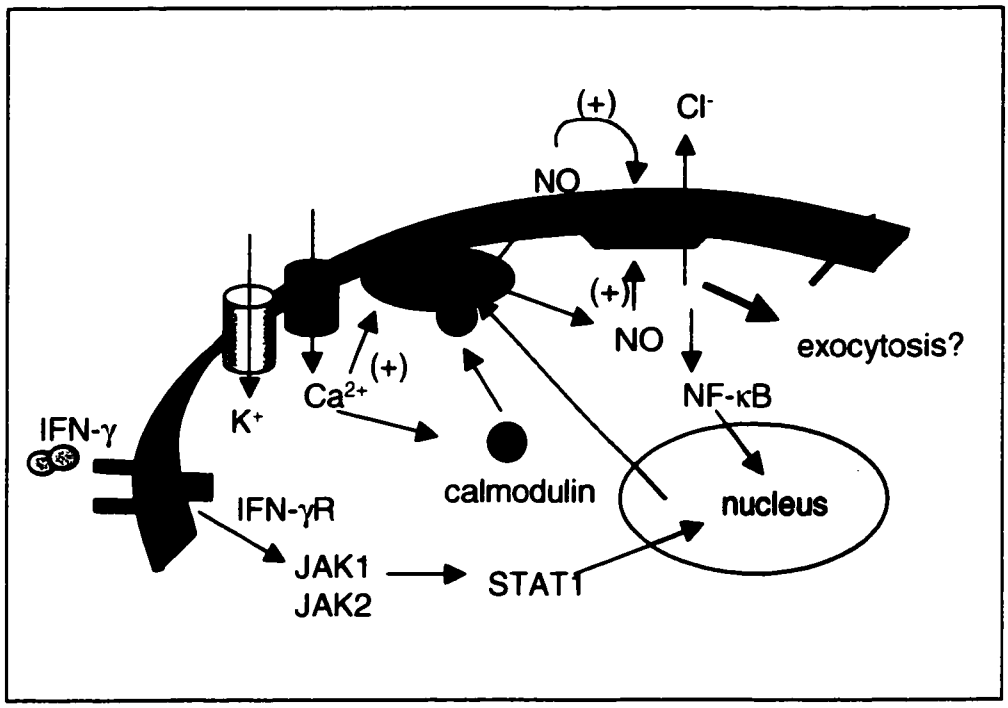


Figure 1.11. IFN-γ mediated changes in gene transcription and expression mediated by NOS. IFN-γ binding to its receptor initiates NOS transcription and increased expression via the JAK/STAT1 pathway. NOS can insert into the membrane and reside in calveoli where it is in close proximity to K^+ and Ca^{2+} channels. Influx of Ca^{2+} ions activates NOS activity. NO production can have inhibitory effects on K^+ channels.

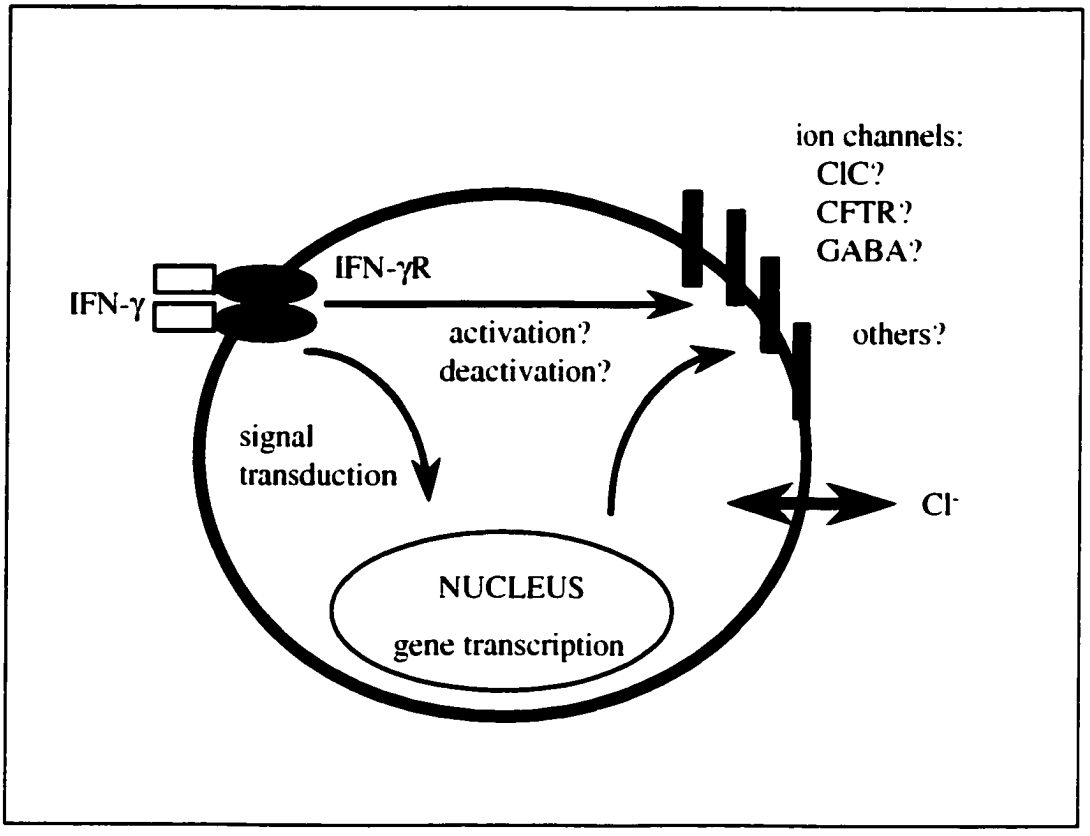


Figure 1.12. Conceptual model of IFN-γ-mediated inhibition of mast cell function and the possible role of ion channels.

References

1. Galli, S. J. 2000. Mast cells and basophils. *Curr. Opin. Hematol. 7*:32.

2. Kirshenbaum, A. S., A. S. Worobec, T. A. Davis, J. P. Goff, T. Semere, and D. D. Metcalfe. 1998. Inhibition of human mast cell growth and differentiation by interferon gamma-1b. *Exp. Hematol. 26*:245.

3. Wills, F. L., M. Gilchrist, and A. D. Befus. 1999. Interferon-gamma regulates the interaction of RBL-2H3 cells with fibronectin through production of nitric oxide. *Immunology 97*:481.

4. Bissonnette, E. Y. and A. D. Befus. 1990. Inhibition of mast cell-mediated cytotoxicity by IFN-alpha/beta and -gamma. *J. Immunol. 145*:3385.

5. Boehm, U., T. Klamp, M. Groot, and J. C. Howard. 1997. Cellular responses to interferon-gamma. *Annu. Rev. Immunol. 15*:749.

6. Bauer, O. and E. Razin. 2000. Mast cell-nerve Interactions. *News Physiol. Sci. 15*:213.

7. Ehrlich, P. 1879. Ueber die specifischen granulationen des blutes. *Arch. Anat. Physiol. 3*:571.

8. Lee, T. D., A. Sterk, T. Ishizaka, J. Bienenstock, and A. D. Befus. 1985. Number and affinity of receptors for IgE on enriched populations of isolated rat intestinal mast cells. *Immunology 55*:363.

9. Lantz, C. S., J. Boesiger, C. H. Song, N. Mach, T. Kobayashi, R. C. Mulligan, Y. Nawa, G. Dranoff, and S. J. Galli. 1998. Role for interleukin-3 in mast-cell and basophil development and in immunity to parasites. *Nature 392*:90.

10. Nakano, T., Y. Kanakura, T. Nakahata, H. Matsuda, and Y. Kitamura. 1987. Genetically mast cell-deficient W/Wv mice as a tool for studies of differentiation and function of mast cells. *Fed. Proc. 46*:1920.

11. Rodewald, H. R., M. Dessing, A. M. Dvorak, and S. J. Galli. 1996. Identification of a committed precursor for the mast cell lineage. *Science 271*:818.

12. Jarboe, D. L. and T. F. Huff. 1989. The mast cell-committed progenitor. II. W/Wv mice do not make mast cell-committed progenitors and S1/S1d fibroblasts do not support development of normal mast cell-committed progenitors. *J. Immunol. 142*:2418.

13. Lin, T. J. and A. D. Befus. 1999. Mast cells and eosinophils in mucosal defenses and pathogenesis. In *Mucosal Immunology*. 2nd ed. P.L. Ogra, J. Mestecky, M.E. Lamm, W. Strober, J. Bienenstock and J.R. McGhee, eds. Academic Press, San Diego, p. 469.

14. Ruitenberg, E. J. and A. Elgersma. 1976. Absense of intestinal mast cell response in congenitally athymic mice during *Trichinella spiralis* infection. *Nature* 258.

15. Irani, A. M., S. S. Craig, G. DeBois, C. O. Elson, N. M. Schechter, and L. B. Schwartz. 1987. Deficiency of the tryptase-positive, chymase-negative mast cell type in gastrointestinal mucosa of patients with defective T lymphocyte function. *J. Immunol. 138*:4381.

16. Arock, M., P. Devillier, G. Luffau, J. J. Guillosson, and M. Renoux. 1989. Histamine-releasing activity of endogenous peptides on mast cells derived from different sites and species. *Int. Arch. Allergy Appl. Immunol. 89*:229.

17. MacDonald, A. J., J. Pick, E. Y. Bissonnette, and A. D. Befus. 1998. Rat mucosal mast cells: the cultured bone marrow-derived mast cell is biochemically and functionally analogous to its counterpart in vivo. *Immunology 93*:533.

18. Gruber, B. L., M. J. Marchese, and R. Kew. 1995. Angiogenic factors stimulate mast-cell migration. *Blood 86*:2488.

19. McCloskey, M. A., Y. Fan, and S. Luther. 1999. Chemotaxis of rat mast cells toward adenine nucleotides. *J. Immunol. 163*:970.

20. Michaloudi, H. C. and G. C. Papadopoulos. 1999. Mast cells in the sheep, hedgehog and rat forebrain. *J. Anat. 195*:577.

21. Rouleau, A., V. Dimitriadou, M. D. Trung Tuong, G. F. Newlands, H. R. Miller, J. C. Schwartz, and M. Garbarg. 1997. Mast cell specific proteases in rat brain: changes in rats with experimental allergic encephalomyelitis. *J. Neural. Transm. 104:* 399.

22. Zhuang, X., A. J. Silverman, and R. Silver. 1999. Distribution and local differentiation of mast cells in the parenchyma of the forebrain. *J. Comp. Neurol. 408*:477.

23. Zhuang, X., A. J. Silverman, and R. Silver. 1997. Mast cell number and maturation in the central nervous system: influence of tissue type, location and exposure to steroid hormones. *Neuroscience 80*:1237.

24. Dimitriadou, V., A. Rouleau, M. D. Trung Tuong, G. J. Newlands, H. R. Miller, G. Luffau, J. C. Schwartz, and M. Garbarg. 1997. Functional relationships between sensory nerve fibers and mast cells of dura mater in normal and inflammatory conditions. *Neuroscience 77*:829.

25. Hough, L. B., S. Jackowski, N. Eberle, K. R. Gogas, N. A. Camarota, and D. Cue. 1988. Actions of the brain-penetrating H2-antagonist zolantidine on histamine dynamics and metabolism in rat brain. *Biochem. Pharmacol. 37*:4707.

26. Rozniecki, J. J., R. Letourneau, M. Sugiultzoglu, C. Spanos, J. Gorbach, and T. C. Theoharides. 1999. Differential effect of histamine 3 receptor-active agents on brain, but not peritoneal, mast cell activation. *J. Pharmacol. Exp. Ther. 290*:1427.

27. Yang, M., C. Chien, and K. Lu. 1999. Morphological, immunohistochemical and quantitative studies of murine brain mast cells after mating. *Brain Res. 846*:30.

28. Silverman, A. J., A. K. Sutherland, M. Wilhelm, and R. Silver. 2000. Mast cells migrate from blood to brain. *J. Neurosci. 20*:401.

29. Zhuang, X., A. J. Silverman, and R. Silver. 1993. Reproductive behavior, endocrine state, and the distribution of GnRH-like immunoreactive mast cells in dove brain. *Horm. Behav. 27*:283.

30. Silverman, A. J., R. P. Millar, J. A. King, X. Zhuang, and R. Silver. 1994. Mast cells with gonadotropin-releasing hormone-like immunoreactivity in the brain of doves. *Proc. Natl. Acad. Sci. U. S. A. 91*:3695.

31. Chen, X. J. and L. Enerback. 2000. Regulation of IgE-receptor expression, IgE occupancy and secretory capacity of mast cells. *APMIS 108*:633.

32. Jouvin, M. H., M. Adamczewski, R. Numerof, O. Letourneur, A. Valle, and J. P. Kinet. 1994. Differential control of the tyrosine kinases Lyn and Syk by the two signaling chains of the high affinity immunoglobulin E receptor. *J. Biol. Chem. 269*:5918.

33. Sada, K., J. Zhang, and R. P. Siraganian. 2001. SH2 domain-mediated targeting, but not localization, of Syk in the plasma membrane is critical for FcepsilonRI signaling. *Blood 97*:1352.

34. Cohen, J. S. and H. A. Brown. 2001. Phospholipases stimulate secretion in RBL mast cells. *Biochemistry 40*:6589.

35. Liu, Y., C. Graham, V. Parravicini, M. J. Brown, J. Rivera, and S. Shaw. 2001. Protein kinase C theta is expressed in mast cells and is functionally involved in Fcepsilon receptor I signaling. *J. Leukoc. Biol. 69*:831.

36. Friis, U. G., T. Johansen, N. A. Hayes, and J. C. Foreman. 1994. IgE-receptor activated chloride uptake in relation to histamine secretion from rat mast cells. *Br. J. Pharmacol. 111*:1179.

37. Mio, M. and K. Tasaka. 1989. Microfilament-associated, local degranulation of rat peritoneal mast cells. *Int. Arch. Allergy Appl. Immunol 88*:369.

38. Tasaka, K., M. Akagi, and K. Miyoshi. 1986. Distribution of actin filaments in rat mast cells and its role in histamine release. *Agents Actions 18*:49.

39. Paumet, F., J. Le Mao, S. Martin, T. Galli, B. David, U. Blank, and M. Roa. 2000. Soluble NSF attachment protein receptors (SNAREs) in RBL-2H3 mast cells: functional role of syntaxin 4 in exocytosis and identification of a vesicle-associated membrane protein 8-containing secretory compartment. *J. Immunol. 164*:5850.

40. Pombo, I., S. Martin-Verdeaux, B. Iannascoli, J. Le Mao, L. Deriano, J. Rivera, and U. Blank. 2001. IgE receptor type I-dependent regulation of a Rab3D-associated kinase. A possible link in the calcium-dependent assembly of SNARE complexes. *J. Biol. Chem.* (In press)

41. Fukuishi, N., M. Sakaguchi, S. Matsuura, C. Nakagawa, R. Akagi, and M. Akagi. 1997. The mechanisms of compound 48/80-induced superoxide generation mediated by A-kinase in rat peritoneal mast cells. *Biochem. Mol. Med. 61*:107.

42. Mousli, M., J. L. Bueb, B. Rouot, Y. Landry, and C. Bronner. 1991. G-proteins as targets for non-immunological histamine releasers. *Agents Actions 33*:81.

43. Mousli, M., T. E. Hugli, Y. Landry, and C. Bronner. 1994. Peptidergic pathway in human skin and rat peritoneal mast cell activation. *Immunopharmacology 27*:1.

44. Suzuki-Nishimura, T., N. Oku, M. Nango, and M. K. Uchida. 1995. PEI6, a new basic secretagogue in rat peritoneal mast cells: characteristics of polyethylenimine PEI6 resemble those of compound 48/80. *Gen. Pharmacol. 26*:1171.

45. Singh, R., P. Kumar, and P. P. Gupta. 2001. Comparative functional characterization of mouse bone marrow-derived mast cells and peritoneal mast cells in response to non-immunological stimuli. *Indian J. Exp. Biol. 39*:323.

46. Lowman, M. A., P. H. Rees, R. C. Benyon, and M. K. Church. 1988. Human mast cell heterogeneity: histamine release from mast cells dispersed from skin, lung, adenoids, tonsils, and colon in response to IgE-dependent and nonimmunologic stimuli. *J. Allergy Clin. Immunol. 81*:590.

47. Hider, R. C. 1988. Honeybee venom: a rich source of pharmacologically active peptides. *Endeavour 12*:60.

48. Irman-Florjanc, T. and F. Erjavec. 1984. The effect of adrenocorticotropin on histamine and 5-hydroxytryptamine secretion from rat mast cells. *Agents Actions 14*:454.

49. Erdei, A., K. Kerekes, and I. Pecht. 1997. Role of C3a and C5a in the activation of mast cells. *Exp. Clin. Immunogenet. 14*:16.

50. Meininger, C. J., H. Yano, R. Rottapel, A. Bernstein, K. M. Zsebo, and B. R. Zetter. 1992. The c-kit receptor ligand functions as a mast cell chemoattractant. *Blood 79*:958.

51. Hartmann, K., B. M. Henz, S. Kruger-Krasagakes, J. Kohl, R. Burger, S. Guhl, I. Haase, U. Lippert, and T. Zuberbier. 1997. C3a and C5a stimulate chemotaxis of human mast cells. *Blood 89*:2863.

52. Nilsson, G., M. Johnell, C. H. Hammer, H. L. Tiffany, K. Nilsson, D. D. Metcalfe, A. Siegbahn, and P. M. Murphy. 1996. C3a and C5a are chemotaxins for human mast cells and act through distinct receptors via a pertussis toxin-sensitive signal transduction pathway. *J. Immunol. 157*:1693.

53. Matsuda, H., N. Watanabe, Y. Kiso, S. Hirota, H. Ushio, Y. Kannan, M. Azuma, H. Koyama, and Y. Kitamura. 1990. Necessity of IgE antibodies and mast cells for manifestation of resistance against larval Haemaphysalis longicornis ticks in mice. *J. Immunol. 144*:259.

54. Malaviya, R., T. Ikeda, E. Ross, and S. N. Abraham. 1996. Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-alpha. *Nature 381*:77.

55. Supajatura, V., H. Ushio, A. Nakao, K. Okumura, C. Ra, and H. Ogawa. 2001. Protective roles of mast cells against enterobacterial infection are mediated by toll-like receptor 4. *J. Immunol. 167*:2250.

56. Askenase, P. W. and R. F. Tsuji. 2000. B-1 B cell IgM antibody initiates T cell elicitation of contact sensitivity. *Curr. Top. Microbiol. Immunol. 252*:171.

57. Malaviya, R. and S. N. Abraham. 2001. Mast cell modulation of immune responses to bacteria. *Immunol. Rev. 179*:16.

58. Holgate, S. T. 1999. The epidemic of allergy and asthma. *Nature 402*:B2.

59. Leung, D. Y. 1997. Immunologic basis of chronic allergic diseases: clinical messages from the laboratory bench. *Pediatr. Res. 42*:559.

60. Renauld, J. C. 2001. New insights into the role of cytokines in asthma. *J. Clin. Pathol. 54*:577.

61. Liu, A. H. 2000. Allergy and asthma: classic TH2 diseases (?). *Allergy Asthma Proc. 21*:227.

62. He, S., Q. Peng, and A. F. Walls. 1997. Potent induction of a neutrophil and eosinophil-rich infiltrate in vivo by human mast cell tryptase: selective enhancement of eosinophil recruitment by histamine. *J. Immunol. 159*:6216.

63. Hamelmann, E. and E. W. Gelfand. 1999. Role of IL-5 in the development of allergen-induced airway hyperresponsiveness. *Int. Arch. Allergy Immunol. 120*:8.

64. Kaplan, A. P. 2001. Chemokines, chemokine receptors and allergy. *Int. Arch. Allergy Immunol. 124*:423.

65. Pan, Z. Z., L. Parkyn, A. Ray, and P. Ray. 2000. Inducible lung-specific expression of RANTES: preferential recruitment of neutrophils. *Am. J. Physiol. Lung Cell Mol. Physiol. 279*:L658.

66. Bates, M. E., V. L. Green, and P. J. Bertics. 2000. ERK1 and ERK2 activation by chemotactic factors in human eosinophils is interleukin 5-dependent and contributes to leukotriene C(4) biosynthesis. *J. Biol. Chem. 275*:10968.

67. Schwarze, J., G. Cieslewicz, E. Hamelmann, A. Joetham, L. D. Shultz, M. C. Lamers, and E. W. Gelfand. 1999. IL-5 and eosinophils are essential for the development of airway hyperresponsiveness following acute respiratory syncytial virus infection. *J. Immunol. 162*:2997.

68. Rumsaeng, V., H. Vliagoftis, C. K. Oh, and D. D. Metcalfe. 1997. Lymphotactin gene expression in mast cells following Fc(epsilon) receptor I aggregation: modulation by TGF-beta, IL-4, dexamethasone, and cyclosporin A. *J. Immunol. 158*:1353.

69. Rumsaeng, V., W. W. Cruikshank, B. Foster, C. Prussin, A. S. Kirshenbaum, T. A. Davis, H. Kornfeld, D. M. Center, and D. D. Metcalfe. 1997. Human mast cells produce the CD4+ T lymphocyte chemoattractant factor, IL-16. *J. Immunol. 159*:2904.

70. Hartmann, K. and B. M. Henz. 2001. Mastocytosis: recent advances in defining the disease. *Br. J. Dermatol. 144*:682.

71. Vliagoftis, H., V. Dimitriadou, W. Boucher, J. J. Roznieckl, I. Correia, S. Raam, and T. C. Theoharides. 1992. Estradiol augments while tamoxifen inhibits rat mast cell secretion. *Int. Arch. Allergy Immunol 98*:398.

72. Larsen, A. E. and R. L. Grier. 1989. Evaluation of canine mast cell tumors for presence of estrogen receptors. *Am. J. Vet. Res. 50*:1779.

73. Pang, X., M. M. Cotreau-Bibbo, G. R. Sant, and T. C. Theoharides. 1995. Bladder mast cell expression of high affinity oestrogen receptors in patients with interstitial cystitis. *Br. J. Urol. 75*:154.

74. Ibrahim, M. Z., A. T. Reder, R. Lawand, W. Takash, and S. Sallouh-Khatib. 1996. The mast cells of the multiple sclerosis brain. *J. Neuroimmunol. 70*:131.

75. Skaper, S. D., L. Facci, S. Romanello, and A. Leon. 1996. Mast cell activation causes delayed neurodegeneration in mixed hippocampal cultures via the nitric oxide pathway. *J. Neurochem. 66*:1157.

76. Nelson, R. B., R. Siman, M. A. Iqbal, and H. Potter. 1993. Identification of a chymotrypsin-like mast cell protease in rat brain capable of generating the N-terminus of the Alzheimer amyloid beta-protein. *J. Neurochem. 61*:567.

77. Scharenberg, A. M. 1999. The inhibitory receptor superfamily: potential relevance to atopy. *Curr. Opin. Immunol 11*:621.

78. Fong, D. C., O. Malbec, M. Arock, J. C. Cambier, W. H. Fridman, and M. Daeron. 1996. Selective in vivo recruitment of the phosphatidylinositol phosphatase SHIP by phosphorylated Fc gammaRIIB during negative regulation of IgE-dependent mouse mast cell activation. *Immunol Lett. 54*:83.

79. Malbec, O., D. C. Fong, M. Turner, V. L. Tybulewicz, J. C. Cambier, W. H. Fridman, and M. Daeron. 1998. Fc epsilon receptor I-associated lyn-dependent phosphorylation of Fc gamma receptor IIB during negative regulation of mast cell activation. *J. Immunol. 160*:1647.

80. Gupta, N., A. M. Scharenberg, D. A. Fruman, L. C. Cantley, J. P. Kinet, and E. O. Long. 1999. The SH2 domain-containing inositol 5'-phosphatase (SHIP) recruits the p85 subunit of phosphoinositide 3-kinase during FcgammaRIIb1-mediated inhibition of B cell receptor signaling. *J. Biol. Chem. 274*:7489.

81. Lobell, R. B., K. F. Austen, and H. R. Katz. 1994. Fc gamma R-mediated endocytosis and expression of cell surface Fc gamma RIIb1 and Fc gamma RIIb2 by mouse bone marrow culture-derived progenitor mast cells. *J. Immunol. 152*:811.

82. Daeron, M., C. Bonnerot, S. Latour, and W. H. Fridman. 1992. Murine recombinant Fc gamma RIII, but not Fc gamma RII, trigger serotonin release in rat basophilic leukemia cells. *J. Immunol. 149*:1365.

83. Hogaboam, C. M., E. Y. Bissonnette, B. C. Chin, A. D. Befus, and J. L. Wallace. 1993. Prostaglandins inhibit inflammatory mediator release from rat mast cells. *Gastroenterology 104*:122.

84. Weston, M. C., N. Anderson, and P. T. Peachell. 1997. Effects of phosphodiesterase inhibitors on human lung mast cell and basophil function. *Br. J. Pharmacol. 121*:287.

85. Sydbom, A. and B. B. Fredholm. 1982. On the mechanism by which theophylline inhibits histamine release from rat mast cells. *Acta Physiol. Scand. 114*:243.

86. Bissonnette, E. Y. and A. D. Befus. 1997. Anti-inflammatory effect of beta 2-agonists: inhibition of TNF-alpha release from human mast cells. *J. Allergy Clin. Immunol. 100*:825.

87. Bissonnette, E. Y., J. A. Enciso, and A. D. Befus. 1995. Inhibition of tumour necrosis factor-alpha (TNF-alpha) release from mast cells by the anti-inflammatory drugs, sodium cromoglycate and nedocromil sodium. *Clin. Exp. Immunol. 102*:78.

88. Devalia, J. L., R. J. Davies, A. D. Befus, E. Y. Bissonnette, F. Levi-Schaffer, D. K. Rainey, and A. A. Norris. 1995. Inhibitory effects of nedocromil sodium on cytokine production from mast and epithelial cells. *Int. Arch. Allergy Immunol. 107*:417.

89. Bissonnette, E. Y., J. A. Enciso, and A. D. Befus. 1995. Interferon and antiallergic drug regulation of histamine and tumor necrosis factor-alpha in rat mast cell subsets. *Int. Arch. Allergy Immunol. 107*:156.

90. Alton, E. W. and A. A. Norris. 1996. Chloride transport and the actions of nedocromil sodium and cromolyn sodium in asthma. *J. Allergy Clin. Immunol. 98*:S102.

91. Reinsprecht, M., I. Pecht, H. Schindler, and C. Romanin. 1992. Potent block of Cl-channels by antiallergic drugs. *Biochem. Biophys. Res. Commun. 188*:957.

92. Romanin, C., M. Reinsprecht, I. Pecht, and H. Schindler. 1991. Immunologically activated chloride channels involved in degranulation of rat mucosal mast cells. *EMBO J. 10*:3603.

93. Lin, T. J. and A. D. Befus. 1997. Differential regulation of mast cell function by IL-10 and stem cell factor. *J. Immunol. 159*:4015.

94. Bissonnette, E. Y., J. A. Enciso, and A. D. Befus. 1997. TGF-beta1 inhibits the release of histamine and tumor necrosis factor-alpha from mast cells through an autocrine pathway. *Am. J. Respir. Cell Mol. Biol. 16*:275.

95. Coleman, J. W., M. G. Buckley, M. R. Holliday, and A. G. Morris. 1991. Interferon-gamma inhibits serotonin release from mouse peritoneal mast cells. *Eur. J. Immunol. 21*:2559.

96. Schreiber, R. D. and M. A. Farrar. 1993. The biology and biochemistry of interferon-gamma and its receptor. *Gastroenterol. Jpn. 28 Suppl*:88.

97. Westermann, J., S. Persin, J. Matyas, P. van der Meide, and R. Pabst. 1993. IFN-gamma influences the migration of thoracic duct B and T lymphocyte subsets in vivo.

Random increase in disappearance from the blood and differential decrease in reappearance in the lymph. *J. Immunol. 150*:3843.

98. Schiff, D. E., J. Rae, T. R. Martin, B. H. Davis, and J. T. Curnutte. 1997. Increased phagocyte Fc gammaRI expression and improved Fc gamma-receptor-mediated phagocytosis after in vivo recombinant human interferon-gamma treatment of normal human subjects. *Blood 90*:3187.

99. Stark, G. R., I. M. Kerr, B. R. Williams, R. H. Silverman, and R. D. Schreiber. 1998. How cells respond to interferons. *Annu. Rev. Biochem. 67:227-64.*227.

100. Kaplan, M. H., A. L. Wurster, and M. J. Grusby. 1998. A signal transducer and activator of transcription (Stat)4-independent pathway for the development of T helper type 1 cells. *J. Exp. Med. 188*:1191.

101. Levine, S. J., T. Wu, and J. H. Shelhamer. 1997. Extracellular release of the type I intracellular IL-1 receptor antagonist from human airway epithelial cells: differential effects of IL-4, IL-13, IFN-gamma, and corticosteroids. *J. Immunol. 158*:5949.

102. Strutz, F., M. Heeg, T. Kochsiek, G. Siemers, M. Zeisberg, and G. A. Muller. 2000. Effects of pentoxifylline, pentifylline and gamma-interferon on proliferation, differentiation, and matrix synthesis of human renal fibroblasts. *Nephrol. Dial. Transplant. 15*:1535.

103. Reano, A., J. Viac, M. H. Richard, and D. Schmitt. 1997. Interferon gamma-induced PNA-binding glycoproteins as markers of human keratinocyte differentiation: biological evidence using protein kinase C agonists, antagonists and retinoic acid. *Arch. Dermatol. Res. 289*:617.

104. Fransson, J., Q. Shen, A. Scheynius, and H. Hammar. 1996. The effect of IFN-gamma on healthy and psoriatic keratinocytes in a skin equivalent model is influenced by the source of the keratinocytes and by their interactions with fibroblasts. *Arch. Dermatol. Res. 289*:14.

105. Wong, C. K., C. Y. Ho, F. W. Ko, C. H. Chan, A. S. Ho, D. S. Hui, and C. W. Lam. 2001. Proinflammatory cytokines (IL-17, IL-6, IL-18 and IL-12) and Th cytokines (IFN-gamma, IL-4, IL-10 and IL-13) in patients with allergic asthma. *Clin. Exp. Immunol. 125*:177.

106. Chung, F. 2001. Anti-inflammatory cytokines in asthma and allergy: interleukin-10, interleukin-12, interferon-gamma. *Mediators. Inflamm. 10*:51.

107. Bodey, K. J., A. E. Semper, A. E. Redington, J. Madden, L. M. Teran, S. T. Holgate, and A. J. Frew. 1999. Cytokine profiles of BAL T cells and T-cell clones obtained from human asthmatic airways after local allergen challenge. *Allergy 54*:1083.

108. Cohn, L., C. Herrick, N. Niu, R. Homer, and K. Bottomly. 2001. IL-4 promotes airway eosinophilia by suppressing IFN-gamma production: defining a novel role for IFN-gamma in the regulation of allergic airway inflammation. *J. Immunol. 166*:2760.

109. Gao, P. S., X. Q. Mao, E. Jouanguy, A. Pallier, R. Doffinger, Y. Tanaka, H. Nakashima, T. Otsuka, M. H. Roberts, T. Enomoto, Y. Dake, M. Kawai, S. Sasaki, S. R. Shaldon, P. Coull, C. N. Adra, Y. Niho, J. L. Casanova, T. Shirakawa, and J. M. Hopkin. 1999. Nonpathogenic common variants of IFNGR1 and IFNGR2 in association with total serum IgE levels. *Biochem. Biophys. Res. Commun. 263*:425.

110. Campbell, D. E., D. J. Hill, and A. S. Kemp. 1998. Enhanced IL-4 but normal interferon-gamma production in children with isolated IgE mediated food hypersensitivity. *Pediatr. Allergy Immunol. 9*:68.

111. Bach, E. A., M. Aguet, and R. D. Schreiber. 1997. The IFN gamma receptor: a paradigm for cytokine receptor signaling. *Annu. Rev. Immunol 15:563-91*.563.

112. Nguyen, H., C. V. Ramana, J. Bayes, and G. R. Stark. 2001. Roles of phosphatidylinositol 3-kinase in interferon-gamma-dependent phosphorylation of STAT1 on serine 727 and activation of gene expression. *J. Biol. Chem. 276*:33361.

113. O'Shea, J. J., L. D. Notarangelo, J. A. Johnston, and F. Candotti. 1997. Advances in the understanding of cytokine signal transduction: the role of Jaks and STATs in immunoregulation and the pathogenesis of immunodeficiency. *J. Clin. Immunol 17*:431.

114. Celada, A. and R. D. Schreiber. 1987. Internalization and degradation of receptor-bound interferon-gamma by murine macrophages. Demonstration of receptor recycling. *J. Immunol. 139*:147.

115. Kontsek, P. and E. Kontseková. 1997. Forty years of interferon. *Acta Virol. (Praha) 41*:349.

116. Larner, A. and N. C. Reich. 1996. Interferon signal transduction. *Biotherapy 8*:175.

117. Bromberg, J. F., C. M. Horvath, Z. Wen, R. D. Schreiber, and J. E. Darnell,Jr. 1996. Transcriptionally active Stat1 is required for the antiproliferative effects of both interferon alpha and interferon gamma. *Proc. Natl. Acad. Sci. U. S. A. 93*:7673.

118. Haque, S. J. and R. G. Williams. 1998. Signal transduction in the interferon system. *Sem. Oncol. 25*:14.

119. Decker, T., P. Kovarik, and A. Meinke. 1997. GAS elements: a few nucleotides with a major impact on cytokine-induced gene expression. *J. Interferon Cytokine. Res. 17*:121.

120. Belich, M. P., R. J. Glynne, G. Senger, D. Sheer, and J. Trowsdale. 1994. Proteasome components with reciprocal expression to that of the MHC-encoded LMP proteins. *Curr. Biol. 4*:769.

121. Min, W., J. S. Pober, and D. R. Johnson. 1996. Kinetically coordinated induction of TAP1 and HLA class I by IFN-g. *J. Immunol. 156*:3174.

122. Mach, B., V. Steimle, E. Martinez-Soria, and W. Reith. 1996. Regulation of MHC class II genes: lessons from a disease. *Annu. Rev. Immunol. 14*:301.

123. Cassatella, M. A., F. Bazzoni, R. M. Flynn, S. Dusi, G. Trinchieri, and F. Rossi. 1990. Molecular basis of interferon-gamma and lipopolysaccharide enchancement of phagocyte respiratory bust capability. Studies on the gene expression of several NADPH oxidase components. *J. Biol. Chem. 265*:20241.

124. Eklund, E. A. and D. G. Skalnik. 1995. Characterization of a gp91-phox promoter element that is required for interferon gamma-induced transcription. *J. Biol. Chem. 270*:8267.

125. Kamijo, R., H. Harada, T. Matsuyama, M. Bosland, D. Gerecitano, D. Shapiro, J. Le, S. I. Koh, T. Kimura, and S. J. Green. 1994. Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. *Science 263*:1612.

126. Meraz, M. A., J. M. White, K. C. Sheehan, E. A. Bach, S. J. Rodig, A. S. Dighe, D. H. Kaplan, J. K. Riley, A. C. Greenlund, D. Campbell, K. Carver-Moore, R. N. Dubois, R. Clark, M. Aguet, and R. D. Schreiber. 1996. Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell 84*:431.

127. Briscoe, J., N. C. Rogers, B. A. Witthuhn, D. Watling, A. G. Harpur, A. F. Wilks, G. R. Stark, J. N. Ihle, and I. M. Kerr. 1996. Kinase-negative mutants of JAK1 can sustain interferon-gamma-inducible gene expression but not an antiviral state. *EMBO J. 15*:799.

128. Gil, M. P., E. Bohn, A. K. O'Guin, C. V. Ramana, B. Levine, G. R. Stark, H. W. Virgin, and R. D. Schreiber. 2001. Biologic consequences of Stat1-independent IFN signaling. *Proc. Natl. Acad. Sci. U. S. A. 98*:6680.

129. Kriebel, P., B. K. Patel, S. A. Nelson, M. J. Grusby, and W. J. LaRochelle. 1999. Consequences of Stat6 deletion on Sis/PDGF- and IL-4-induced proliferation and transcriptional activation in murine fibroblasts. *Oncogene 18*:7294.

130. Durbin, J. E., R. Hackenmiller, M. C. Simon, and D. E. Levy. 1996. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell 84*:443.

131. Ramana, C. V., M. P. Gil, Y. Han, R. M. Ransohoff, R. D. Schreiber, and G. R. Stark. 2001. Stat1-independent regulation of gene expression in response to IFN-gamma. *Proc. Natl. Acad. Sci. U. S. A. 98*:6674.

132. Gilchrist, M., M. Savoie, N. S. Hirji, O. Nohara, F. L. Wills, J. L. Wallace, and A. D. Befus. 2002. Nitric oxide synthase and nitric oxide production in in-vivo derived mast cells. *J. Leukoc. Biol.* (In press)

133. Forsythe, P., M. Gilchrist, M. Kulka, and A. D. Befus. 2001. Mast cells and nitric oxide: control of production, mechanisms of response. *Int. Immunopharmacol. 1*:1525.

134. Eastmond, N. C., E. M. Banks, and J. W. Coleman. 1997. Nitric oxide inhibits IgE-mediated degranulation of mast cells and is the principal intermediate in IFN-gamma-induced suppression of exocytosis. *J. Immunol. 159*:1444.

135. Kroncke, K. D., K. Fehsel, C. Suschek, and V. Kolb-Bachofen. 2001. Inducible nitric oxide synthase-derived nitric oxide in gene regulation, cell death and cell survival. *Int. Immunopharmacol. 1*:1407.

136. Nafziger, J., M. Arock, J. J. Guillosson, and J. Wietzerbin. 1990. Specific high-affinity receptors for interferon-gamma on mouse bone marrow-derived mast cells: inhibitory effect of interferon-gamma on mast cell precursors. *Eur. J. Immunol. 20*:113.

137. Bissonnette, E. Y., B. Chin, and A. D. Befus. 1995. Interferons differentially regulate histamine and TNF-alpha in rat intestinal mucosal mast cells. *Immunology 86*:12.

138. Frandji, P., C. Tkaczyk, C. Oskeritzian, J. Lapeyre, R. Peronet, B. David, J. G. Guillet, and S. Mecheri. 1995. Presentation of soluble antigens by mast cells: upregulation by interleukin-4 and granulocyte/macrophage colony-stimulating factor and downregulation by interferon-gamma. *Cell Immunol. 163*:37.

139. Poncet, P., M. Arock, and B. David. 1999. MHC class II-dependent activation of CD4+ T cell hybridomas by human mast cells through superantigen presentation. *J. Leukoc. Biol. 66*:105.

140. Okayama, Y., D. D. Hagaman, M. Woolhiser, and D. D. Metcalfe. 2001. Further characterization of FcgammaRII and FcgammaRIII expression by cultured human mast cells. *Int. Arch. Allergy Immunol. 124*:155.

141. George, A. L., Jr., L. Bianchi, E. M. Link, and C. G. Vanoye. 2001. From stones to bones: the biology of ClC chloride channels. *Curr. Biol. 11*:R620.

142. Jentsch, T. J. 1996. Chloride channels: a molecular perspective. *Curr. Opin. Neurobiol. 6*:303.

143. Bormann, J. 2000. The 'ABC' of GABA receptors. *Trends. Pharmacol. Sci. 21*:16.

144. Burt, D. R. and G. L. Kamatchi. 1991. $GABA_A$ receptor subtypes: from pharmacology to molecular biology. *FASEB J. 5*:2916.

145. Schofield, P. R., M. G. Darlison, N. Fujita, D. R. Burt, F. A. Stephenson, H. Rodriguez, L. Rhee, J. Ramachandran, V. Reale, T. A. Glencorse, P. H. Seeburg, and E. A. Barnard. 1987. Sequence and functional expression of the GABA-A receptor shows a ligand-gated receptor superfamily. *Nature 328*:221.

146. Gyenes, M., Q. Wang, T. T. Gibbs, and D. H. Farb. 1994. Phosphorylation factors control neurotransmitter and neuromodulator actions at the gamma-aminobutyric acid type A receptor. *Mol. Pharmacol. 46*:542.

147. Huang, C. S. and T. Narahashi. 1997. The role of phosphorylation in the activity and mercury modulation of GABA-induced currents in rat neurons. *Neuropharmacology 36*:1631.

148. Leidenheimer, N. J., S. J. McQuilkin, L. D. Hahner, P. Whiting, and R. A. Harris. 1992. Activation of protein kinase C selectivity inhibits the gamma-aminobutyric $acid_A$ receptor, role of desensitization. *Mol. Pharmacol. 41*:1116.

149. Leidenheimer, N. J., T. K. Machu, S. Endo, R. W. Olsen, R. A. Harris, and M. D. Browning. 1991. Cyclic cAMP-dependent protein kinase decreases gamma-aminobutyric $acid_A$ receptor-mediated $^{36}Cl^-$ uptake by brain microsacs. *J. Neurochem. 57*:722.

150. Olsen, R. W. and A. J. Tobin. 1990. Molecular biology of $GABA_A$ receptors. *FASEB J. 4*:1469.

151. Suzuki-Nishimura, T., T. Sano, and M. K. Uchida. 1989. Effects of benzodiazepines on serotonin release from rat mast cells. *Eur. J. Pharmacol. 167*:75.

152. Bidri, M., B. Royer, G. Averlant, G. Bismuth, J. J. Guillosson, and M. Arock. 1999. Inhibition of mouse mast cell proliferation and proinflammatory mediator release by benzodiazepines. *Immunopharmacology 43*:75.

153. Sano, T., M. K. Uchida, and T. Suzuki-Nishimura. 1990. The effects of clozapine on rat mast cells are different from those of benzodiazepines. *Gen. Pharmacol. 21*:559.

154. Jentsch, T. J., K. Steinmeyer, and G. Schwarz. 1990. Primary structure of Torpedo marmorata chloride channel isolated by expression cloning in Xenopus oocytes. *Nature 348*:510.

155. Jentsch, T. J. and W. Gunther. 1997. Chloride channels: an emerging molecular picture. *Bioessays 19*:117.

156. Purdy, M. D. and M. C. Wiener. 2000. Expression, purification, and initial structural characterization of YadQ, a bacterial homolog of mammalian ClC chloride channel proteins. *FEBS Lett. 466*:26.

157. Barbier-Brygoo, H., M. Vinauger, J. Colcombet, G. Ephritikhine, J. Frachisse, and C. Maurel. 2000. Anion channels in higher plants: functional characterization, molecular structure and physiological role. *Biochim. Biophys. Acta 1465*:199.

158. Huang, M. E., J. C. Chuat, and F. Galibert. 1994. A voltage-gated chloride channel in the yeast Saccharomyces cerevisiae. *J. Mol. Biol. 242*:595.

159. Jentsch, T. J., T. Friedrich, A. Schriever, and H. Yamada. 1999. The CLC chloride channel family. *Pflugers Arch. 437*:783.

160. Steinmeyer, K., R. Klocke, C. Ortland, M. Gronemeier, H. Jockusch, S. Grunder, and T. J. Jentsch. 1991. Inactivation of muscle chloride channel by transposon insertion in myotonic mice. *Nature 354*:304.

161. Igarashi, T., W. Gunther, T. Sekine, J. Inatomi, H. Shiraga, S. Takahashi, J. Suzuki, N. Tsuru, T. Yanagihara, M. Shimazu, T. J. Jentsch, and R. V. Thakker. 1998. Functional characterization of renal chloride channel, CLCN5, mutations associated with Dent's disease. *Kidney Int. 54*:1850.

162. Thakker, R. V. 1999. Chloride channels in renal disease. *Adv. Nephrol. Necker. Hosp. 29*:289.

163. Konrad, M., M. Vollmer, H. H. Lemmink, L. P. van den Heuvel, N. Jeck, R. Vargas-Poussou, A. Lakings, R. Ruf, G. Deschenes, C. Antignac, L. Guay-Woodford, N. V. Knoers, H. W. Seyberth, D. Feldmann, and F. Hildebrandt. 2000. Mutations in the chloride channel gene CLCNKB as a cause of classic Bartter syndrome. *J. Am. Soc. Nephrol. 11*:1449.

164. Fahlke, C. 2000. Molecular mechanisms of ion conduction in ClC-type chloride channels: lessons from disease-causing mutations. *Kidney Int. 57*:780.

165. Kornak, U., D. Kasper, M. R. Bosl, E. Kaiser, M. Schweizer, A. Schulz, W. Friedrich, G. Delling, and T. J. Jentsch. 2001. Loss of the ClC-7 chloride channel leads to osteopetrosis in mice and man. *Cell 104*:205.

166. Steinmeyer, K., C. Ortland, and T. J. Jentsch. 1991. Primary structure and functional expression of a developmentally regulated skeletal muscle chloride channel. *Nature 354*:301.

167. Smith, R. L., C. L. Clayton, C. L. Wilcox, K. W. Escudero, and K. J. Staley. 1995. Differential expression of an inwardly rectifying chloride conductance in rat brain neurons: a potential mechanism for cell-specific modulation of postsynaptic inhibition. *J. Neurosci. 15*:4057.

168. Schwappach, B., S. Stobrawa, M. Hechenberger, K. Steinmeyer, and T. J. Jentsch. 1998. Golgi localization and functionally important domains in the NH2 and COOH terminus of the yeast ClC putative chloride channel Gef1p. *J. Biol. Chem. 273*:15110.

169. Duan, D., S. Cowley, B. Horowitz, and J. R. Hume. 1999. A serine residue in ClC-3 links phosphorylation-dephosphorylation to chloride channel regulation by cell volume. *J. Gen. Physiol. 113*:57.

170. Shimada, K., X. Li, G. Xu, D. E. Nowak, L. A. Showalter, and S. A. Weinman. 2000. Expression and canalicular localization of two isoforms of the ClC-3 chloride channel from rat hepatocytes. *Am. J. Physiol. Gastrointest. Liver Physiol. 279*:G268.

171. Wang, L., L. Chen, and T. J. Jacob. 2000. The role of ClC-3 in volume-activated chloride currents and volume regulation in bovine epithelial cells demonstrated by antisense inhibition. *J. Physiol. 524*:63.

172. Kawasaki, M., M. Suzuki, S. Uchida, S. Sasaki, and F. Marumo. 1995. Stable and functional expression of the CIC-3 chloride channel in somatic cell lines. *Neuron 14*:1285.

173. Friedrich, T., T. Breiderhoff, and T. J. Jentsch. 1999. Mutational analysis demonstrates that ClC-4 and ClC-5 directly mediate plasma membrane currents. *J. Biol. Chem. 274*:896.

174. Lamb, F. S., G. H. Clayton, B. X. Liu, R. L. Smith, T. J. Barna, and B. C. Schutte. 1999. Expression of CLCN voltage-gated chloride channel genes in human blood vessels. *J. Mol. Cell Cardiol. 31*:657.

175. Kawasaki, M., T. Fukuma, K. Yamauchi, H. Sakamoto, F. Marumo, and S. Sasaki. 1999. Identification of an acid-activated Cl(-) channel from human skeletal muscles. *Am. J. Physiol. 277*:C948.

176. Gunther, W., A. Luchow, F. Cluzeaud, A. Vandewalle, and T. J. Jentsch. 1998. ClC-5, the chloride channel mutated in Dent's disease, colocalizes with the proton pump in endocytotically active kidney cells. *Proc. Natl. Acad. Sci. U. S. A. 95*:8075.

177. Buyse, G., T. Voets, J. Tytgat, C. De Greef, G. Droogmans, B. Nilius, and J. Eggermont. 1997. Expression of human pICln and ClC-6 in Xenopus oocytes induces an identical endogenous chloride conductance. *J. Biol. Chem. 272*:3615.

178. Brandt, S. and T. J. Jentsch. 1995. ClC-6 and ClC-7 are two novel broadly expressed members of the CLC chloride channel family. *FEBS Lett. 377*:15.

179. Hill, P. B., R. J. Martin, and H. R. Miller. 1996. Characterization of whole-cell currents in mucosal and connective tissue rat mast cells using amphotericin-B-perforated patches and temperature control. *Pflugers Arch. 432*:986.

180. Reinsprecht, M., M. H. Rohn, R. J. Spadinger, I. Pecht, H. Schindler, and C. Romanin. 1995. Blockade of capacitive Ca2+ influx by Cl- channel blockers inhibits secretion from rat mucosal-type mast cells. *Mol. Pharmacol. 47*:1014.

181. Hoth, M., C. Fasolato, and R. Penner. 1993. Ion channels and calcium signaling in mast cells. *Ann. N. Y. Acad. Sci. 707*:198.

182. Kulka, M., M. Gilchrist, M. Duszyk, and A. D. Befus. 2002. Expression and functional characterization of CFTR in mast cells. *J. Leukoc. Biol.* (In press)

183. Duffy, S. M., M. L. Leyland, E. C. Conley, and P. Bradding. 2001. Voltage-dependent and calcium-activated ion channels in the human mast cell line HMC-1. *J. Leukoc. Biol. 70*:233.

184. Riordan, J. R., J. M. Rommens, B. Kerem, N. Alon, R. Rozmahel, Z. Grzelczak, J. Zielenski, S. Lok, N. Plavsic, and J. L. Chou. 1989. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science 245*:1066.

185. Schreiber, R., A. Hopf, M. Mall, R. Greger, and K. Kunzelmann. 1999. The first-nucleotide binding domain of the cystic-fibrosis transmembrane conductance regulator is important for inhibition of the epithelial Na+ channel. *Proc. Natl. Acad. Sci. U. S. A. 96*:5310.

186. Gadsby, D. C. and A. C. Nairn. 1999. Control of CFTR channel gating by phosphorylation and nucleotide hydrolysis. *Physiol. Rev. 79*:S77.

187. Liedtke, C. M. and T. S. Cole. 1998. Antisense oligonucleotide to PKC-? alters cAMP-dependent stimulation of CFTR in Calu-3 cells. *Am. J. Physiol. 275*:C1357.

188. Schultz, B. D., A. K. Singh, D. C. Devor, and R. J. Bridges. 1999. Pharmacology of CFTR chloride channel activity. *Physiol. Rev. 79*:S109.

189. Stutts, M. J., J. G. Fitz, A. M. Paradiso, and R. C. Boucher. 1994. Multiple modes of regulation of airway epithelial chloride secretion by extracellular ATP. *Am. J. Physiol. 267*:C1442.

190. Schwiebert, E. M., D. J. Benos, M. E. Egan, M. J. Stutts, and W. B. Guggino. 1999. CFTR is a conductance regulator as well as a chloride channel. *Physiol. Rev. 79*:S145.

191. Schreiber, R., R. Nitschke, R. Greger, and K. Kunzelmann. 1999. The cystic fibrosis transmembrane conductance regulator activates aquaporin 3 in airway epithelial cells. *J. Biol. Chem. 274*:11811.

192. Vennekens, R., D. Trouet, A. Vankeerberghen, T. Voets, H. Cuppens, J. Eggermont, J. J. Cassiman, G. Droogmans, and B. Nilius. 1999. Inhibition of volume-regulated anion channels by expression of the cystic fibrosis transmembrane conductance regulator. *J. Physiol. (Lond) 515*:75.

193. Lee, M. G., W. C. Wigley, W. Zeng, L. E. Noel, C. R. Marino, P. J. Thomas, and S. Muallem. 1999. Regulation of Cl-/ HCO3- exchange by cystic fibrosis transmembrane conductance regulator expressed in NIH 3T3 and HEK 293 cells. *J. Biol. Chem. 274*:3414.

194. Braunstein, G. M., R. M. Roman, J. P. Clancy, B. A. Kudlow, A. L. Taylor, V. G. Shylonsky, B. Jovov, K. Peter, T. Jilling, I. I. Ismailov, D. J. Benos, L. M. Schwiebert, J. G. Fitz, and E. M. Schwiebert. 2001. Cystic fibrosis transmembrane conductance regulator facilitates ATP release by stimulating a separate ATP release channel for autocrine control of cell volume regulation. *J. Biol. Chem. 276*:6621.

195. Hallows, K. R., V. Raghuram, B. E. Kemp, L. A. Witters, and J. K. Foskett. 2000. Inhibition of cystic fibrosis transmembrane conductance regulator by novel interation with the metabolic sensor AMP-activated protein kinase. *J. Clin. Invest. 105*:1711.

196. Short, D. B., K. W. Trotter, D. Reczek, S. M. Kreda, A. Bretscher, R. C. Boucher, M. J. Stutts, and S. L. Milgram. 1998. An apical PDZ protein anchors the cystic fibrosis transmembrane conductance regulator to the cytoskeleton. *J. Biol. Chem. 273*:19797.

197. Boockfor, F. R., R. A. Morris, D. C. DeSimone, D. M. Hunt, and K. B. Walsh. 1998. Sertoli cell expression of the cystic fibrosis transmembrane conductance regulator. *Am. J. Physiol. 274*:C922.

198. Dong, Y. J., A. C. Chao, K. Kouyama, Y. P. Hsu, R. C. Bocian, R. B. Moss, and P. Gardner. 1995. Activation of CFTR chloride current by nitric oxide in human T lymphocytes. *EMBO J. 14*:2700.

199. Wong, K. R., A. E. Trezise, S. Bryant, G. Hart, and J. I. Vandenberg. 1999. Molecular and functional distributions of chloride conductances in rabbit ventricle. *Am. J. Physiol. 277*:H1403.

200. Zhang, A. L. and G. M. Roomans. 1999. Multiple intracellular pathways for regulation of chloride secretion in cultured pig tracheal submucosal gland cells. *Eur. Respir. J. 13*:571.

201. Weyler, R. T., K. A. Yurko-Mauro, R. Rubenstein, W. J. Kollen, W. Reenstra, S. M. Altschuler, M. Egan, and A. E. Mulberg. 1999. CFTR is functionally active in GnRH-expressing GT1-7 hypothalamic neurons. *Am. J. Physiol. 277*:C563.

202. Hart, P., J. D. Warth, P. C. Levesque, M. L. Collier, Y. Geary, B. Horowitz, and J. R. Hume. 1996. Cystic fibrosis gene encodes a cAMP-dependent chloride channel in heart. *Proc. Natl. Acad. Sci. U. S. A. 93*:6343.

203. Premack, B. A. and P. Gardner. 1991. Role of ion channels in lymphocytes. *J. Clin. Immunol. 11*:225.

204. Loffing, J., B. D. Moyer, D. Reynolds, and B. A. Stanton. 1999. PBA increases CFTR expression but at high doses inhibits Cl(-) secretion in Calu-3 airway epithelial cells. *Am. J. Physiol. 277*:L700.

205. Moyer, B. D., D. Loffing-Cueni, J. Loffing, D. Reynolds, and B. A. Stanton. 1999. Butyrate increases apical membrane CFTR but reduces chloride secretion in MDCK cells. *Am. J. Physiol. 277*:F271.

206. Chanson, M., I. Scerri, and S. Suter. 1999. Defective regulation of gap junctional coupling in cystic fibrosis pancreatic duct cells. *J. Clin. Invest. 103*:1677.

207. Doull, I. J., H. C. Ryley, P. Weller, and M. C. Goodchild. 2001. Cystic fibrosis-related deaths in infancy and the effect of newborn screening. *Pediatr Pulmonol. 31*:363.

208. Eggermont, E. and K. Boeck. 1991. Small-intestine abnormalities in cystic fibrosis patients. *Eur J. Pediatr 1991*:824.

209. Riordan, J. R. 1999. Therapeutic strategies for treatment of CF based on knowledge of CFTR. *Pediatr. Pulmonol. Suppl. 18:83-7*:83.

210. Kopito, R. R. 1999. Biosynthesis and degradation of CFTR. *Physiol. Rev. 79*:S167.

211. Meacham, G. C., Z. Lu, S. King, E. Sorscher, A. Tousson, and D. M. Cyr. 1999. The Hdj-2/Hsc70 chaperone pair facilitates early steps in CFTR biogenesis. *EMBO J. 18*:1492.

212. Ostedgaard, L. S., B. Zeiher, and M. J. Welsh. 1999. Processing of CFTR bearing the P574H mutation differs from wild-type and deltaF508-CFTR. *J. Cell Sci. 112*:2091.

213. Loo, M. A., T. J. Jensen, L. Cui, Y. Hou, X. B. Chang, and J. R. Riordan. 1998. Perturbation of Hsp90 interaction with nascent CFTR prevents its maturation and accelerates its degradation by the proteasome. *EMBO J. 17*:6879.

214. Pind, S., J. R. Riordan, and D. B. Williams. 1994. Participation of the endoplasmic reticulum chaperone calnexin (p88, IP90) in the biogenesis of the cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem. 269*:12784.

215. Zhang, F., F. Kartner, and G. L. Lukacs. 1998. Limited proteolysis as a probe for arrested conformational maturation of the F508 CFTR. *Nat. Struct. Biol. 5*:180.

216. Moss, R. B., R. C. Bocian, Y. P. Hsu, Y. J. Dong, M. Kemna, T. Wei, and P. Gardner. 1996. Reduced IL-10 secretion by CD4+ T lymphocytes expressing mutant cystic fibrosis transmembrane conductance regulator (CFTR). *Clin. Exp. Immunol. 106*:374.

217. Graff, I., A. Schram-Doumont, and C. Szpirer. 1991. Defective protein kinase C-mediated actions in cystic fibrosis neutrophils. *Cell Signal. 3*:259.

218. Crawford, I., P. C. Maloney, P. L. Zeitlin, W. B. Guggino, S. C. Hyde, H. Turley, K. C. Gatter, A. Harris, and C. F. Higgins. 1991. Immunocytochemical localization of the cystic fibrosis gene product CFTR. *Proc. Natl. Acad. Sci. U. S. A. 88*:9262.

219. Gray, M. A., J. P. Winpenny, D. J. Porteous, J. R. Dorin, and B. E. Argent. 1994. CFTR and calcium-activated chloride currents in pancreatic duct cells of a transgenic CF mouse. *Am. J. Physiol. 266*:C213.

220. Ameen, N., J. Alexis, and P. Salas. 2000. Cellular localization of the cystic fibrosis transmembrane conductance regulator in mouse intestinal tract. *Histochem. Cell Biol. 114*:69.

221. Ameen, N., T. Ardito, M. Kashgarian, and C. Marino. 2000. A unique subset of rat and human intestinal villus cells express the cystic fibrosis transmembrane conductance regulator. *Gastroenterology 108*:1016.

222. Tebbutt, S. J., C. J. Wardle, D. F. Hill, and A. Harris. 1995. Molecular analysis of the ovine cystic fibrosis transmembrane conductance regulator gene. *Proc. Natl. Acad. Sci. U. S. A. 92*:2293.

223. Meyer, G., S. Doppierio, P. Vallin, and L. Daffonchio. 1996. Effect of frusemide on Cl- channel in rat peritoneal mast cells. *Eur. Respir. J. 9*:2461.

224. McCloskey, M. A. and Y. X. Qian. 1994. Selective expression of potassium channels during mast cell differentiation. *J. Biol. Chem. 269*:14813.

225. Qian, Y. X. and M. A. McCloskey. 1993. Activation of mast cell K+ channels through multiple G protein-linked receptors. *Proc. Natl. Acad. Sci. U. S. A. 90*:7844.

226. McCloskey, M. A. and M. D. Cahalan. 1990. G protein control of potassium channel activity in a mast cell line. *J. Gen. Physiol. 95*:205.

227. Gericke, M., O. Dar, G. Droogmans, I. Pecht, and B. Nilius. 1995. Immunological stimulation of single rat basophilic leukemia RBL-2H3 cells co-activates Ca(2+)-entry and K(+)-channels. *Cell Calcium 17*:71.

228. Parekh, A. B. 1998. Voltage-dependent conductance changes in a nonvoltage-activated sodium current from a mast cell line. *J. Membr. Biol. 165*:145.

229. Kaplan, M. R., D. B. Mount, and E. Delpire. 1996. Molecular mechanisms of NaCl cotransport. *Annu. Rev. Physiol. 58*:649.

230. Hancox, J. C. and A. J. Levi. 1995. Na-Ca exchange tail current indicates voltage dependence of the Cai transient in rabbit ventricular myocytes. *J. Cardiovasc. Electrophysiol. 6*:455.

231. Kameyama, M., M. Kakei, R. Sato, T. Shibasaki, H. Matsuda, and H. Irisawa. 1984. Intracellular Na^+ activates a K^+ channel in mammalian cardiac cells. *Nature 309*:354.

232. Pilatus, U. and I. Pecht. 1993. 86Rb+ ion fluxes in resting and immunologically stimulated mucosal mast cells. *Eur. J. Immunol. 23*:1125.

233. Vieytes, M. R., A. Alfonso, M. J. Bujan, M. C. Louzao, A. Cavado, L. F. de la Cruz, and L. M. Botana. 1991. Changes in rat mast cell responses in sodium-free media. Lack of demonstrable sodium channel activity. *Agents Actions 33*:94.

234. Duffy, S. M., W. J. Lawley, E. C. Conley, and P. Bradding. 2001. Resting and activation-dependent ion channels in human mast cells. *J. Immunol. 167*:4261.

235. Redrup, A. C., J. C. Foreman, N. A. Hayes, and F. L. Pearce. 1997. Fc epsilon RI-mediated chloride uptake by rat mast cells: modulation by chloride transport inhibitors in relation to histamine secretion. *Br. J. Pharmacol. 122*:1188.

236. Lindau, M. 1989. Patch-clamp capacitance measurements: a tool for investigating the second messengers regulating exocytosis. *J. Protein Chem. 8*:438.

237. Salvatore, C. A., S. L. Tilley, A. M. Latour, D. S. Fletcher, B. H. Koller, and M. A. Jacobson. 2000. Disruption of the A(3) adenosine receptor gene in mice and its effect on stimulated inflammatory cells. *J. Biol. Chem. 275*:4429.

238. Lau, H. Y. and S. P. Wan. 2000. Inhibition of compound 48/80 induced histamine release from mast cells by chloride channel blockers is affected by methods of drug preincubation. *Inflamm. Res. 49*:S21.

239. Martens, J. R., R. Navarro-Polanco, E. A. Coppock, A. Nishiyama, L. Parshley, T. D. Grobaski, and M. M. Tamkun. 2000. Differential targeting of Shaker-like potassium channels to lipid rafts. *J. Biol. Chem. 275*:7443.

240. Luck, G., W. Hoch, C. Hopf, and D. Blottner. 2000. Nitric oxide synthase (NOS-1) coclustered with agrin-induced AChR-specializations on cultured skeletal myotubes. *Mol. Cell Neurosci. 16*:269.

241. Jilling, T., I. Y. Haddad, S. H. Cheng, and S. Matalon. 1999. Nitric oxide inhibits heterologous CFTR expression in polarized epithelial cells. *Am. J. Physiol. 277*:L89.

242. Jin, J. S., D. W. Wilde, R. C. Webb, and L. G. D'Alecy. 1996. Calcium channel activity increased by plasma from ischemic hindlimbs of rats: role of an endogenous NO synthase inhibitor. *Am. J. Physiol. 270*:H1484.

243. Abderrahmane, A., D. Salvail, M. Dumoulin, J. Garon, A. Cadieux, and E. Rousseau. 1998. Direct activation of K(Ca) channel in airway smooth muscle by nitric oxide: involvement of a nitrothiosylation mechanism? *Am. J. Respir. Cell Mol. Biol. 19*:485.

244. Jeremy, R. W. and H. McCarron. 2000. Effect of hypercholesterolemia on Ca(2+)-dependent K(+) channel-mediated vasodilatation in vivo. *Am. J. Physiol. Heart Circ. Physiol. 279*:H1600.

245. Okayama, Y., A. S. Kirshenbaum, and D. D. Metcalfe. 2000. Expression of a functional high-affinity IgG receptor, Fc gamma RI, on human mast cells: Up-regulation by IFN-gamma. *J. Immunol. 164*:4332.

246. Tkaczyk, C., I. Villa, R. Peronet, B. David, and S. Mecheri. 1999. FcepsilonRI-mediated antigen endocytosis turns interferon-gamma-treated mouse mast cells from inefficient into potent antigen-presenting cells. *Immunology 97*:333.

247. Selvaraj, N. G., E. Omi, G. Gibori, and M. Rao. 2000. Janus kinase 2 (JAK2) regulates prolactin-mediated chloride transport in mouse mammary epithelial cells through tyrosine phosphorylation of Na^+-K^+-$2Cl^-$. *Mol. Endocrin. 14*:2054.

Chapter 2

Interferon-gamma modulates chloride ion flux in mast cells

Introduction

Mast cells (MC) are found widely distributed in tissues and contribute to regulation of inflammatory responses and ongoing modulation of the vasculature (1,2). When an allergen enters the body, its multivalent epitopes cross-link IgE molecules bound to MC surface Fcε receptors, mobilizing second messengers such as inositol 1,4,5-triphosphate or sphingosine-1-phosphate resulting in Ca^{2+} release from internal stores (3-5). The resulting depletion of Ca^{2+} stores activates Ca^{2+} channels in plasma membranes and initiates entry of extracellular Ca^{2+}, further increasing intracellular Ca^{2+} concentration ($[Ca]_i$) to levels necessary for MC exocytosis (6,7). High intracellular Ca^{2+} results in degranulation and release of preformed mediators such as histamine, proteases, and TNF, as well as newly-synthesized mediators including prostaglandin D_2 and leukotrienes (8,9).

When influx of Ca^{2+} depolarizes the membrane beyond electrochemical and concentration equlibrium for Cl^- (E_{Cl}), Cl^- follows Ca^{2+} into the cell and may balance the inward positive charge, allowing maximum increases in intracellular Ca^{2+} concentration (10-13). The electrochemical equilibrium for Cl^- is calculated from the Nernst equation to be approximately –60 mV in MC (14). At least two independent studies have estimated intracellular $[Cl^-]$ to be approximately 13.4 to 29 mM (11,14) suggesting that in physiological buffer, this electrochemical gradient would cause an influx of Cl^- into the cell when Cl^- channels are open. The Cl^- channel blocker, 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) inhibits both Cl^- current and mediator release from MC (15) suggesting that Cl^- flux may be important during MC activation. Anti-allergic drugs such as cromolyn and nedocromil inhibit mediator release and Cl^- ion flux in MC (16,17).

Interferon-gamma (IFN-γ), an important cytokine in host defense inhibits mediator release from MC (18-21) by mechanisms poorly understood. One possibility is that IFN-γ inhibits mediator release from MC by changing the phenotype of the cell such that Cl^- ion flux is altered. We hypothesized that resting MC Cl^- flux was altered in IFN-γ-treated MC. Therefore, the aim of this study was to characterize Cl^- ion transport in resting MC and its sensitivity to IFN-γ treatment. *In* vivo-derived rat peritoneal MC

(PMC) and rat cultured MC (RCMC) were treated with IFN-γ and changes in Cl^- uptake were measured. As well, indirect factors that may affect changes in Cl^- flux were also measured such as changes in cell volume and surface charge. These studies reveal that IFN-γ treatment of MC decreases Cl^- uptake in resting MC, perhaps reflecting profound phenotypic changes.

Materials and Methods

Materials and Reagents

The fluorescent indicators N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE) and 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ) were purchased from Molecular Probes (Eugene, OR). Triton X-100, solubilized L-α-phosphatidylcholine lipids from soybeans, heparinase and poly(diallyldimethyl-ammonium chloride) (Cat-floc; MW= 400 000 g/mol), silicone oil (density of 1.05 g/mL), phenylmethylsulfonyl fluoride (PMSF), 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), sodium nitroferricyanide (SNP), and toluidine blue (TB) were purchased from Sigma (St. Louis, MO).

Rats and PMC Isolation

Male Sprague Dawley rats (300-350 g; Charles River, St. Constant, Quebec, Canada) were housed in a pathogen-free viral antibody-free facility. For MC sensitization, rats were infected with 3000 *Nippostrongylus brasiliensis* as previously described (22). Peritoneal MC (PMC) were isolated from sensitized rats $\geq$ 4 weeks after infection. Rats were sacrificed by cervical dislocation under anesthesia and PMC were isolated by the following procedure: 20 mL of ice-cold Hepes Tyrode's buffer (HTB) was injected into the peritoneal cavity and massaged gently for 30 sec; the peritoneum was opened and the buffer collected with a transfer pipette and kept on ice or at 4°C for all subsequent procedures. Following centrifugation at 200 g for 5 min the cell pellet was resuspended in 5 mL of fresh HTB and layered on top of a 30%/80% Percoll gradient. The gradient was centrifuged at 500 g for 20 min and the highly enriched MC were

collected from the pellet (23). PMC were >98% pure and >96% viable as measured by trypan blue exclusion.

Worm antigen used to stimulate PMC was prepared as follows: rats were infected with 6000 *Nippostrongylus brasiliensis* as previously described (22). Two weeks after infection, rats were sacrificed by cervical dislocation under anesthesia and adult worms were isolated from the small intestines. Adult worms were incubated in HTB at 1000 worm equivalents (WE)/mL for 24 hr at 37°C. Supernatants were isolated and stored at -20°C.

Cell Culture

The rat MC line, RCMC 1.11.2 (kindly provided by B. Chan and A. Froese, Winnipeg, Manitoba), was established from Wistar-ICI rats (24). It was cultured in RPMI 1640 medium containing 5% FBS (Gibco BRL), 100 U/mL penicillin, 100 μg/mL streptomycin and 10 mM Hepes. The cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C.

$^{36}Cl^-$ Flux Measurements

Changes in $[Cl^-]_i$ were measured by incubating 1 x 10^6 cells/mL MC suspension with 8.7 mM $Na^{36}Cl$ (ICN; stock = 1.07 M, 0.867 mCi/mL or 12.0 mCi/g) in flux buffer (137 mM NaCl, 4 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 20 mM HEPES, 1 mg/mL BSA, 1 mg/ml glucose) at 37°C for 30 min. The incubation was terminated by transferring 100 μL of the cell suspension onto 120 μL of silicone oil in long, thin Eppendorf tubes. The tubes were centrifuged in a microfuge at 18,000 *g* for 30 sec and then placed into a freezing methanol bath until frozen. The bottom of each tube was cut off and placed into a scintillation vial with 48 mM NaOH. Each scintillation vial was vortexed for 1 min after which 5 mL of scintillation fluid was added and the vial was counted using a Beckman scintillation counter. $^{36}Cl^-$ uptake was calculated based on the specific activity of $^{36}Cl^-$ in the extracellular medium. The specific activity was calculated as follows:

$$\frac{[\text{extracellular } Cl^- \text{ added}] + [{}^{36}Cl^- \text{ added}]}{\text{radioactivity of added } {}^{36}Cl^-} = \text{specific activity (nmole/cpm)}$$

All values of $^{36}Cl^-$ uptake were corrected for $^{36}Cl^-$ trapped in the extracellular space. $^{36}Cl^-$ trapped in the extracellular space was determined by measuring cpm at time zero, immediately after the addition of $^{36}Cl^-$ (50 $\pm$ 10.2 cpm). Intracellular Cl^- concentration was calculated under the assumption that intracellular water is 1.3 $\mu L/10^6$ cells (14). Details of calculations are in Appendix A.

Cell Volume Measurements Using Flow Cytometry

Single cell suspensions of 10^5 PMC and RCMC were washed with HTB and resuspended in PBS at 1 x 10^6 cells/300 µL. Forward scatter and side scatter measurements were obtained using a Becton Dickinson flow cytometer to assess cell volume.

Liposome Preparation

Membrane proteins from RCMC were isolated by suspending 80 x 10^6 cells in solution A (60 mM mannitol, 5 mM HEPES, 175 µg/mL PMSF). Cell suspension in solution A was homogenized in an Ultrasonic homogenizer on ice for approximately 5 min. The homogenate was placed in a microfuge tube and spun at 1000 g for 15 min at 4°C. The supernatant was removed and spun for 1 hr at 100 000*g* at 4°C. The pellet, which contains membrane proteins, was resuspended at 4 mg/mL (as measured using a BioRad protein concentration calculation kit) and immediately placed on ice or stored at –70°C.

Asolectin lipids (Sigma) were resuspended at 50 mg/mL in a buffer containing 100 mM Na-gluconate, 45 mM *N*-methyl-D-glucamine gluconate (NMG-gluconate), 5 mM K-gluconate and 10 mM Hepes (pH 7.4), with 0.02% NaN_3 and 1.5 % CHAPS. Lipid aliquots were mixed with proteins and 100 µl of 100 mM SPQ (final volume 1 mL, containing 2 mg protein, 10 mg lipid and 10 mM SPQ). The mixture was incubated for 20 min at 4°C, followed by the removal of CHAPS and extravesicular SPQ by gel filtration on a Sephadex G-50-80 column (1.5 x 90 cm^2). During reconstitution, SPQ was trapped within the vesicles, and the dye-loaded vesicles eluted in the void volume. The

diameter of the vesicles was 176 ± 35 nm (mean ± S.D., n=3) as estimated by laser light scattering using a Brookhaven BI-90 particle size analyser.

Liposomes were added to a cuvette containing E buffer (10 mM HEPES-Tris, 0.02% NaN_3, 50 mM KCl, 100 mM Na-gluconate, pH 7.4) and fluorescence readings were made in a SLM 8000C spectrofluorimeter (SLM, Urbana, IL, USA) at 37°C. At indicated times valinomycin (4 μM final concentration), a potassium ionophore, was added to facilitate the flow of K^+ ions into the liposome.

MQAE Loading and Fluorescent Measurements of Intact MC to Monitor Cl^- Flux

Isolated RCMC and PMC were resuspended at 2 x 10^6 cells/mL in loading buffer (130 mM Na-gluconate, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES) containing 10 mM MQAE. Osmolarity of the loading buffer was 279 mOsmol/kg. Cells were incubated with MQAE for 5 min at 37°C, determined to be optimal for loading. Cells were washed twice with loading buffer and resuspended in loading buffer before they were placed in the spectrofluorimeter.

In the presence of gentle stirring an initial fluorescence reading of RCMC and PMC at 460 nm was taken. After the initial reading, in the presence of gentle stirring, 50 mM KCl or NaCl (final concentration in 2 mL) was added and the fluorescent measurements taken every 0.1 second. At the end of the experiment, 10 μL of Triton X-100 was added to lyse the cells. The intracellular [Cl^-] was calculated using the Stern-Volmer equation:

$$(F_o/F) - 1 = K_{sv}\,[Cl]_i$$

where F_o is the fluorescence intensity without halide, F is the fluorescence intensity in the presence of quencher, and K_{sv} is the Stern-Volmer constant. The K_{sv} for MQAE and SPQ in solution is 200 and 118 respectively but K_{sv} for Cl^- quenching in cells is often reduced by at least a factor of 10 or more (see Appendix A). The absorption and fluorescence emission wavelength for MQAE is 350 nm and 460 nm respectively. The absorption and fluorescence emission wavelength for SPQ is 344 nm and 443 nm respectively (Molecular Probes specifications). The bandwidth utilized was ± 5 nm.

Antigen Stimulation and β-hexosaminidase assay

PMC were resuspended in HTB (140 mM [Cl^-]) and stimulated with either 48/80 or 10 worm equivalents (WE) per mL of antigen for 30 min unless indicated otherwise. PMC were pelleted at 200 g and the supernatant was removed. Pellet and supernatant samples were assayed for β-hexosaminidase (β-hex) by hydrolysis of the fluorescent substrate 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (Sigma M-2133). One unit of enzyme cleaves 1 μmol of substrate/hr at 37°C (23). Pellet samples were frozen and thawed three times to lyse cells (total cell lysate). Pellet and supernatant samples (50 μL) were added in duplicate to wells in a microtitre plate followed by 1 mM substrate (50 μL) and the mixture was incubated at 37°C for 2 hr. The reaction was terminated by the addition of 100 μL of 0.2 M Tris base and the optical density was read at 450 nm (excitation 356 nm). The OD_{450nm} for blank wells, containing only substrate and Tris base, was automatically subtracted from the sample OD_{450nm} and the percentage β-hex release was calculated by the formula:

$$\frac{OD_{450nm} \text{ of substrate samples}}{OD_{450nm} \text{ of pellet samples} + OD_{450nm} \text{ of supernatant samples}} \times 100 = \% \text{ release}$$

The spontaneous release (in HTB) was subtracted from all samples to give % specific release.

Determination of cell surface charge

Cell surface charge measurements were performed as described previously (25). RCMC and PMC were washed with 0.25 M sucrose by centrifugation (200 *g*, 5 min), resuspended in 0.25 M sucrose at approximately 1 x 10^6 cells/mL. Cells were incubated with 20μL of 0.1% (w/v) toluidine blue (TB) and the absorbance at 525 nm (bound) and at 630 nm (non-bound) was recorded on a Beckmann spectrophotometer. The cells were titrated using 0.02% (w/v) Cat-floc solution (CFC; MW = 400 000 g/mol) which displaces TB at the cell surface, producing a shift in the maximum absorbance from 525 nm to 630 nm. The ratio $A_{630}/(A_{525} + A_{630})$ was used to calculate a relative amount of

non-bound TB in the cell suspension. The amount of CFC necessary to displace TB was calculated according to the equation:

$$\frac{V_e C_t}{V} = [SC\text{-}TB] + [SC^-]$$

or $v[TB^+] = K_a (V_e + v)$ at the equivalence point.

where V is the total volume of the reaction, C_t is the concentration of CFC, v is the volume of CFC added and V_e is the volume of titrant added at equivalence point. The plot of $v[TB^+]$ versus v is a straight line, where K_a is the slope and V_e is the intercept with the X-axis. The total negative charge of the cell suspension was determined from the equivalence volume V_e, using 33.9 μeq/mg as a CFC charge concentration (see Appendix B). This result was converted to the number of charges per cell using cell concentration and Avagadro's number.

Charge density (mC/m^2) was calculated assuming PMC diameter of 19.6 μm (26) and a RCMC diameter of 9.7 μm (24).

Statistical Analysis

Where appropriate, results are expressed as means ± SEM. Values were analyzed by ANOVA or student t test as indicated and significant values represent $P<0.01$.

Results

IFN-γ downregulates MC β-hexosaminidase release

Previous studies have shown that IFN-γ downregulates MC release of preformed mediators such as serotonin and histamine and newly synthesized mediators such as arachidonic acid and TNF (18-21). In these studies, doses of IFN-γ used were comparatively high (> 100 U/mL). Therefore, initial experiments were conducted to determine if the inhibitory effect of IFN-γ also occurred at lower, and arguably more physiological concentrations of 10, 50 and 100 U/mL. PMC were isolated from rats sensitized with *Nippostrongylus brasiliensis* and enriched to >99% purity. PMC were pretreated with 10, 50, 100 and 1000 U/mL of IFN-γ for 24 hr, washed, stimulated with

antigen for 30 min and the release of the stored mediator, β-hex was measured. MC viability was >96%.

MC stimulated with 25 WE/mL of antigen released 31.5 ± 2.4 % of stored β-hex. Spontaneous β-hex release of unstimulated MC was 6.4 ± 0.7 %. Fig 2.1 shows that PMC pretreated with IFN-γ released 32% less β-hex (than untreated) even at the relatively low dose of 10 U/mL (27.6 ± 3.7 %). IFN-γ dose-dependently inhibits β-hex release when MC were stimulated with antigen. Inhibition of β-hex release reached a maximum at 100 U/mL (70.2 ± 2.1 % inhibition) and remained high even with 1000 U/mL of IFN-γ (71.8 ± 1.3 % inhibition). MC pretreated with 10 U/mL of IFN-γ and stimulated with a lower dose of antigen (10 WE/mL) showed a 53.5 ± 11.9 % inhibition of β-hex release.

MC and MC membranes are capable of Cl^- ion transport

To determine whether resting PMC and RCMC were capable of Cl^- ion transport and whether IFN-γ treatment had any effect on the rate of Cl^- ion flux, PMC and RCMC were loaded with 10 mM of MQAE, a membrane-permeable fluorescent dye quenched in the presence of Cl^- ions.

PMC and RCMC were loaded with MQAE in the presence of low $[Cl^-]$ (4 mM) so that quenching by $[Cl^-]_i$ was decreased. The ability of exogenously added KCl to quench the MQAE dye was then measured in a spectrofluorometer in real time. The rate of quenching is a measure of the Cl^- flux across the plasma membrane. A representative experiment of untreated RCMC (n=10) is shown in Fig 2.2A. When the MQAE-loaded cells are placed in the spectrofluorometer, they have a high initial fluorescence (arbitrary fluorescence units). Upon the addition of 40 μL of 2.5 M KCl (50 mM final concentration), the MQAE fluorescence is quenched in less than 5 min, causing a dramatic drop in fluorescence (a reduction of 50 %; Fig 2.2A). Triton X-100 was added at the end of the experiment to lyse the cells and quench the remaining intracellular MQAE (Fig 2.2A). Using the Stern-Volmer equation and a K_{sv} of 20, the intracellular $[Cl^-]$ is calculated to be 16.5 mM ± 2.5 (n=8).

IFN-γ treated MC showed a different quenching profile (Fig 2.2B). Upon the addition of KCl (50 mM final concentration) to IFN-γ-treated, MQAE-loaded MC, there was a gradual decrease (a reduction of 2 %) in fluorescence as the Cl^- ions entered the cell and quenched the MQAE (Fig 2.2B). Therefore, the rate of Cl^- influx of IFN-γ treated MC is slower than Cl^- influx in untreated MC (Fig 2.2A and 2.2B). The Stern-Volmer equation estimates the intracellular [Cl^-] for IFN-γ treated cells is 3.33 mM $\pm$ 1.4.

MQAE is a membrane permeable fluorochrome that may leak out of the cell during flux measurements. Similarly, the concentration of MQAE within each cell population is difficult to predict and therefore these measurements are only rough estimates of Cl^- flux in the cell population as a whole. Therefore, to ensure that MQAE quenching observed in Fig 2.2A and B was not due to fluorochrome leakage and to reduce variations in background fluorescence readings, membrane proteins from RCMC were isolated and used to construct liposomes. These liposomes were made in the presence of SPQ such that the inside of the resulting liposome contained 10 mM SPQ. Liposomes were placed into a cuvette containing 50 mM NaCl buffer (time=0) and fluorescence measurements were taken for 150 sec (Fig 2.3).

In the presence of 50 mM NaCl, the SPQ fluorescence was quenched gradually as Cl^- ions moved into the liposome, presumably via MC proteins present in the liposome membrane. At the indicated time, 4 μM valinomycin, a K^+ ionophore, was added to facilitate the influx of K^+ ions ($[K]_i$=12.5 mM and $[K]_e$=50 mM) and increased the electrochemical gradient for Cl^- ions ($[Cl^-]_i$=0 mM and $[Cl^-]_e$=50 mM). The resulting gradient, activated further Cl^- ion influx into the liposome and resulted in quenching of SPQ (Fig 2.3). Triton lysed the liposomes and quenched the remaining SPQ present within the lipsosome. Liposomes made in the absence of protein were incapable of quenching with NaCl.

IFN-γ reduces Cl^- uptake at equilibrium

Measurements using fluorochromes revealed a discernable IFN-γ effect on the rate of Cl^- ion transport and suggested that perhaps IFN-γ-treated MC displayed altered Cl^- uptake. To determine whether IFN-γ effected Cl^- uptake in MC, Cl^- uptake was measured

before and after IFN-γ treatment. Treated and untreated PMC and RCMC were incubated with ^{36}Cl for 30 min (enough time to reach equilibrium (11)) and washed. The amount of radioactivity present in the cells was measured in a scintillation counter and was an indication of Cl^- uptake at equilibrium. Fig 2.4A shows that IFN-γ treatment at 10, 100, 800 and 1000 U/mL decreases the Cl^- uptake of *in vivo*-derived PMC from 50.2 ± 2.6 nmole/10^6 in untreated cells to 28.5 ± 2.1 nmole/10^6, 27.7 ± 1.2 nmole/10^6, 24.4 ± 1.7 nmole/10^6, and 36.2 ± 3.2 nmole/10^6 respectively. A time course analysis of PMC Cl^- uptake shows that IFN-γ treatment does not have an effect at the earlier treatment points of 10 min and 2 hr (45.5 ± 6.2 nmole/10^6 in untreated cells compared to 62.8 ± 2.0 nmole/10^6 and 31.3 ± 7.1 nmole/10^6 respectively) but decreases Cl^- uptake at 20 and 24 hr (17.2 ± 3.0 nmole/10^6 and 22.7 ± 1.8 nmole/10^6 respectively; Fig 2.4B).

Similarly, measurements using RCMC show that IFN-γ treatment for 24 hr decreases Cl^- uptake by up to 50% (Fig 2.4C). IFN-γ treatment of RCMC at 100, 400 800 and 1000 U/mL decreases the Cl^- uptake from 38.4 ± 2.7 nmole/10^6 in untreated cells to 28.1 ± 4.0 nmole/10^6, 23.0 ± 2.6 nmole/10^6, 21.2 ± 2.4 nmole/10^6, and 20.6 ± 3.1 nmole/10^6 respectively (Fig 2.4C). Like the PMC in Fig 2.4B, a time course analysis of Cl^- uptake by RCMC 0-shows that IFN-γ does not have an effect at the earlier treatment points of 10 min and 30 min of IFN-γ treatment (37.0 ± 5.4 nmole/10^6 in untreated cells compared to 29.0 ± 2.6 nmole/10^6 and 32.8 ± 5.1 nmole/10^6 respectively), but decreases Cl^- uptake at 2, 20 and 24 hr (20.9 ± 3.4 nmole/10^6, 17.5 ± 2.0 nmole/10^6 and 19.4 ± 3.2 nmole/10^6 respectively; Fig 2.4D). Furthermore, PMC have 26% higher uptake of Cl^- at rest than RCMC.

IFN-γ reduces Cl^- uptake within first 30 min

The Cl^- uptake data in Fig 4 suggested that IFN-γ might inhibit the immediate uptake of Cl^- within the first 30 min of incubation with $Na^{36}Cl$. RCMC were treated with IFN-γ (800 U/mL for 24 hr) or sham treated for 24 hr and then incubated in the $Na^{36}Cl$-containing buffer (see methods) for 1 to 30 min (Fig 2.5). Compared to untreated cells, IFN-γ inhibits Cl^- influx at all time points. At 30 min, the untreated MC have a

significantly higher uptake of Cl^- (40.1 $\pm$ 1.3 nmoles/10^6 cells) than IFN-γ-treated cells (29.5 $\pm$ 5.9 nmoles/10^6 cells; $P<0.01$).

Changes in Cl^- uptake are inhibitable by DIDS but not by nitric oxide

Cl^- flux may be dependent upon anion channel function. Therefore, to determine whether anion channels were involved in Cl^- uptake, MC were preincubated with DIDS (30 μM), a general anion channel inhibitor, for 15 min, and then Cl^- uptake was measured. MC were washed and incubated with $^{36}Cl^-$ for 30 min. After incubation period, PMC were spun through oil to remove excess $^{36}Cl^-$ and radioactivity of cell pellets was measured in a scintillation counter (Fig 2.6A). DIDS inhibited Cl^- uptake by 54 % (from 50.6 $\pm$ 3.0 nmole/10^6 cells to 23.4 $\pm$ 3.2 nmole/10^6 cells). IFN-γ inhibited Cl^- uptake by 55.1 $\pm$ 1.8 %. However, despite their independent effects, when DIDS and IFN were used together, they decreased Cl^- uptake by 23.9 $\pm$ 0.8 % compared to untreated cells ($P< 0.01$).

One of the messengers of IFN-γ signaling is nitric oxide (NO). IFN-γ upregulates nitric oxide synthase (NOS) resulting in an increase in NO production (27). To determine whether IFN-γ-mediated inhibition of $^{36}Cl^-$ uptake was NO-mediated, MC were preincubated with SNP (50 μM), a NO donor for 30 min (Fig 2.6B). The SNP-treated MC showed no discernable change in Cl^- uptake compared to untreated cells.

To determine the effect of antigen-stimulation on $^{36}Cl^-$ uptake, PMC were either pretreated with IFN-γ (800 U/mL) for 24 hr and then incubated in buffer containing antigen (10 WE/mL) and $^{36}Cl^-$. After 30 min, cells were spun through oil and the radioactivity of the cell pellets was measured. IFN-γ treated MC show a net decrease of $^{36}Cl^-$ uptake (24.0 $\pm$ 3.3 nmole/10^6 cells) compared to untreated cells (53.1 $\pm$ 7.8 nmole/10^6 cells). Antigen-stimulated PMC (after 30 min) show a net decrease in $^{36}Cl^-$ uptake (28.2 $\pm$ 0.4 nmole/10^6 cells). IFN-γ treated (24 hr) PMC stimulated with antigen (30 min) also show a net decrease in $^{36}Cl^-$ (24.2 $\pm$ 2.6 nmole/10^6 cells) compared to untreated.

IFN-γ changes in Cl^- uptake are independent of cell volume changes

Changes in net intracellular ion content and Cl^- uptake can be dependent upon changes in cell volume. Thus, a decrease in Cl^- uptake may reflect a decrease in cell volume. Therefore, forward scatter and side scatter measurements were taken of untreated PMC and RCMC (Fig 2.7A and C) and the same cells treated with IFN-γ (Fig 2.7B and D respectively). These measurements show that there is little change in cell volume between IFN-γ treated and untreated populations. Therefore, it is unlikely that observed changes in Cl^- uptake with IFN-γ treatment reflect changes in cell volume.

Cell size measurements were confirmed using a coulter counter since this instrument provides a different kind of volume measurement. While the flow cytometer measures granularity (side scatter) and size (forward scatter), the coulter counter measures the impedance created when a cell displaces a volume of ions proportionate to its volume. Coulter counter measurements in Fig 2.8 support the flow cytometry results that there is no detectable change in cell volume after IFN-γ treatment.

IFN-γ decreases cell surface charge

Interferons can change fluidity of the cell membrane (28) and alter the cholesterol/phospholipid ratio (29). Cl^- ion flux through Cl^- channels is largely dependent upon other ions in the vicinity of the channels, charge of the residues within the pore opening and the charge of the surrounding membrane (30). Thus, flux of Cl^- ions and changes in $[Cl^-]_i$ could be affected by changes in the surface charge of the cell. To measure the surface charge of PMC and RCMC before and after IFN-γ treatment, cells were titrated with the polycationic colloid Cat-Floc using TB as an indicator. Cell surface charge determination was performed with 1×10^6 cells with 10 μM TB.

Titrations show that RCMC treated with 100 U/mL of IFN-γ for 24 hr have a lower surface charge density (9.3 ± 1.6 mC/m^2) than untreated cells (32.8 ± 10.0 mC/m^2; Fig 2.9A and C). Treatment with 10 U/mL of IFN-γ had no significant effect on surface charge density (25.2 ± 11.8 mC/m^2). Similarly, PMC treated with 100 U/mL of IFN-γ for 24 hr also have a lower surface charge density (32.1 ± 1.9 mC/m^2) than untreated cells

(46.75 $\pm$ 6.40 mC/m^2; Fig 2.9B and D). Colloid titration is a general measurement of net surface charge density in a cell population and therefore this difference reflects an overall change in the entire cell population. Calculations of surface charge density assumed a PMC diameter of 19.6 μm (26) and a RCMC diameter of 9.7 μm (24).

Discussion

Our data shows that IFN-γ alters quenching of MC loaded with a [Cl^-] sensitive fluorochrome and inhibits $^{36}Cl^-$ uptake of MC compared to untreated MC. This effect is abrogated by DIDS but unaffected by NO. IFN-γ inhibition of $^{36}Cl^-$ uptake is independent of changes in cell volume but is accompanied by a decrease in cell surface charge. Therefore, this study characterized the effect of IFN-γ on Cl^- flux, an elusive and complex component of MC function.

IFN-γ has several effects on MC function including dose-dependent inhibition of β-hex release. We postulated that IFN-γ also modulated Cl^- flux in resting MC and we have shown that IFN-γ inhibits Cl^- uptake, an effect sensitive to the anion channel inhibitor, DIDS. DIDS inhibits a variety of anion channels, including Cl^- channels (31,32). Therefore, a possible mechanism of IFN-γ inhibition of Cl^- influx is via modulation of anion channels, including Cl^- channels.

Cl^- channels regulate cell volume, membrane potential, pH and osmolarity and are classified according to their mechanism of activation. Cl^- currents in resting rat MC have been measured by patch clamp and $^{36}Cl^-$ influx studies (11,33,34). A recent study of a human MC line show that MC express a Cl^- current activated by hypotonicity, suggesting the presence of ClC3 (35). Resting MC express outwardly rectifying voltage- and Ca^{2+}-dependent Cl^- fluxes (35). Our results support these studies and indicate that resting MC display a Cl^- current. MC loaded with MQAE show quenching of this fluorochrome in the presence of extracellular Cl^-, indicating that Cl^- ions can transverse the membrane and quench the fluorochrome. Quenching of MQAE in untreated MC occurs in a rapid manner. This pattern suggests the presence of channels (or transporters) capable of shuttling Cl^- ions, including specific or non-specific anion channels.

IFN-γ-treated MC show a slower rate of $^{36}Cl^-$ uptake compared to untreated MC, perhaps reflecting changes in channel activity and/or expression. However, antigen-stimulated MC also show a decreased net $^{36}Cl^-$ uptake after 30 min. Antigen stimulation decreases MC volume and changes cell shape and granule content (36) and decreases in cell size may ultimately decrease the amount of Cl^- ions present in the cytoplasm. Therefore, decreased $^{36}Cl^-$ uptake may not be a consequence of secretion but the result of cell volume changes. Friis et al., however, found that antigen stimulation increases the rate of $^{36}Cl^-$ uptake in MC but results in the same amount (approximately 30 nmole/10^6 cells) of $^{36}Cl^-$ uptake after 30 min as untreated cells (11). These authors suggest that Cl^- uptake is not a consequence of secretion since removal of extracellular Ca^{2+} produces a marked reduction of antigen-stimulated histamine secretion but does not change $^{36}Cl^-$ uptake. In our study we also observed that antigen-stimulated MC took up approximately 30 nmole/10^6 cells of $^{36}Cl^-$ after 30 min. However, untreated cells took up between 55-60 nmole/10^6 cells of $^{36}Cl^-$ after 30 min, twice more than in the Friis study (11). These differences could reflect slight differences in isolation technique or stimulation procedures.

Liposomes made to contain membrane proteins from MC are also capable of transporting Cl^- ions and quenching SPQ. The addition of valinomycin to the liposomes (100 μM final concentration) induced a quenching of SPQ. Valinomycin is a repeating cyclic molecule made of L-lactate, L-valine, D-hydroxyisovalerate and D-valine residues, which form a K^+ binding pore (37). This pore undergoes a conformational change upon binding K^+, which facilitates the unloading of K^+ ions into the cytoplasm (38). This process is dependent upon the concentration gradient of K^+ ions. Liposomes were made in buffer containing 5 mM K+ ions (I1 buffer) and when the liposomes were added to the cuvette buffer containing 50 mM K^+ (E buffer) valinomycin facilitated the influx K^+ into the liposome according to its concentration gradient. A build-up of K^+ within the liposome created an electrochemical gradient and facilitated the entry of Cl^- ions. Therefore, MC membrane proteins incorporated into the liposome (deficient in intracellular signaling molecules) transported Cl^- ions across the lipid membrane. Proteins incorporated into the liposome may include both plasma membrane and other

membrane proteins in the cell. Specific Cl^- channels may be one group of proteins capable of transporting Cl^- in these liposomes.

ClC are voltage-gated Cl^- channels expressed on the plasma membrane and on membranes of intracellular organelles. RT-PCR shows that RCMC express mRNA for ClC 2,3,5 and 7, while PMC express mRNA for ClC 7 (Chap 3). ClC 7 is a broadly expressed but poorly characterized ClC member (39) and is expressed by both PMC and RCMC. ClC 2, expressed only in RCMC, is involved with cell volume regulation and is thought to be ubiquitously expressed (40). However, ClC 2 is not expressed by PMC, a tissue-type MC, residing in the peritoneal cavity (Chap 3). ClC 3 is Ca^{2+}-sensitive and is mainly expressed in neuronal cells (Chap 3) (41). ClC 5 is the Cl^- channel mutated in Dent's disease and is mainly expressed in endocytic vesicles of proximal tubule cells of the kidney (42). MC express other Cl^- channels such as the cystic fibrosis transmembrane conductance regulator (CFTR; Chap 5) (43) and mRNA for subunits comprising the γ-aminobutyric acid receptor ($GABA_AR$) Cl^- channel (Chap 4). Either of these important Cl^- channels could also be responsible for the observed decrease in $[Cl^-]_i$ with IFN-γ treatment although CFTR is not inhibited by external DIDS (44).

IFN-γ decrease of Cl^- flux may involve changes in either Cl^- channel (or other Cl^--permeable channel) function and/or expression (Fig 10). IFN-γ modulates the function of a variety of cell types by regulating gene expression via activation of signal transducers and activators of transcription 1 (STAT1) (45). It is possible that IFN-γ regulates the expression of various ion channels, including Cl^- channels. In epithelial cells, IFN-γ downregulates CFTR expression and Cl^- current (46) and we have shown that MC express CFTR (43). CFTR is a cAMP-activated Cl^- channel that belongs to the gene family of ATP-binding cassette (ABC) transporters which generally function as active transporters driven by ATP hydrolysis (47). CFTR acts as a "conductance regulator," coordinating an ensemble of transmembrane ion fluxes in polarized epithelia (48). Several plasma membrane transport proteins are modulated by CFTR, including EnaC, an outwardly rectifying Cl^- channel, Ca^{2+}-activated and volume-activated Cl^- channels, the ROMK K^+ channel; anion exchangers and the aquaporin 3 water channel (48,49). MC express an outwardly rectifying Cl^- channel that may be a target of CFTR (50). The other

mechanism of IFN-γ-mediated action may be via direct modulation of ion channels. IFN-γ ligation of the IFN-γ receptor activates janus kinase activity which has been shown to modulate the Na^+-K^+-$2Cl^-$ cotransporter via tyrosine phosphorylation (51).

IFN-γ decreased in MC surface charge may also potentiate changes in Cl^- flux. Changes in surface charge of a cell can be caused by changes in fatty acid content, sialic acid content or protein expression (52). IFN-γ-induced changes in protein expression on the cell surface could consequently alter surface charge (Fig 2.10). However, it is also possible that IFN-γ alters plasma membrane fatty acid content and membrane fluidity. IFN-γ treatment can change the fatty acid content of the macrophage cell membrane increasing membrane fluidity (53). IFN-γ-mediated changes in plasma membrane cholesterol/phospholipid ratio can result in growth arrest or apoptosis in some cell types (29). IFN-γ-mediated changes in membrane fluidity could potentially effect the stoichiometric requirements for ClC opening since the integrity of the channel pore is largely dependent on the hydrophobic residues in the transmembrane regions, which comprise the pore.

It is difficult to speculate on the potentially complex interaction between IFN-γ and Cl^- in MC. However, our data have shown that MC are capable of transporting Cl^- ions and that IFN-γ decreases MC Cl^- uptake, independently of volume changes (Fig 2.10). IFN-γ-mediated decrease in Cl^- uptake is accompanied by a decrease in surface charge, possibly reflecting a change in plasma membrane composition and fluidity.

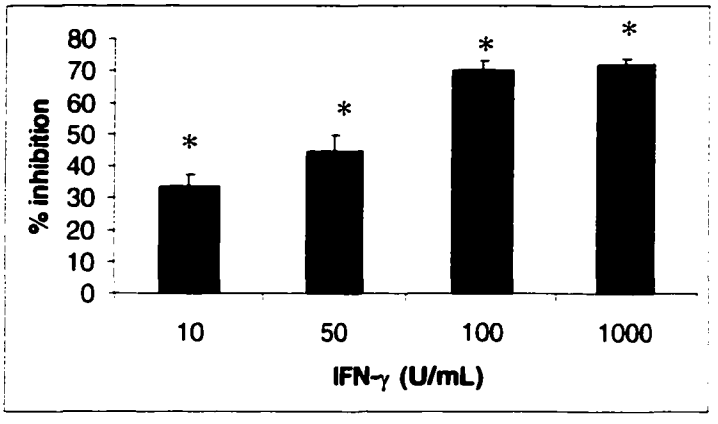


Figure 2.1. IFN-γ inhibition of β-hexosaminidase (β-hex) release from antigen-stimulated mast cells. PMC were isolated as described and treated with either 10, 50, 100 or 1000 U/mL of IFN-γ for 24 hr. After the treatment period, PMC were stimulated with antigen (25 WE/mL) for 30 min and β-hex release was measured. Antigen-induced release was 31.5 ± 2.4 %. These data represent n=3. (* represents significance of $P < 0.01$ compared to untreated)

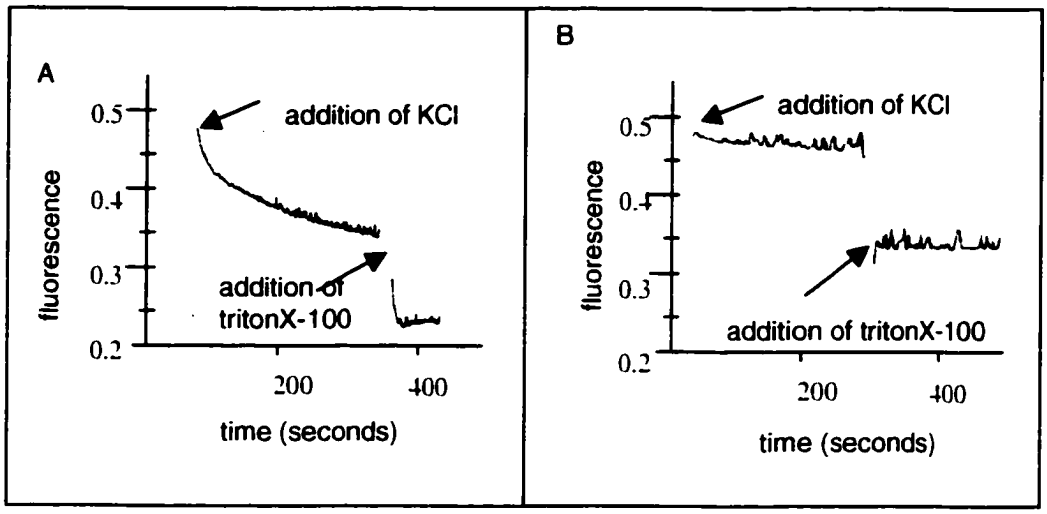


Figure 2.2. Cl^- flux measurements of intact MC. (A) Untreated RCMC loaded with MQAE show a gradual rate of fluorescent quenching (n=10). Rate of MQAE quenching is indicated by the slope of the change in fluorescence (n=3). (B) IFN-γ-treated RCMC loaded with MQAE show a very low rate of fluorescent quenching (n=10). Fluorescence units are arbitrary.

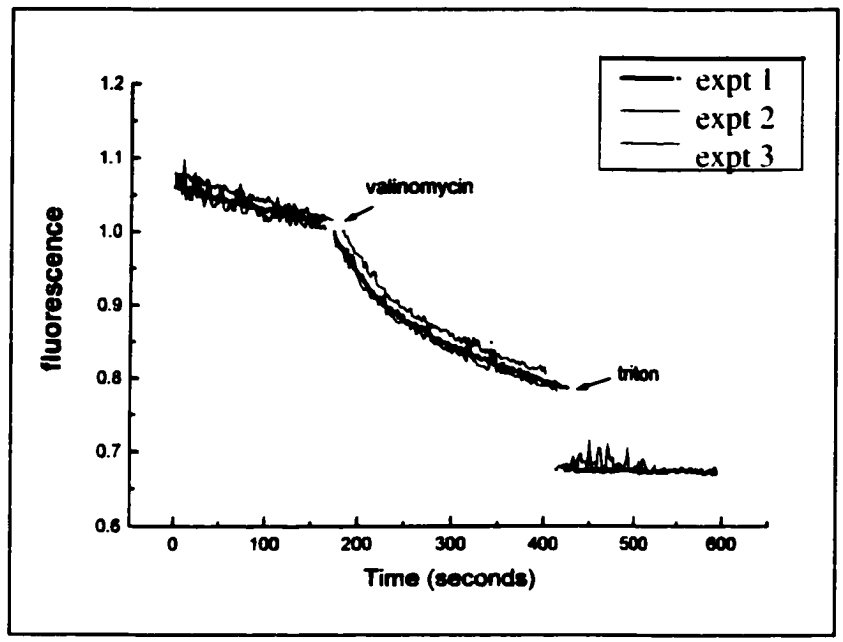


Figure 2.3. Cl^- flux measurements of liposomes containing RCMC membrane proteins. Liposomes placed in a buffer containing Cl ions and treated with valinomycin (4 μM) show a gradual decrease in fluorescence which indicates a slow rate of SPQ quenching. The three lines indicate three separate experiments (n=3). Liposomes made without MC protein did not show fluorescence quenching. Fluorescence units are arbitrary.

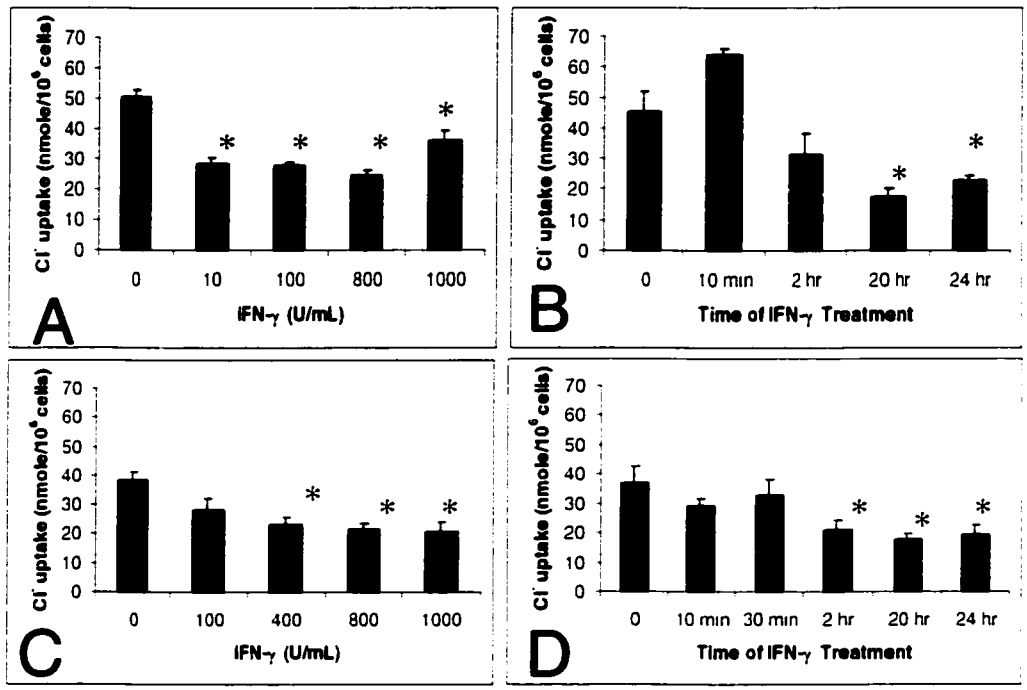


Figure 2.4. IFN-γ decreases Cl^- uptake. (A) PMC and RCMC (C) were isolated and treated with the indicated doses of IFN-γ for 24 hr. After treatment, MC were washed and incubated with ^{36}Cl for 30 min. Following this incubation, PMC were spun through oil to remove excess $^{36}Cl^-$ and radioactivity of cell pellets was measured in a scintillation counter. (B) PMC and RCMC (D) were isolated and treated with IFN-γ (800 U/mL) for the indicated times (n=5). * values are significant $P<0.01$ compared to untreated.

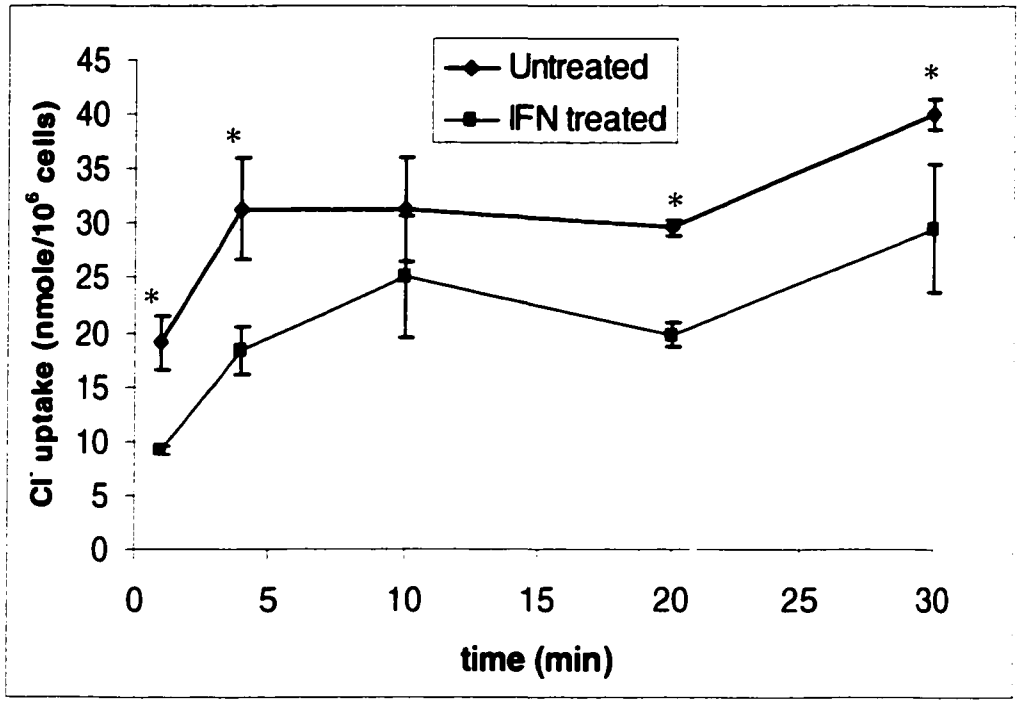


Figure 2. 5. IFN-γ decreases rate of $^{36}Cl^-$ uptake. RCMC were treated with 800 U/mL of IFN-γ for 24 hr washed and incubated with ^{36}Cl for 30 minutes. After incubation period, PMC were spun through oil to remove excess ^{36}Cl and radioactivity of cell pellets was measured in a scintillation counter. (n=5). * Values at each time point for untreated and IFN-γ-treated cells were compared using student t test and are significant ($P<0.01$) where indicated.

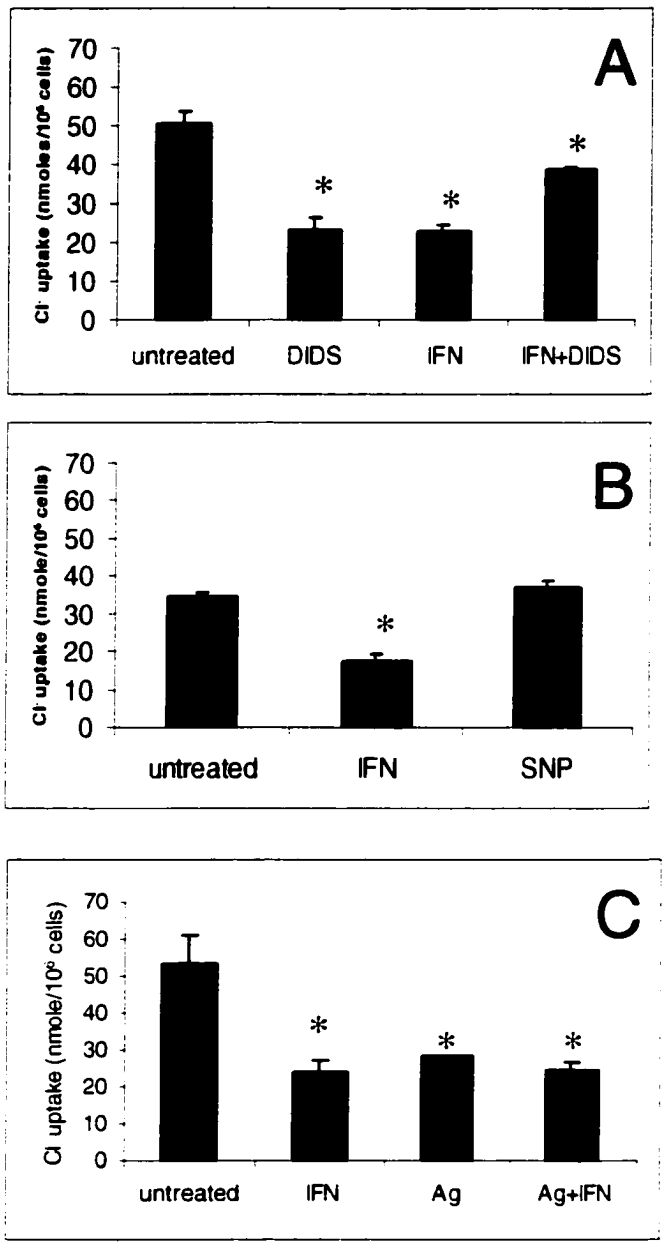


Figure 2.6. IFN-γ effect on Cl^- uptake is DIDS sensitive. (A) Peritoneal mast cells (PMC) were treated with IFN-γ (800 U/mL) for 24 hr, DIDS (30 μM) for 15 min or both (IFN–γ for 24 hr then DIDS for 15 min) and ^{36}Cl uptake was measured. MC were washed and incubated with ^{36}Cl for 30 min. After incubation period, PMC were spun through oil to remove excess ^{36}Cl and radioactivity of cell pellets was measured in a scintillation counter. (n=5). (B) Rat cultured mast cells (RCMC) were treated with IFN-γ (800 U/mL) for 24 hr or the NO donor SNP (50 μM) for 30 min and ^{36}Cl uptake was measured (n=3). (C) PMC were either pretreated with IFN-γ (800 U/mL for 24 hr) or untreated, then incubated with antigen (10 WE/mL) and ^{36}Cl for 30 min (n=3). * values are significant $P<0.01$ compared to untreated.

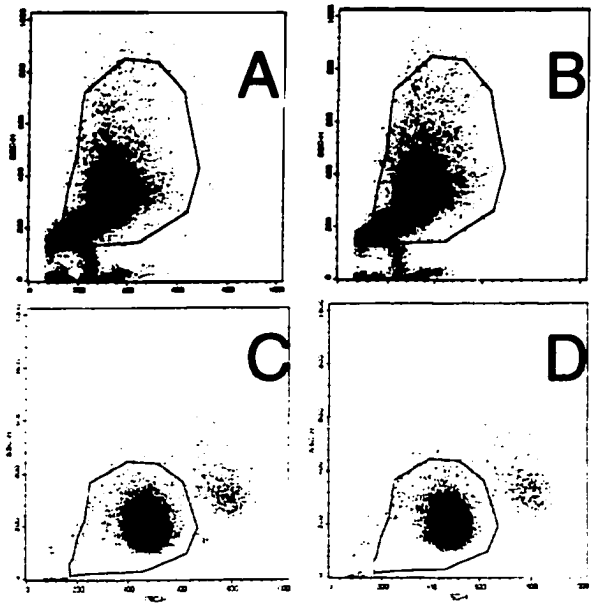


Figure 2.7. IFN-γ does not alter MC volume. PMC (A) volume does not change with IFN-γ treatment (B; 800 U/mL for 24 hr) as measured by light scatter on a flow cytometer. Untreated RCMC (C) and IFN-γ treated RCMC (D; 800 U/mL for 24 hr) also show no difference in volume as measured by light scatter on a flow cytometer. (n=5).

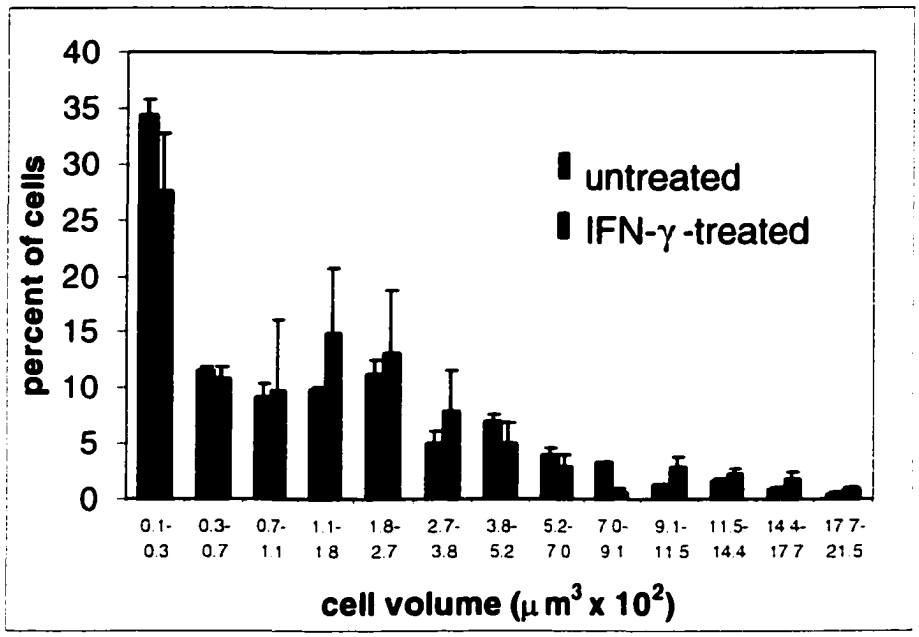


Figure 2.8. IFN-γ does not alter MC volume. RCMC were isolated and treated with IFN-γ (800 U/mL) for 24 hr or remained untreated. The cells were washed and placed in 0.9% saline and their size was counted in a coulter counter based on charge displacement (n=5).

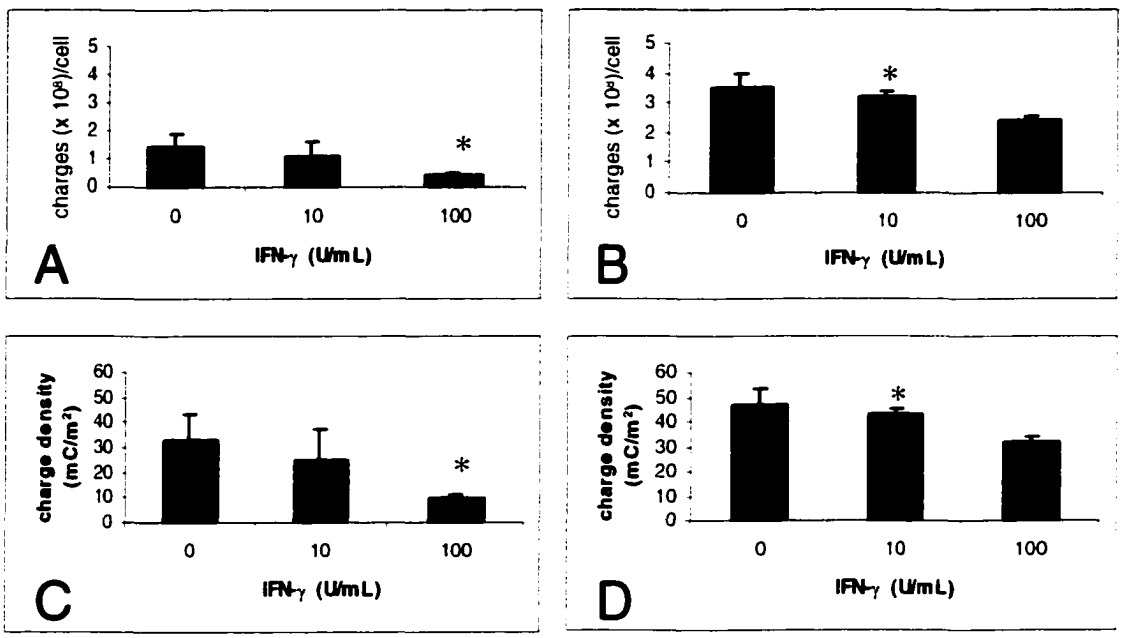


Figure 2.9. Surface charge measurements of untreated and IFN-γ-treated mast cells using the colloid titration method. (A) Surface charge of IFN-γ treated (0, 10 and 100 U/mL for 24 hr) RCMC (n=6). (B) Surface charge of IFN-γ treated (0, 10 and 100 U/mL for 24 hr) PMC (n=5). (C) Surface charge of RCMC shown in mC/m^2 (n=5). (D) Surface charge of PMC shown in mC/m^2 (n=5). * values are significant P>0.01.

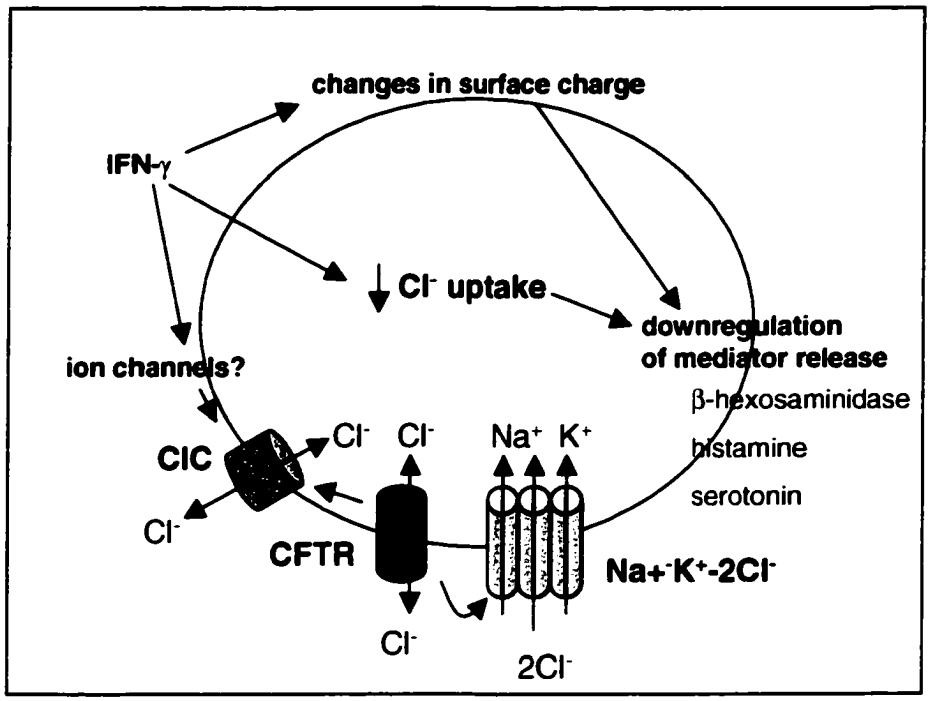


Figure 2.10. Conceptual model of IFN-γ effects on mast cell phenotypic changes. IFN-γ treatment of mast cells results in profound changes in Cl^- ion flux which decreases $[Cl^-]_i$ and may affect the mast cell's ability to release mediators. IFN-γ may influence ion channel expression and or function. These ion channels may include ClC, CFTR and/or the Na+-K+-2Cl- cotransporter. There is cross-talk between ion channels and IFN–γ modulation of one ion channel may have a cascade effect on others.

References

1. Lin, T. J. and A. D. Befus. 1999. Mast cells and eosinophils in mucosal defenses and pathogenesis. In *Mucosal Immunology*. 2nd ed. P.L. Ogra, J. Mestecky, M.E. Lamm, W. Strober, J. Bienenstock and J.R. McGhee, eds. Academic Press, San Diego, p. 469.

2. Hamelmann, E., K. Tadeda, A. Oshiba, and E. W. Gelfand. 1999. Role of IgE in the development of allergic airway inflammation and airway hyperresponsiveness--a murine model. *Allergy 54*:297.

3. Matthews, G., E. Neher, and R. Penner. 1989. Second messenger-activated calcium influx in rat peritoneal mast cells. *J. Physiol. (Lond) 418:105-30*:105.

4. Choi, O. H., J. H. Kim, and J. P. Kinet. 1996. Calcium mobilization via sphingosine kinase in signalling by the Fc epsilon RI antigen receptor. *Nature 380*:634.

5. Nakata, Y. and I. Hide. 1998. Calcium signaling and protein kinase C for TNF-alpha secretion in a rat mast cell line. *Life Sci. 62*:1653.

6. Zhang, L. and A. McCloskey. 1999. Immunoglobulin E receptor-activated calcium conductance in rat mast cells. *J. Physiol. 483*:59.

7. Suzuki, H., M. Takei, M. Yanagida, T. Nakahata, T. Kawakami, and H. Fukamachi. 1997. Early and late events in Fc epsilon RI signal transduction in human cultured mast cells. *J. Immunol. 159*:5881.

8. Penner, R., G. Matthews, and E. Neher. 1988. Regulation of calcium influx by second messengers in rat mast cells. *Nature 334*:499.

9. Lane, S. J. and T. H. Lee. 1996. Mast cell effector mechanisms. *J. Allergy Clin. Immunol. 98*:S67.

10. Reinsprecht, M., M. H. Rohn, R. J. Spadinger, I. Pecht, H. Schindler, and C. Romanin. 1995. Blockade of capacitive Ca2+ influx by Cl- channel blockers inhibits secretion from rat mucosal-type mast cells. *Mol. Pharmacol. 47*:1014.

11. Friis, U. G., T. Johansen, N. A. Hayes, and J. C. Foreman. 1994. IgE-receptor activated chloride uptake in relation to histamine secretion from rat mast cells. *Br. J. Pharmacol. 111*:1179.

12. Hoth, M., C. Fasolato, and R. Penner. 1993. Ion channels and calcium signaling in mast cells. *Ann. N. Y. Acad. Sci. 707*:198.

13. Meyer, G., S. Doppierio, P. Vallin, and L. Daffonchio. 1996. Effect of frusemide on Cl- channel in rat peritoneal mast cells. *Eur. Respir. J. 9*:2461.

14. Jensen, T. B., U. G. Friis, and T. Johansen. 1998. Role of physiological HCO3-buffer on intracellular pH and histamine release in rat peritoneal mast cells. *Pflugers Arch. 436*:357.

15. Romanin, C., M. Reinsprecht, I. Pecht, and H. Schindler. 1991. Immunologically activated chloride channels involved in degranulation of rat mucosal mast cells. *EMBO J. 10*:3603.

16. Alton, E. W. and A. A. Norris. 1996. Chloride transport and the actions of nedocromil sodium and cromolyn sodium in asthma. *J. Allergy Clin. Immunol. 98*:S102.

17. Reinsprecht, M., I. Pecht, H. Schindler, and C. Romanin. 1992. Potent block of Cl-channels by antiallergic drugs. *Biochem. Biophys. Res. Commun. 188*:957.

18. Bissonnette, E. Y., B. Chin, and A. D. Befus. 1995. Interferons differentially regulate histamine and TNF-alpha in rat intestinal mucosal mast cells. *Immunology 86*:12.

19. Bissonnette, E. Y., J. A. Enciso, and A. D. Befus. 1995. Interferon and antiallergic drug regulation of histamine and tumor necrosis factor-alpha in rat mast cell subsets. *Int. Arch. Allergy Immunol. 107*:156.

20. Holliday, M. R., E. M. Banks, R. J. Dearman, I. Kimber, and J. W. Coleman. 1994. Interactions of IFN-gamma with IL-3 and IL-4 in the regulation of serotonin and arachidonate release from mouse peritoneal mast cells. *Immunology 82*:70.

21. Coleman, J. W., M. G. Buckley, M. R. Holliday, and A. G. Morris. 1991. Interferon-gamma inhibits serotonin release from mouse peritoneal mast cells. *Eur. J. Immunol. 21*:2559.

22. Ramaswamy, K. and D. Befus. 1993. IgE antibody responses in bronchoalveolar spaces of rats infected with Nippostrongylus brasiliensis. *Exp. Parasitol. 76*:23.

23. MacDonald, A. J., J. Pick, E. Y. Bissonnette, and A. D. Befus. 1998. Rat mucosal mast cells: the cultured bone marrow-derived mast cell is biochemically and functionally analogous to its counterpart in vivo. *Immunology 93*:533.

24. Chan, B. M., C. S. Ra, W. N. Hu, K. McNeill, H. R. Miller, D. Befus, J. P. Kinet, and A. Froese. 1990. Characterization of rat tissue cultured mast cells. *Eur. J. Immunol. 20*:185.

25. Thethi, K., P. Jurasz, A. J. MacDonald, A. D. Befus, S. F. Man, and M. Duszyk. 1997. Determination of cell surface charge by photometric titration. *J. Biochem. Biophys. Methods 34*:137.

26. Lee, T. D., A. Sterk, T. Ishizaka, J. Bienenstock, and A. D. Befus. 1985. Number and affinity of receptors for IgE on enriched populations of isolated rat intestinal mast cells. *Immunology 55*:363.

27. Wills, F. L., M. Gilchrist, and A. D. Befus. 1999. Interferon-gamma regulates the interaction of RBL-2H3 cells with fibronectin through production of nitric oxide. *Immunology 97*:481.

28. Kuhry, J. G., P. Poindron, and G. Laustriat. 1983. Evidence for early fluidity changes in the plasma membranes of interferon treated L cells, from fluorescence anisotropy data. *Biochem. Biophys. Res. Commun. 110*:88.

29. Guha, A., R. P. Mason, D. Primorac, G. Konopaske, J. Miller, P. Sinha, T. N. Tulenko, and A. Hand. 1997. Interferon-induced growth arrest is mediated by membrane structural changes. *J. Interferon Cytokine. Res. 17*:701.

30. Yellen, G. 1998. The moving parts of voltage-gated ion channels. *Q. Rev. Biophys. 31*:239.

31. Kanno, T. and T. Takishima. 1990. Chloride and potassium channels in U937 human monocytes. *J. Membr. Biol. 116*:149.

32. Dietrich, J. and M. Lindau. 1994. Chloride channels in mast cells: block by DIDS and role in exocytosis. *J. Gen. Physiol. 104*:1099.

33. Hill, P. B., R. J. Martin, and H. R. Miller. 1996. Characterization of whole-cell currents in mucosal and connective tissue rat mast cells using amphotericin-B-perforated patches and temperature control. *Pflugers Arch. 432*:986.

34. Matthews, G., E. Neher, and R. Penner. 1989. Chloride conductance activated by external agonists and internal messengers in rat peritoneal mast cells. *J. Physiol. 418:131-44.*131.

35. Duffy, S. M., M. L. Leyland, E. C. Conley, and P. Bradding. 2001. Voltage-dependent and calcium-activated ion channels in the human mast cell line HMC-1. *J. Leukoc. Biol. 70*:233.

36. Perretti, M., S. Nuti, and L. Parente. 1989. Investigation of rat mast cell degranulation using flow cytometry. *J. Pharmacol. Methods 23*:187.

37. Krasne, S., G. Eisenman, and G. Szabo. 1971. Freezing and melting of lipid bilayers and the mode of action of nonactin, valinomycin and gramicidin. *Science 174*:412.

38. Lauger, P. 2001. Kinetic properties of ion carriers and channels. *J. Memb. Biol. 57*:163.

39. Brandt, S. and T. J. Jentsch. 1995. ClC-6 and ClC-7 are two novel broadly expressed members of the CLC chloride channel family. *FEBS Lett. 377*:15.

40. Schwappach, B., S. Stobrawa, M. Hechenberger, K. Steinmeyer, and T. J. Jentsch. 1998. Golgi localization and functionally important domains in the NH2 and COOH terminus of the yeast ClC putative chloride channel Gef1p. *J. Biol. Chem. 273*:15110.

41. Kawasaki, M., M. Suzuki, S. Uchida, S. Sasaki, and F. Marumo. 1995. Stable and functional expression of the CIC-3 chloride channel in somatic cell lines. *Neuron 14*:1285.

42. Gunther, W., A. Luchow, F. Cluzeaud, A. Vandewalle, and T. J. Jentsch. 1998. ClC-5, the chloride channel mutated in Dent's disease, colocalizes with the proton pump in endocytotically active kidney cells. *Proc. Natl. Acad. Sci. U. S. A. 95*:8075.

43. Kulka, M., M. Gilchrist, M. Duszyk, and A. D. Befus. 2002. Expression and functional characterization of CFTR in mast cells. *J. Leukoc. Biol.* (In press)

44. Schultz, B. D., A. K. Singh, D. C. Devor, and R. J. Bridges. 1999. Pharmacology of CFTR chloride channel activity. *Physiol. Rev. 79*:S109.

45. Bach, E. A., M. Aguet, and R. D. Schreiber. 1997. The IFN gamma receptor: a paradigm for cytokine receptor signaling. *Annu. Rev. Immunol 15:563-91*.563.

46. Besancon, F., G. Przewlocki, I. Baro, A. S. Hongre, D. Escande, and A. Edelman. 1994. Interferon-gamma downregulates CFTR gene expression in epithelial cells. *Am. J. Physiol. 267*:C1398.

47. Sheppard, D. N. and M. J. Welsh. 1999. Structure and function of the CFTR chloride channel. *Physiol. Rev. 79*:S23.

48. Schwiebert, E. M., D. J. Benos, M. E. Egan, M. J. Stutts, and W. B. Guggino. 1999. CFTR is a conductance regulator as well as a chloride channel. *Physiol. Rev. 79*:S145.

49. Schreiber, R., R. Nitschke, R. Greger, and K. Kunzelmann. 1999. The cystic fibrosis transmembrane conductance regulator activates aquaporin 3 in airway epithelial cells. *J. Biol. Chem. 274*:11811.

50. Duffy, S. M., W. J. Lawley, E. C. Conley, and P. Bradding. 2001. Resting and activation-dependent ion channels in human mast cells. *J. Immunol. 167*:4261.

51. Selvaraj, N. G., E. Omi, G. Gibori, and M. Rao. 2000. Janus kinase 2 (JAK2) regulates prolactin-mediated chloride transport in mouse mammary epithelial cells through tyrosine phosphorylation of Na^+-K^+-$2Cl^-$. *Mol. Endocrin. 14*:2054.

52. Mehrishi, J. N. 1970. Cell surface charge increase by both tumor inhibitory and tumor growth promoting polyanions. *Nature 228*:364.

53. Darmani, H., J. L. Harwood, and S. K. Jackson. 1993. Effect of interferon-gamma on membrane conformation in the macrophage-like cell line P388D. *J. Interferon Res. 13*:427.

Chapter 3

Mast cells express chloride channels of the ClC family[1]

[1] Authors. M. Kulka and D. Befus. This chapter was submitted to Inflammation Research. I contributed to this work by carrying out all the experimental procedures and writing the manuscript.

Introduction

Mast cells (MC), the major effector cell of immediate hypersensitivity reactions, are derived from the bone marrow and reside in tissues. MC express a high affinity Fc receptor for IgE (FcεRI) and contain numerous mediator-filled granules (1). Aggregation of MC FcεRI by antigen initiates generation of arachidonic acid, hydrolysis of phosphatidyl inositides, protein tyrosine phosphorylation, increase in intracellular free Ca^{2+} concentration $[Ca^{2+}]_i$ and mediator release (2,3). An increase in intracellular free $[Ca^{2+}]_i$ is initiated by release of Ca^{2+} from intracellular stores. Depletion of Ca^{2+} stores leads to concomitant activation of Cl^- and Ca^{2+} currents resulting in influx of extracellular Ca^{2+} and a transient membrane depolarization (3,4). Patch clamp studies show that Ca^{2+} and Cl^- currents are intimately linked in stimulus-secretion coupling since Cl^- channel blockers inhibit not only the Cl^- current but also the Ca^{2+} current (4). Similarly, interferon-gamma (IFN-γ) which downregulates MC mediator release has been shown to modulate Cl^- flux in MC (Chap 2). Although the role of Ca^{2+} and Cl^- in MC degranulation has been studied, the expression of specific Cl^- channels that facilitate Cl^- flux in MC is poorly understood.

The largest and most ubiquitously expressed family of Cl^- channels is the ClC. Members of this family function as voltage-gated and volume-activated channels and are highly conserved during evolution (5). Passive movement of Cl^- through ClC facilitates several processes such as transepithelial salt transport (6), electrical excitability (7), cell volume regulation (8) and acidification of internal and external compartments (9). However, each member of the ClC family has a unique set of characteristics and performs a specific function.

A human mast cell line, HMC-1, expresses a strong outwardly rectifying voltage-dependent Cl^- conductance characteristic of ClC-4 or ClC-5 and expresses mRNA for ClC-3 and ClC-5 (10). Rat peritoneal mast cells and bone marrow-derived mast cells also express a strong outwardly rectifying Cl^- conductance that is sensitive to DIDS and is temperature dependent (11,12). Since previous studies had demonstrated Cl^- currents in MC, we hypothesized that MC expressed one or more members of the ClC family. The

aim of this study was to characterize the expression of ClC in rat peritoneal mast cells (PMC) and a rat cultured mast cell line (RCMC) and determine the effect of IFN-γ on their expression.

Materials and Methods

Rats and PMC Isolation

Male Sprague Dawley rats (300-350 g; Charles River, St. Constant, Quebec, Canada) were housed in a pathogen-free viral antibody-free facility. Peritoneal MC (PMC) were isolated from rats sacrificed by CO_2 asphyxiation. PMC were isolated by the following procedure: 20 mL of ice-cold Hepes Tyrode's buffer (HTB) was injected into the peritoneal cavity and massaged gently for 30 sec; the peritoneum was opened and the buffer collected with a transfer pipette and kept on ice or at 4°C for all subsequent procedures. Following centrifugation at 200 g for 5 min the cell pellet was resuspended in 5 mL of fresh HTB and layered on top of a 30%/80% Percoll gradient. The gradient was centrifuged at 500 g for 20 min and the highly enriched MC were collected from the pellet (13). PMC were >98% pure and >99% viable as measured by trypan blue exclusion.

Cell Culture

The rat MC line, RCMC 1.11.2 (kindly provided by B. Chan and A. Froese, Winnipeg, Manitoba), was established from Wistar-ICI rats (14) and cultured in RPMI 1640 medium containing 5% fetal bovine serum (FBS; Gibco BRL), 100 U/mL penicillin, 100 μg/mL streptomycin and 10 mM Hepes. The cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RNA was extracted from PMC and RCMC cells as described previously (15). The RNA obtained from the PMC was treated with 1 U/mL of heparinase for 2 hr at room

temperature because high concentrations of contaminating heparin markedly decrease the efficiency of the RT-PCR procedure (16).

Genomic DNA was digested by incubating 10 μg of total RNA with 5 U of DNAse (amplification grade; GIBCO BRL), 10X DNAse buffer (GIBCO BRL) 10 U of RNAse inhibitor (GIBCO BRL) and RNAse-free H_2O for 15 min at room temperature. After 15 min incubation, 25 mM EDTA was added and the sample was heated at 65°C for 20 min to inactivate the enzyme.

Treated RNA (1 μg) was incubated with 0.5 μg oligo(dT) at 70°C for 10 min in a thermocycler. A reverse transcriptase master mix was added to the RNA. The master mix contained First Strand Buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, pH 8.3, Gibco/BRL), 10 mM DTT, 10 mM of each dNTP, Sigma sterile water and 200 U M-MLV RT enzyme (Gibco/BRL). This mixture was incubated at 37°C for 1 hr, then 70°C for 10 min. To test for RT reaction, a duplicate of each sample was reverse transcribed in the presence of [α-^{32}P]dCTP (1 μCi/μL). Unincorporated [α-^{32}P]dCTP was separated from labelled cDNA sample by paper chromatography, cDNA sample was cut out, 6 mL of scintillation fluid was added and radioactivity was measured on a Beckman scintillation counter. Based on scintillation counter readings, equal quantities of cDNA were used for PCR reactions.

The PCR mix contained 1X PCR buffer (20 mM Tris-Cl, 50 mM KCl, pH 8.4, Gibco/BRL), 0.8 mM dNTP mix, 20 μM antisense primer, 20 μM sense primer, 1.5 mM $MgCl_2$ (Gibco/BRL), 1 μg of cDNA and 2.5 units of Taq DNA polymerase. The primer sequences used are summarized in Table 3.1. The mixture was amplified at the annealing temperature indicated for 25-35 cycles. The product was analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. A 1kb ladder (Gibco/BRL) was used to estimate product size.

Each set of ClC primers were tested on kidney or muscle RNA and PCR conditions for each primer were optimized using four variables: (1) final primer concentration (10-100 μM), (2) $MgCl_2$ concentration (1.0-3.0 mM), (3) annealing temperature (55-63°C) and cycle number (25-35 cycles). In each case, 15-20 μM final primer concentration, 1.5 mM $MgCl_2$ and 35 cycles was optimal for detection of ClC

mRNA. Optimal annealing temperatures for each set of ClC primers varied and are listed on Table 1.

Cloning and Sequencing of PCR products

To confirm that RT-PCR was specifically amplifying ClC mRNA, we cloned and sequenced the PCR products from MC (ClC-2, 3, 4, 5 and 7), kidney (ClC-6) and muscle (ClC-1) RNA. The PCR product from each PCR reaction was cloned into the pCR 2.1® (Invitrogen, Carlsbad, Ca, USA) vector using T4 DNA ligase and then used to transform "one shot" *E. coli* cells (Invitrogen INVaF'). The *E. coli* cells were grown for 3 d, the DNA was isolated using a Sigma miniprep isolation kit and the insert was sequenced on the ABI PRISM sequencer model 2.1.1 and analyzed by BLAST (17).

The ClC-1 and ClC-6 PCR products, amplified from muscle and kidney RNA had the expected sequence sizes and had 100 % homology to the rat ClC sequences. The ClC-2, 3, 4, 5 and 7 PCR products amplified from MC RNA were also of the expected size and had 100 % homology to rat ClC genes.

Results

MC express chloride channels of the ClC family

Primers to the major ClC family members (1-7) were designed and RT-PCR conditions for ClC primers were optimized using rat kidney and muscle RNA. Expected product sizes and optimal annealing temperatures for the ClC primers are listed in Table 1. At optimal conditions, PCR results indicate that rat kidney tissue expresses ClC 2-7. The PCR products are of predicted sizes and the sequences were confirmed to be correct both by comparison to 1 Kb ladder (see Table 1; Fig 1A) and by sequencing of the PCR product (see methods). As expected, ClC1 was found in muscle but not kidney RNA at the expected size of 656 bp (Fig 1B).

To characterize the Cl^- channel expression of rat MC, RT-PCR analysis was performed on total RNA from RCMC and PMC. RCMC (Fig 2A) express mRNA for ClC 2, 3, 4, 5 and 7 as shown by PCR products of the expected sizes. However, PMC

express mRNA for only ClC 7 (Fig 2B). Sequence analysis confirms PCR products are of the expected size and they match the sequence for rat ClC mRNA.

IFN-γ does not modulate ClC mRNA expression

To determine if IFN-γ modulated ClC mRNA expression, RT-PCR, using carefully controlled conditions, was conducted on IFN-γ-treated (100-800 U/mL for 24 hr) RCMC and PMC. Total RNA was reverse transcribed and ^{32}P was used to measure the RT reaction in IFN-γ and untreated samples (described in methods). Based on scintillation counter readings, equal quantities of cDNA were used for PCR reactions. PCR revealed no difference in ClC expression between IFN-γ treated and untreated samples.

To optimize conditions and to control for pipetting error, IFN-γ-treated and untreated cDNA was serially diluted and PCR amplified using 25, 30 and 35 cycles. ClC band intensities of IFN-γ and untreated samples were compared and showed no detectable difference.

Discussion

For the first time, we characterized ClC expression in two types of rat MC; freshly isolated PMC and a RCMC cell line. Primers were designed to the seven non-kidney-specific ClC members (1-7) and RT-PCR was used to amplify PMC and RCMC RNA. Our results show that RCMC express mRNA for ClC-2, 3, 4, 5 and 7, while PMC express mRNA for ClC-7. Sequence analysis of these PCR products shows that amplified products match published rat ClC mRNA sequences. IFN-γ, at the doses studied (100-800 U/mL), has no effect on ClC mRNA expression as observed by PCR.

ClC is the largest gene family of Cl^- channels. At least nine mammalian isoforms exist and there are homologues in a wide range of other species including bacteria, *Drosophila*, *Saccharomyces* (yeast), *Neurospora* (mold), *Arabidopsis* (plant) *and Caenorhabditis* (worm) suggesting that ClC channels mediate cellular events that are

essential for life (18). Mutations in some of these ClC can cause severe diseases with profound pathophysiology (18).

ClC-1 is specifically expressed in skeletal muscle and accounts for approximately 75 % of the ionic conductance of the resting sarcoplasmic membrane (19). Our RT-PCR confirms that rat muscle expresses ClC-1 while rat kidney, PMC and RCMC do not express ClC-1.

In contrast, ClC-2 is widely expressed with strong expression in brain neurons and lung epithelia (20). ClC-2 can be activated by cell swelling, arachidonic acid and acidic extracellular pH and is involved in limiting intracellular Cl^- concentration (21,22). In epithelial cells, ClC-2 channel activity is modulated by transforming growth factor-alpha (TGF-α) (23). RCMC express ClC-2 but PMC do not, suggesting that volume regulation and/or Cl^- concentration might be regulated differently in these cell types. This difference could be due to phenotypic differences between the connective tissue-type PMC and the mucosal tissue-type RCMC (14). Connective tissue and mucosal MC differ in protease expression (24), responsiveness to secretagogues (25), cytokines and anti-allergic drugs (26,27).

RCMC also express ClC-3, 4 and 5 while PMC do not. ClC-3 is Ca^{2+}-sensitive, volume regulated Cl^- channel that maintains a variety of cellular functions during osmotic perturbation (28). ClC-3 channels are opened by protein kinase C (PKC) activation and are expressed in epithelial cells, hepatocytes and neuronal cells (29-31). ClC-3, ClC-4 and ClC-5 belong to the same branch in the ClC family and are highly homologous (32). ClC-4 is expressed in human skeletal and vascular smooth muscle and is activated by external pH changes, inhibited by DIDS and has concensus sequences for protein kinase A (PKA) phosphorylation (33,34). ClC-5 is mainly expressed in endocytic vesicles of proximal tubule cells of the kidney and mutation results in Dent's disease (35-37). ClC-6-mediated currents are inhibited by extracellular cAMP or the Cl^- channel inhibitor NPPB (38) and there is some evidence that ClC-6 is expressed in vascular smooth muscle (33). ClC-7 resides in late endosomal and lysosomal compartments (39) and is important in bone resorption by osteoclasts (40). Interestingly, ClC-7 is expressed by both PMC and RCMC suggesting that ClC-7 is present in endosomal compartments in both cell

types. It would be advantageous to determine which MC endosomal compartments (i.e. granules) express ClC-7.

MC also express other channels capable of transporting Cl^- such as the cystic fibrosis transmembrane conductance regulator (CFTR; (41)) and the Cl^-/HCO_3^- exchanger (42). The function of these Cl^- channels may be important in MC degranulation since the Cl^-/HCO_3^- exchanger is also sensitive to DIDS (42). Although CFTR is not sensitive to DIDS, the CFTR inhibitor, diphenylamine-2-carboxylate (DPC; 1 μM) decreases antigen-stimulated β- hexosaminidase release from PMC by 31 $\pm$ 6.7% (41). Therefore, ClC as well as other Cl^- channels may cooperatively coordinate important MC functions.

Several questions about ClC expression in mast cells remain unanswered. Firstly, differences in ClC expression between RCMC and PMC might reflect tissue-specific phenotypic heterogeneity and further characterization of MC from other tissues is required. PCR analysis suggests that IFN-γ has no effect on ClC mRNA expression although a more quantitative analysis using Northern blot or real-time PCR may reveal small changes in expression. Specific ClC protein expression and function will need to be characterized. Specific ClC channel inhibitors would be invaluable in delineating the role of specific ClC in MC function. Although these inhibitors are currently unavailable, antisense to ClC-5 has successfully been used to block human ClC-5 expression (43) and similar approaches to the other ClC in MC might prove useful. In addition, ClC knock-out mice have been developed recently and have provided a vast amount of knowledge about the role of ClC-7 and ClC-3 in osteopetrosis and brain neuro-degenerative disease (40). Regulation of ClC expression and function in MC would offer insights into their function. Compounds such as TGF-α, arachidonic acid and hydrogen peroxide regulate ClC expression in epithelial cells (21,23,43) and the same may be true in MC.

In conclusion, rat MC express several Cl^- channels that may be important in MC functions including mediator secretion. Specifically, MC express several members of the ClC family which are involved in diverse role in other cell types and may be involved in similar processes in MC.

Table 3.1. Primer sequences, expected products and annealing temperatures.

Name of Primer Sequence			Expected product (bp)	T_m (°C)
ClC-1	sense	TGTGGAACGCTCAGAACTGCAGTC	656	61
ClC-1	antisense	TCTAGTGCCAAGACACCTCTGAGC		
ClC-2	sense	CTGCGACTGGCACTGCACCGAAC	487	63
ClC-2	antisense	CACTCGCCAGGGAGATTCGGACC		
ClC-3	sense	GCTGCTGATGTCATGAGACCTCG	235	63
ClC-3	antisense	CCCGAGAACTGCCAACAACACCT		
ClC-4	sense	GAGACTCGGAGCGTCTCATCGG	276	63
ClC-4	antisense	CTGTTGGCAGGCAGCTCAGGAG		
ClC-5	sense	TGCTGACTGTCCTTACTCAG	269	63
ClC-5	antisense	CAGGATGTTCCGAAGCTTCA		
ClC-6	sense	TCTGTGCACTCCTCCACCGATGTC	345	56
ClC-6	antisense	TGGCTGCACTCCTCCACCGATGTC		
ClC-7	sense	ATGAGCACGCCTGTGACCTGCCTG	377	63
ClC-7	antisense	CGAGGAAGAGATGCCTCCTGTGGC		
β-actin	sense	CCATGTACGTAGCCATCCA	625	46
β-actin	antisense	GATAGAGCCACCAATCCAC		

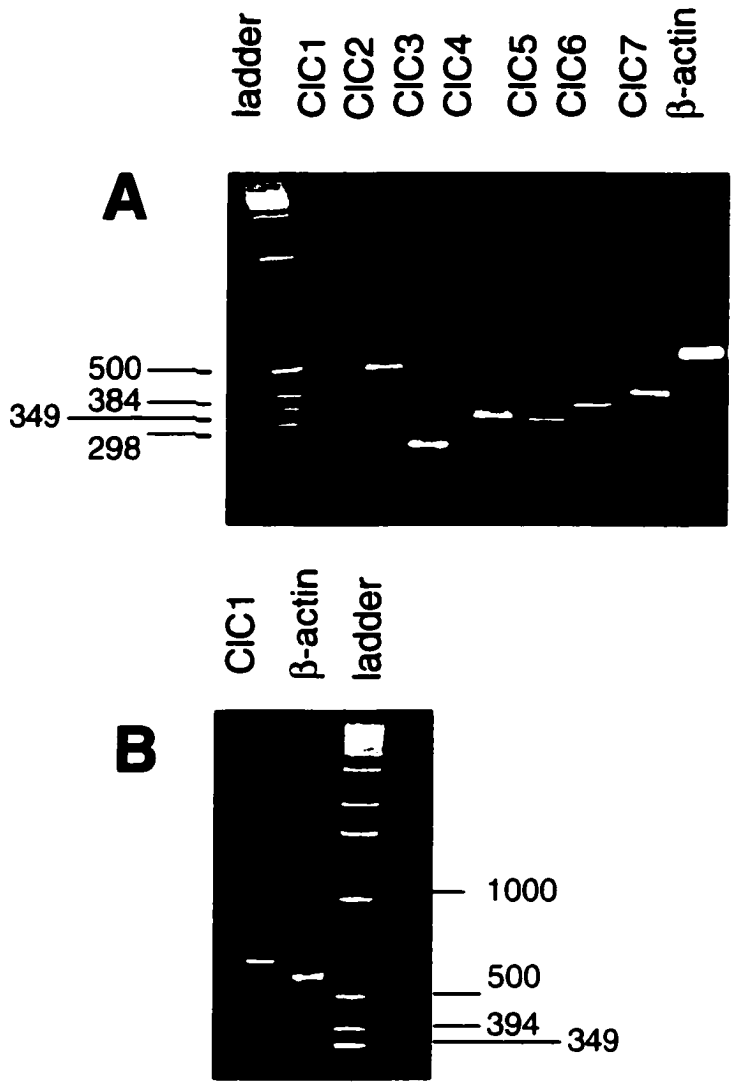


Figure 3.1. RT-PCR showing ClC expression in rat kidney (A) and muscle (B; one representative experiment from n=4).

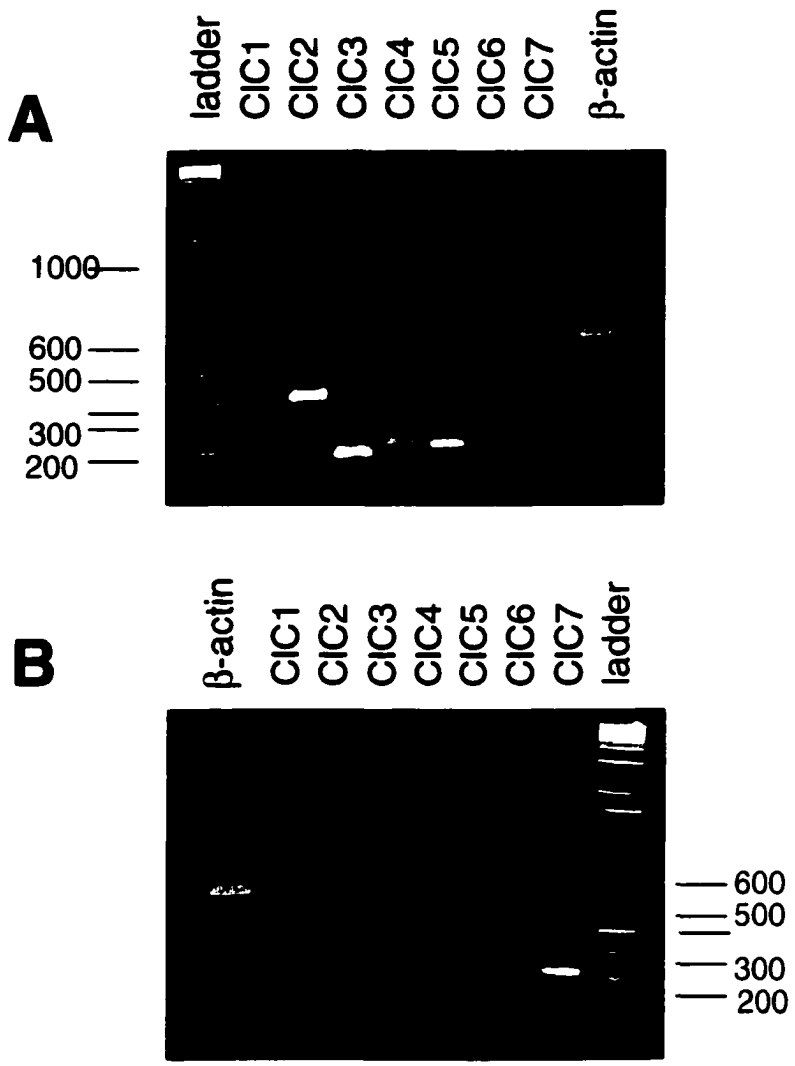


Figure 3.2. (A) RT-PCR showing ClC expression in rat cultured mast cells. β-actin is shown as a control. Rat cultured mast cell total RNA was isolated as described in methods and amplified using specific primers (see Table 1) (one representative experiment from n=5). (B) RT-PCR showing ClC expression in peritoneal mast cells. β-actin is shown as a control. Peritoneal mast cell total RNA was isolated as described in methods and amplified using specific primers (see Table 1) (one representative experiment from n=5).

References

1. Lin, T. J. and A. D. Befus. 1999. Mast cells and eosinophils in mucosal defenses and pathogenesis. In *Mucosal Immunology*. 2nd ed. P.L. Ogra, J. Mestecky, M.E. Lamm, W. Strober, J. Bienenstock and J.R. McGhee, eds. Academic Press, San Diego, p. 469.

2. Hirasawa, N., Y. Sato, Y. Fujita, and K. Ohuchi. 2000. Involvement of a phosphatidylinositol 3-kinase-p38 mitogen activated protein kinase pathway in antigen-induced IL-4 production in mast cells. *Biochim. Biophys. Acta 1456*:45.

3. Lloret, S. and J. J. Moreno. 1995. Ca2+ influx, phosphoinositide hydrolysis, and histamine release induced by lysophosphatidylserine in mast cells. *J. Cell Physiol. 165*:89.

4. Janiszewski, J., J. D. Huizinga, and M. G. Blennerhassett. 1992. Mast cell ionic channels: significance for stimulus-secretion coupling. *Can. J. Physiol. Pharmacol. 70*:1.

5. Jentsch, T. J. and W. Gunther. 1997. Chloride channels: an emerging molecular picture. *Bioessays 19*:117.

6. Reeves, W. B. and T. E. Andreoli. 1996. Chloride channels in renal epithelial cells. *Curr. Opin. Nephrol. Hypertens. 5*:406.

7. Komwatana, P., A. Dinudom, J. A. Young, and D. I. Cook. 1994. Characterization of the Cl- conductance in the granular duct cells of mouse mandibular glands. *Pflugers Arch. 428*:641.

8. Stegen, C., I. Matskevich, C. A. Wagner, M. Paulmichl, F. Lang, and S. Broer. 2000. Swelling-induced taurine release without chloride channel activity in Xenopus laevis oocytes expressing anion channels and transporters. *Biochim. Biophys. Acta 1467*:91.

9. Blaisdell, C. J., R. D. Edmonds, X. T. Wang, S. Guggino, and P. L. Zeitlin. 2000. pH-regulated chloride secretion in fetal lung epithelia. *Am. J. Physiol. Lung Cell Mol. Physiol. 278*:L1248.

10. Duffy, S. M., M. L. Leyland, E. C. Conley, and P. Bradding. 2001. Voltage-dependent and calcium-activated ion channels in the human mast cell line HMC-1. *J. Leukoc. Biol. 70*:233.

11. Hill, P. B., R. J. Martin, and H. R. Miller. 1996. Characterization of whole-cell currents in mucosal and connective tissue rat mast cells using amphotericin-B-perforated patches and temperature control. *Pflugers Arch. 432*:986.

12. Matthews, G., E. Neher, and R. Penner. 1989. Chloride conductance activated by external agonists and internal messengers in rat peritoneal mast cells. *J. Physiol. 44*:131.

13. MacDonald, A. J., J. Pick, E. Y. Bissonnette, and A. D. Befus. 1998. Rat mucosal mast cells: the cultured bone marrow-derived mast cell is biochemically and functionally analogous to its counterpart in vivo. *Immunology 93*:533.

14. Chan, B. M., C. S. Ra, W. N. Hu, K. McNeill, H. R. Miller, D. Befus, J. P. Kinet, and A. Froese. 1990. Characterization of rat tissue cultured mast cells. *Eur. J. Immunol. 20*:185.

15. Gilchrist, M., A. J. MacDonald, I. Neverova, B. Ritchie, and A. D. Befus. 1997. Optimization of the isolation and effective use of mRNA from rat mast cells. *J. Immunol. Methods 201*:207.

16. Tsai, M., M. Miyamoto, S. Y. Tam, Z. S. Wang, and S. J. Galli. 1995. Detection of mouse mast cell-associated protease mRNA. Heparinase treatment greatly improves RT-PCR of tissues containing mast cell heparin. *Am. J. Pathol. 146*:335.

17. Iwagaki, H., M. Marutaka, T. Yoshino, L. Cao, I. Sakuma, M. Uomoto, Y. Takeuchi, and N. Tanaka. 1996. Gamma-interferon upregulates transferrin receptors and decreases membrane microviscosity in K562 cells. *Res. Commun. Mol. Pathol. Pharmacol. 92*:191.

18. Maduke, M., C. Miller, and J. A. Mindell. 2000. A decade of ClC chloride channels: structure, mechanism, and many unsettled questions. *Annu. Rev. Biophys. Biomol. Struct. 29*:411.

19. Steinmeyer, K., C. Ortland, and T. J. Jentsch. 1991. Primary structure and functional expression of a developmentally regulated skeletal muscle chloride channel. *Nature 354*:301.

20. Smith, R. L., C. L. Clayton, C. L. Wilcox, K. W. Escudero, and K. J. Staley. 1995. Differential expression of an inwardly rectifying chloride conductance in rat brain neurons: a potential mechanism for cell-specific modulation of postsynaptic inhibition. *J. Neurosci. 15*:4057.

21. Cuppoletti, J., K. P. Tewari, A. M. Sherry, E. Y. Kupert, and D. H. Malinowska. 2001. ClC-2 Cl- channels in human lung epithelia: activation by arachidonic acid, amidation, and acid-activated omeprazole. *Am. J. Physiol. Cell Physiol. 281*:C46.

22. Schwappach, B., S. Stobrawa, M. Hechenberger, K. Steinmeyer, and T. J. Jentsch. 1998. Golgi localization and functionally important domains in the NH2 and COOH terminus of the yeast ClC putative chloride channel Gef1p. *J. Biol. Chem. 273*:15110.

23. Bali, M., J. Lipecka, A. Edelman, and J. Fritsch. 2001. Regulation of ClC-2 chloride channels in T84 cells by TGF-alpha. *Am. J. Physiol. Cell Physiol. 280*:C1588.

24. Befus, A. D., B. Chin, J. Pick, S. Evans, S. Osborn, and J. Forstrom. 1995. Proteinases of rat mast cells. Peritoneal but not intestinal mucosal mast cells express mast cell proteinase 5 and carboxypeptidase A. *J. Immunol. 155*:4406.

25. Galli, S. J. 2000. Mast cells and basophils. *Curr. Opin. Hematol. 7*:32.

26. Zheng, Y. L., B. M. Chan, E. S. Rector, I. Berczi, and A. Froese. 1991. Establishment and characterization of hybrid rat mast cells. *Exp. Cell Res. 194*:301.

27. Austen, K. F. and J. A. Boyce. 2001. Mast cell lineage development and phenotypic regulation. *Leuk. Res. 25*:511.

28. Duan, D., S. Cowley, B. Horowitz, and J. R. Hume. 1999. A serine residue in ClC-3 links phosphorylation-dephosphorylation to chloride channel regulation by cell volume. *J. Gen. Physiol. 113*:57.

29. Shimada, K., X. Li, G. Xu, D. E. Nowak, L. A. Showalter, and S. A. Weinman. 2000. Expression and canalicular localization of two isoforms of the ClC-3 chloride channel from rat hepatocytes. *Am. J. Physiol. Gastrointest. Liver Physiol. 279*:G268.

30. Wang, L., L. Chen, and T. J. Jacob. 2000. The role of ClC-3 in volume-activated chloride currents and volume regulation in bovine epithelial cells demonstrated by antisense inhibition. *J. Physiol. 524*:63.

31. Kawasaki, M., M. Suzuki, S. Uchida, S. Sasaki, and F. Marumo. 1995. Stable and functional expression of the ClC-3 chloride channel in somatic cell lines. *Neuron 14*:1285.

32. Friedrich, T., T. Breiderhoff, and T. J. Jentsch. 1999. Mutational analysis demonstrates that ClC-4 and ClC-5 directly mediate plasma membrane currents. *J. Biol. Chem. 274*:896.

33. Lamb, F. S., G. H. Clayton, B. X. Liu, R. L. Smith, T. J. Barna, and B. C. Schutte. 1999. Expression of CLCN voltage-gated chloride channel genes in human blood vessels. *J. Mol. Cell Cardiol. 31*:657.

34. Kawasaki, M., T. Fukuma, K. Yamauchi, H. Sakamoto, F. Marumo, and S. Sasaki. 1999. Identification of an acid-activated Cl(-) channel from human skeletal muscles. *Am. J. Physiol. 277*:C948.

35. Thakker, R. V. 1999. Chloride channels in renal disease. *Adv. Nephrol. Necker. Hosp. 29*:289.

36. Igarashi, T., W. Gunther, T. Sekine, J. Inatomi, H. Shiraga, S. Takahashi, J. Suzuki, N. Tsuru, T. Yanagihara, M. Shimazu, T. J. Jentsch, and R. V. Thakker. 1998. Functional

characterization of renal chloride channel, CLCN5, mutations associated with Dent's disease. *Kidney Int. 54*:1850.

37. Gunther, W., A. Luchow, F. Cluzeaud, A. Vandewalle, and T. J. Jentsch. 1998. ClC-5, the chloride channel mutated in Dent's disease, colocalizes with the proton pump in endocytotically active kidney cells. *Proc. Natl. Acad. Sci. U. S. A. 95*:8075.

38. Buyse, G., T. Voets, J. Tytgat, C. De Greef, G. Droogmans, B. Nilius, and J. Eggermont. 1997. Expression of human pICln and ClC-6 in Xenopus oocytes induces an identical endogenous chloride conductance. *J. Biol. Chem. 272*:3615.

39. Brandt, S. and T. J. Jentsch. 1995. ClC-6 and ClC-7 are two novel broadly expressed members of the CLC chloride channel family. *FEBS Lett. 377*:15.

40. Kornak, U., D. Kasper, M. R. Bosl, E. Kaiser, M. Schweizer, A. Schulz, W. Friedrich, G. Delling, and T. J. Jentsch. 2001. Loss of the ClC-7 chloride channel leads to osteopetrosis in mice and man. *Cell 104*:205.

41. Kulka, M., M. Gilchrist, M. Duszyk, and A. D. Befus. 2002. Expression and functional characterization of CFTR in mast cells. *J. Leukoc. Biol.* (In press)

42. Vilarino, N., M. R. Vieytes, J. M. Vieites, and L. M. Botana. 1998. Role of HCO3- ions in cytosolic pH regulation in rat mast cells: evidence for a new Na+-independent, HCO3--dependent alkalinizing mechanism. *Biochem. Biophys. Res. Commun. 253*:320.

43. Weng, T. X., L. Mo, H. L. Hellmich, A. S. Yu, T. Wood, and N. K. Wills. 2001. Expression and regulation of ClC-5 chloride channels: effects of antisense and oxidants. *Am. J. Physiol. Cell Physiol. 280*:C1511.

Chapter 4

Mast cells express subunits of the gamma-aminobutyric acid receptor ($GABA_AR$)

Introduction

Gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the vertebrate brain, mediates neuronal inhibition by binding to GABA type A ($GABA_AR$) and type B receptors ($GABA_BR$; (1)). GABA ligation of $GABA_AR$ opens an integral ion channel selectively permeable to Cl^-. $GABA_AR$ are mainly expressed on neurons although recent evidence has also demonstrated expression on T lymphocytes (2). Ligation of T cell $GABA_AR$ inhibits T cell proliferation and cytotoxicity (2,3). These observations suggest that GABA and $GABA_AR$ may modulate immune responses.

$GABA_AR$ also contains binding sites for pharmaceutically significant drugs such as benzodiazepines (BDZ) that interact allosterically with the GABA agonist site or the receptor channel (4). BDZ are the most frequently prescribed psychotropic drugs used to treat generalized anxiety disorder and insomnia (5). High affinity binding sites for BZD are present in a wide range of tissues other than the brain and appear to be pharmacologically distinct from central nervous system receptors. Peripheral BZD receptors are expressed on monocytes (6), lymphocytes (7-11), platelets (9), neutrophils (12) and mast cells (13).

BDZ receptors/binding sites appear to mediate a host of specific interactions between the nervous and immune systems. Lymphocytes isolated from patients with migraine (9) show increased expression of peripheral BDZ receptors while lymphocytes isolated from patients with generalized anxiety disorder show decreased expression of peripheral BDZ receptors (6). Interleukin-1 (IL-1) decreases BDZ binding site expression while phorbol myristate acetate (PMA) increases BDZ binding site expression in a murine thymoma cell line (14). For the most part, BDZ compounds inhibit immune cell functions. For example, the peripheral BDZ receptor agonist, Ro5-4864, inhibits natural killer cell activity (15) and spontaneous IgA secretion by peripheral blood mononuclear cells (16). However, BDZ compounds can also activate immune cells. The central BDZ receptor agonist, alprazolam activates lymphocyte proliferation (17) and the peripheral BDZ receptor agonist, diazepam, activate neutrophil migration (18).

Therefore, the regulatory role of BDZ receptors and BDZ compounds on immune functions is diverse.

BZD binding sites have been reported and characterized on rat peritoneal mast cells (PMC), although little is known regarding the biological effect of BDZ on MC function (13). The BDZ Ro5-4864 (10^{-6} M) has no effect on histamine or serotonin release in either unstimulated PMC or those stimulated by compound 48/80 or substance P (13). However, midzolam and diazepam (10^{-6} to 10^{-4} M) decrease proliferation, β-hexosaminidase (β-hex) release, tumor necrosis factor (TNF) release and nitrite production from bone marrow-derived MC (BMMC) (19). Ligation of BDZ binding sites by some diazepam derivatives on rat PMC inhibits calcium influx generated by concanavalin A, possibly by inhibiting calcium channels and calmodulin activation (20). It is unknown whether BDZ specifically bind GABAR.

$GABA_AR$ or $GABA_BR$ expression on MC has never been reported and the effect of GABA on MC function is unknown. However, since BDZ had an inhibitory effect on MC function, we hypothesized that GABA may also have a modulatory effect on MC mediator release and that MC may express mRNA and protein for some of the subunits for the $GABA_AR$.

The central hypothesis of this thesis is that interferon-gamma (IFN-γ), an immunomodulatory cytokine, has effects on chloride and chloride channels in MC. IFN-γ may regulate expression of BDZ binding sites and/or $GABA_AR$ subunits. Certainly, the pro-inflammatory cytokine, IL-1, decreases BDZ binding sites in a mouse thymoma cell line (14). IFN-γ inhibits a number of MC functions including clonal proliferation, differentiation, TNF-mediated cytotoxicity, adhesion and mediator release (21-25). These effects appear to be transcription/translation dependent and are probably the consequence of several changes in MC gene expression. Therefore, the postulate that $GABA_AR$ subunits expression is modulated by IFN-γ (see Chap1, aim #2) was also tested.

Materials and Methods

Rats and PMC Isolation

Male Sprague Dawley rats (300-350 g; Charles River, St. Constant, Quebec, Canada) were housed in a pathogen-free viral antibody-free facility. For MC sensitization, rats were infected with 3000 *Nippostrongylus brasiliensis* as previously described (26). Peritoneal MC (PMC) were isolated from sensitized rats $\geq$ 4 weeks after infection. Rats were sacrificed by cervical dislocation under anesthesia and PMC were isolated by the following procedure: 20 mL of ice-cold Hepes Tyrode's buffer (HTB) was injected into the peritoneal cavity and massaged gently for 30 sec; the peritoneum was opened and the buffer collected with a transfer pipette and kept on ice or at 4°C for all subsequent procedures. Following centrifugation at 200 g for 5 min the cell pellet was resuspended in 5 mL of fresh HTB and layered on top of a 30%/80% Percoll gradient. The gradient was centrifuged at 500 g for 20 min and the highly enriched MC were collected from the pellet (27). PMC were >98% pure and >96% viable as measured by trypan blue exclusion.

Worm antigen used to stimulate PMC was prepared as follows: rats were infected with 6000 *Nippostrongylus brasiliensis* as previously described (26). Two weeks after infection, rats were sacrificed by cervical dislocation under anesthesia and adult worms were isolated from the small intestines. Adult worms were incubated in HTB at 1000 worm equivalents (WE)/mL for 24 hr at 37°C. Supernatants were isolated and stored at -20°C.

Stimulation and β-hexosaminidase assay

PMC were resuspended in HTB (140 mM [Cl^-]) and stimulated with either 48/80 or 10 WE per mL of antigen for 30 min unless indicated otherwise (28). PMC were pelleted at 200 g and the supernatant was removed. Pellet and supernatant samples were assayed for β-hexosaminidase (β-hex) by hydrolysis of the fluorescent substrate 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (Sigma M-2133). One unit of enzyme

cleaves 1 μmol of substrate/hr at 37°C (27). Pellet and supernatant samples (50 μL) were added in duplicate wells in a microtitre plate followed by 1 mM substrate (50 μL) and the mixture was incubated at 37°C for 2 hr. The reaction was terminated by the addition of 100 μL of 0.2 M Tris base and the optical density was read at 450 nm (excitation 356 nm). The OD_{450nm} for blank wells, containing only substrate and Tris base, was automatically subtracted from the sample OD_{450nm} and the percentage β-hex release was calculated by the formula:

$$\frac{OD_{450nm} \text{ of substrate samples}}{OD_{450nm} \text{ of pellet samples} + OD_{450nm} \text{ of supernatant samples}} \times 100 = \% \text{ release}$$

The spontaneous release (in HTB) was subtracted from all samples to give % specific release.

Cell Culture

The rat MC line, RCMC 1.11.2 (kindly provided by B. Chan and A. Froese, Winnipeg, Manitoba), was established from Wistar-ICI rats (29) and cultured in RPMI 1640 medium containing 5% fetal bovine serum (FBS; Gibco BRL), 100 U/mL penicillin, 100 μg/mL streptomycin and 10 mM Hepes. The cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C.

S1 Nuclease Protection Assay

Total RNA was isolated from RCMC as described previously ((30), Chap5). Approximately 4 μg of total RNA was obtained from 1 x 10^6 cells and each RNA sample had an A_{260}/A_{280} of 1.7 to 2. γ-^{32}P-labelled oligonucleotide probes for $GABA_AR$ subunits α (1-5), β (1 and 3) and γ (1-3) and β-actin were the generous gift of Dr. Alan Bateson (Department of Pharmacology, University of Alberta) and were designed as previously described (31). The sequences of the oligodeoxyribnucleotide probes are presented in Table 4.1.

RCMC RNA (10 μg) was hybridized to an excess of each oligonucleotide probe (0.003 pmol of each oligonucleotide per μg total RNA). The RNA/oligoncleotide mixture was precipitated by adding 0.1 vol 3M sodium acetate, pH 5.0 and 2.5 vol 95% (v/v) ethanol and incubated on ice for 30 min. The mixture was centrifuged at 4°C for 30 min at 10,000 *g* in a microfuge, the supernatant was discarded and the pellet was washed with 0.5 mL of ice-cold 75% (v/v) ethanol. The pellet was briefly air-dried then resuspended in 30 μL of S1 hybridization buffer (4.5 mM zinc sulfate, 50 mM sodium acetate, pH 4.2, 0.3 M NaCl in DEPC-treated H_2O and filter sterilized) to a final probe concentration of 1 pmol/mL. Mixture incubated at room temperature for 10 min, 90°C for 7 min, immediately chilled in ice water, then incubated overnight at 70°C. S1 nuclease enzyme (120 U/mL final concentration) and denatured salmon sperm DNA (10 μg/mL in S1 nuclease buffer) was added to the RNA/oligonucleotide mix and incubated at 37° for 15 min. The reaction was stopped by adding 49 μL of S1 stop buffer (4M ammonium acetate, 0.1 M EDTA, pH 8.0 made in DEPC-treated H_2O) and 870 μL of 95% (v/v) ethanol and incubating on ice for 30 min. The mixture was centrifuged at 4°C for 30 min at 10,000*g* to recover the protected oligonucleotides. Protected oligonucleotide samples were washed with 500 μL of 75% ice-cold (v/v) ethanol and resuspended in 6 μL of DEPC H_2O. Samples were loaded onto a 10% acrylamide sequencing gel and run at 43 W (constant power mode) for about 1.5-2 hr. The gel was dried under vacuum at 80°C for approx 1 hr and exposed to Biomax film at room temperature for 2 to 3 days.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RNA was extracted from PMC, RCMC and rat brain cells as described previously (30). The RNA obtained from the PMC was treated with 1 U/mL of heparinase for 2 hr at room temperature because high concentrations of contaminating heparin markedly decrease the efficiency of the RT-PCR procedure (32).

Treated RNA (1 μg) was incubated with 0.5 μg oligo(dT) at 70°C for 10 min in a thermocycler. A reverse transcriptase master mix was added to the RNA. The master mix contained First Strand Buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, pH 8.3, Gibco/BRL), 10 mM DTT, 10 mM of each dNTP, Sigma sterile water and 200 U M-

MLV RT enzyme (Gibco/BRL). This mixture was incubated at 37°C for 1 hr, then 70°C for 10 min.

The polymerase chain reaction master mix contained 1X PCR buffer (20 mM Tris-Cl, 50 mM KCl, pH 8.4, Gibco/BRL), 0.8 mM dNTP mix, 20 μM antisense $GABA_AR\alpha1$ primer (5'-AGCTATACCCCTAACTTAGCCAGG-3') which binds bp 1174-1197 of the *Rattus rattus* $GABA_AR\alpha1$ gene (NCBI accession # L08490.1), 20 μM of sense $GABA_AR\alpha1$ primer (5'-AGAAAGCGATTCTCAGTGCAGAGG-3') which binds bp 1455-1478 of the *Rattus rattus* $GABA_AR\alpha1$ gene (expected product size 304 bp), 1.5 mM $MgCl_2$ (Gibco/BRL), sterile Sigma water, 2 μL of cDNA and 2.5 units of Taq DNA Polymerase (Gibco/BRL). The mixture was amplified at an annealing temperature of 56°C for 35 cycles. The β-actin sense (5'-CCATGTACGTAGCCATCCA-3') and antisense (5'-GATAGAGCCACCAATCCAC-3') primers had an annealing temperature of 46°C (expected product size 625 bp). Product was analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. A 1kb ladder (Gibco/BRL) was used to estimate product size.

Western Immunoblotting

RCMC and PMC were isolated, washed with PBS and 1 x 10^6 cells were lysed with 2X sample buffer (0.5 mL 1M Tris-Cl, 1 mL DTT, 2 mL 10% SDS, 1 mL glycerol, 0.5 mL 0.12 % bromophenol blue) supplemented with 2% β-mercaptoethanol.

Approximately 40 μg of whole cell extracts were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% milk (Carnation NF milk powder) in TBS-Tween for 24 hr at room temperature and then blotted with primary antibodies, anti-$GABA_AR\alpha$ (Chemicon, MAB339) or isotype control purified mouse IgG_1 κ-isotype standard (anti-TNP; Serotec, 03001D) for 1 hr at room temperature. The membranes were washed with TBS-Tween 3X and then blotted with the secondary antibody for 1 hr. The secondary antibody was sheep anti-mouse IgG conjugated to HRP (Amersham Life Science, catalogue # NA 9310).

The nitrocellulose membranes were developed with chemiluminescence reagent (NEN Life Technologies, catalogue # NEL 101) for 1 min and placed into an

autoradiography cassette containing high performance chemiluminescence film (Amersham Life Science, catalogue # RPN2103H). The film was exposed for 30 min.

Statistical Analysis

Each set of treatments for β-hex and radioligand binding experiments were analyzed by ANOVA and significance values indicate $P<0.01$.

Results

MC express $GABA_AR$ subunit mRNA

To determine $GABA_AR$ subunit expression in MC, γ-^{32}P-labelled oligonucleotides were hybridized to total MC RNA using the S1 nuclease protection assay. The principle of the simultaneous multiprobe S1 nuclease protection assay is simple. The labeled oligonucleotide probe, complementary to the mRNA of $GABA_AR$ subunits, is added in molar excess to a solution of total RNA under conditions that will allow hybridization to proceed. Excess unhybridized oligonucleotide molecules are removed by digestion with S1 nuclease. Oligonucleotide molecules that are protected from digestion, by virtue of being part of a double-stranded DNA:RNA hybrid, are visualized as a band by autoradiography following denaturing polyacrylamide gel electrophoresis. The amount of radioactivity detected in the band (i.e. intensity of band) is directly proportional to the number of molecules of the specific mRNA under investigation in that sample.

The S1 nuclease protection assay indicates that MC express mRNA for $GABA_AR$ subunits α (1-5), β (1 and 3) and γ (1-3) but there was no detectable signal for β-2 (Fig 4.1). Oligonucleotide probe for β-actin was the internal control for RNA loading and is also shown (Fig 4.1). Yeast tRNA was used as a negative control and showed no bands. MC RNA shows strong bands for α-3, γ-2, (Fig 4.1A) and β-1 (Fig 4.1B). There are multiple bands below the α-4 subunit, probably the result of over digestion. These oligonucleotides designed for and tested on rat brain total RNA and showed the requisite subunits. Reverse transcriptase-polymerase chain reaction was used to confirm $GABA_AR\alpha1$ subunit expression in RCMC and PMC. As expected, RCMC, PMC and rat brain show a band at 304 bp corresponding to $GABA_AR\alpha1$ (Fig 4.1C).

MC express the $GABA_AR$ α-1 subunit protein

To determine if the $GABA_AR$ subunit mRNA were translated to protein in MC, RCMC and PMC protein lysate was resolved on a 10% polyacrylamide gel and blotted with a polyclonal antibody recognizing the α chain of $GABA_AR$. The immunoblot shows that rat brain lysate, RCMC (lysate prepared from 2 x 10^5 and 4 x 10^5 cells) express a band at 53 kDa corresponding to $GABA_AR$ α-1 chain (Fig 4.2). PMC do not express these bands, suggesting that PMC do not express the α-1 subunit protein. In RCMC lysate, the anti- $GABA_AR$α mAb also detects bands at 85 kDa and 45-50 kDa. Immunoblotting with an mouse IgG_1 isotype control mAb also shows the bands at 85 kDa and 45-50 kDa suggesting that these are unspecific.

GABA has no effect on spontaneous release of MC β-hexosaminidase

Since BDZ can modulate MC mediator release, we wanted to determine if GABA had a similar effect. Purified PMC were washed, treated with GABA (10 to 1000 μM) for 10 min and spontaneous β-hex release was measured. Spontaneous β-hex release in the absence of GABA (4.0 ± 0.9 %) was subtracted from all values. Fig 4.3 shows that GABA has no significant effect on β-hex release on resting MC.

GABA has no effect on 48/80-induced release of MC β-hexosaminidase

To determine if GABA affected MC β-hex release induced by a secretogogue, PMC were pretreated with GABA (10 to 1000 μM) at 37°C for 10 min and then stimulated with 48/80 (0.75 μg/mL) for 30 min (Fig 4.3). 48/80 alone induced 67.1 ± 5.0 % β-hex release (Fig 4.4). GABA has no significant effect on 48/80-induced β-hex release since GABA (10, 50, 100 and 1000 μM) pretreated PMC released 61.8 ± 8.1, 64.8 ± 10.6, 67.0 ± 8.7 and 67.4 ± 7.9 % β-hex respectively.

GABA inhibits antigen-induced release of MC β-hexosaminidase

To determine if GABA affected antigen-activated MC β-hex release, PMC were pretreated with GABA (10 to 1000 μM) at 37°C for 10 min and stimulated with antigen (10 WE/mL) and incubated for 30 min (Fig 4.5). Antigen alone induced 15.5 ± 2.3 % β-

hex release. Pretreatment with GABA (10 to 1000 μM) inhibited β-hex release by 30 to 35 % ($P<0.01$; Fig 4.5).

IFN-γ upregulates $GABA_AR$ subunit mRNA

In an effort to determine the effect of IFN-γ on $GABA_AR$ mRNA, total RNA from untreated and IFN-γ-treated (100 U/mL) RCMC were compared using the S1 nuclease assay. Preliminary results indicate that IFN-γ treatment (100 U/mL for 24 hr) upregulates expression of α1, α3, α4, α5, β1, β2, β3, γ1, γ2 and γ3 $GABA_AR$ subunits (Fig 4.6). The β-actin internal control, however, remains unchanged between the treated and untreated RCMC RNA.

Discussion

These preliminary results show that MC express mRNA for $GABA_AR$ subunits α (1-5), β (1 and 3) and γ (1-3) and protein for the α1 subunit. This is the first study to demonstrate $GABA_AR$ subunit expression in rat MC. This is also the first study to demonstrate IFN-γ upregulation of $GABA_AR$ subunit expression in MC. Although previous studies had shown that BDZ can inhibit MC functions, this study shows that GABA inhibits antigen-dependent β-hex release from MC but has no effect on resting MC or 48/80-stimulated MC.

MC mediator release is dependent upon chloride flux yet the expression of specific chloride channels in MC is poorly known. There are three major classes of chloride channels in mammalian cells; the volume and voltage-gated chloride channels (ClC), the cystic fibrosis transmembrane conductance regulator (CFTR) and the $GABA_AR$. In these preliminary studies we wanted to determine whether mast cells (MC) expressed mRNA and protein for $GABA_A$ subunits and whether GABA had an effect on MC mediator release.

For the first time, we have shown that mast cells express GABAR subunit mRNA as well as protein. Radiolabeled oligonucleotides specific for GABAR subunits hybridized with mast cell total RNA show that RCMC express message for subunits α1,

α3, α4, α5, β1, β2, β3, γ1, γ2 and γ3. Immunoblotting with an anti-α1 antibody indicates that RCMC express a 53 kDa protein that corresponds to α1 protein. The presence of the α1 subunit protein in MC suggests the presence of additional subunits since the α1 subunit requires a β subunit for assembly into the $GABA_AR$ in cerebellar granule neurons (33). This is the first study to demonstrate $GABA_AR$ subunit expression in rat peritoneal and cultured MC. However, mouse splenic T cells express $GABA_AR$ subunit mRNA and protein and ligation of these receptors inhibits anti-CD3 and antigen-specific T cell proliferation (2). GABA pretreatment of T cells inhibits their cytotoxicty (3). However, GABA pretreatment of target cells (mastocytoma cells) potentiats T cell cytoxicity. This study suggests that GABA may differentially modulate cytotoxic functions (perhaps via production of TNF) and antigen presentation.

Preliminary S1 nuclease assay suggests that IFN-γ upregulates mRNA expression of α1, α3, α4, α5, β1, β2, β3, γ1, γ2 and γ3 $GABA_AR$ subunits. However, these are preliminary experiments and it is unknown whether all of the subunits expressed as mRNA are expressed as surface protein or whether these subunits comprise a functional $GABA_AR$ chloride channel capable of conducting a Cl^- current. In order to determine whether MC express a functional $GABA_AR$, patch clamp measurements of Cl^- current in response to $GABA_AR$ agonists and antagonists need to be performed.

MC activation is regulated by chloride movement across the plasma membrane and activation of GABAR chloride channels could have an important regulatory effect on mast cell secretion. Accordingly, we tested whether different GABA concentrations would modulate β-hex release from activated (48/80 or antigen) MC. Resting mast cells were unaffected by various concentrations of GABA (10 to 1000 μM). Moreover, GABA had no effect on 48/80 stimulated mast cell degranulation even at high doses (1000 μM GABA). Interestingly, low doses of GABA inhibited antigen-stimulated release of β-hex by as much as 35% (10 μM GABA). A previous study of GABA effects on guinea pig MC and macrophages shows that GABA (10 to 1000 μM) inhibits 10 to 19 % of antigen-stimulated histamine release (34). However, GABA has no effect on macrophage release of platelet-activating factor (PAF) induced by formyl-methionyl-leucyl-

phenylalanine (fMLP). This suggests that GABA inhibition of MC mediator release is MC-specific and may involve MC-specific pathways.

Radioligand binding experiments must be done to determine if GABA binds to MC. Ideally, specific and non-specific binding is calculated using a range of concentrations of several drugs and the data is presented as a Scatchard plot. The K_d is calculated from a line fitting the curve of specific binding. Therefore, these analyses may fully characterize the nature of GABA binding to MC membranes.

The ability of a neuronal product to regulate MC function may be an important link between the immune and nervous system. There is evidence that neuronal products can regulate MC functions such as migration and mediator release (35-37). MC express the high affinity receptor for nerve growth factor (NGF), and NGF is a strong chemoattractant for peritoneal MC, inducing severe morphological changes and rearrangements of F-actin (35,38). In the presence of lysophosphatidylserine, NGF also stimulates serotonin release from MC (37).

Interestingly, peripheral BDZ receptor ligands have an anti-inflammatory effect on mouse models of inflammation. BDZ derivatives inhibit prostaglandin E_2 and IL-6 production and leukocyte recruitment in a mouse air-pouch model of local inflammation (39). PK11195 and Ro5-4864 inhibit mouse paw edema induced by IL-13 and IL-6 (40). Although Ro5-4864 (10^{-6} M) has no effect on histamine or serotonin release in either unstimulated PMC or those stimulated by compound 48/80 or substance P (13), MC activated by pro-inflammatory cytokines may be sensitive to Ro5-4864 inhibition in this paw edema model. GABA inhibition of MC mediator release may similarly regulate inflammatory responses.

Therefore, this study opens new avenues for $GABA_A$ studies in non-neuronal cells. GABA inhibition of MC mediator release may be mediated via $GABA_AR$. Detailed characterization of $GABA_AR$ subunit expression in MC and their ability to form a functional Cl^- channel, capable of altering MC Cl^- will prove valuable. Furthermore, it would be interesting to determine whether IFN-γ upregulation of $GABA_AR$ subunits alters $GABA_AR$ affinity for various ligands and effects receptor Cl^- conductance. It may

be that the GABA subunit promoters contain IFN-γ response elements capable of initiating transcription. This is the first study to show cytokine regulation of the $GABA_AR$. GABA may be another neurostransmitter capable of mediating the link between the nervous and immune systems.

Table 4.1. Sequences of the oligodeoxyribonucleotide probes complementary to the $GABA_AR$ subunits used in the S1 nuclease protection assay.

$GABA_AR$ subunit	Sequence (5'-3')
α-1	GGGGTCACCCCTGGCTAAGTTAGGGGTATA GCTGGTTGC
α-2	AGATTCGGGGCGTAGTTGGCAACGGCTACAGCA
α-3	CTCAGCAGGACTGTCTTGCACATAAGTGGTCTTGGGGGAAGCAATCACTG
α-4	CAAGTCGCCAGGCACAGGACGTGCAGGAGG
α-5	CACAGCATTCCCAGTCCCGCCTGGAAGCTGCTCCTTTGGGA
β-1	ATGGCAACCATCACAGGAAAAGAGAGAAGCCCCAAACTCTCTCGA
β-3	CTGAATTCCTGGTGTCACCAACGCTGCCTGCAACCTCATTCATTTCATTGTGAAC
γ-1	GCAGTCTTCAAAGCAACAGAAAAAGGTAGCACAGTCTTTGCCCTCCAAGC
γ-2	GTTCATTTGGATCGTTGCTGATCTGGGACGGAT
γ-3	AGAGGGTGCTTAAGGCTTATTCGATCAGGAATCCATCTTGTTGAATCTGGATGT
β-actin	CTGGTGGCGGGTGTGGACCGGGACGGAGGAGCTGCAA

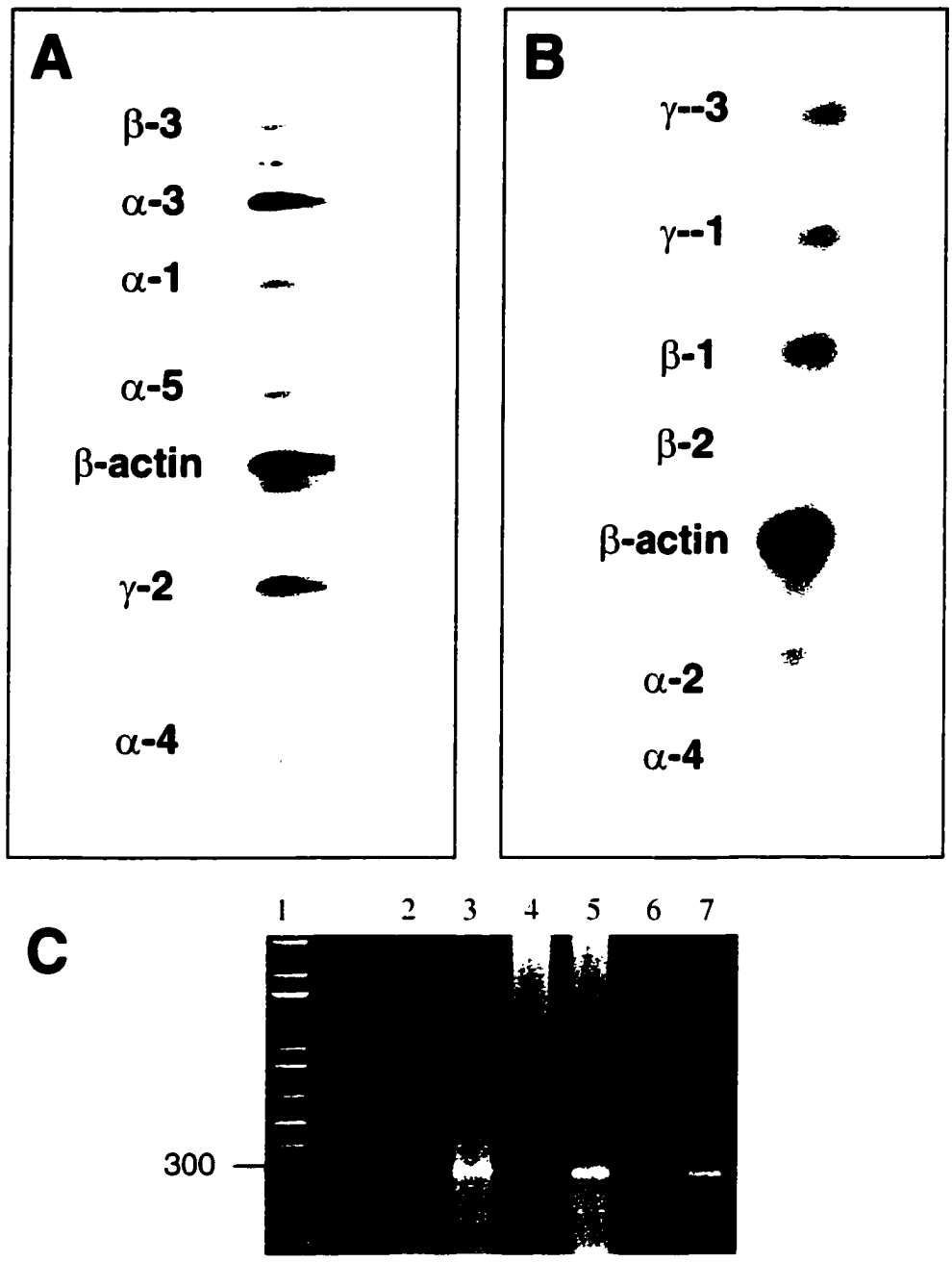


Figure 4.1. S1 nuclease assay shows $GABA_AR$ mRNA is expressed by rat cultured mast cells (RCMC). Radiolabeled oligonucleotides complementary to $GABA_AR$ subunit mRNA were hybridized to RCMC total RNA and treated with S1 nuclease (A and B). (C) RT-PCR analysis was used to confirm $GABA_AR\alpha R$ subunit expression in RCMC and peritoneal mast cells (PMC). Lane 1, DNA ladder; lane 2, RCMC RNA (no RT); lane 3, RCMC cDNA; lane 4, PMC RNA (no RT); lane 5, PMC cDNA; lane 6, rat brain RNA (no RT); lane 7, rat brain cDNA. (These are representative experiments of n=3).

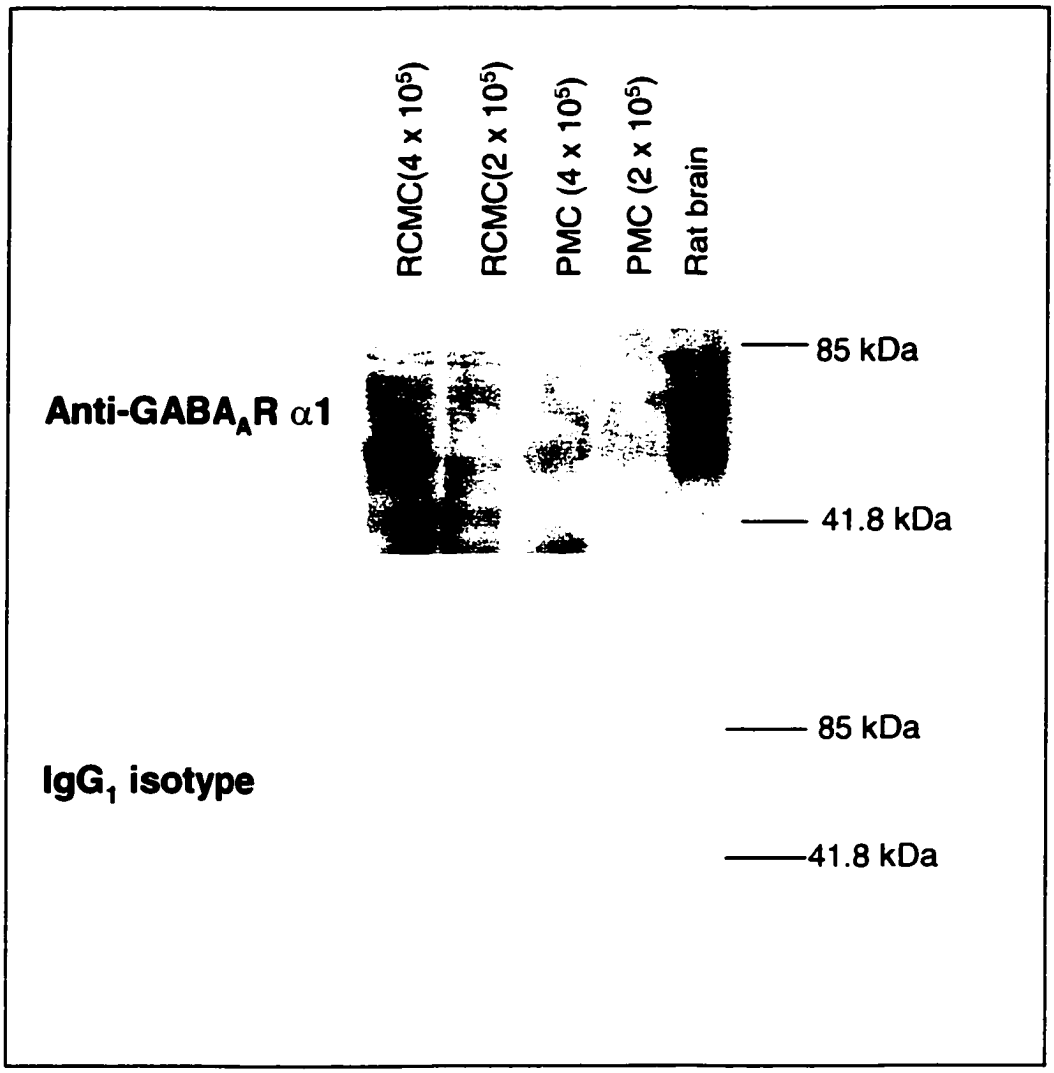


Figure 4.2. Peritoneal mast cells (PMC) and rat cultured mast cells (RCMC) express $GABA_AR$ α-1 subunit. Lanes were loaded with RCMC and PMC lysate from 2.0 x 10^5 and 4.0 x 10^5 cells on a 10% polyacrylamide gel and blotted with anti-$GABA_AR$α-1 subunit mAb. The blots were treated with chemiluminescent reagent and exposed to film for 30 min. (This is a representative experiment of n=3).

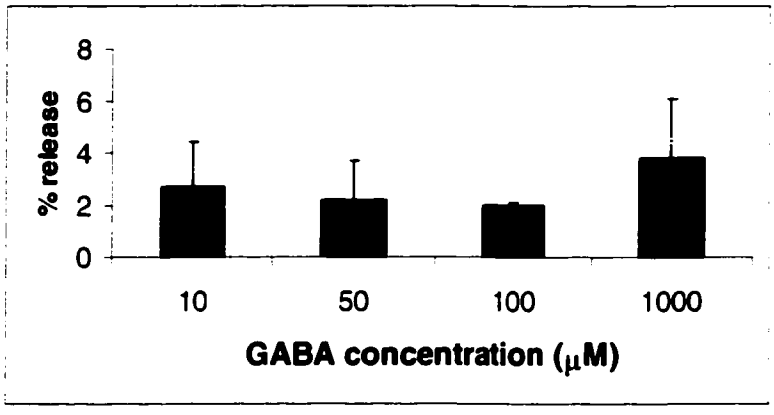


Figure 4.3. γ-aminobutyric acid (GABA) has no effect on resting mast cell β-hexosaminidase release. Peritoneal mast cells were isolated, placed in HTB and treated with different concentrations of γ-aminobutyric acid (GABA, 10 to 1000 μM) in 10 min (minus spontaneous release, n=6).

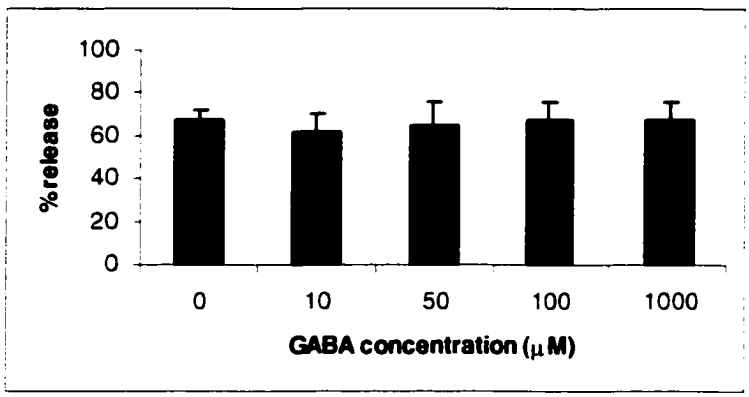


Figure 4.4. γ -aminobutyric acid (GABA) has no effect on 48/80 stimulated β-hexosaminidase release. Peritoneal mast cells were isolated and pretreated with GABA (10 to 1000 μM) for 10 min, stimulated with 48/80 (0.75 μg/mL) for 30 min and β-hexosaminidase release was measured. (Values indicated are minus spontaneous release, n=5).

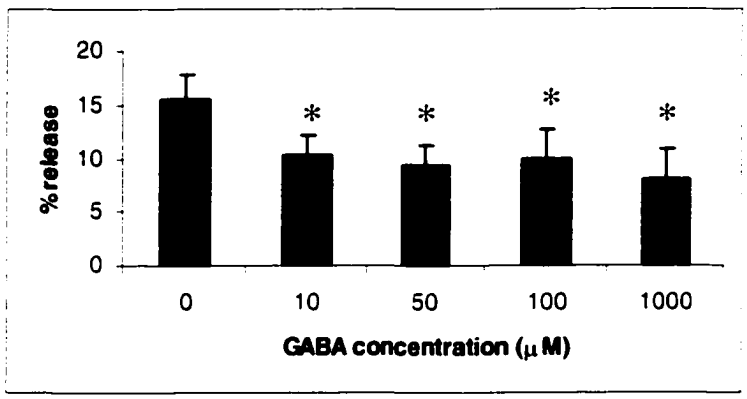


Figure 4.5. γ -aminobutyric acid (GABA) inhibits antigen-stimulated β-hexosaminidase release. Peritoneal mast cells were isolated and pretreated with GABA (10 to 1000 μM) for 10 min. stimulated with antigen (10 U/mL) for 30 min and β-hexosiminidase release was measured. (Values indicated are minus spontaneous release, n=5). * values significant compared to untreated ($P<0.01$).

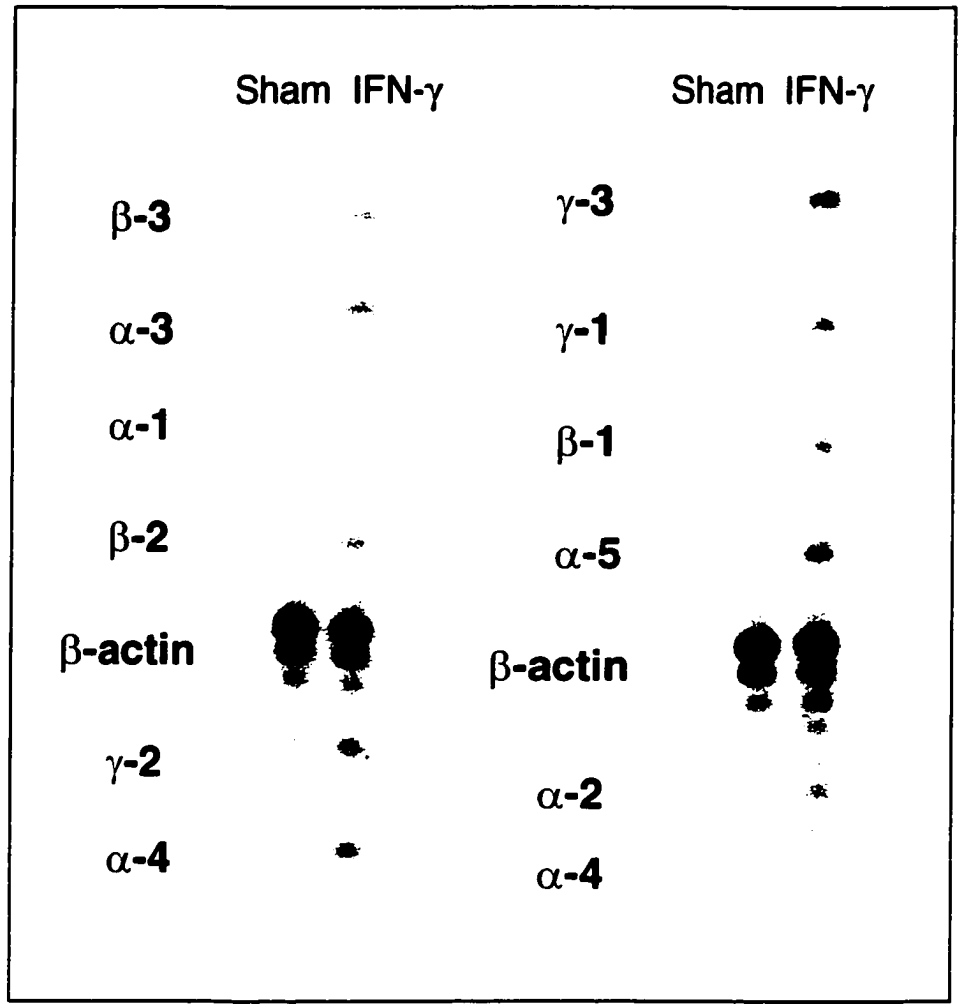


Figure 4.6. IFN-γ upregulates mRNA expression of several $GABA_AR$ subunits. Radiolabeled oligonucleotides complementary to $GABA_AR$ subunit mRNA were hybridized to rat cultured mast cell total RNA and treated with S1 nuclease. (This is a representative experiment of n=3).

References

1. Kuriyama, K. 1994. Cerebral GABA receptors. *Alcohol Alcohol Suppl. 2*:181.

2. Tian, J., C. Chau, T. G. Hales, and D. L. Kaufman. 1999. GABA(A) receptors mediate inhibition of T cell responses. *J.Neuroimmunol. 96*:21.

3. Bergeret, M., M. Khrestchatisky, E. Tremblay, A. Bernard, A. Gregoire, and C. Chany. 1998. GABA modulates cytotoxicity of immunocompetent cells expressing GABAA receptor subunits. *Biomed. Pharmacother. 52*:214.

4. Kardos, J. 1999. Recent advances in GABA research. *Neurochem. Int. 34*:353.

5. Schousboe, A. 1999. Pharmacologic and therapeutic aspects of the developmentally regulated expression of GABA(A) and GABA(B) receptors: cerebellar granule cells as a model system. *Neurochem. Int. 34*:373.

6. Rocca, P., A. M. Beoni, C. Eva, P. Ferrero, E. Zanalda, and L. Ravizza. 1998. Peripheral benzodiazepine receptor messenger RNA is decreased in lymphocytes of generalized anxiety disorder patients. *Biol. Psychiatry 43*:767.

7. Rocca, P., A. M. Beoni, C. Eva, P. Ferrero, G. Maina, F. Bogetto, and L. Ravizza. 2000. Lymphocyte peripheral benzodiazepine receptor mRNA decreases in obsessive-compulsive disorder. *Eur. Neuropsychopharmacol. 10*:337.

8. Maeda, S., T. Miyawaki, T. Nakanishi, M. Takigawa, and M. Shimada. 1998. Peripheral type benzodiazepine receptor in T lymphocyte rich preparation. *Life Sci. 63*:1423.

9. Pavese, N., G. Giannaccini, L. Betti, S. Ferrari, E. Bonanni, U. Bonuccelli, L. Murri, and A. Lucacchini. 2000. Peripheral-type benzodiazepine receptors in human blood cells of patients affected by migraine without aura. *Neurochem. Int. 37*:363.

10. Cahard, D., X. Canat, P. Carayon, C. Roque, P. Casellas, and G. Le Fur. 1994. Subcellular localization of peripheral benzodiazepine receptors on human leukocytes. *Lab. Invest. 70*:23.

11. Alexander, B. E., E. Roller, and U. Klotz. 1992. Characterization of peripheral-type benzodiazepine binding sites on human lymphocytes and lymphoma cell lines and their role in cell growth. *Biochem. Pharmacol. 44*:269.

12. Caldiroli, E., F. Marino, M. Cosentino, F. De Ponti, A. M. Fietta, A. Mazzone, A. Zibetti, S. Lecchini, and G. M. Frigo. 1998. Peripheral benzodiazepine receptor expression on leukocytes and neutrophil function during anticonvulsant monotherapy. *Pharmacology 57*:215.

13. Miller, L. G., A. Lee-Paritz, D. J. Greenblatt, and T. C. Theoharides. 1988. High-affinity benzodiazepine binding sites on rat peritoneal mast cells and RBL-1 cells: binding characteristics and effects on granule secretion. *Pharmacology 36*:52.

14. Moynagh, P. N., L. A. O'Neill, and D. C. Williams. 1993. Interleukin-1 and phorbol myristate acetate modulate the peripheral-type benzodiazepine receptor in lymphocytes and glial cells. *Biochem. Pharmacol. 46*:821.

15. Bessler, H., B. Caspi, M. Gavish, M. Rehavi, J. Hart, and R. Weizman. 1997. Peripheral-type benzodiazepine receptor ligands modulate human natural killer cell activity. *Int. J. Immunopharmacol. 19*:249.

16. Bessler, H., B. Caspi, M. Gavish, M. Rehavi, and A. Weizman. 1997. Significant inhibition of spontaneous IgA secretion by selective peripheral-type benzodiazepine receptor ligands. *Clin. Neuropharmacol. 20*:215.

17. Fride, E., R. Meng, P. Skolnick, and P. K. Arora. 1992. Immunoenhancing effects of alprazolam, a benzodiazepine receptor agonist. *Ann. N. Y. Acad. Sci. 650:*132.

18. Marino, F., S. Cattaneo, M. Cosentino, E. Rasini, L. Di Grazia, A. M. Fietta, S. Lecchini, and G. Frigo. 2001. Diazepam stimulates migration and phagocytosis of human neutrophils: possible contribution of peripheral-type benzodiazepine receptors and intracellular calcium. *Pharmacology 63*:42.

19. Bidri, M., B. Royer, G. Averlant, G. Bismuth, J. J. Guillosson, and M. Arock. 1999. Inhibition of mouse mast cell proliferation and proinflammatory mediator release by benzodiazepines. *Immunopharmacology 43*:75.

20. Suzuki-Nishimura, T., T. Sano, and M. K. Uchida. 1989. Effects of benzodiazepines on serotonin release from rat mast cells. *Eur J. Pharmacol. 167*:75.

21. Wills, F. L., M. Gilchrist, and A. D. Befus. 1999. Interferon-gamma regulates the interaction of RBL-2H3 cells with fibronectin through production of nitric oxide. *Immunology 97*:481.

22. Kirshenbaum, A. S., A. S. Worobec, T. A. Davis, J. P. Goff, T. Semere, and D. D. Metcalfe. 1998. Inhibition of human mast cell growth and differentiation by interferon gamma-1b. *Exp. Hematol. 26*:245.

23. Nafziger, J., M. Arock, J. J. Guillosson, and J. Wietzerbin. 1990. Specific high-affinity receptors for interferon-gamma on mouse bone marrow-derived mast cells: inhibitory effect of interferon-gamma on mast cell precursors. *Eur. J. Immunol. 20*:113.

24. Coleman, J. W., M. G. Buckley, M. R. Holliday, and A. G. Morris. 1991. Interferon-gamma inhibits serotonin release from mouse peritoneal mast cells. *Eur. J. Immunol. 21*:2559.

25. Bissonnette, E. Y., B. Chin, and A. D. Befus. 1995. Interferons differentially regulate histamine and TNF-alpha in rat intestinal mucosal mast cells. *Immunology 86*:12.

26. Ramaswamy, K. and D. Befus. 1993. IgE antibody responses in bronchoalveolar spaces of rats infected with Nippostrongylus brasiliensis. *Exp. Parasitol. 76*:23.

27. MacDonald, A. J., J. Pick, E. Y. Bissonnette, and A. D. Befus. 1998. Rat mucosal mast cells: the cultured bone marrow-derived mast cell is biochemically and functionally analogous to its counterpart in vivo. *Immunology 93*:533.

28. Ramaswamy, K. and D. Befus. 1993. Pulmonary inflammation and immune responses during the course of Nippostrongylus brasiliensis infection: lymphocyte subsets in bronchoalveolar lavage fluids of rats. *Parasite Immunol. 15*:281.

29. Chan, B. M., C. S. Ra, W. N. Hu, K. McNeill, H. R. Miller, D. Befus, J. P. Kinet, and A. Froese. 1990. Characterization of rat tissue cultured mast cells. *Eur. J. Immunol. 20*:185.

30. Gilchrist, M., A. J. MacDonald, I. Neverova, B. Ritchie, and A. D. Befus. 1997. Optimization of the isolation and effective use of mRNA from rat mast cells. *J. Immunol. Methods 201*:207.

31. Tanay, V. A.-M. I., B. P. Tancowny, T. A. Glencorse, and A. N. Bateson. 1997. The quantitative analysis of multiple mRNA species using oligonucleotide probes in an S1 nuclease protection assay. *Mol. Biotech. 7*:217.

32. Tsai, M., M. Miyamoto, S. Y. Tam, Z. S. Wang, and S. J. Galli. 1995. Detection of mouse mast cell-associated protease mRNA. Heparinase treatment greatly improves RT-PCR of tissues containing mast cell heparin. *Am. J. Pathol. 146*:335.

33. Kumar, M., X. Luo, P. L. Quirk, and R. E. Siegel. 2001. Antisense suppression of $GABA_A$ receptor ? subunit levels in cultured cerebellar granule neurons demonstrates their importance in receptor expression. *J. Neurochem. 77*:211.

34. Gentilini, G., S. Franchi-Micheli, S. Mugnai, D. Bindi, and L. Zilletti. 1995. GABA-mediated inhibition of the anaphylactic response in the guinea-pig trachea. *Br. J.Pharmacol. 115*:389.

35. Sawada, J., A. Itakura, A. Tanaka, T. Furusaka, and H. Matsuda. 2000. Nerve growth factor functions as a chemoattractant for mast cells through both mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling pathways. *Blood 95*:2052.

36. Marshall, J. S., R. H. Stead, C. McSharry, L. Nielsen, and J. Bienenstock. 1990. The role of mast cell degranulation products in mast cell hyperplasia. I. Mechanism of action of nerve growth factor. *J.Immunol 144*:1886.

37. Horigome, K., J. C. Pryor, E. D. Bullock, and E. M. Johnson,Jr. 1993. Mediator release from mast cells by nerve growth factor. Neurotrophin specificity and receptor mediation. *J.Biol. Chem. 268*:14881.

38. Tam, S. Y., M. Tsai, M. Yamaguchi, K. Yano, J. H. Butterfield, and S. J. Galli. 1997. Expression of functional TrkA receptor tyrosine kinase in the HMC-1 human mast cell line and in human mast cells. *Blood 90*:1807.

39. Fruscella, P., M. Sottocorno, M. Di Braccio, L. Diomede, N. Piccardi, A. Cagnotto, G. Grossi, M. Romano, T. Mennini, and G. Roma. 2001. 1,5-Benzodiazepine tricyclic derivatives exerting anti-inflammatory effects in mice by inhibiting interleukin-6 and prostaglandinE(2) production. *Pharmacol. Res. 43*:445.

40. Torres, S. R., T. S. Frode, G. M. Nardi, N. Vita, R. Reeb, P. Ferrara, R. M. Ribeiro-do-Valle, and R. C. Farges. 2000. Anti-inflammatory effects of peripheral benzodiazepine receptor ligands in two mouse models of inflammation. *Eur J.Pharmacol. 408*:199.

Chapter 5

Expression and Functional Characterization of CFTR in Mast Cells[1]

[1] Authors, M. Kulka, M. Gilchrist, M Duszyk and A.D. Befus. A version of this chapter was published in *Journal of Leukocyte Biology*. 2002. I contributed to this work by carrying out every experiment and writing the manuscript.

Introduction

The product of the cystic fibrosis (CF) gene is the cystic fibrosis transmembrane conductance regulator (CFTR) which functions as a cAMP-regulated Cl^- channel in the apical membrane of secretory epithelial cells (1). While CFTR expression has been generally considered to be epithelial cell specific, evidence for CFTR expression and/or function has also been observed in other cell types, including lymphocytes, Sertoli cells, heart muscle cells, tracheal submucosal gland cells and hypothalmic neurons (2-6). Although Cl^- transport has been generally implicated in the modulation of membrane potential in several cell types, lymphocyte activation, $CD8^+$ T cell-mediated cytotoxicity and volume regulation, the physiological relevance of CFTR expression in non-epithelial cells is poorly understood (3,7,8).

Cl^- transport has also been implicated in mast cell (MC) activation and degranulation (9,10). Cations such as K^+ and Ca^{2+} play a key role in many MC functions (11) and recent patch clamp and pharmacological studies have shown that Cl^- conductance is an important component of MC activation and secretion (12-14). MC are important effector cells in inflammatory diseases such as asthma and allergy. Following antigen-mediated clustering of IgE bound to the high affinity Fcε receptor (FcεRI), MC release mediators such as histamine and arachidonic acid metabolites that cause immediate bronchial smooth muscle constriction, bronchial edema and mucus hypersecretion (15-18). Ion transport studies suggest that MC mediator release is a multiphasic process. Following activation, the influx of Ca^{2+} from both intracellular and extracellular sources leads to the activation of Ca^{2+}-dependent enzymes and G-proteins initiating the fusion of the granule membrane with the plasma membrane (13,19-23). This Ca^{2+} flux also activates Cl^- channels that balance whole cell current and allow a sufficient increase in intracellular Ca^{2+} to initiate Ca^{2+}-dependent events (12,24). Experiments using pharmacological blockers provide evidence that MC exocytosis (which ultimately results in release of histamine and other mediators) is dependent on channels that are Cl^--selective (12). In fact, drugs that prevent MC secretion such as furosemide and cromolyn block Cl^- channel activity (25).

The identity of Cl^- channels expressed in MC is poorly known. However, cAMP-activated Cl^- conductances have been measured in rat MC suggesting the possible expression of CFTR (24). In epithelial cells, CFTR is one of the major Cl^- channels controlling Cl^- flux across the apical membrane. We postulate that MC express CFTR mRNA and protein and that the function of CFTR is important for MC activation and subsequent mediator release.

Materials and Methods

Materials and Reagents

The pharmacological reagents used in these experiments were: 4,4'-diisothiocyano-2,2'-stilbenedisulphonate (DIDS; Sigma D-3514), diphenylamine-2-carboxylate (DPC; ICN 193703), 8-bromo-cAMP (Sigma), 8-(4-chlorophenylthio)-cAMP (CPT-cAMP; Sigma) and forskolin (Sigma). DIDS blocks Cl^- channels and Cl^- transporters by modifying amino groups, but does not affect CFTR Cl^- transport (26,27). DPC blocks Cl^- flux via CFTR and the Cl^-/HCO_3 co-transporter by binding to the channel pore (28).

Rats and PMC Isolation

Male Sprague Dawley rats (300-350 g; Charles River, St. Constant, Quebec, Canada) were housed in a pathogen-free, viral antibody-free facility. For MC sensitization, rats were infected with 3000 *Nippostrongylus brasiliensis* as previously described (29). Peritoneal mast cells (PMC) were isolated from sensitized rats $\geq$ 4 weeks after infection. Rats were sacrificed by cervical dislocation under anesthesia and PMC were isolated by the following procedure: 20 mL of ice-cold Hepes Tyrode's buffer (HTB) was injected into the peritoneal cavity and massaged gently for 30 s; the peritoneum was opened and the buffer collected with a transfer pipette and kept on ice or at 4°C for subsequent procedures. Following centrifugation at 200 g the cell pellet was resuspended in 5 mL of fresh HTB and layered on top of a 30%/80% Percoll gradient.

The gradient was centrifuged at 500 g for 20 min and the highly enriched MC were collected from the pellet (30). PMC were >98% pure and >96% viable.

Worm antigen used to stimulate PMC was prepared as follows: rats were infected with 6000 *Nippostrongylus brasiliensis* as previously described (29). Two weeks after infection, rats were sacrificed by cervical dislocation under anesthesia and adult worms were isolated from the small intestines. Adult worms were incubated in HTB at 1000 worm equivalents (WE)/mL for 24 hr at 37°C. Supernatants were isolated and stored at -20 °C.

Cell Culture

The rat MC line, RCMC 1.11.2 (kindly provided by A. Froese, Winnipeg, Manitoba), was established from Wistar-ICI rats (31) and cultured in a humidified atmosphere of 5% CO_2 in air at 37°C. RCMC were placed in RPMI 1640 medium containing 5% FBS (Gibco BRL), 100 U/mL penicillin, 100 μg/mL streptomycin and 10 mM Hepes. The human lung carcinoma epithelial A549 and human lung edenocarcinoma CALU-3 cell lines were grown in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin and incubated in the same conditions as above.

Stimulation and β-hexosaminidase assay

PMC were either pre-treated with ion channel inhibitors for 5 min or untreated. The ion channel inhibitors used were DIDS (20 μM or 80μM) and DPC (0.5 mM or 1.0 mM) in HTB. DIDS is an irreversible inhibitor of anion channels, including Cl^- channels (26,27). DPC is a reversible inhibitor of both CFTR and a bicarbonate exchanger. PMC were resuspended in several different buffers: complete buffer (containing 140 mM NaCl, 4.3 mM $Na_2PO_4{\cdot}2H_20$, 1.2 mM K_2PO_4, 1.2 mM $MgCl_2$, 5 mM KCl, 0.5 mM $CaCl_2$, 1 mM EGTA, 4 mM glucose, 0.1% BSA), chloride-reduced buffer (11.4 mM NaCl, 127 mM NaGluconate, 4.3 mM $Na_2PO_4{\cdot}2H_20$, 1.2 mM K_2PO_4, 1.2 mM $MgCl_2$, 5 mM KCl, 0.5 mM $CaCl_2$, 1 mM EGTA, 4 mM glucose, 0.1% BSA), bromide-enhanced buffer (11.4 mM NaCl, 127 mM NaBr, 4.3 mM $Na_2PO_4{\cdot}2H_20$, 1.2 mM K_2PO_4, 1.2 mM $MgCl_2$, 5 mM KCl, 0.5 mM $CaCl_2$, 1 mM EGTA, 4 mM glucose, 0.1% BSA) and iodide-enhanced buffer

(11.4 mM NaCl, 127 mM NaI, 4.3 mM $Na_2PO_4{\cdot}2H_20$, 1.2 mM K_2PO_4, 1.2 mM $MgCl_2$, 5 mM KCl, 0.5 mM $CaCl_2$, 1 mM EGTA, 4 mM glucose, 0.1% BSA). The PMC were stimulated with either 48/80 or 10 worm equivalents (WE) of *Nippostrongylus brasiliensis* worm antigen for 10 min at 37°C (32). PMC were pelleted at 200*g* and the supernatant was removed. Pellet and supernatant samples were assayed for β-hexosaminidase (β-hex) by hydrolysis of the fluorescent substrate 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (Sigma M-2133). One unit of enzyme cleaves 1 μmol of substrate/hr at 37°C (30). Pellet and supernatant samples (50 μL) were added in duplicate wells in a microtitre plate followed by 1 mM substrate (50 μL) and the mixture was incubated at 37°C for 2 hr. The reaction was terminated by the addition of 100 μL of 0.2 M Tris base and the optical density was read at 450 nm (excitation 356 nm). The OD_{450nm} for blank wells, containing only substrate and Tris base, was automatically subtracted from the sample OD_{450nm} and the percentage β-hex release was calculated by the formula:

$$\frac{OD_{450nm} \text{ of substrate samples}}{OD_{450nm} \text{ of pellet samples} + OD_{450nm} \text{ of supernatant samples}} \times 100 = \% \text{ release}$$

The spontaneous release (in HTB) was subtracted from all samples to give % specific release.

Reverse Transcripase-Polymerase Chain Reaction (RT-PCR), Cloning and Sequence Analysis

RNA was extracted from PMC and RCMC using the modified Chomczynski and Sacchi method (33). Briefly, 10^6 to 10^7 cells were homogenized with solution D (4 M guanidinium thiocyanate, 0.5% sodium n-laurylsarcosine, 1 M sodium citrate and 0.1 mM 2-mercaptoethanol). To the homogenate, 2 M NaOAc, water saturated phenol and chloroform-isoamyl alcohol was added. The aqueous phase was removed and treated with solution D once again. The RNA was precipitated with absolute ethanol and washed with 70% ethanol and air-dried. The RNA obtained from PMC was treated with 1 U/mL

of heparinase for 2 hr at room temperature to remove contaminating heparin because high heparin concentrations markedly decrease the efficiency of the RT-PCR procedure (34).

Genomic DNA was digested by incubating 10 μg of total RNA with 5 U of DNAse (amplification grade; GIBCO BRL), 10X DNAse buffer (GIBCO BRL) 10 U of RNAse inhibitor (GIBCO BRL) and RNAse-free H_2O for 15 min at room temperature. After 15 min incubation, 25 mM EDTA was added and the sample was heated at 65°C for 20 min to inactivate the enzyme.

Treated RNA (1 μg) was incubated with 0.5 μg oligo(dT) at 70°C for 10 min in a thermocycler. A reverse transcriptase master mix was added to the RNA. The master mix contained First Strand Buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, pH 8.3, Gibco/BRL), 10 mM DTT, 10 mM of each dNTP, Sigma sterile water and 200 U M-MLV RT enzyme (Gibco/BRL). This mixture was incubated at 37°C for 1 hr, then 70°C for 10 min.

The polymerase chain reaction master mix contained 1X PCR buffer (20 mM Tris-Cl, 50 mM KCl, pH 8.4, Gibco/BRL), 0.8 mM dNTP mix, 20 μM antisense CFTR primer (5' GGT GTC CTA TTC ACC TCA AGT TCT CTG 3') which binds bp 901-927 of the *Rattus norvegicus* CFTR gene (NCBI accession # X95927.1), 20 μM of sense CFTR primer (5' CTC TGT AGA CCA TAC TGG CCT TGA AC 3') which binds bp 618-643 of the *Rattus norvegicus* CFTR gene, 1.5 mM $MgCl_2$ (Gibco/BRL), sterile Sigma water, 2 μL of cDNA and 2.5 units of Taq DNA Polymerase (Gibco/BRL). The mixture was amplified at an annealing temperature of 56°C for 35 cycles. The product was analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. The conditions of use for the primers specific for CFTR were optimized using rat lung RNA.

The 309 bp CFTR PCR product was cloned into the pCR 2.1® (Invitrogen, Carlsbad, Ca, USA) vector using T4 DNA ligase and then used to transform "super competent" *E.coli* cells. The *E.coli* cells were grown for 3 d, the DNA was isolated using a Promega isolation kit and the plasmid was sequenced on the ABI PRISM sequencer model 2.1.1 and analyzed by BLAST (35). The sequence obtained was 92% homologous

to the CFTR gene. The remaining 8% was due to an inability of the sequencer to determine the nucleic acid in the selected position.

Flow Cytometry

Single cell suspensions of 10^5 cells were fixed with 5% formalin for 5 min. The fixation reaction was stopped by adding PBS/1% BSA. Whenever antibodies that recognized an intracellular portion of the CFTR protein (C-terminus or R domain) were used, cells were permeabilized and blocked with PBS/0.1% saponin/5% dried milk for 24 hr at 4°C. Otherwise, cells were blocked with PBS/5% dried milk for 24 hr at 4°C. Blocked cells were incubated with primary mAb (mouse anti-CFTR C-terminus, Genzyme, 2503-01; mouse monoclonal anti-CFTR extracellular domain, Affinity Bioreagents, MA1-935; or mouse monoclonal anti-CFTR R domain, Genzyme, 1660-01) for 1 hr in either PBS/0.1% saponin/5% dried milk or PBS/5% dried milk. Cells were washed twice with either PBS/0.1%/5% dried milk or PBS/5% dried milk and incubated with the appropriate secondary antibody (rabbit anti-mouse IgG:FITC, Serotec, STAR 38 or goat anti-mouse IgM:FITC, Biosource AM14708) for an additional hr. The isotypes antibody used for comparison was purified mouse IgG_1 κ-isotype standard (anti-TNP) (Serotec, catalogue # 03001D) and mouse IgM isotype control anti-TNP (Pharmingen 03081D).

Western Immunoblotting and Immunocytochemistry

RCMC, PMC or CALU-3 cells were isolated, washed with PBS and 1 x 10^6 cells were lysed with 2X sample buffer (0.5 mL 1M Tris-Cl, 1 mL DTT, 2 mL 10% SDS, 1 mL glycerol, 0.5 mL 0.12 % bromophenol blue) supplemented with 2% β-mercaptomethanol.

Before staining with Ab, 30-40 μg of whole cell extracts were separated by 8% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% milk in TBS-Tween for 24 hr at room temperature and then stained with primary antibodies, anti-CFTR (R domain, Genzyme, 1660-01) and isotype control purified mouse IgG_1 κ-isotype standard (anti-TNP; Serotec, 03001D) for 1 hr at room

temperature. The membranes were washed with TBS-Tween 3X and then stained with the secondary antibody for 1 hr. The secondary antibody was sheep anti-mouse IgG conjugated to HRP (Amersham Life Science, catalogue # NA 9310).

The nitrocellulose membranes were developed with chemiluminescence reagent (NEN Life Technologies, catalogue # NEL 101) for 1 min and placed into an autoradiography cassette containing high performance chemiluminescence film (Amersham Life Science, catalogue # RPN2103H). The film was exposed for 30 min.

Immunocytochemistry was performed on cytospins of RCMC cells according to instructions of commercial kit (Vectastain ABC kit PK-6200) as follows. Fifty μL aliquots of RCMC and PMC (1 x 10^6 cells/mL) cell suspension were cytospun onto slides. Slides were air dried and then stained with primary Ab (extracellular domain specific mAb) and isotype (mouse IgM) in PBS for 45 min in humidified atmosphere at room temperature. Slides were washed with PBS for 5 min. Biotinylated Ab solution from the commercial kit was added for 30 min. Slides were once again washed for 5 min in PBS. Developing reagent was added for 30 min and washed away for 5 min. Chromagen was added for 5 min or until color developed. Slides were washed again and fixed with ethanol, cleaned in xylene and mounted with Permount (Sigma). Images were photographed using a phase contrast microscope at 625 X magnification.

^{36}Cl Efflux Assay

To determine Cl^- secretion attributable to CFTR, a ^{36}Cl efflux assay was used (36). This assay is based on the principle that CFTR Cl^- secretion is activated by cyclic AMP. This assay requires repeated washing of cells and measurement of ^{36}Cl present in the medium before and after addition of a cAMP analogue.

RCMC were seeded at 70-95% confluence in a 25 cm^2 flask and washed 3X with Ringer's solution (HCO_3^-, phosphate-buffered 109 mM NaCl Ringer's solution supplemented with 28 mM lactate). Thirty μL of $^{36}Cl^-$ solution (sodium salt from ICN; 1 μCi/μL; 1 mCi=37 kBq) were diluted in 5 mL of Ringer's solution and added to the flask containing the cells. The flask was incubated for 2 to 3 hr in at 37°C. All assays were performed at 37°C. At time 0, the flask was washed with Ringer's solution. A fresh 2

mL aliquot of Ringer's solution was added immediately to the flask and the measurement of Cl^- efflux rate was started. This process was repeated every 15 sec for 1 min, when Ringer's solution with forskolin (2.5 μM), 8-bromo-cAMP (250 μM), and 8-(4-chlorophenylthio)-cAMP (CPT-cAMP; 250 μM) was added, incubated with the cells for 15 sec and removed. These compounds were not added again for the remaining 4 min of the efflux run. At the end of the run, 50 mM NaOH was added to lyse the cells and to determine the radioactive counts remaining in the cells. Each sample was diluted in scintillation cocktail and its radioactivity was measured in a scintillation counter. The fraction of intracellular ^{36}Cl remaining in the cells during each time point was calculated and the time-dependent rates of ^{36}Cl efflux were calculated as $\ln(^{36}Cl_{t=1}/^{36}Cl_{t=2})/(t_1-t_2)$, where ^{36}Cl is the percent intracellular Cl at time t and t_1 and t_2 are successive time points. (37).

Statistical Analysis

One way analysis of variance (ANOVA) was performed on all β-hex and ^{36}Cl efflux data assuming $P<0.01$ unless otherwise stated.

Results

Mast cells express message and protein for CFTR

To determine whether MC expressed CFTR, specific primers were designed for rat CFTR and reverse transcriptase-polymerase chain reaction (RT-PCR) was used to screen two MC populations for CFTR expression. Fig 1 shows that cDNA preparations made from RCMC, *in vivo*-derived PMC and rat lung total RNA contain CFTR mRNA. The 309 bp PCR product represents the fragment size expected from the published rat cDNA sequence (NCBI accession number X95927.1). RCMC and PMC total RNA preparations (without RT) do not show any amplification, indicating that the primers are specifically amplifying CFTR mRNA not contaminating genomic DNA. PCR amplification was also performed using primers specific for β-actin to ensure cDNA

quality. RCMC, PMC and lung cDNA express the 560 bp β-actin product and, as expected, RCMC, PMC and lung RNA (without RT) do not.

To ensure that the CFTR primers were specifically amplifying CFTR cDNA, PCR products from different RNA preparations were amplified using CFTR primers and inserted into a pCR vectors and cloned in *E.coli.* Sequencing showed that the PCR products had 100% homology to region 763-927 of the *Rattus norvegicus* CFTR gene (Fig 2).

To determine the presence of CFTR protein in rat MC, a mouse monoclonal anti-CFTR C-terminus, a mouse monoclonal anti-CFTR extracellular domain and a mouse monoclonal anti-CFTR R domain antibody was used in flow cytometry to screen RCMC and PMC for CFTR expression. To ensure that these antibodies recognized native CFTR protein, CALU-3 submucosal airway epithelial cells, which express large amounts of CFTR protein, were used as a positive control and A549 lung epithelial cells, which do not express CFTR protein, were used as a negative control. As expected, CALU-3 cells were positive for CFTR protein expression using antibodies recognizing the C-terminus and extracellular domain, while A549 cells were negative (Fig 3). RCMC and PMC were also positive for CFTR expression using the C-terminus and extracellular domain antibodies since the anti-CFTR antibody shows a shift in expression from the isotype control antibodies (Fig 3). The antibody to the C terminus of CFTR used in this experiment has been used by others to demonstrate that cAMP induces the recruitment of CFTR from cytoplasmic pools to the apical plasma membrane [39,40]. The protocol in this study involved permeabilization of the MC before immunostaining. Thus, the C-terminus antibody is capable of detecting intracellular as well as surface expression of CFTR. However, the antibody recognizing the R-domain did not show a shift even in the CALU-3 cells, suggesting that in the flow assay this antibody does not recognize native CFTR protein. Since previous studies have shown that the R-domain antibody can recognize CFTR in western immunoblotting, we extracted cell lysates from PMC, RCMC and CALU-3, resolved them by SDS-PAGE and immunoblotted using this antibody. Western immunoblotting indicates that RCMC, PMC and CALU-3 express a CFTR protein of approximately 170 kDa as expected (Fig 4).

Immunocytochemistry was also performed to determine the localization of CFTR protein expression in RCMC and PMC using the mAB specific for the extracellular domain of CFTR. Fig 5 shows that CFTR protein expression is indeed localized to the periphery of the mast cell (Fig 5A) with strongest staining at the cell membrane. Greater than 90% of the cells observed were positive for CFTR expression. RCMC stained with isotype control antibody (IgM) show very little staining (Fig 5B). PMC likewise stained with anti-CFTR (extracellular domain) mAb also show staining although it is not localized to the periphery but rather scattered throughout the cell in small pockets (Fig 5C). More than 90% of the cells observed were positive for CFTR expression. The isotype control for PMC was negative (Fig 5D).

Elevated cAMP Levels Induce Cl^- Secretion in MC

To assess the Cl^- channel function of CFTR in MC, paired $^{36}Cl^-$ efflux assays with and without a membrane-permeable cAMP agonist mixture (2.5 μM forskolin, 250 μM 8-bromo-cAMP, and 250 μM CPT-cAMP) were performed in RCMC according to the procedure adapted from Schwiebert et al., 1998 (36). Results indicated that, similar to CALU-3 cells (which express wild-type CFTR), RCMC show a recognizable Cl^- flux when cAMP agonists were added (1 min; Fig 6). The cAMP-dependent Cl^- flux is sensitive to DPC (Fig 6A) since the addition of DPC to the agonist mixture reduced the Cl^- flux by 50%. The A549 epithelial cell line does not express CFTR and does not show an increased Cl^- flux after addition of cAMP analogs above basal rates. These findings indicate that the RCMC CFTR protein is a functioning Cl^- channel since the cells display a DPC-sensitive, cAMP-dependent Cl^- current.

Release of β-Hex from Mast Cells is CFTR and chloride channel dependent

Ion Selectivity

CFTR has a characteristic ion permeability (Br>Cl>I), which can be used to distinguish it from other Cl^- channels. We postulated that if CFTR regulated mediator release from PMC, the ion content of the buffer would also be important in mediator release. The ability of PMC to release granule-associated β-hex in buffers containing Cl^-,

Br^- and I^- ions was measured (Fig 7) following stimulation with either 48/80 (0.75 μg/mL) or antigen (10 WE). Under physiological conditions (140 mM NaCl), PMC stimulated with Ag released 9.9 ± 0.9 % of their stored β-hex (minus spontaneous release 3.5 ± 0.1%). This value was considered to be 100% release in these experiments and all subsequent values were calculated as a percentage of this (Fig 7A). PMC stimulated with Ag in buffer containing NaGluconate (127 mM) or NaI (127 mM) released considerably less β-hex (42.4 ± 9.7 % and 20.7 ± 2.1 % of maximum respectively), whereas PMC in NaBr (127 mM) buffer released almost maximum levels of β-hex (81.3 ± 8.6 %) suggesting that both Br^- and Cl^- ions can facilitate maximum PMC release and that the presence of gluconate or I^- ions cannot compensate for their absence.

Fig 7B shows that in the presence of a physiological concentration of Cl^- ions (140 mM), PMC stimulated with 48/80 released a substantial amount of β-hex (58.4 ± 3.7 % of amount stored in cell) which represents maximum release possible in these experiments and subsequent values were calculated in relation to this maximum release. PMC stimulated with 48/80 in buffer containing NaGluconate (127 mM) or NaI (127 mM) released considerably less β-hex (30.6 ± 4.5 % and 11.9 ± 12.1 % respectively), whereas PMC suspended in NaBr (127 mM) buffer were unaffected, releasing almost the same amount of β-hex (85.1 ± 2.1 %) as cells in physiological NaCl buffer. These data suggest that in the absence of Cl^- or Br^- ions there is a significant decrease in PMC mediator release and that the presence of I^- ions cannot compensate. Gluconate is a large negatively charged molecule that has been postulated to compensate for Cl^- ions in some assays yet, gluconate does not restore PMC β-hex release. Since β-hex release is dependent on Br^- or Cl^- but not on I^-, this supports the contention that CFTR-specific Cl^- ion transport is important in PMC mediator release when stimulated with 48/80 or Ag.

Mast cell mediator release in the presence of the chloride channel inhibitor DIDS, DPC and glibenclamide

To determine if CFTR-specific Cl^- transport is important for MC mediator release, we used pharmacological inhibitors of different ion channels to determine the importance of ion flux and particular channels in β-hex release by PMC. Whereas RCMC contain

few granules and are not activated by conventional MC stimuli such as antigen (data not shown), PMC are readily activated by both antigen (Ag) and 48/80 (a potent activator of G-proteins). PMC were pretreated with the Cl^- channel inhibitor DIDS and the CFTR channel inhibitors DPC and glibenclamide for 5 min before activation by either 48/80 (0.75 μg/mL) or antigen (10 worm equivalents/mL). Thirty min after activation, β-hex release was measured (Fig 8, 9 and 10). DPC, DIDS and glibenclamide alone had no effect on spontaneous β-hex release from PMC. DIDS, a Cl^- channel inhibitor, dose dependently inhibited antigen-stimulated and 48/80 stimulated PMC β-hex release (Fig 8, A and B). The lowest concentration of DIDS (10 μM) inhibited 38 ± 13.2% of antigen-stimulated β-hex release, whereas the highest concentration of DIDS (800 μM) inhibited 138 ± 0.1%, making the IC_{50} (concentration that inhibits release by 50%) of antigen-stimulated β-hex release between 20-40 μM (Fig 8A). The lowest concentration of DIDS that significantly inhibited 48/80-stimulated β-hex release was 40 μM (inhibited release by 39 ± 17.2%; Fig 8B). Thus, the IC_{50} of 48/80-stimulated PMC was between 80-100 μM, which is higher than the IC_{50} of antigen-stimulated PMC. This difference in IC_{50} between antigen- and 48/80-stimulated PMC may reflect the potencies and differing mechanisms of activation of the two stimuli.

DPC, a more specific inhibitor of CFTR Cl^- channels, decreased antigen-stimulated PMC β-hex release by 31 ± 6.7% (1 μM; Fig 7A) but had little effect on 48/80 stimulated release (Fig 9B). The IC_{50} of antigen-stimulated β-hex release was between 0.1-0.5 mM DPC.

Glibenclamide, a sulfonylureal used in the treatment of ischemia, has previously been shown to block epithelial CFTR channels (38). In patch clamp experiments, glibenclamide inhibits CFTR Cl^- current with an IC_{50} of 22-38 μM with 100 μM glibenclamide causing nearly complete inhibition (39). Thus, β-hex release was measured from PMC treated with glibenclamide (5 min) and activated with either 48/80 or Ag (Fig 10). Glibenclamide (3, 30 and 300 μM) had no effect on 48/80- or antigen-stimulated PMC β-hex release (Fig 10A). The differences in sample means are not significant as tested by ANOVA ($P=0.202$ with $F=1.69$).

Discussion

Cl^- channels regulate cell volume, membrane potential, pH and osmolarity and can be classified according to their mechanism of activation. Cl^- channels are involved in degranulation of rat mucosal MC by maintaining MC resting membrane potential, counteracting the depolarization of the cell during degranulation and maintaining the driving force for Ca^{2+} entry (24,40). Because Cl^- channels in MC are poorly understood, our aim was to characterize and determine the role of CFTR, a complex Cl^- channel, in the release of MC mediators.

This study found that CFTR mRNA and protein is expressed in both *in vivo*-derived and cultured rat MC. PCR shows that PMC and RCMC express mRNA for CFTR and that this amplification product is, in fact, from the rat CFTR gene. Flow cytometry data, western blot analysis and immunostaining shows that as many as 80% of RCMC and PMC are positive for CFTR, which is similar in intensity to the CALU-3 epithelial cell line.

Our data also show that MC stimulated with membrane permeable analogues of cAMP can induce Cl^- secretion resulting in a net efflux of ^{36}Cl. This data coincides with previous observations in MC, which found that internally applied cAMP induces a Cl^- current within 10-30 sec of activation (41,42). Despite its structural similarity to members of the ATP binding cassette family, CFTR is a Cl^-channel (43,44) whose activity is under complex regulation by phosphorylation, nitric oxide and cytoplasmic ATP (3,43,45,46). In all cells in which CFTR is expressed, it is regulated by cAMP-dependent phosphorylation (1,47,48). CFTR-dependent Cl^- current is controlled by cAMP-dependent internal messengers which phosphorylate serine residues on the regulatory (R) domain of the CFTR molecule and cause a conformational change allowing the Cl^- channel to be opened (49). Elevated cAMP levels can also induce the activation of other ion channels such as cAMP-sensitive K^+ channels capable of hyperpolarizing the membrane and allowing for Cl^- flux through open Cl^- channels. Our data shows that elevation of intracellular [cAMP] in MC activates a net outward Cl^- flux

which is partly inhibited by DPC. This supports the hypothesis that MC express a functional CFTR, capable of conducting Cl^- ions.

Although our data clearly shows that MC express a CFTR Cl^- channel, the role of CFTR in MC function is less clear. Pharmacologic inhibition of Cl^- channels and ion replacement studies are often the best tools available to study the importance of particular ions in cell functions and in this study these methods indicate that Cl^- and Cl^- channels are important in MC β-hex release. PMC stimulated with 48/80 (0.75 μg/mL) in the presence of low [Cl^-] (16 mM) released less β-hex then in the presence of physiological levels of Cl^- (140 mM) suggesting that Cl^- ion exchange is important for 48/80 stimulated secretion. A previous report concluded that MC exocytosis was not dependent upon activation of Cl^- current by 48/80 (50). However, in that study, MC were activated with a high concentration of 48/80 (10 μg/mL) and the investigators suggested that Cl^- current may enhance secretion at suboptimal stimulation (50). In our study, we used a lower concentration of 48/80 (0.75 μg/mL) and at this dosage, our results suggest that PMC mediator release is Cl^- ion dependent.

The CFTR Cl^- channel displays ion selectivity and permeability such that substitution of Br^- or I^- for Cl^- in the buffer of a patch clamp system can alter the current through the channel. Normally, in the wild type CFTR channel, the sequence of anion permeability through the channel pore is Br>Cl>I and is strongly regulated by a "selectivity filter" close to the cytoplasmic end of the pore (51). In an experimental system where Br^- not I^- ions can partially substitute for Cl^-, the presence of a functional CFTR channel is suspected. PMC stimulated with 48/80 or antigen in a buffer containing Br^- instead of Cl^- ions released 85.1% of maximum β-hex, whereas PMC stimulated in a buffer containing I^- ions released 11.9% of maximum β-hex, suggesting a Br>I selectivity. Based on this selectivity, a CFTR-dependent ion flux is suspected to be present during MC β-hex release.

The presence of a functional CFTR channel is further characterized by its sensitivity to a variety of pharmacologic inhibitors known to block CFTR function. CFTR Cl^- channel *activity* (as measured by patch clamp) in epithelial cells can be blocked with DPC and glibenclamide, but not with the broad anion exchanger inhibitor DIDS

(52). In our study, DPC blocked β-hex release from PMC stimulated with antigen but the effect of glibenclamide is more difficult to interpret. Glibenclamide did not block β-hex release from PMC stimulated with antigen or 48/80. Glibenclamide's inability to effectively block degranulation may be a reflection of (1) 48/80 and antigen have different mechanisms of activation and (2) glibenclamide is also an inhibitor of Ca^{2+}-activated Cl^- channels and ATP-activated K^+ channels, the blockade of which may modify Cl^- currents (53).

The broad range inhibitor of several ion channels, DIDS, also reduced the release of β-hex from 48/80-stimulated PMC indicating that the role of Cl^- channels (and other ion channels in general) other than CFTR in mediator release should not be ruled out. In fact, CFTR can directly regulate ion flux through DIDS-sensitive channels and blocking these channels would affect CFTR's ability to affect the flow of ions across the plasma membrane (54).

Ag-stimulated and 48/80-stimulated mast cells appear to be differentially sensitive to the ion inhibitors. Ag activates PMC through the $F_c\varepsilon$ receptor (55), whereas 48/80 is a cation which complexes with the negatively charged sialic acid residues in the cell membrane and non-specifically activates phospholipase C (56). Thus, the difference in sensitivity to DPC, DIDS and glibenclamide of antigen- and 48/80-stimulated PMC is a reflection of the different modes of stimuli. Yet, the IC_{50} of DIDS treated PMC stimulated with 48/80 is ten times higher than the IC_{50} of DIDS treated PMC stimulated with antigen. Therefore, 48/80 stimulation of PMC might be less dependent upon DIDS-sensitive Cl^- flux. DPC inhibits antigen-stimulated but not 48/80-stimulated β-hex which suggests that Cl^- flux via CFTR might be more important at suboptimal stimulation which occurs via antigen-IgE-$F_c\varepsilon$ receptor complexes. The role of other DIDS-sensitive channels in β-hex release is possible. Both DIDS and DPC can block the Cl^-/HCO_3^- exchanger, which is an alkalinizing mechanism responsible for maintaining internal pH in MC. DIDS inhibition of the Cl^-/HCO_3^- exchanger would alter intracellular pH and indirectly inhibit CFTR-dependent ion flux (9,52).

Proposed Model for Cl^- flux in mast cells

MC resting membrane potential is regulated by two types of ion channels: an inwardly rectifying K^+ current and an outwardly rectifying Cl^- current (57). Upon stimulation by antigen, a small increase in membrane conductance occurs and causes an influx of extracellular Ca^{2+}. Ca^{2+} entry is driven by a hyperpolarized membrane. However, the resting potential of rat PMC is unstable as measured in the permeabilized patch configuration and an inward Ca^{2+} current would easily depolarize the cell. It has been proposed that Cl^- channels open and clamp the membrane potential at the electromotive force of Cl^- ions (E_{Cl}) (20), allowing for Ca^{2+} influx driven by a hyperpolarized membrane.

In electrophysiological studies (14), an outward membrane current (corresponding to the influx of Cl^-) is observed in rat PMC when they are stimulated with substance P or compound 48/80. These studies demonstrate the existence of hyperpolarization resulting from Cl^- influx into the cell, which creates a driving force to facilitate the entry of Ca^{2+} required to initiate histamine secretion. When rat MC are stimulated with antigen, there is a large increase in the rate of Cl^- uptake into the cell consistent with the hypothesis that overall Cl^- influx is important for MC exocytosis (24). The putative Cl^- channel blocker DIDS dose-dependently inhibits the antigen-stimulated histamine secretion but does not inhibit the antigen-stimulated increase of Cl^- uptake. These results indicate that initial rises in $[Cl]_i$ might be mediated by channels other than DIDS-sensitive Cl^- channels, such as a co-transporter or CFTR. The Na/K/2Cl-cotransporter inhibitor, furosemide, abolishes the increased antigen-induced Cl^- uptake but does not affect antigen-induced histamine release (58). Our studies show that CFTR is likely the DIDS-insensitive Cl^- channel that is important in this first step of MC exocytosis.

One of the major symptoms of CF is massive inflammation in the lung. The mechanism of this inflammation is unexplained, although the absence of functional CFTR protein is thought to play a role. Traditionally, the CFTR mutation has been thought to be most influential in disrupting epithelial cell function. P. aeruginosa is the most common infection found in lungs of CF patients and aberrant CFTR function correlates with increased levels of apical asioloGM1, asialylated glycolipids that function

as *P. aeruginosa* receptors (59). However, CFTR might also be an important regulator of non-epithelial cell function particulary in cells involved in immunity. $CD4^+$ T lymphocytes expressing mutant CFTR and stimulated with concanavalin A secrete 45% less IL-10 compared to T lymphocytes from healthy controls (60). Recruitment of neutrophils to sites of infection in CF patients is upregulated yet ineffective at clearing bacterial infection (61,62). Opsonic quality of naturally occuring antibodies to *P. aeruginosa* is markedly decreased in chronically infected CF patients (63). PMC pretreated with *P. aeruginosa* isolates from patients with CF show a decrease in release of histamine when stimulated with A23187 by at least 47% (64). This would suggest that bacterial infections can regulate mast cell secretion. In fact, mast cells secrete histamine, β-hexosaminidase and serotonin in response to gram negative rods (65,66), a process that may be lacking in patients with CF.

Furthermore, there is evidence that CF cells may be less sensitive to mast cell mediators. For example, tracheal gland cells isolated from cystic fibrosis patients show a smaller peak in $[Ca^{2+}]_i$ in response to histamine than tracheal gland cells from normal subjects (67). Therefore, mast cell function as well as responses to mast cell regulation of innate immune responses may be fundamentally altered in CF. This report offers insight into mechanisms regulating MC exocytosis and suggests that epithelial cells may not be the only cell type responsible for the CFTR defective phenotype seen in many CF patients and CFTR knockout mice.

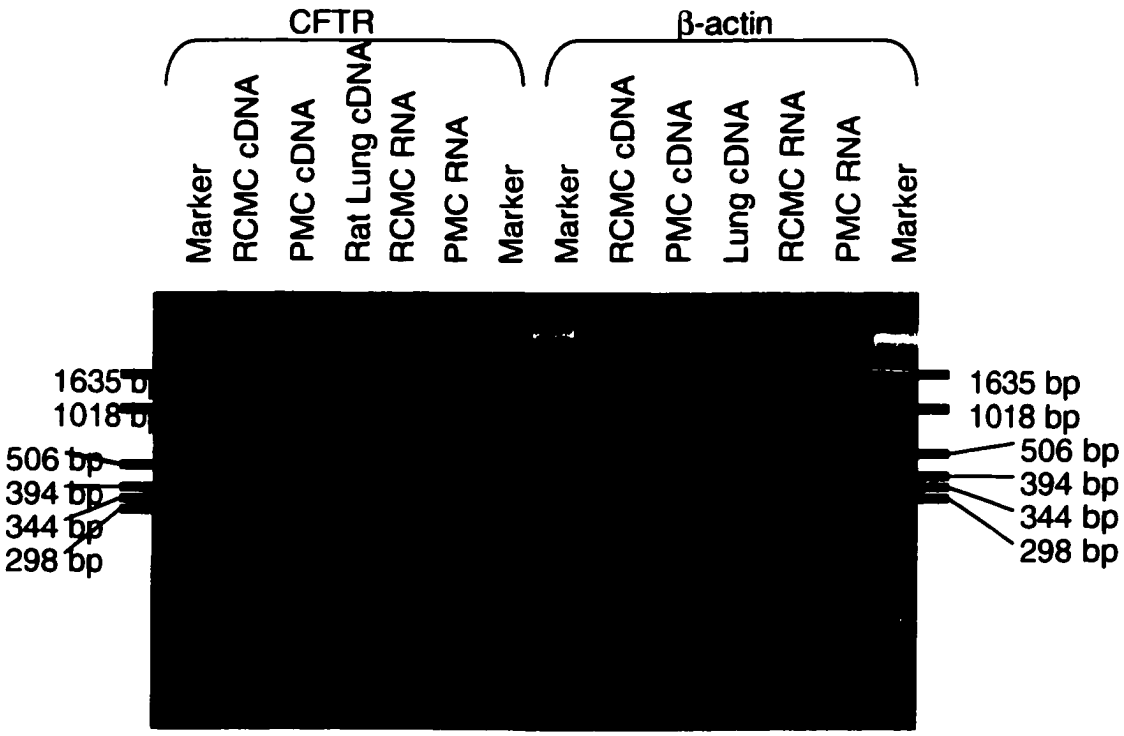


Fig. 5.1. RT-PCR showing CFTR mRNA expression by RCMC, PMC and rat lung cells. RCMC, PMC and lung RNA was reverse transcribed into cDNA. Subsequent cDNA samples were PCR amplified through 35 cycles using CFTR specific primers (lane 2-4 from left). PCR amplification was also conducted on RCMC, PMC and rat lung RNA preparations (without reverse transcription) to control for possible genomic contamination (lanes 5 and 6). RCMC, PMC and lung cDNAs show the expected band of 309 bp representing CFTR but RCMC, PMC and rat lung RNA that has also undergone PCR amplification does not contain the CFTR product. β-actin amplification (35 cycles) of the same RCMC, PMC and lung samples is also shown as a control for RNA quality. RCMC, PMC and rat lung cDNAs contain the expected 560 bp product (lanes 9-11) while the RNA controls do not (lanes 12 and 13). Lanes 1, 6, 7 and 14 contain a 1 kb marker for comparison). These results are representative of experiments on 10 separate RNA preparations.

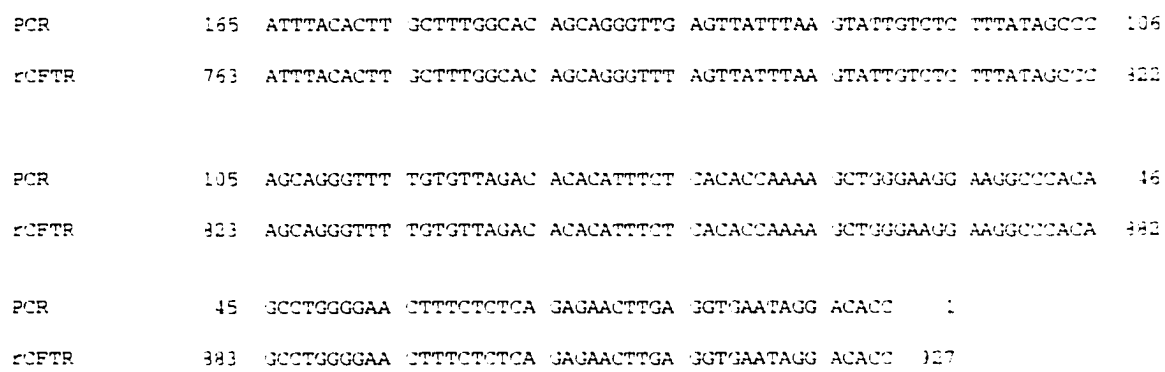


Fig. 5.2. Comparison of PCR amplification product (PCR; 1-165) with the published sequence of the rat CFTR gene sequence (rCFTR; 763-927). The comparison shows 100% homology to the rat CFTR gene. Three PCR products from independent preparations of RNA were cloned and sequenced by the ABI PRISM sequencer model 2.1.1 and analyzed by BLAST.

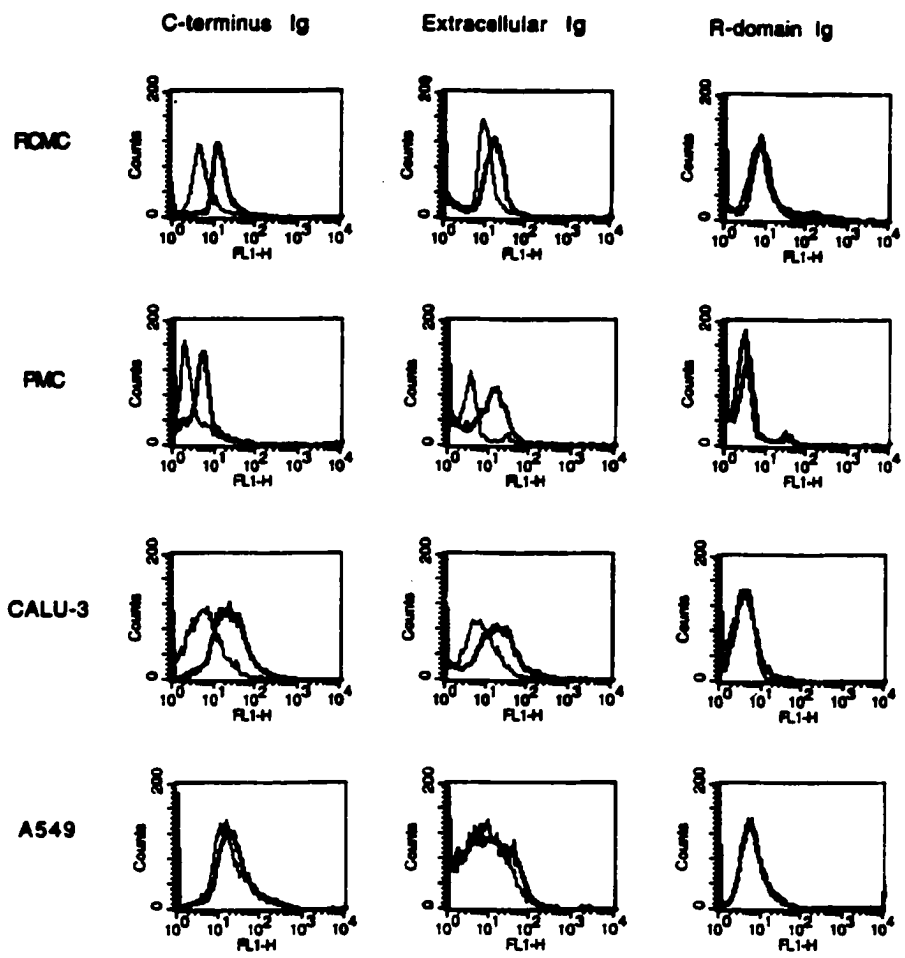


Fig. 5.3. Flow cytometry showing expression of CFTR on RCMC (first row), PMC (second row), CALU-3 epithelial cells (third row) and A549 epithelial cells (fourth row) using the three mAb recognizing three regions of the CFTR protein (C-terminus, extracellular domain and R domain) as labeled above. In each flow diagram, thin line represents fluorescence values obtained using the appropriate isotype control (see methods) and dark line represents fluorescence values obtained using anti-CFTR antibody indicated. Fluorescence values (FL1-H) are in log units on the x-axis and the cell counts are on the y-axis. Cells were incubated with monoclonal anti-CFTR (5-10 μg/mL) and isotype antibody (5-10 μg/mL) for 1 hr at room temperature.

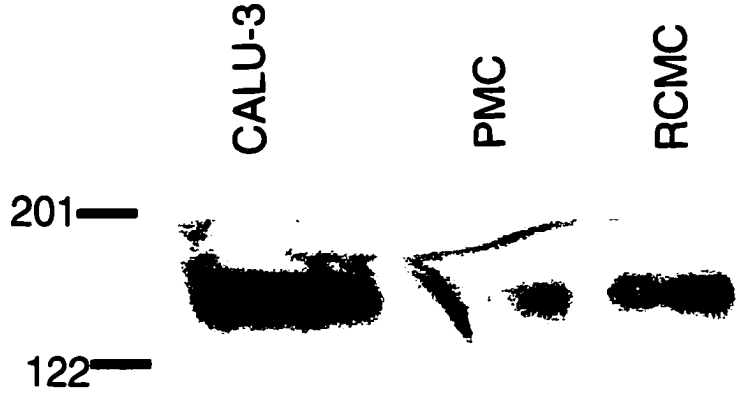


Fig. 5.4. Western blot analysis of CALU-3 (lane 1), PMC (lane 2) and RCMC (lane 3) cell lysates shows CFTR protein expression. Cell pellets were lysed in the presence of 2% β-mercaptoethanol and SDS and then resolved by 8% acrylamide SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane and stained with anti-CFTR (R-domain specific) mAb for 1 hr at room temperature (1/1000 dilution). This experiment is representative of 3 separate experiments on independently isolated cell pellets.

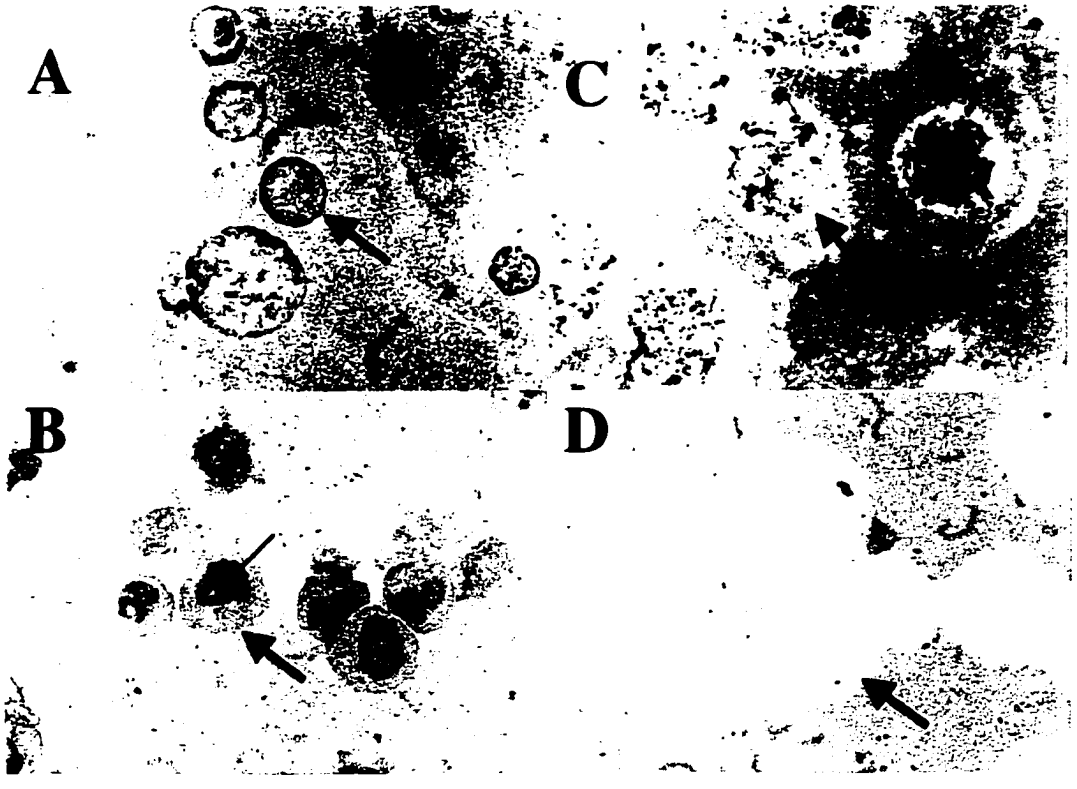


Fig. 5.5. Immunocytochemistry of RCMC **(A and B)** and PMC **(C and D)** showing expression of CFTR protein. **(A)** RCMC (at 5 x 10^4 cells/mL) cytospins were prepared as described and stained with anti-CFTR antibody (extracellular domain specific) at 1/100 dilution for 1 hr at room temperature. **(B)** RCMC (at 5 x 10^4 cells/mL) cytospins were prepared as described and stained with IgM isotype antibody at 1/100 dilution for 1 hr at room temperature. Cells were also counter-stained with hemotoxylin to make nuclei more visible. **(C)** PMC (at 5 x 10^4 cells/mL) cytospins were prepared as described and stained with anti-CFTR antibody (extracellular domain specific) at 1/100 dilution for 1 hr at room temperature. **(D)** PMC (at 5 x 10^4 cells/mL) cytospins were prepared as described and stained with IgM isotype antibody at 1/100 dilution for 1 hr at room temperature. Results are typical of 2 experiments. Thick arrows indicate individual cells. Thin arrow indicates nucleus.

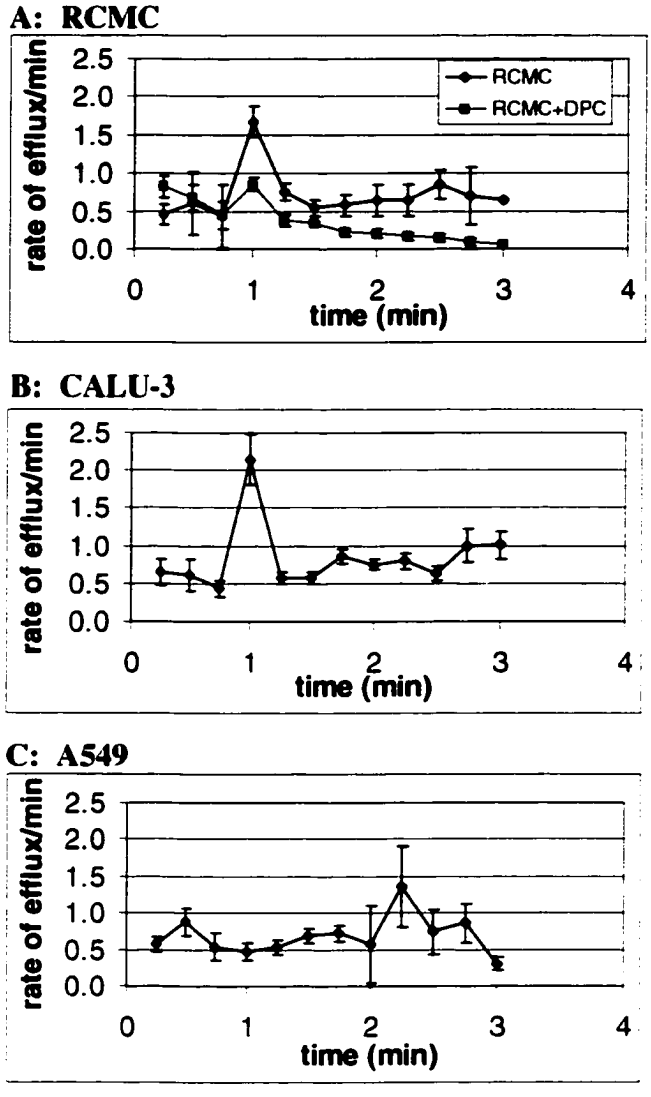
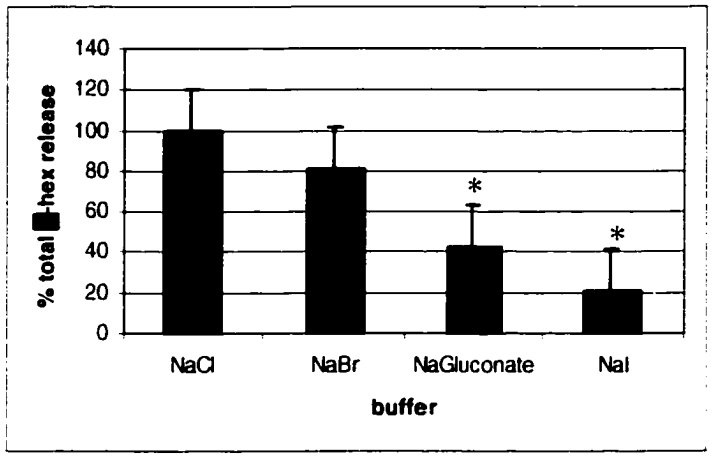


Fig. 5.6. ^{36}Cl Efflux Assay showing ^{36}Cl release from RCMC (**A**), CALU-3 (**B**) and A549 (**C**). Cells were grown to confluence and loaded with ^{36}Cl for 2 to 3 hr at 37°C. The unincorporated ^{36}Cl was discarded and the cells were washed every 15 sec to measure the rate of ^{36}Cl release over time. At time 60 s (1 min $\pm$ 5 sec), cAMP agonists were added for 15 sec. CALU-3 express wild-type CFTR protein and A549 do not express CFTR. (n=7, $P<0.01$, t_0 vs t_{60})

A: PMC stimulated with antigen



B: PMC stimulated with 48/80

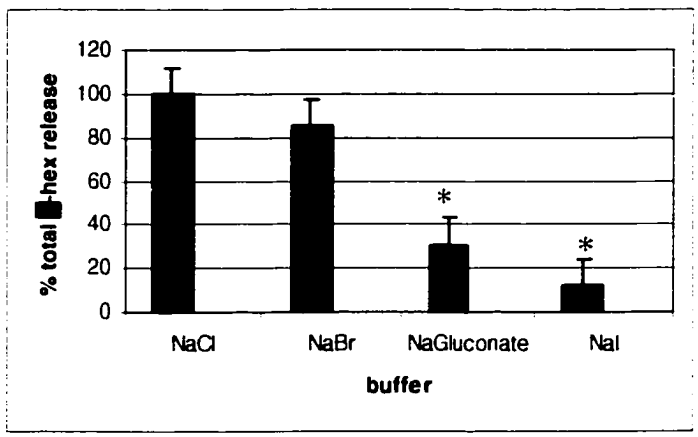
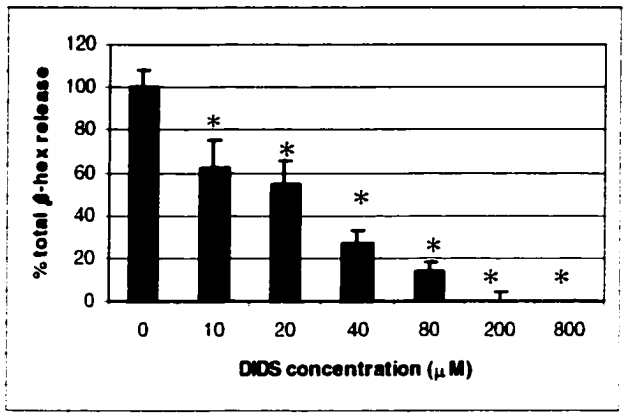


Fig. 5.7. β-hexosaminidase release of stimulated PMC in different buffers. PMC were placed in different buffers containing specific ions (Cl, Br, Gluconate or I) and then stimulated with either antigen or 48/80 at 37°C. **(A)** PMC were stimulated with antigen (10 worm equivalents/mL) for 10 minutes. **(B)** PMC were stimulated with 48/80 (0.75 µg/mL) for 10 min. Significance values are calculated in relation to antigen or 48/80 stimulated % total release and represent $P<0.01$. (n=5 separate experiments)

A: PMC stimulated with antigen



B: PMC stimulated with 48/80

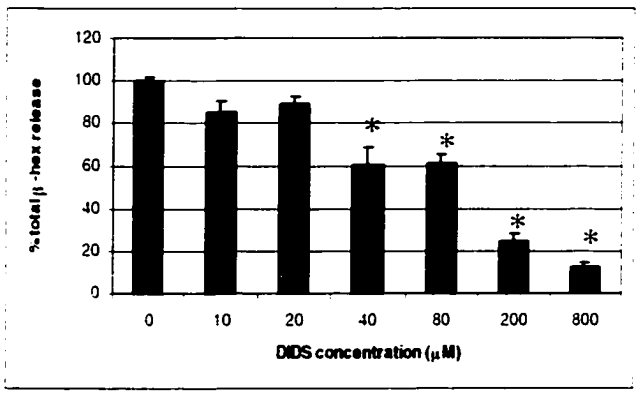
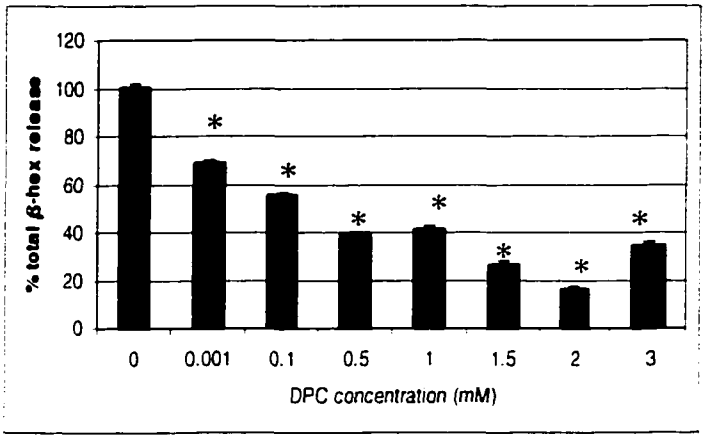


Fig. 5.8. β-hexosaminidase release from stimulated PMC treated with DIDS, a pharmacological inhibitor of chloride channels. PMC were treated with DIDS (chloride channels inhibitor; 10–800 μM) at 37°C for 5 min in physiological buffer containing 140 mM NaCl. **(A)** PMC were treated with DIDS for 5 min and stimulated with antigen (10 worm equivalents/mL) for 10 min. **(B)** PMC were treated with DIDS then stimulated with 48/80 (0.75 μg/mL) for 10 minutes. Significance values are calculated in relation to antigen or 48/80 stimulated % total release and represent $P<0.01$. (n=5 separate experiments)

A: PMC stimulated with antigen



B: PMC stimulated with 48/80

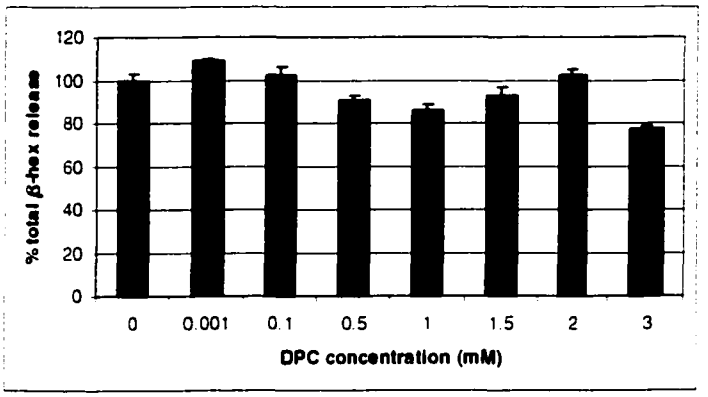
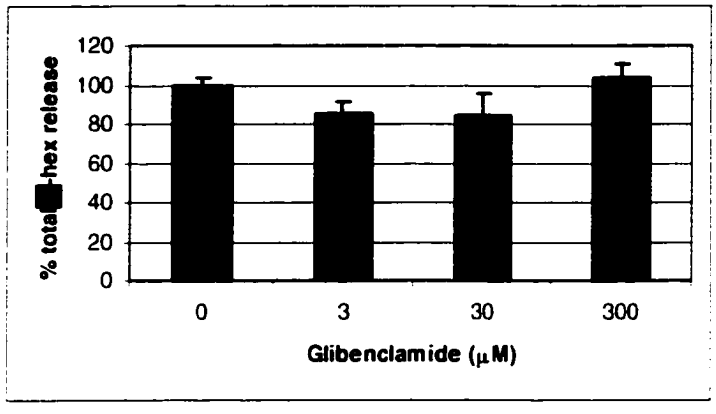


Fig. 5.9. β-hexosaminidase release from stimulated PMC treated with DPC. PMC were treated with DPC (CFTR chloride channel inhibitor; 0.001-3.0 mM) at 37°C for 5 min in physiological buffer containing 140 mM NaCl. **(A)** PMC were treated with DPC for 5 min and stimulated with antigen (10 worm equivalents/mL) for 10 min. **(B)** PMC were treated with DPC then stimulated with 48/80 (0.75 µg/mL) for 10 min. Significance values are calculated in relation to antigen or 48/80 stimulated % total release and represent $P<0.01$. (n=5 separate experiments)

A: PMC stimulated with antigen



B: PMC stimulated with 48/80

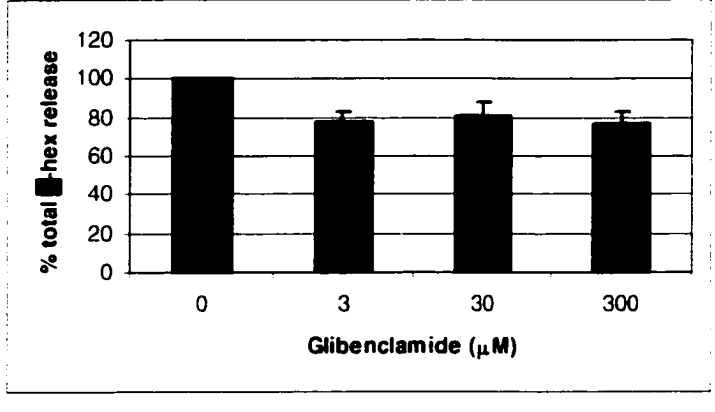


Fig. 5.10. β-hexosaminidase release from stimulated PMC treated with glibenclamide, a pharmacological inhibitor of CFTR channels. PMC were treated with glibenclamide (CFTR channel inhibitor; 3–300 μM) at 37°C for 5 min in physiological buffer containing 140 mM NaCl. **(A)** PMC were treated with glibenclamide for 5 min and stimulated with antigen (10 worm equivalents/mL) for 10 min. **(B)** PMC were treated with glibenclamide then stimulated with 48/80 (0.75 μg/mL). (n=6 separate experiments)

References

1. Sheppard, D. N. and M. J. Welsh. 1999. Structure and function of the CFTR chloride channel. *Physiol. Rev. 79*:S23.

2. Boockfor, F. R., R. A. Morris, D. C. DeSimone, D. M. Hunt, and K. B. Walsh. 1998. Sertoli cell expression of the cystic fibrosis transmembrane conductance regulator. *Am. J. Physiol. 274*:C922.

3. Dong, Y. J., A. C. Chao, K. Kouyama, Y. P. Hsu, R. C. Bocian, R. B. Moss, and P. Gardner. 1995. Activation of CFTR chloride current by nitric oxide in human T lymphocytes. *EMBO J. 14*:2700.

4. Wong, K. R., A. E. Trezise, S. Bryant, G. Hart, and J. I. Vandenberg. 1999. Molecular and functional distributions of chloride conductances in rabbit ventricle. *Am. J. Physiol. 277*:H1403.

5. Zhang, A. L. and G. M. Roomans. 1999. Multiple intracellular pathways for regulation of chloride secretion in cultured pig tracheal submucosal gland cells. *Eur. Respir. J. 13*:571.

6. Weyler, R. T., K. A. Yurko-Mauro, R. Rubenstein, W. J. Kollen, W. Reenstra, S. M. Altschuler, M. Egan, and A. E. Mulberg. 1999. CFTR is functionally active in GnRH-expressing GT1-7 hypothalamic neurons. *Am. J. Physiol. 277*:C563.

7. Hart, P., J. D. Warth, P. C. Levesque, M. L. Collier, Y. Geary, B. Horowitz, and J. R. Hume. 1996. Cystic fibrosis gene encodes a cAMP-dependent chloride channel in heart. *Proc. Natl. Acad. Sci. U. S. A. 93*:6343.

8. Premack, B. A. and P. Gardner. 1991. Role of ion channels in lymphocytes. *J. Clin. Immunol. 11*:225.

9. Vilarino, N., M. R. Vieytes, J. M. Vieites, and L. M. Botana. 1998. Role of HCO3- ions in cytosolic pH regulation in rat mast cells: evidence for a new Na+-independent, HCO3--dependent alkalinizing mechanism. *Biochem. Biophys. Res. Commun. 253*:320.

10. Jensen, T. B., U. G. Friis, and T. Johansen. 1998. Role of physiological HCO3-buffer on intracellular pH and histamine release in rat peritoneal mast cells. *Pflugers Arch. 436*:357.

11. Heidelberger, R., C. Heinemann, E. Neher, and G. Matthews. 1994. Calcium dependence of the rate of exocytosis in a synaptic terminal. *Nature 371*:513.

12. Reinsprecht, M., M. H. Rohn, R. J. Spadinger, I. Pecht, H. Schindler, and C. Romanin. 1995. Blockade of capacitive Ca2+ influx by Cl- channel blockers inhibits secretion from rat mucosal-type mast cells. *Mol. Pharmacol. 47*:1014.

13. Matthews, G., E. Neher, and R. Penner. 1989. Second messenger-activated calcium influx in rat peritoneal mast cells. *J. Physiol. (Lond) 418:105-30*:105.

14. Matthews, G., N. Erwin, and R. Penner. 1989. Chloride conductance activated by external agonists and internal messengers in rat peritoneal mast cells. *J. Physiol. 418*:131.

15. Takagi, M., T. Nakahata, T. Kubo, M. Shiohara, K. Koike, A. Miyajima, K. Arai, S. Nishikawa, K. M. Zsebo, and A. Komiyama. 1992. Stimulation of mouse connective tissue-type mast cells by hemopoietic stem cell factor, a ligand for the c-kit receptor. *J. Immunol. 148*:3446.

16. Ying, S., L. T. Barata, Q. Meng, J. A. Grant, J. Barkans, S. R. Durham, and A. B. Kay. 1998. High-affinity immunoglobulin E receptor (Fc?RI)-bearing eosinophils, mast cells, macrophages and Langerhans' cells in allergen-induced late-phase cutaneous reactions in atopic subjects. *Immunology 93*:281.

17. Darmani, H., J. L. Harwood, and S. K. Jackson. 1995. Differential effects of interferon-gamma and -beta on fatty acid turnover, lipid bilayer fluidity and TNF-alpha release in murine macrophage J774.2 cells. *Int. J. Exp. Pathol. 76*:331.

18. Lane, S. J. and T. H. Lee. 1996. Mast cell effector mechanisms. *J. Allergy Clin. Immunol. 98*:S67.

19. Suzuki, H., M. Takei, M. Yanagida, T. Nakahata, T. Kawakami, and H. Fukamachi. 1997. Early and late events in Fc epsilon RI signal transduction in human cultured mast cells. *J. Immunol. 159*:5881.

20. Penner, R., G. Matthews, and E. Neher. 1988. Regulation of calcium influx by second messengers in rat mast cells. *Nature 334*:499.

21. Beaven, M. A., J. Rogers, J. P. Moore, T. R. Hesketh, G. A. Smith, and J. C. Metcalfe. 1984. The mechanism of the calcium signal and correlation with histamine release in 2H3 cells. *J. Biol. Chem. 259*:7129.

22. Baram, D., R. Adachi, O. Medalia, M. Tuvim, B. F. Dickey, Y. A. Mekori, and R. Sagi-Eisenberg. 1999. Synaptotagmin II negatively regulates Ca2+-triggered exocytosis of lysosomes in mast cells. *J. Exp. Med. 189*:1649.

23. Pinxteren, J. A., A. J. O'Sullivan, P. E. Tatham, and B. D. Gomperts. 1998. Regulation of exocytosis from rat peritoneal mast cells by G protein beta gamma-subunits. *EMBO J. 17*:6210.

24. Friis, U. G., T. Johansen, N. A. Hayes, and J. C. Foreman. 1994. IgE-receptor activated chloride uptake in relation to histamine secretion from rat mast cells. *Br. J. Pharmacol. 111*:1179.

25. Hinder, F., J. Meyer, M. Booke, J. S. Ehardt, J. R. Salsbury, L. D. Traber, and D. L. Traber. 1998. Endogenous nitric oxide and the pulmonary microvasculature in healthy sheep and during systemic inflammation. *Am. J. Respir. Crit. Care Med. 157*:1542.

26. Pace, C. S., J. T. Tarvin, and J. S. Smith. 1983. Stimulus-secretion coupling in beta-cells: modulation by pH. *Am. J. Physiol. 244*:E3.

27. Passow, H., H. Fasold, E. M. Gartner, B. Legrum, W. Ruffing, and L. Zaki. 1980. Anion transport across the red blood cell membrane and the conformation of the protein in Band 3. *Ann. N. Y. Acad. Sci. 341:361-83*:361.

28. Lu, L., N. Okada, S. Nakatani, and K. Yoshikawa. 1995. Eicosapentaenoic acid-induced changes in membrane fluidity and cell adhesion molecules in cultured human keratinocytes. *Br. J. Dermatol. 133*:217.

29. Ramaswamy, K. and D. Befus. 1993. IgE antibody responses in bronchoalveolar spaces of rats infected with Nippostrongylus brasiliensis. *Exp. Parasitol. 76*:23.

30. MacDonald, A. J., J. Pick, E. Y. Bissonnette, and A. D. Befus. 1998. Rat mucosal mast cells: the cultured bone marrow-derived mast cell is biochemically and functionally analogous to its counterpart in vivo. *Immunology 93*:533.

31. Chan, L. N., Y. W. Chung, P. S. Leung, C. Q. Liu, and H. C. Chan. 1999. Activation of an adenosine 3',5'-cyclic monophosphate-dependent Cl- conductance in response to neurohormonal stimuli in mouse endometrial epithelial cells: the role of cystic fibrosis transmembrane conductance regulator. *Biol. Reprod. 60*:374.

32. Ramaswamy, K. and D. Befus. 1993. Pulmonary inflammation and immune responses during the course of Nippostrongylus brasiliensis infection: lymphocyte subsets in bronchoalveolar lavage fluids of rats. *Parasite Immunol. 15*:281.

33. Gilchrist, M., A. J. MacDonald, I. Neverova, B. Ritchie, and A. D. Befus. 1997. Optimization of the isolation and effective use of mRNA from rat mast cells. *J. Immunol. Methods 201*:207.

34. Tsai, M., M. Miyamoto, S. Y. Tam, Z. S. Wang, and S. J. Galli. 1995. Detection of mouse mast cell-associated protease mRNA. Heparinase treatment greatly improves RT-PCR of tissues containing mast cell heparin. *Am. J. Pathol. 146*:335.

35. Iwagaki, H., M. Marutaka, T. Yoshino, L. Cao, I. Sakuma, M. Uomoto, Y. Takeuchi, and N. Tanaka. 1996. Gamma-interferon upregulates transferrin receptors and decreases membrane microviscosity in K562 cells. *Res. Commun. Mol. Pathol. Pharmacol. 92*:191.

36. Schwiebert, E. M., M. M. Morales, S. Devidas, M. E. Egan, and W. B. Guggino. 1998. Chloride channel and chloride conductance regulator domains of CFTR, the cystic fibrosis transmembrane conductance regulator. *Proc. Natl. Acad. Sci. U. S. A. 95*:2674.

37. Hallows, K. R. and P. A. Knauf. 1994. Regulatory volume decrease in HL-60 cells: importance of rapid changes in permeability of Cl- and organic solutes. *Am. J. Physiol. 267*:C1045.

38. Sheppard, D. N. and M. J. Welsh. 1992. Effect of ATP-sensitive K+ channel regulators on cystic fibrosis transmembrane conductance regulator chloride currents. *J. Gen. Physiol. 100*:573.

39. Yamazaki, J. and J. R. Hume. 1997. Inhibitory effects of glibenclamide on cystic fibrosis transmembrane regulator, swelling-activated, and Ca(2+)-activated Cl- channels in mammalian cardiac myocytes. *Circ. Res. 81*:101.

40. Romanin, C., M. Reinsprecht, I. Pecht, and H. Schindler. 1991. Immunologically activated chloride channels involved in degranulation of rat mucosal mast cells. *EMBO J. 10*:3603.

41. Nafziger, J., M. Arock, J. J. Guillosson, and J. Wietzerbin. 1990. Specific high-affinity receptors for interferon-gamma on mouse bone marrow-derived mast cells: inhibitory effect of interferon-gamma on mast cell precursors. *Eur. J. Immunol. 20*:113.

42. Matthews, G., E. Neher, and R. Penner. 1989. Chloride conductance activated by external agonists and internal messengers in rat peritoneal mast cells. *J. Physiol. (Lond) 418*:131.

43. Anderson, M. P., R. J. Gregory, S. Thompson, D. W. Souza, S. Paul, R. C. Mulligan, A. E. Smith, and M. J. Welsh. 1991. Demonstration that CFTR is a chloride channel by alteration of its anion selectivity. *Science 253*:202.

44. Sheppard, D. N. and L. S. Ostedgaard. 1996. Understanding how cystic fibrosis mutations cause a loss of Cl-channel function. *Mol. Med. Today 2*:290.

45. Sugita, M., Y. Yue, and J. K. Foskett. 1998. CFTR Cl- channel and CFTR-associated ATP channel: distinct pores regulated by common gates. *EMBO J. 17*:898.

46. Steagall, W. K., T. J. Kelley, R. J. Marsick, and M. L. Drumm. 1998. Type II protein kinase A regulates CFTR in airway, pancreatic, and intestinal cells. *Am. J. Physiol. 274*:C819.

47. Leung, G. P. and P. Y. Wong. 2000. Activation of cystic fibrosis transmembrane conductance regulator in rat epididymal epithelium by genistein [In Process Citation]. *Biol. Reprod. 62*:143.

48. Seibert, F. S., X. B. Chang, A. A. Aleksandrov, D. M. Clarke, J. W. Hanrahan, and J. R. Riordan. 1999. Influence of phosphorylation by protein kinase A on CFTR at the cell surface and endoplasmic reticulum. *Biochim. Biophys. Acta 1461*:275.

49. Ma, J., J. Zhao, M. L. Drumm, J. Xie, and P. B. Davis. 1997. Function of the R domain in the cystic fibrosis transmembrane conductance regulator chloride channel. *J. Biol. Chem. 272*:28133.

50. Dietrich, J. and M. Lindau. 1994. Chloride channels in mast cells: block by DIDS and role in exocytosis. *J. Gen. Physiol. 104*:1099.

51. Cheung, M. and M. H. Akabas. 1997. Locating the anion-selectivity filter of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel. *J. Gen. Physiol. 109*:289.

52. Ballard, S. T., L. Trout, Z. Bebok, E. J. Sorscher, and A. Crews. 1999. CFTR involvement in chloride, bicarbonate, and liquid secretion by airway submucosal glands. *Am. J. Physiol. 277*:L694.

53. Iwagaki, H., M. Marutaka, M. Nezu, T. Suguri, N. Tanaka, and K. Orita. 1994. Cell membrane fluidity in K562 cells and its relation to receptor expression. *Res. Commun. Mol. Pathol. Pharmacol. 85*:141.

54. Chabot, H., M. F. Vives, A. Dagenais, C. Grygorczyk, Y. Berthiaume, and R. Grygorczyk. 1999. Downregulation of epithelial sodium channel (ENaC) by CFTR co-expressed in Xenopus oocytes is independent of Cl- conductance. *J. Membr. Biol. 169*:175.

55. Takagi, M., T. Nakahata, K. Koike, T. Kobayashi, K. Tsuji, S. Kojima, T. Hirano, A. Miyajima, K. Arai, and T. Akabane. 1989. Stimulation of connective tissue-type mast cell proliferation by crosslinking of cell-bound IgE. *J. Exp. Med. 170*:233.

56. Adamczyk-Engelmann, P. and H. Bader. 1989. The role of membrane bound sialic acid of rat mast cells in histamine release induced by compound 48/80 and derivatives as well as calcium. *Agents Actions 28*:34.

57. Hill, P. B., R. J. Martin, and H. R. Miller. 1996. Characterization of whole-cell currents in mucosal and connective tissue rat mast cells using amphotericin-B-perforated patches and temperature control. *Pflugers Arch. 432*:986.

58. Friis, U. G., H. A. Praetorius, T. Knudsen, and T. Johansen. 1997. Role of the Na+/K+-ATPase in regulating the membrane potential in rat peritoneal mast cells. *Br. J. Pharmacol. 122*:599.

59. Darmani, H., J. L. Harwood, and S. K. Jackson. 1993. Effect of interferon-gamma on membrane conformation in the macrophage-like cell line P388D. *J. Interferon Res. 13*:427.

60. Moss, R. B., R. C. Bocian, Y. P. Hsu, Y. J. Dong, M. Kemna, T. Wei, and P. Gardner. 1996. Reduced IL-10 secretion by CD4+ T lymphocytes expressing mutant cystic fibrosis transmembrane conductance regulator (CFTR). *Clin. Exp. Immunol. 106*:374.

61. Brennan S, Cooper D, and Sly PD. 2001. Directed neutrophil migration to IL-8 is increased in cystic fibrosis: a study of the effect of erythromycin. *Thorax 56*:62.

62. Tabary O, Zahm JM, Hinnrasky J, Couetil JP, Cornillet P, Guenounou M, Gaillard D, Puchelle E, and Jacquot J. 1998. Selective up-regulation of chemokine IL-8 expression in cystic fibrosis bronchial gland cells in vivo and in vitro. *Am. J. Pathol. 153*:921.

63. Tosi MF, Zakem-Cloud H, Demko CA, Schreiber JR, Stern RC, Konstan MW, and Berger M. 1995. Cross-sectional and longitudinal studies of naturally occurring antibodies to *Pseudomonas aeruginosa* in cystic fibrosis indicate absence of antibody-mediated protection and decline in opsonic quality after infection. *J. Infect. Dis. 172*:453.

64. Friedl P, Konig B, and Konig W. 1992. Effects of mucoid and non-mucoid Pseudomonas aeruginosa isolates from cystic fibrosis patients on inflammatory mediator release from human polymorphonuclear granulocytes and rat mast cells. *Immunology* 76:86.

65. Wedemeyer J, Tsai M, and Galli SJ. 2001. Roles of mast cells and basophils in innate and acquired immunity. *Curr. Opin. Immunol. 12*:624.

66. Harville BA, and Dreyfus LA. 1996. Release of serotonin from RBL-2H3 cells by the Escherichia coli peptide toxin STb. *Peptides 17*:363.

67. Yamamoto J, Watanabe S, Hirose M, Osada T, Ra C, and Sato N. 1999. Role of mast cells as a trigger of inflammation in Helicobacter pylori infection. *J. Physiol. Pharmacol. 50*:17.

Chapter 6

Interferon-γ upregulates cystic fibrosis transmembrane conductance regulator (CFTR) expression in mast cells

Introduction

Interferon-γ (IFN-γ) is a member of a family of inducible secretory proteins produced largely by activated T lymphocytes and natural killer (NK) cells (1). IFN-γ is capable of regulating the transcription and expression of more than 200 genes and is considered to be a major modulator of immune cell function. With regard to mast cells (MC), IFN-γ can inhibit proliferation, TNF-mediated cytotoxicity, cell differentiation and mediator release (2-4).

MC are granulated leukocytes resident in the lung that are important in innate immunity as well as allergic inflammation. Upon activation by antigen-mediated crosslinking of surface IgE, MC release several pro-inflammatory mediators including histamine and leukotrienes leading to bronchoconstriction and recruitment of lymphocytes, neutrophils and eosinophils to the airways (5).

The mechanism of IFN-γ-mediated downregulation of MC function is thought to involve an indirect mechanism via IFN-γ-mediated production of nitric oxide (NO) (6,7). IFN-γ induces nitric oxide synthase production (NOS) in MC as well as some of the regulatory proteins associated with increased NO production (8,9). Through cell surface glycosaminoglycans, MC can also present IFN-γ to macrophages inducing them to produce NO (10) that in turn can inhibit mediator release from MC (6). In fact, some investigators believe that MC do not express an IFN-γR and MC respond to mediators released from contaminating cells that express IFN-γ. However, the downstream targets of NO in the MC that ultimately result in a decrease in mediator secretion are unknown.

Cl^- flux through Cl^- channels plays an important role in mast cell activation and subsequent mediator release (11,12). Patch clamp studies show that pharmacologic agents such as nedocromil sodium and sodium cromoglycate that downregulate MC mediator release inhibit Cl^- ion currents (13). Recently, we have shown that IFN-γ (100 U/mL for 24 hr) also alters MC Cl^- ion flux and decreases the rate of Cl^- influx in resting cells possibly by modulation of Cl^- channel expression (Chap 2-5). Threfore, a mechanism of IFN-γ mediated downregulation of MC function may involve regulation of Cl^- channel function and/or expression.

MC express a variety of Cl^- channels including members of the ClC family (Chap 2) and the cystic fibrosis transmembrane conductance regulator (CFTR; Chap 5, (14)). CFTR is a phosphorylation, cyclic AMP (cAMP) activated Cl^- channel that is central for controlling transepithelial salt transport, fluid flow and ion concentrations in the intestine, pancreas and sweat glands (15). Various mutations in CFTR lead to the pathological symptoms associated with cystic fibrosis (16,17).

In epithelial cells, IFN-γ downregulates expression of CFTR by post-transcriptional destabilization of its mRNA (18). Decreased CFTR expression results in a significant decrease in CFTR-mediated Cl^- current (18). We hypothesized that MC CFTR expression might also be regulated by IFN-γ in an NO-dependent mechanism. We treated rat peritoneal mast cells (PMC) and a rat cultured mast cell line (RCMC) with IFN-γ and determined CFTR expression using RT-PCR, flow cytometry and western blotting. The mechanism of IFN-γ regulation of CFTR in mast cells was also investigated using inhibitors of the NO-cGMP pathway.

Materials and Methods

Rats and PMC Isolation

Male Sprague Dawley rats (300-350 g; Charles River, St. Constant, Quebec, Canada) were housed in a pathogen-free viral antibody-free facility. Rats were sacrificed by cervical dislocation under anesthesia and PMC were isolated by the following procedure: 20 mL of ice-cold Hepes Tyrode's buffer (HTB) was injected into the peritoneal cavity and massaged gently for 30 sec; the peritoneal cavity was opened and the buffer collected with a transfer pipette and kept on ice or at 4°C for all subsequent procedures. Following centrifugation at 200 g for 5 min the cell pellet was resuspended in 5 mL of fresh HTB and layered on top of a 30%/80% Percoll gradient. The gradient was centrifuged at 500 g for 20 min and the highly enriched MC were collected from the pellet (19). PMC were >98% pure and >96% viable as measured by trypan blue exclusion.

Cell Culture

The rat MC line, RCMC 1.11.2 (kindly provided by B. Chan and A. Froese, Winnipeg. Manitoba), was established from Wistar-ICI rats (20) and cultured in RPMI 1640 medium containing 5% FBS (Gibco BRL), 100 U/mL penicillin, 100 μg/mL streptomycin and 10 mM Hepes. The cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. NR8383, a rat macrophage cell line, were grown in F12 media (Gibco BRL) supplemented with 15% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 10 mM Hepes. NR8383 cells were also incubated in a humidified atmosphere of 5% CO_2 in air at 37°C.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), Cloning and Sequence Analysis

RNA was extracted from PMC and RCMC using the modified Chomczynski and Sacchi method (21). Briefly, 10^6 to 10^7 cells were homogenized with solution D (4 M guanidinium thiocyanate, 0.5% sodium n-laurylsarcosine, 1 M sodium citrate and 0.1 mM 2-mercaptoethanol). To the homogenate, 2 M NaOAc, water saturated phenol and chloroform-isoamyl alcohol was added. The aqueous phase was removed and treated with solution D once again. The RNA was precipitated with absolute ethanol and washed with 70% ethanol and air-dried. The RNA obtained from PMC was treated with 1 U/mL of heparinase for 2 hr at room temperature to remove contaminating heparin because high heparin concentrations markedly decrease the efficiency of the RT-PCR procedure (22).

Genomic DNA was digested by incubating 10 μg of total RNA with 5 U of DNAse (amplification grade; GIBCO BRL), 10X DNAse buffer (GIBCO BRL) 10 U of RNAse inhibitor (GIBCO BRL) and RNAse-free H_2O for 15 min at room temperature. After 15 min incubation, 25 mM EDTA was added and the sample was heated at 65°C for 20 min to inactivate the DNAse.

Treated RNA (1 μg) was incubated with 0.5 μg oligo(dT) at 70°C for 10 min in a thermocycler. A reverse transcriptase master mix was added to the RNA. The master mix contained First Strand Buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, pH 8.3, Gibco/BRL), 10 mM DTT, 10 mM of each dNTP, Sigma sterile water and 200 U M-

MLV RT enzyme (Gibco/BRL). This mixture was incubated at 37°C for 1 hr, then 70°C for 10 min. To test for effiency of reaction, a duplicate of each sample was reverse transcribed in the presence of [α-^{32}P]dCTP (1 μCi/μL). Unincorporated [α-^{32}P]dCTP was separated from labelled cDNA sample by paper chromatography, cDNA sample was cut out, 6 mL of scintillation fluid was added and radioactivity was measured on a Beckman scintillation counter.

The polymerase chain reaction master mix contained 1X PCR buffer (20 mM Tris-Cl, 50 mM KCl, pH 8.4, Gibco/BRL), 0.8 mM dNTP mix, 20 μM antisense CFTR primer (5' GGT GTC CTA TTC ACC TCA AGT TCT CTG 3') which binds bp 901-927 of the *Rattus norvegicus* CFTR gene (NCBI accession # X95927.1), 20 μM of sense CFTR primer (5' CTC TGT AGA CCA TAC TGG CCT TGA AC 3') which binds bp 618-643 of the *R. norvegicus* CFTR gene, 1.5 mM $MgCl_2$ (Gibco/BRL), RNAse-free H_2O, 2 μL of cDNA and 2.5 units of Taq DNA Polymerase (Gibco/BRL). The mixture was amplified at an annealing temperature of 56°C for 35 cycles. The product was analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. The conditions of use for the primers specific for CFTR were optimized using rat lung RNA. The 2,5 OAS sense (5'-TCCTTCCAAACACCAGCT TCCG-3') and antisense (5'-GGATCAGGATTGCTGTAGAGGC-3') primers (expected product size 309 bp) were also optimized using rat lung RNA and 56°C was the optimal annealing temperature. The β-actin sense (5'-CCATGTACGTAGCCATCCA-3') and antisense (5'-GATAGAGCCACCAATCCAC-3') primers were also optimized using rat lung RNA and had an annealing temperature of 46°C. The IFN-γRα sense (5'-GTGTCCACGCTGAATTCCAA-3') and antisense (5'-TTGGAATTCAGCGTGGACAC-3') primers were optimized using NR8383 RNA (expected product size 331 bp) and had an annealing temperature of 55°C.

The 309 bp CFTR PCR product was cloned into the pCR 2.1® (Invitrogen, Carlsbad, Ca, USA) vector using T4 DNA ligase and then used to transform "super competent" *E.coli* cells. The *E.coli* cells were grown for 3 d, the DNA was isolated using a Promega isolation kit and the plasmid was sequenced on the ABI PRISM sequencer

model 2.1.1 and analyzed by BLAST (23). The sequence obtained was 100% homologous to the CFTR gene.

Flow Cytometry

Single cell suspensions of 10^5 cells were blocked with PBS/5% rat serum for 1 hr. Blocked cells were incubated with primary mAb (mouse monoclonal anti-CFTR extracellular domain, Affinity Bioreagents, MA1-935) for 1 hr in PBS/5% mouse serum. Cells were washed twice with PBS and incubated with the secondary antibody (goat anti-mouse IgM:FITC, Biosource AM14708) for an additional hr. The isotype antibody used for comparison was mouse IgM isotype control anti-TNP (Pharmingen 03081D).

Western Immunoblotting and Immunocytochemistry

RCMC and PMC were isolated, washed with PBS and 1 x 10^6 cells were lysed with 2X sample buffer (0.5 mL 1M Tris-Cl, 1 mL DTT, 2 mL 10% SDS, 1 mL glycerol, 0.5 mL 0.12 % bromophenol blue) supplemented with 2% β-mercaptoethanol.

Whole cell lysates (30-40 μg) were separated by 8% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% milk in TBS-Tween for 24 hr at room temperature and then stained with primary antibodies, anti-CFTR (R domain, Genzyme, 1660-01) and isotype control purified mouse IgG_1 κ-isotype standard (anti-TNP; Serotec, 03001D) for 1 hr at room temperature. The membranes were washed with TBS-Tween 3X and then stained with the secondary antibody for 1 hr. The secondary antibody was sheep anti-mouse IgG conjugated to HRP (Amersham Life Science, catalogue # NA 9310).

The nitrocellulose membranes were developed with chemiluminescence reagent (NEN Life Technologies, catalogue # NEL 101) for 1 min and exposed to high performance chemiluminescence film (Amersham Life Science, catalogue # RPN2103H) for 30 min.

Immunocytochemistry was performed on cytospins of RCMC cells according to instructions of a commercial kit (Vectastain ABC kit PK-6200) as follows. Fifty μL aliquots of RCMC and PMC (1 x 10^6 cells/mL) cell suspension were cytospun onto

slides. Slides were air dried and then incubated with primary Ab (extracellular domain specific mAb) and isotype (mouse IgM) in PBS for 45 min in humidified atmosphere at room temperature. Slides were washed with PBS for 5 min. Biotinylated Ab solution from the commercial kit was added for 30 min. Slides were once again washed for 5 min in PBS. Developing reagent was added for 30 min and washed away for 5 min. Chromagen was added for 5 min or until color developed. Slides were washed again and fixed with ethanol, cleaned in xylene and mounted with Permount (Sigma). Images were photographed using a phase contrast microscope at 625 X magnification.

Results

IFN-γ upregulates CFTR mRNA in MC

To determine the effect of IFN-γ on CFTR mRNA expression, RCMC were treated with IFN-γ (10 to 1000 U/mL) for 24 hr, RNA was extracted and subjected to RT-PCR analysis using CFTR primers. The RCMC RNA was also analyzed using primers recognizing the IFN-γ-upregulated gene, 2,5 oligoadenylate synthase (2,5 OAS), and β-actin. Figure 6.1 shows that IFN-γ dose-dependently increases CFTR mRNA with as little as 10 U/mL of IFN-γ and reaches a maximum induction at 800 U/mL (Fig 6.1A). In comparison, 2,5 OAS mRNA is induced in RCMC at 200 U/mL of IFN-γ and reaches a maximum induction at 800 U/mL. β-actin mRNA levels are stable in these treatments. Similarly with PMC, IFN-γ dose dependently upregulates CFTR mRNA with 10 U/mL to 1000 U/mL of IFN-γ, reaching a maximum induction of CFTR mRNA at 1000 U/mL of IFN-γ. 2,5 OAS mRNA is induced in PMC only at 1000 U/mL of IFN-γ for 24 hr.

Figure 6.2 shows that IFN-γ induces CFTR mRNA upregulation in RCMC and PMC as early as 3 hr of treatment and continues until 24 hr of treatment. In contrast, 2,5 OAS mRNA is slightly upregulated in RCMC at 6 to 12 hr of 100 U/mL IFN-γ treatment and maximal induction of 2,5 OAS mRNA occurs at 24 hr treatment. In PMC, 2,5 OAS mRNA is upregulated following 24 hr treatment with 1000 U/mL IFN-γ.

IFN-γ upregulates CFTR protein in MC

To determine the effect of IFN-γ on the expression of CFTR protein, RCMC protein lysate was resolved on SDS-PAGE and blotted with anti-CFTR monoclonal antibody. IFN-γ dose-dependently upregulates CFTR protein in RCMC (Fig 6.3A & 6.3B) and PMC (Fig 6.3C & 6.3D). Densitometry analysis indicates that 10, 100 and 1000 U/mL of IFN-γ treatment increases CFTR protein on RCMC 3.8, 4.2 and 5.0 times respectively (Fig 6.3B). Similarly, IFN-γ upregulates CFTR protein in PMC. Densitometry analysis indicates that 10, 100 and 1000 U/mL of IFN-γ upregulates CFTR protein on PMC 1.3, 1.5 and 1.7 times respectively (Fig 6.3D).

IFN-γ upregulates CFTR surface protein

To determine if CFTR surface protein expression was altered by IFN-γ treatment, we used a monoclonal (mAb) to the extracellular epitope of CFTR and flow cytometry to measure CFTR surface expression on RCMC. At 12 hr of treatment, IFN-γ increased basal surface CFTR expression by 27.4 $\pm$ 4.6 % (Fig 6.4C). At 24 and 48 hr, IFN-γ upregulated basal CFTR expression by 114.3 $\pm$ 6.9 and 171.8 $\pm$ 0.9 % respectively (Fig 6.4C). However, at 72 hr of IFN-γ treatment, CFTR expression was below basal levels, representing a change in expression of -7.1 ± 0.8 % (Fig 6.4C).

IFN-γ increased CFTR surface expression on RCMC as measured by flow cytometry (Fig 6.4B) since 10, 100 and 1000 U/mL of IFN-γ increased surface CFTR expression by 73.5 $\pm$ 6.0, 117.6 $\pm$ 3.6 and 125.4 $\pm$ 3.0 % respectively (Fig 6.4D). Immunocytochemistry analysis confirmed that IFN-γ-treated (100 U/mL for 24 hr) MC showed stronger staining for CFTR compared to untreated MC (Fig 6.5). Untreated RCMC showed modest staining for CFTR on the cell periphery (Fig 6.5A). However, IFN-γ-treated RCMC showed CFTR staining on the periphery as well as in the cytoplasm (Fig 6.5B). Untreated PMC showed speckled CFTR staining throughout the cell (Fig 6.5C) and IFN-γ treatment increased the magnitude of the CFTR staining and it appeared more uniform throughout the cell.

IFN-γ-mediated CFTR mRNA upregulation is unaffected by L-NMMA

The mechanism of IFN-γ-mediated downregulation of MC function has been proposed to be an indirect mechanism dependent upon nitric oxide (NO) production (6). To determine whether IFN-γ-mediated upregulation of CFTR was mediated via production of NO by NOS enzymes, RCMC were treated with IFN-γ (100 U/mL), an inhibitor of NOS (L-NMMA; 100 μM) or L-NMMA and IFN-γ together for 24 hr. RNA from treated cells was isolated and analyzed for CFTR, 2,5 OAS and β-actin mRNA expression by RT-PCR. L-NMMA did not affect IFN-γ-induced CFTR mRNA levels but decreased basal CFTR mRNA expression (Fig 6.6A). In contrast, L-NMMA had no effect on 2,5 OAS basal or IFN-γ-induced mRNA expression. To determine if exogenous NO affected CFTR expression, RCMC were treated with 10 or 50 μM GSNO, an NO donor, for 24 hr (Fig 6.6B). CFTR mRNA expression was upregulated by both 10 and 50 μM GSNO. Similarly, 2,5 OAS expression was upregulated by 10 and 50 μM GSNO.

MC express IFN-γRα

It has been suggested that IFN-γ effects on MC are indirect and that MC do not express the IFN-γR (10). To determine whether MC expressed the IFN-γ receptor (IFN–γR), primers were designed that are complementary to region 367-398 and 676-698 of the *R. norvegicus* IFN-γRα gene and RT-PCR was used to amplify RCMC and the in vivo-derived PMC RNA (Fig 6.7A). RCMC, PMC and the control macrophage cell line, NR8383, show a band corresponding to IFN-γRα at the expected size of 313 bp.

To ensure that the IFN-γR primers were specifically amplifying IFN-γR cDNA, PCR products from different RNA preparations were amplified using IFN-γR primers and inserted into a pCR vectors and cloned in *E.coli*. Sequence analysis of the PCR product confirmed that region 367-698 of the *R. norvegicus* IFN-γR mRNA was amplified.

To determine the presence of IFN-γRα protein in rat MC, a rabbit polyclonal anti-IFN-γRα was used in western immunobloting to screen RCMC and PMC for IFN-γRα expression and the macrophage cell line NR8383 was used as a control. Both RCMC and PMC express the 91 kDa IFN-γRα protein (Fig 6.7B).

Discussion

Our results show that IFN-γ upregulates CFTR mRNA and protein. Time course analysis shows that CFTR is an early IFN-γ-inducible gene since IFN-γ upregulates CFTR mRNA expression as early as 3 hr of treatment. Furthermore, upregulation of CFTR protein expression occurs on the cell surface at 12 to 48 hr but decreases to basal levels at 72 hr of treatment. IFN-γ upregulation of CFTR is independent of endogenous NO production and cannot be inhibited by the NOS inhibitor L-NMMA. However, exogenous NO, by the NO donor GSNO, upregulates CFTR mRNA expression.

The capacity of IFN-γ to enhance host defense is due to its capacity to stimulate specific microbicidal pathways of phagocytes, upregulate class II MHC antigen expression and prime various cells that release inflammatory mediators. To date, these effects are all transcription/translation dependent resulting in altered protein expression. The signals that result in altered gene expression begin when IFN-γ binds the IFN-γR. The IFN-γR is composed of two α and two β subunits, which are constitutively associated with janus kinase-1 and 2 (JAK1 and JAK2). When IFN-γ binds the α subunit homodimer, the β subunits are brought into close proximity and allow JAK1 and JAK2 to cross-phosphorylate one another. Phosphorylation activates the JAKs and allows them to phosphorylate the receptor at a tyrosine residue on the β subunit, creating a binding site for two signal transducer and activator of transcription-1 (STAT1) proteins. Once bound to the IFN-γRβ chain, the STAT1 proteins are phosphorylated and form a homodimer capable of translocating the nuclear envelope and initiating the transcription of genes possessing the interferon-gamma response element (GRE) sequences in their promoter. The expression of all IFN-γ-responsive genes are regulated via the JAK/STAT pathway. In this study we clearly show that MC express IFN-γRα mRNA and protein and are therefore capable of responding to IFN-γ via the IFN-γR.

IFN-γ-inducible genes can be classified according to the time during IFN-γ treatment at which their expression is induced. The expression of immediate, early or primary response genes, such as the ubiquitin cross-reactive protein (24) and platelet-activating factor receptor (25), usually occurs within the first 6 hr of IFN-γ treatment and

may disappear shortly thereafter. Intermediate IFN-γ-inducible genes, such as gamma-inducible factor 1 (26) are expressed 6 to 24 hr. Late IFN-γ inducible genes, such as 2,5 OAS, are expressed after 24 hr or more of IFN-γ treatment (27).

Although IFN-γ inhibits MC proliferation, differentiation and mediator release (such as histamine, serotonin and the proinflammatory mediator tumor necrosis factor (TNF)), the genes responsible for these changes are poorly understood. Possible targets of IFN-γ modulation are the early events in MC mediator release. A common theme in MC activation, whether resulting in mediator release or chemotaxis, involves an increase in intracellular calcium (28-30). Increase in calcium is followed by influx of extracellular Cl^-, which maintains the driving force for entry of extracellular calcium (31) and facilitates maximal MC mediator release (32). Patch clamp and ^{36}Cl studies have also shown that extracellular Cl^- is required for maximal MC degranulation (12) and Cl^- channel blockers inhibit both Ca^{2+} influx and MC mediator release (33).

In gut epithelial cells (T84), IFN-γ decreases Cl^- efflux in response to vasoactive intestinal polypeptide (VIP) and cholera toxin, as well as changing cell membrane protein composition (34). The decrease in Cl^- efflux is likely facilitated by upregulation of CFTR, since IFN-γ downregulates CFTR expression and inhibits Cl^- currents in gut epithelial cells (18). Conversely, in MC, IFN-γ causes a net decrease in intracellular Cl^- concentration by decreasing the rate of Cl^- influx (Chap 2).

Our data shows that IFN-γ dose-dependently upregulates CFTR mRNA and protein in MC. In PMC and RCMC, even the relatively low dose of 10 U/mL of IFN-γ induced CFTR mRNA expression. In comparison, 200 U/mL of IFN-γ induced 2,5 OAS expression in RCMC and 1000 U/mL of IFN-γ induced 2,5 OAS expression in PMC.

In both PMC and RCMC, IFN-γ induced CFTR mRNA expression as early as 3 hr of treatment suggesting that CFTR is an early IFN-γ-inducible gene, whereas 2,5 OAS expression is induced at 24 hr, confirming it as a late gene (Fig 6.2). These results are consistent with the observation that 2,5 OAS is a "late" IFN-γ inducible gene (35). In a human glioma cell line, IFN-γ induces 2,5 OAS mRNA expression only after 24 hr treatment because IFN-γ 2,5 OAS induction occurs through protein synthesis dependent induction of the interferon-gamma response factor-1 (IRF-1) (27). This observation

suggests that CFTR mRNA upregulation may be independent of IRF-1 induction and may not require protein synthesis.

Flow cytometry and immunoblot analysis using a mAb to CFTR showed that IFN-γ upregulated CFTR protein, both in cell lysates and on the surface of MC. These effects can be seen as early as 12 hr of treatment suggesting that CFTR mRNA translation and expression on the surface is a relatively quick process. In fact, pulse chase experiments in epithelial cells show that translation and expression of the fully glycosylated form of CFTR (170-180 kDa) occurs within 5 hr (36).

One of the intermediates of IFN-γ signaling in MC is NO. Our data shows that exogenous NO (GSNO) upregulates CFTR mRNA expression (compared to untreated cells) and blocking constitutive NO production using a NOS inhibitor (L-NMMA) downregulated basal levels of CFTR mRNA expression but had no effect on IFN-γ-mediated upregulation of CFTR. This data suggests that basal CFTR expression may be sensitive to endogenous NO production, but IFN-γ–mediated upregulation of CFTR occurs via an NO-independent pathway, namely, the JAK/STAT pathway. In rat mast cells, IFN-γ induces the expression of nitric oxide synthase (iNOS) resulting in the constitutive release of small quantities of NO for several hours (6,8). It may be that this small constitutive production of NO helps maintain production and turnover of CFTR protein. Certainly, NO appears to be an important regulator of CFTR expression and function in other cell types. For example, NO activates CFTR Cl^- current in T cells (37) and downregulates CFTR expression in kidney epithelial cells by as much as 43 % through a sGC pathway (38).

Our data suggests that in MC, CFTR expression is modulated by at least two distinct pathways. One pathway involves upregulation of CFTR expression by IFN-γ via the IFN-γR and the JAK/STAT pathway. The other pathway affecting CFTR expression is dependent on NO production, cGS and cGMP, which may maintain constitutive levels of CFTR protein. Therefore, in diseases where IFN-γ or NO levels are high, CFTR expression is maybe downregulated in epithelial cells but upregulated in MC. In such a case, IFN-γ may alter MC functions such as mediator release and affect MC regulation of immune responses.

However, IFN-γ-mediated downregulation of MC mediator release may occur by means other than modulation of CFTR expression. MC express a number of other Cl^- channels including ClC, subunits of the $GABA_AR$, CFTR and possibly bicarbonate exchangers ((14,39-41), Chap 3-5). Any of these Cl^- channels/exchangers could also be the target of IFN-γ-mediated modulation. For example, IFN-γ increases channel activities of DIDS-sensitive and forskolin-activated outwardly rectifying Cl^- channels (ORCC) in a human bronchial epithelial cell line (BEAS-2B) at 24 hr of treatment (42). MC exocytosis is a dynamic process requiring the cooperation of several ion channels and IFN-γ most likely alters many of these channels.

This study shows that IFN-γ upregulates CFTR expression in MC independently of NO and cGS. This is a novel finding and this effect is opposite to that observed for epithelial cells. Thus, IFN-γ-mediated inhibition of MC functions may involve upregulation of CFTR expression. Increased CFTR expression in MC may alter their Cl^- flux, resulting in decreased intracellular Cl^- concentration and inhibition of MC mediator release.

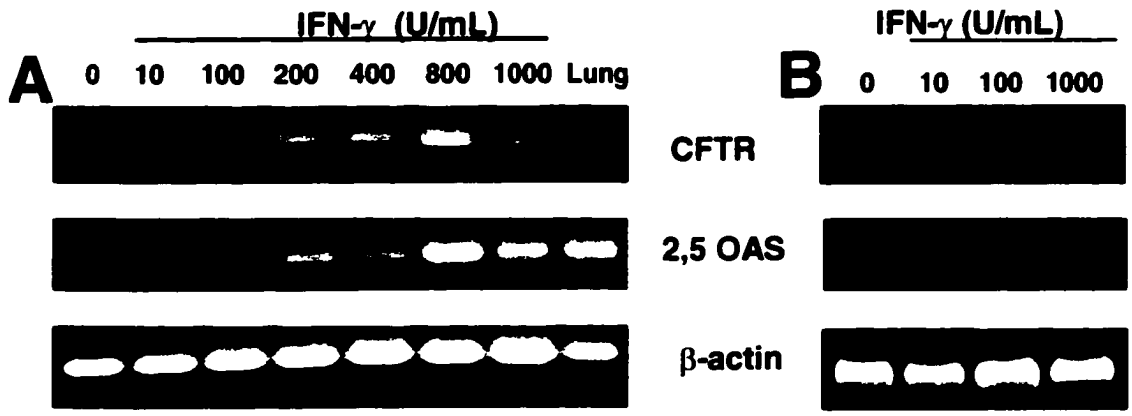


Figure 6.1. RT-PCR shows that IFN-γ (24 hr) upregulates CFTR mRNA in mast cells. (A) Dose response of IFN-γ on CFTR, 2.5 OAS and β-actin mRNA expression in RCMC. CFTR PCR product is 309 bp, 2.5 OAS PCR product is 445 bp and β-actin PCR product is 560 bp (n=5). (B) Dose response of IFN-γ treatment on CFTR, 2.5 OAS and β-actin mRNA in PMC (n=5).

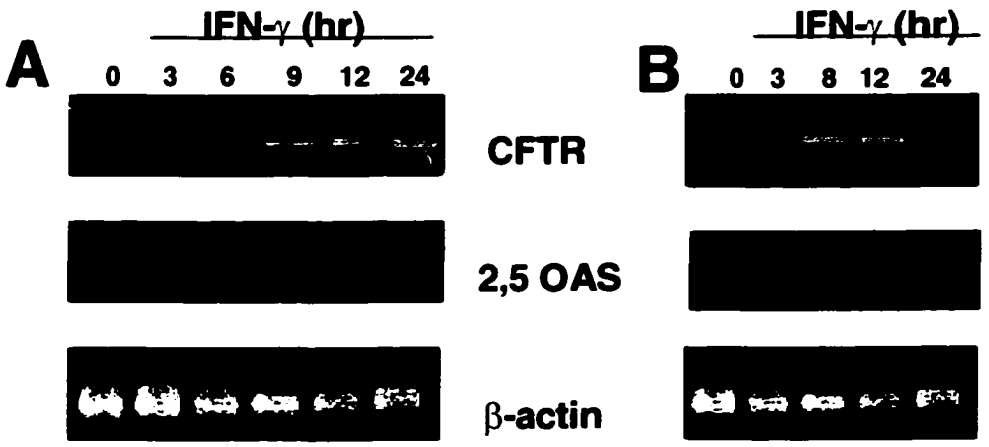


Figure 6.2. RT-PCR shows that IFN-γ upregulates CFTR mRNA in mast cells. (A) Time course of IFN-γ (100 U/mL) effect on CFTR, 2,5 OAS and β-actin mRNA expression in RCMC. CFTR PCR product is 309 bp, 2,5 OAS PCR product is 445 bp and β-actin PCR product is 560 bp (n=5). (B) Timecourse of IFN-γ treatment on CFTR (100 U/mL IFN-γ), 2,5 OAS (1000 U/mL IFN-γ) and β-actin (100 U/mL IFN-γ) mRNA in PMC (n=5).

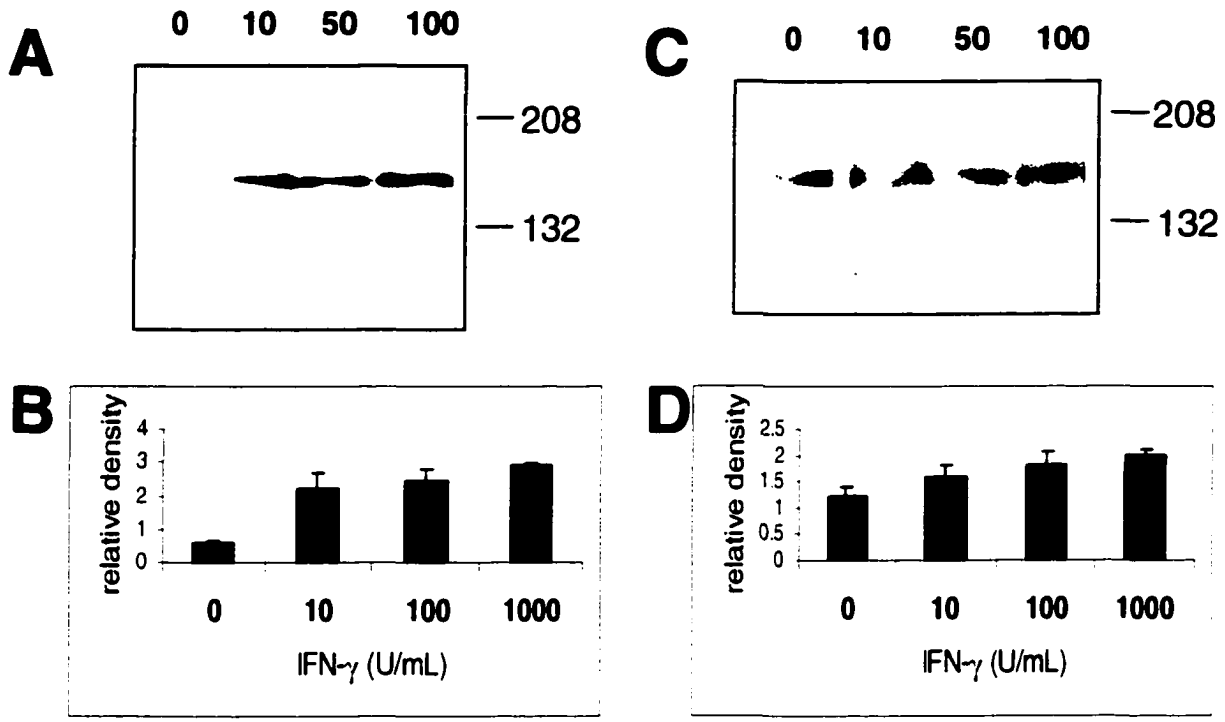


Figure 6.3. Western blot analysis shows that IFN-γ upregulates CFTR protein in rat cultured mast cells (RCMC) and peritoneal mast cells (PMC). (A) Dose response of IFN-γ treatment on CFTR protein in RCMC. 100 000 cells or 5 μg of RCMC protein was loaded in each lane (representative experiment of n=3). (B) Densitometry analysis of IFN-γ upregulation of CFTR protein (n=3). Western blot analysis shows that IFN-γ upregulates CFTR protein in PMC. (C) Dose response of IFN-γ treatment on CFTR protein in PMC. 100 000 cells or 5 μg of PMC protein was loaded in each lane (n=3). (D) Densitometry analysis of IFN-γ upregulation of CFTR protein in PMC (n=3).

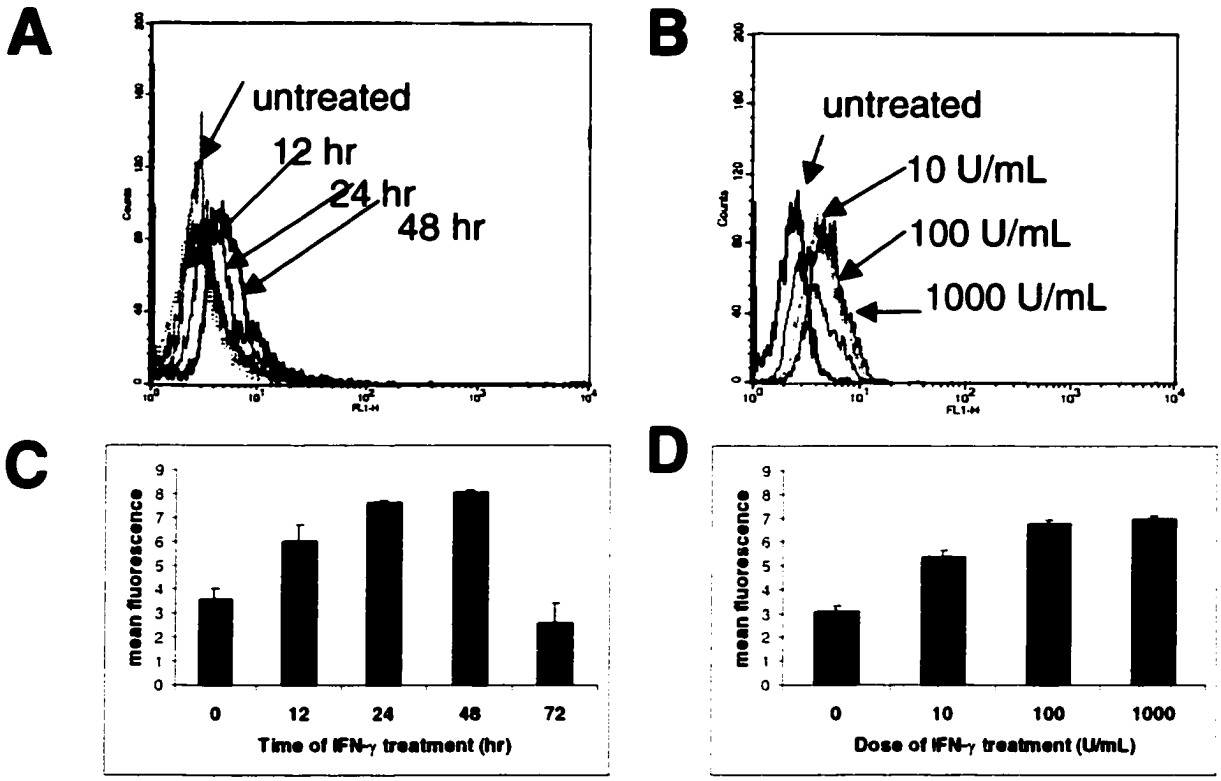


Figure 6.4. Flow cytometry analysis shows that IFN-γ upregulates CFTR surface protein in rat cultured mast cells (RCMC). (A) Time course showing IFN-γ (100 U/mL) upregulation of CFTR expression at 12, 24, 48 and 72 hr of treatment (representative of 3 experiments). (B) Dose response showing upregulation of CFTR with 10, 100 and 1000 U/mL of IFN-γ treatment for 24 hr (representative of 3 experiments). (C) Time course results expressed as mean fluorescent units (n=3). (D) Dose response results expressed as mean fluorescent units (n=3).

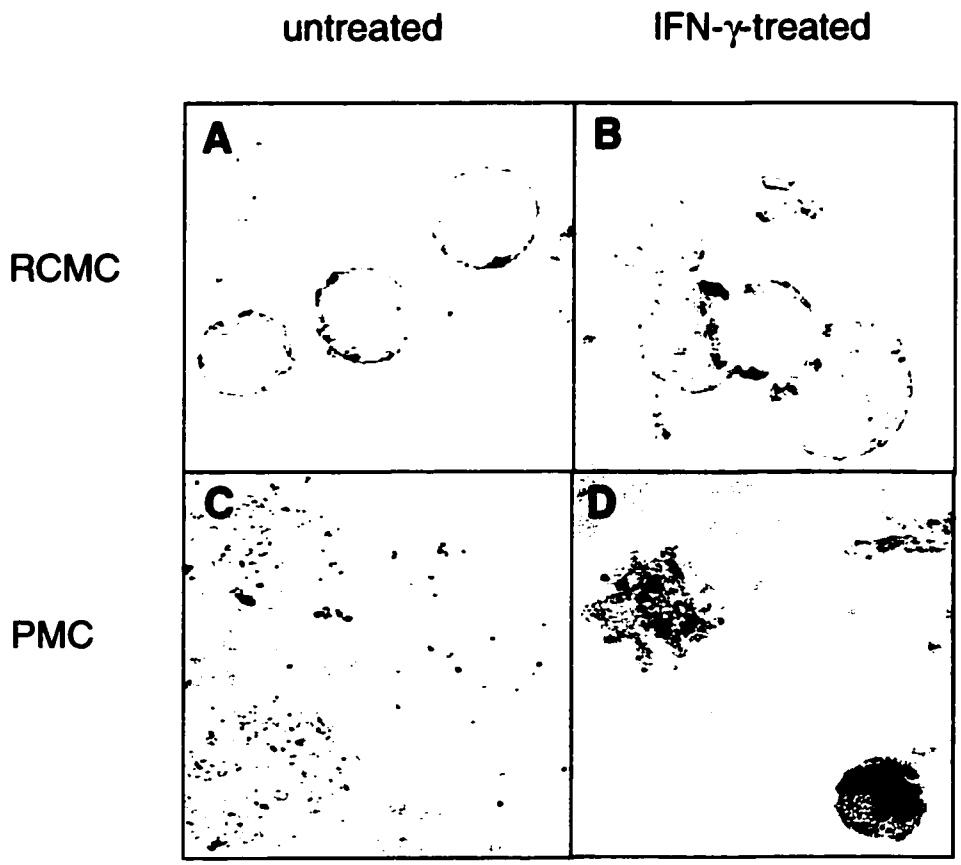


Figure 6.5. IFN-γ treatment increases CFTR protein expression in mast cells. Immunocytochemistry with anti-CFTR (extracellular domain specific) shows stronger staining for IFN-γ-treated (100 U/mL for 24 hr) RCMC (B) and PMC (D) compared to untreated RCMC (A) and PMC (B:). 625 X magnification (n=3).

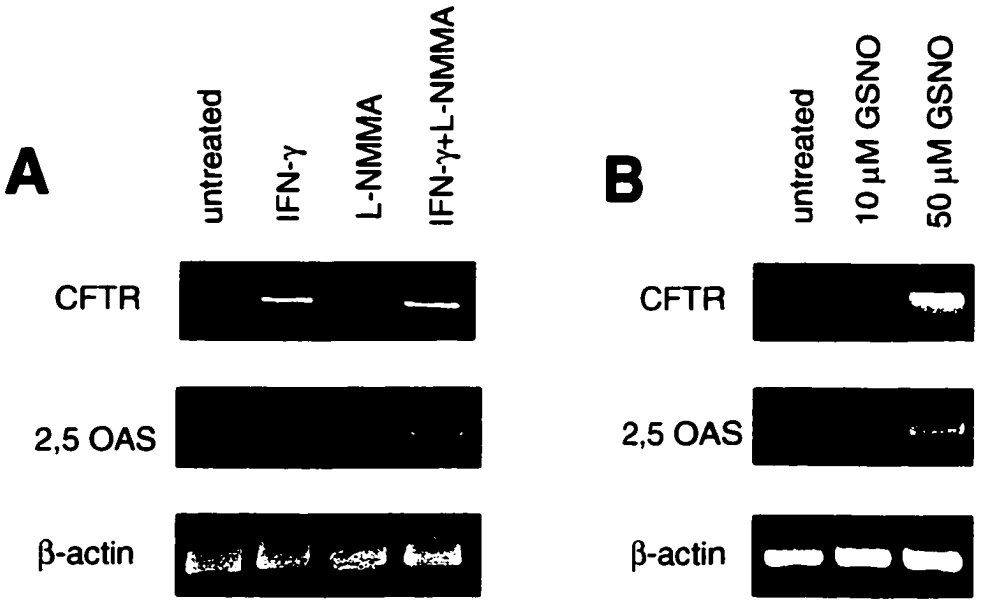


Figure 6.6. IFN-γ-mediated CFTR mRNA upregulation is NO independent. (A) RCMC were treated with a NOS inhibitor, IFN-γ (100 U/mL), L-NMMA (100μM) or IFN-γ and L-NMMA for 24 hr. RT-PCR analysis was performed using CFTR and β-actin primers (n=3). (B) RCMC were treated with an NO donor, GSNO, at different concentrations for 24 hr and RT-PCR analysis was performed using CFTR and β-actin primers (n=3).

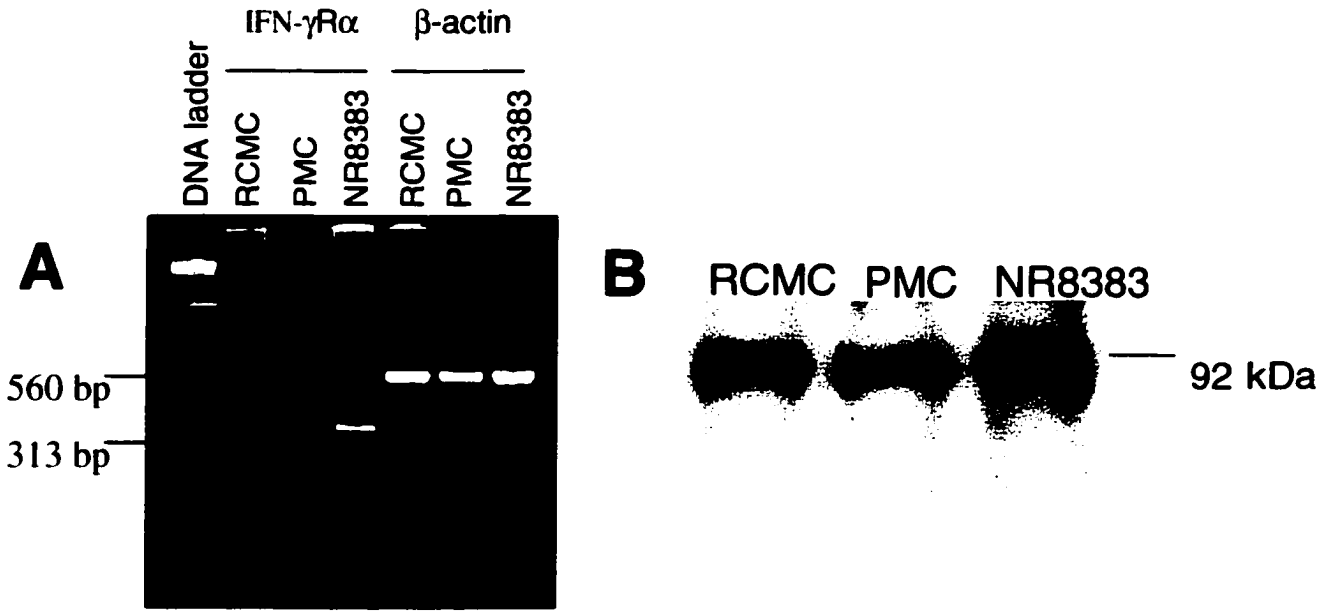


Figure 6.7. Mast cells express IFN-γRα mRNA and protein. (A) RT-PCR analysis of rat cultured mast cells (RCMC), peritoneal mast cells (PMC) and NR8383 RNA showing IFN-γRα product at 313 bp. β-actin is shown as control (560 bp; n=3). (B) Western blot analysis of IFN-γRα protein expression (91 kDa) in NR8383, PMC and RCMC (n=3).

References

1. Novelli, F., M. Giovarelli, R. Reber-Liske, G. Virgallita, G. Garotta, and G. Forni. 1991. Blockade of physiologically secreted IFN-gamma inhibits human T lymphocyte and natural killer cell activation. *J. Immunol. 147*:1445.

2. Enciso, J. A., E. Y. Bissonnette, and A. D. Befus. 1996. Regulation of mRNA levels of TNF-alpha and the alpha chain of the high-affinity receptor for IgE in mast cells by IFN-gamma and alpha/beta. *Int. Arch. Allergy Immunol. 110*:114.

3. Holliday, M. R., E. M. Banks, R. J. Dearman, I. Kimber, and J. W. Coleman. 1994. Interactions of IFN-gamma with IL-3 and IL-4 in the regulation of serotonin and arachidonate release from mouse peritoneal mast cells. *Immunology 82*:70.

4. Kirshenbaum, A. S., A. S. Worobec, T. A. Davis, J. P. Goff, T. Semere, and D. D. Metcalfe. 1998. Inhibition of human mast cell growth and differentiation by interferon gamma-1b. *Exp. Hematol. 26*:245.

5. Louis, R., L. C. Lau, A. O. Bron, A. C. Roldaan, M. Radermecker, and R. Djukanovi. 2000. The relationship between airways inflammation and asthma severity. *Am. J. Respir. Crit. Care Med. 161*:9.

6. Eastmond, N. C., E. M. Banks, and J. W. Coleman. 1997. Nitric oxide inhibits IgE-mediated degranulation of mast cells and is the principal intermediate in IFN-gamma-induced suppression of exocytosis. *J. Immunol. 159*:1444.

7. Wills, F. L., M. Gilchrist, and A. D. Befus. 1999. Interferon-gamma regulates the interaction of RBL-2H3 cells with fibronectin through production of nitric oxide. *Immunology 97*:481.

8. Gilchrist, M., M. Savoie, N. S. Hirji, O. Nohara, F. L. Wills, J. L. Wallace, and A. D. Befus. 2002. Nitric oxide synthase and nitric oxide production in in-vivo derived mast cells. *J. Leukoc. Biol.* (In press)

9. Koranteng, R. D., R. J. Dearman, I. Kimber, and J. W. Coleman. 2000. Phenotypic variation in mast cell responsiveness to the inhibitory action of nitric oxide. *Inflamm. Res. 49*:240.

10. Brooks, B., D. M. Briggs, N. C. Eastmond, D. G. Fernig, and J. W. Coleman. 2000. Presentation of IFN-gamma to nitric oxide-producing cells: a novel function for mast cells. *J. Immunol. 164*:573.

11. Matthews, G., E. Neher, and R. Penner. 1989. Chloride conductance activated by external agonists and internal messengers in rat peritoneal mast cells. *J. Physiol. 44*.131.

12. Friis, U. G., T. Johansen, N. A. Hayes, and J. C. Foreman. 1994. IgE-receptor activated chloride uptake in relation to histamine secretion from rat mast cells. *Br. J. Pharmacol. 111*:1179.

13. Heinke, S., G. Szucs, A. Norris, G. Droogmans, and B. Nilius. 1995. Inhibition of volume-activated chloride currents in endothelial cells by chromones. *Br. J. Pharmacol. 115*:1393.

14. Kulka, M., M. Gilchrist, M. Duszyk, and A. D. Befus. 2002. Expression and functional characterization of CFTR in mast cells. *J. Leukoc. Biol.* (In press)

15. Al-Nakkash, L. and T. C. Hwang. 1999. Activation of wild-type and deltaF508-CFTR by phosphodiesterase inhibitors through cAMP-dependent and -independent mechanisms. *Pflugers Arch. 437*:553.

16. Sheppard, D. N. and L. S. Ostedgaard. 1996. Understanding how cystic fibrosis mutations cause a loss of Cl-channel function. *Mol. Med. Today 2*:290.

17. Griesenbach, U., D. M. Geddes, and E. W. Alton. 1999. The pathogenic consequences of a single mutated CFTR gene. *Thorax 54*:S19.

18. Besancon, F., G. Przewlocki, I. Baro, A. S. Hongre, D. Escande, and A. Edelman. 1994. Interferon-gamma downregulates CFTR gene expression in epithelial cells. *Am. J. Physiol. 267*:C1398.

19. MacDonald, A. J., J. Pick, E. Y. Bissonnette, and A. D. Befus. 1998. Rat mucosal mast cells: the cultured bone marrow-derived mast cell is biochemically and functionally analogous to its counterpart in vivo. *Immunology 93*:533.

20. Chan, B. M., C. S. Ra, W. N. Hu, K. McNeill, H. R. Miller, D. Befus, J. P. Kinet, and A. Froese. 1990. Characterization of rat tissue cultured mast cells. *Eur. J. Immunol. 20*:185.

21. Gilchrist, M., A. J. MacDonald, I. Neverova, B. Ritchie, and A. D. Befus. 1997. Optimization of the isolation and effective use of mRNA from rat mast cells. *J. Immunol. Methods 201*:207.

22. Tsai, M., M. Miyamoto, S. Y. Tam, Z. S. Wang, and S. J. Galli. 1995. Detection of mouse mast cell-associated protease mRNA. Heparinase treatment greatly improves RT-PCR of tissues containing mast cell heparin. *Am. J. Pathol. 146*:335.

23. Iwagaki, H., M. Marutaka, T. Yoshino, L. Cao, I. Sakuma, M. Uomoto, Y. Takeuchi, and N. Tanaka. 1996. Gamma-interferon upregulates transferrin receptors and decreases membrane microviscosity in K562 cells. *Res. Commun. Mol. Pathol. Pharmacol. 92*:191.

24. Haas, A., P. Ahrens, P. M. Bright, and H. Ankel. 1987. Interferon induces a 15-kilodalton protein exhibiting marked homology to ubiquitin. *J. Biol. Chem. 262*:11315.

25. Ouellet, S., S. Gingras, S. Turcotte, and M. Rola-Pleszczynski. 1994. Priming of monocytes with interferon-gamma is associated with up-regulation of expression of the receptor for platelet-activating factor. *Ann. N. Y. Acad. Sci. 744*:184.

26. Weihua, X., V. Kolla, and D. V. Kalvakolanu. 1997. Interferon-gamma-induced transcription of the murine ISGF3g (p48) gene is mediated by novel factors. *Proc. Natl. Acad. Sci. U. S. A. 94*:103.

27. Floyd-Smith, G., Q. Wang, and G. C. Sen. 1999. Transcriptional induction of the p69 isoform of 2',5'-oligoandenylate synthase by interferon-? and interferon-gamma involves three regulatory elements and interferon-stimulated gene factor 3. *Experimental Cell Research 246*:138.

28. Penner, R. 1988. Multiple signalling pathways control stimulus-secretion coupling in rat peritoneal mast cells. *Proc. Natl. Acad. Sci. U. S. A. 85*:9856.

29. Penner, R. and E. Neher. 1988. Secretory responses of rat peritoneal mast cells to high intracellular calcium. *FEBS Lett. 226*:307.

30. McCloskey, M. A., Y. Fan, and S. Luther. 1999. Chemotaxis of rat mast cells toward adenine nucleotides. *J. Immunol. 163*:970.

31. Dietrich, J. and M. Lindau. 1994. Chloride channels in mast cells: block by DIDS and role in exocytosis. *J. Gen. Physiol. 104*:1099.

32. Romanin, C., M. Reinsprecht, I. Pecht, and H. Schindler. 1991. Immunologically activated chloride channels involved in degranulation of rat mucosal mast cells. *EMBO J. 10*:3603.

33. Reinsprecht, M., M. H. Rohn, R. J. Spadinger, I. Pecht, H. Schindler, and C. Romanin. 1995. Blockade of capacitive Ca2+ influx by Cl- channel blockers inhibits secretion from rat mucosal-type mast cells. *Mol. Pharmacol. 47*:1014.

34. Holmgren, J., J. Fryklund, and H. Larsson. 1989. Gamma-interferon-mediated down-regulation of electrolyte secretion by intestinal epithelial cells: a local immune mechanism? *Scand. J. Immunol. 30*:499.

35. Corrias, M. V., G. Gribaudo, F. Guarnaccia, and M. Ponzoni. 1995. Induction of 2,5 OAS gene expression and activity is not sufficient for IFN-gamma-induced neuroblastoma cell differentiation. *Int. J. Cancer 17*:223.

36. Bebok, Z., C. Mazzochi, S. A. King, J. S. Hong, and E. J. Sorscher. 1998. The mechanism underlying cystic fibrosis transmembrane conductance regulator transport from the endoplasmic reticulum to the proteasome includes Sec61 β and a cytosolic, deglycosylated intermediary. *J. Biol. Chem. 273*:29873.

37. Dong, Y. J., A. C. Chao, K. Kouyama, Y. P. Hsu, R. C. Bocian, R. B. Moss, and P. Gardner. 1995. Activation of CFTR chloride current by nitric oxide in human T lymphocytes. *EMBO J. 14*:2700.

38. Jilling, T., I. Y. Haddad, S. H. Cheng, and S. Matalon. 1999. Nitric oxide inhibits heterologous CFTR expression in polarized epithelial cells. *Am. J. Physiol. 277*:L89.

39. Duffy, S. M., M. L. Leyland, E. C. Conley, and P. Bradding. 2001. Voltage-dependent and calcium-activated ion channels in the human mast cell line HMC-1. *J. Leukoc. Biol. 70*:233.

40. Duffy, S. M., W. J. Lawley, E. C. Conley, and P. Bradding. 2001. Resting and activation-dependent ion channels in human mast cells. *J. Immunol. 167*:4261.

41. Vilarino, N., M. R. Vieytes, J. M. Vieites, and L. M. Botana. 1999. Modulatory effect of HCO3- on rat mast cell exocytosis: cross-talks between bicarbonate and calcium. *Biochem. Biophys. Res. Commun. 260*:71.

42. Ge, N., Y. Nakamura, Y. Nakaya, and S. Sone. 2001. Interferon-gamma activates outwardly rectifying chloride channels in the human bronchial epithelial cell line BEAS-2B. *J. Med. Invest. 48*:97.

Chapter 7

Antisense oligodeoxynucleotides inhibit expression of cystic fibrosis transmembrane conductance regulator (CFTR)

Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-activated and protein kinase regulated Cl^- channel expressed in various cell types. In cystic fibrosis (CF), defective CFTR expression results in chronic pulmonary disease, pancreatic insufficiency, elevated sweat electrolytes and male sterility (1). The role of CFTR in non-epithelial cells has been largely characterized by comparing Cl^- currents and secretory mechanisms in cells from cystic fibrosis (CF) and normal patients (2).

Recently, we have shown that mast cells (MC), important effector cells in allergic disease, also express CFTR (3). Although the role of CFTR as a Cl^- channel and modulator of complex signal transduction in epithelial cells has been well documented, the role of CFTR in MC functions is poorly characterized. The CFTR blocker, diphenylamine-2-carboxylate (DPC), inhibits antigen-stimulated MC mediator release (3) but DPC is not a specific blocker of CFTR channels and can block other ion channels with varying effects. For example, DPC can block the cell volume-activated Cl^- channel, ClC2 (4) but not ClC5, expressed in intracellular vesicles (5).

At least two studies have used antisense oligonucleotides to CFTR mRNA to specifically inhibit CFTR-mediated functions (6,7). Antisense oligonucleotides designed against the first 12 codons of CFTR mRNA completely inhibit cell-cycle dependent Cl^- permeability when transfected into a B lymphocyte cell line (7). Also, antisense oligonucleotides have been used successfully to reduce CFTR expression and cAMP-activated Cl^- current in the colonic T84 epithelial cell line (6). The use of antisense oligonucleotides to block gene expression relys on the ability of cells to internalize small DNA species by endocytosis or pinocytosis (8,9).

To determine the role of CFTR in MC, we treated a human MC line (HMC-1) with antisense oligodeoxynucleotides (ODN) to CFTR. Preliminary results indicate that antisense treatment decreases CFTR protein expression and may alter release of a particular mediator, matrix metalloproteinase-9 (MMP-9).

Materials and Methods

Oligonucleotide Design

ODN were designed as described in Gardner et al. (6). Briefly, antisense ODN used in this study were a pair of adjoining 18-mers (5'-CAGAGGCGACCTCTGCAT-3' and 5'-GACAACGCTGGCCTTTTC-3') that are complementary to nucleotides 1-18 and 19-36 of CFTR mRNA (nucleotide 1 begins with the AUG codon). A pair of sense ODN (5'-ATGCAGAGGTCGCCTCTG-3' and 5'- GAAAAGGCCAGCGTTGTC-3') that have a sequence corresponding to that of the mRNA and a pair of missense ODN (5'-CAGCGGCGACCGATGCAG-3' and 5'-GACAACTCTGGACGTTTA-3') that have the same sequence as the antisense pair except for the presence of four mismatches in each, were used as controls. Each pair of ODN was synthesized with a modified phosphodiester bonds (phosphorothioate backbone), which renders the ODN nuclease resistant and has been shown to improve internalization (10).

Cell Culture and Treatment

The rat MC line, RCMC 1.11.2 (kindly provided by B. Chan and A. Froese, Winnipeg, Manitoba), was established from Wistar-ICI rats (11) and cultured in RPMI 1640 medium containing 5% FBS (Gibco BRL), 100 U/mL penicillin, 100 μg/mL streptomycin and 10 mM Hepes. The cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. The human MC line, HMC-1 were cultured in Iscove's medium (Gibco BRL) containing 10% FBS (Gibco BRL), 100 U/mL penicillin, 100 μg/mL streptomycin and 1.2 mM monothioglycerol (Sigma). The cells were also incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. T84, a colonic epithelial cell line, were grown in a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) FBS (Gibco BRL), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. The human lung carcinoma epithelial A549 and human lung adenocarcinoma CALU-3 cell lines were grown in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin and incubated in the same conditions as above.

The following procedure was found to be optimal for treatment of MC with ODN. ODN treatment was started at 50% confluency. The growth medium was removed, growth medium without FBS was added and ODN were added at 20 μM each for 30 min at 37°C. After incubation, FBS was returned to the medium. The same procedure was repeated every 12 hr for 48 hr or 72 hr.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), Cloning and Sequence Analysis

RNA was extracted from HMC-1 and T84 using Trizol (Gibco BRL). Genomic DNA was digested by incubating 10 μg of total RNA with 5 U of DNAse (amplification grade; GIBCO BRL), 10X DNAse buffer (GIBCO BRL) 10 U of RNAse inhibitor (GIBCO BRL) and RNAse-free H_2O for 15 min at room temperature. After 15 min incubation, 25 mM EDTA was added and the sample was heated at 65°C for 20 min to inactivate the DNAse.

RNA (1 μg) was incubated with 0.5 μg oligo(dT) at 70°C for 10 min in a thermocycler. A reverse transcriptase master mix was added to the RNA. The master mix contained First Strand Buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, pH 8.3, Gibco/BRL), 10 mM DTT, 10 mM of each dNTP, Sigma sterile water and 200 U M-MLV RT enzyme (Gibco/BRL). This mixture was incubated at 37°C for 1 hr, then 70°C for 10 min. To test for RT reaction, a duplicate of each sample was reverse transcribed in the presence of [α-^{32}P]dCTP (1 μCi/μL). Unincorporated [α-^{32}P]dCTP was separated from labelled cDNA sample by paper chromatography, cDNA sample was cut out, 6 mL of scintillation fluid was added and radioactivity was measured on a Beckman scintillation counter.

The polymerase chain reaction master mix contained 1X PCR buffer (20 mM Tris-Cl, 50 mM KCl, pH 8.4, Gibco/BRL), 0.8 mM dNTP mix, 20 μM antisense CFTR primer (5'-GGAATCACACTGAGTGGAGGTCAAC-3') which binds bp 1765 to 1789 of the human CFTR gene (NCBI accession # 004980.1), 20 μM of sense CFTR primer (5'-GGCACATCAGAATCCTCTTCGATG-3') which binds bp 2301 to 2320 of the human CFTR gene (expected product size = 550 bp), 1.5 mM $MgCl_2$ (Gibco/BRL),

sterile Sigma water, 2 μL of cDNA and 2.5 units of Taq DNA Polymerase (Gibco/BRL). The mixture was amplified at an annealing temperature of 50°C for 35 cycles. The product was analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. The conditions of use for the primers specific for CFTR were optimized using T84 RNA. The β-actin sense (5'-CCATGTACGTAGCCATCCA-3') and antisense (5'-GATAGAGCCACCAATCCAC-3') primers were also were used as a control (annealing temperature of 46°C).

The 550 bp CFTR PCR product was cloned into the pCR 2.1® (Invitrogen, Carlsbad, Ca, USA) vector using T4 DNA ligase and then used to transform "one shot" *E. coli* cells (Invitrogen INVaF'). The *E. coli* cells were grown for 3 d, the DNA was isolated using a Sigma miniprep isolation kit and the insert was sequenced on the ABI PRISM sequencer model 2.1.1 and analyzed by BLAST (12). The sequence obtained was 100% homologous to the CFTR gene.

Flow Cytometry

Single cell suspensions of 10^5 cells were isolated and blocked with PBS/5% mouse serum for 1 hr. Blocked cells were incubated with primary monoclonal antibody (mAb. anti-CFTR extracellular domain, Affinity Bioreagents, MA1-935) for 1 hr in PBS/5% mouse serum. Cells were washed twice with PBS and incubated with the secondary antibody (goat anti-mouse IgM:FITC, Biosource AM14708) for an additional hr. The isotype antibody used for comparison was mouse IgM isotype control anti-TNP (Pharmingen 03081D).

Western Immunoblotting

RCMC, PMC or CALU-3 cells were isolated, washed with PBS and 1 x 10^6 cells were lysed with 2X sample buffer (0.5 mL 1M Tris-Cl, 1 mL DTT, 2 mL 10% SDS, 1 mL glycerol, 0.5 mL 0.12 % bromophenol blue) supplemented with 2% β-mercaptoethanol.

Whole cell extracts (30-40 μg) were separated by 8% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% milk in TBS-

Tween for 24 hr at room temperature and then stained with primary antibodies, anti-CFTR (R domain, Genzyme, 1660-01) and isotype control purified mouse IgG_1 κ-isotype standard (anti-TNP; Serotec, 03001D) for 1 hr at room temperature. The membranes were washed with TBS-Tween 3X and then stained with the secondary antibody for 1 hr. The secondary antibody was sheep anti-mouse IgG conjugated to HRP (Amersham Life Science, catalogue # NA 9310).

The nitrocellulose membranes were developed with chemiluminescence reagent (NEN Life Technologies, catalogue # NEL 101) for 1 min and placed into an autoradiography cassette containing high performance chemiluminescence film (Amersham Life Science, catalogue # RPN2103H). The film was exposed for 30 min.

Zymography

HMC-1 (2 x 10^6 cells/mL) were incubated in serum-free medium for 24 h at 37°C. Supernatants were collected and electrophoresed on a SDS-10% polyacrylamide gel containing 1 mg/mL gelatin under non-reducing conditions as described previously (13). After electrophoresis, SDS was removed by incubation in 2.5 % Triton X-100 for 20 min, three times. The gels were incubated for 24 h at 37°C in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ and 1 μM $ZnCl_2$. The gels were then stained with Coomassie Brilliant Blue G-250 solution, and destained in 10% acetic acid/25% methanol. Gelatinolytic enzyme activity was detected by the appearance of negatively stained bands. Supernatant from PMA-stimulated rat fibroblasts (previously shown to contain high levels of MMP-9 and MMP-2 activity) was used as a positive control and comparison (14).

Mouse BM cultures

B6-Cftr (-/-) and B6-Cftr (+/+) mice were generated at the University of North Carolina by insertion of a stop codon in exon 10 of the Cftr gene (15). These mice have been backcrossed to C57BL/6 mice and have a homozygous C57BL/6 background. Bones from these mice was the kind gift of Dr. Danuta Radzioch from McGill University.

Eight to ten week old mice were sacrificed as described previously (16) and the limbs were shipped in phosphate-buffered saline to the University of Alberta.

Bone marrow (BM) was aseptically flushed from femurs and tibias into RPMI 1640 medium containing 4 mM L-glutamine, 50 μM β-mercaptoethanol, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.1 mM nonessential amino acids (complete RPMI). The cell suspension was washed twice in complete RPMI by centrifugation at 200*g* for 10 min and finally resuspended in complete RPMI containing 10% FBS and 10 ng/mL recombinant mouse IL-3. Cell suspensions (2 x 10^5 cells/mL) were placed in 50 cm^2 flasks and incubated at 37°C in a 5% CO_2 humidified atmosphere. Cells were centrifuged and resuspended in fresh media every seven days to achieve a final concentration of 2 x 10^5 cells/mL. At designated times, aliquots of cultured cells were stained with toluidine blue and examined (at 650 X magnification) for metachromatic staining.

Bone marrow-derived mast cells (BMMC) were sensitized with 10 μg/mL of mouse IgE (anti-dinitropyridine; DNP; Sigma) for 2 hr at 37°C, washed once and stimulated with DNP-human serum albumin (Sigma; 5 to 20 ng/mL) for 30 min at 37°C. BMMC were pelleted at 200*g* and the supernatant was removed. Pellet and supernatant samples were assayed for β-hexosaminidase (β-hex) by hydrolysis of the fluorescent substrate 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (Sigma M-2133). One unit of enzyme cleaves 1 μmol of substrate/hr at 37°C (17). Pellet and supernatant samples (50 μL) were added in duplicate wells in a microtitre plate followed by 1 mM substrate (50 μL) and the mixture was incubated at 37°C for 2 hr. The reaction was terminated by the addition of 100 μL of 0.2 M Tris base and the optical density was read at 450 nm (excitation 356 nm). The OD_{450nm} for blank wells, containing only substrate and Tris base, was automatically subtracted from the sample OD_{450nm} and the percentage β-hex release was calculated by the formula:

$$\frac{OD_{450nm} \text{ of supernatant samples}}{OD_{450nm} \text{ of pellet samples} + OD_{450nm} \text{ of supernatant samples}} \times 100 = \% \text{ release}$$

The spontaneous release (in HTB) was subtracted from all samples to give % specific release.

Results

HMC-1 express CFTR

We had previously shown that rat peritoneal mast cells (PMC) and a rat cultured mast cell line (RCMC) express CFTR (3). To determine if human mast cells expressed CFTR, we isolated cell lysates from HMC-1, human bronchial epithelial cells (Calu-3) that express CFTR and human epithelial cells (A549) that do not express CFTR. RCMC lysates are also shown for comparison. HMC-1, RCMC and Calu-3 express a ~ 180 kDa CFTR protein, whereas A549 do not (Fig 7.1A).

Similarly, flow cytometry was used to determine CFTR surface expression in A549, Calu-3 and HMC-1 cells. Calu-3 and HMC-1 cells express surface CFTR, whereas A549 do not (Fig 7.1B).

CFTR antisense oligodeoxynucleotides inhibit CFTR expression in T84

Antisense ODN were designed as previously described (6) and had complementary sequences to nucleotides 1-18 and 19-36 of CFTR mRNA. Control ODN were also designed that had the same sequence as CFTR mRNA (sense) and the same sequence as the antisense ODN except for 4 mismatches (missense). Since the use of these ODN to inhibit CFTR expression in T84 cells (6) had previously been published, we tested the ability of these ODN to inhibit T84 CFTR expression. In our hands, antisense ODN almost completely inhibited CFTR mRNA expression in T84 cells (Fig 7.2A). CFTR antisense ODN also inhibited CFTR protein expression in T84 while untreated, missense and sense ODN treated T84 showed normal expression of CFTR protein (Fig 7.2B). Flow cytometry showed that CFTR antisense ODN inhibits surface expression of CFTR in T84 (Fig 7.2C and D).

Antisense oligodeoxynucleotides inhibit CFTR expression in HMC-1

Antisense ODN were tested for their ability to inhibit CFTR expression in MC. HMC-1 cells were treated with 20 μM of antisense ODN every 12 hr for a total of 6 times. At 72 hr, cells were isolated as described in methods. Antisense ODN inhibited CFTR expression while missense and sense ODN had no effect on CFTR expression (Fig 7.3).

PMA release of MMP-9 is sensitive to glibenclamide

HMC-1 cells release MMP-9 when activated with PMA (13). To determine the effect of various ion channel inhibitors on PMA-induced MMP-9 release, HMC-1 were stimulated with PMA (50 ng/mL) for 24 hr in the presence of DIDS (10 μM), DPC (1 mM) and glibenclamide (30 μM). Zymography analysis of these supernatants revealed that PMA induced release of a major 92 kDa lytic band representing MMP-9 activity (Fig 7.4). DIDS and DPC had no discernable effect on the release of MMP-9 activity (Fig 7.4). However, glibenclamide inhibited the release of MMP-9 activity (Fig 7.4A).

C5a and C3a induce HMC-1 migration and increases in intracellular Ca^{2+} (18). To determine if C5a would also induce MMP-9 release and if this release was sensitive to DIDS, DPC or glibenclamide, HMC-1 were incubated with C5a in the presence of the inhibitors for 24 hr. C5a did not induce MMP-9 release and basal release of MMP-9 was not sensitive to DIDS, DPC or glibenclamide (Fig 7.4B).

Antisense oligodeoxynucleotides have no effect on PMA-induced MMP-9 release

To determine the effect of CFTR antisense ODN on MC mediator release, HMC-1 were pretreated with antisense, missense and sense ODN for 72 hr and stimulated with PMA (50 ng/mL). Supernatants were collected and tested for MMP-9 activity (Fig 7.5). CFTR antisense ODN treated HMC-1 release the same amounts of MMP-9 (Fig 7.5) as missense, sense and untreated HMC-1.

Bone marrow-derived mast cells from CFTR knock-out mice grow normally and release β-hexosaminidase

To determine if CFTR expression was important for mediator release in another cell type, BMMC were grown from CFTR knock-out (-/-) and wild-type (+/+) and mediator release was measured (Fig 7.6). BM cells isolated from both CFTR (-/-) and CFTR (+/+) mice grew in the presence of 10 ng/mL of mouse recombinant IL-3 and produced mononuclear cells that stained metachromatically with toluidine blue (BMMC; Fig 7.6A). After three weeks in culture, CFTR (-/-) BM cultures had 36.0 $\pm$ 15.0 (x 10^6) BMMC total while BM cultures from CFTR (+/+) had 37.1 $\pm$ 22.5 (x 10^6) BMMC total (Fig 7.6A).

At three to four weeks of culture, BMMC were isolated, sensitized with mouse IgE (anti-DNP) for 2 hr and stimulated with DNP-human serum albumin (5 to 20 ng/mL) for 30 min (Fig 7.6B). BMMC from both CFTR (-/-) and CFTR (+/+) responded to FcεR-mediated stimulated and released β-hex. BMMC from CFTR (-/-) mice released 16 $\pm$ 0.7 % β-hex when stimulated with 10 ng/mL of DNP-human serum albumin. BMMC from CFTR (+/+) mice released 12.1 $\pm$ 2.7 % β-hex when stimulated with 10 ng/mL of DNP-human serum albumin. These are preliminary experiments from two sets of independently grown BMMC cultures.

Discussion

This study has shown that a human mast cell (HMC-1) expresses CFTR, confirming earlier findings that rat mast cell express a CFTR channel activated by cAMP analogues (3). We have also shown that, like studies in the T84 epithelial cell line, antisense ODN to CFTR mRNA are capable of inhibiting expression of CFTR in MC. Flow cytometry shows that ODN inhibit surface CFTR protein expression on both T84 and HMC-1 cells.

To determine whether inhibition of CFTR expression by antisense ODN affects MC functions, we used a MMP-9 release assay. Unfortunately, these HMC-1 cells do not

express FcεR and contain small amounts of histamine and are therefore difficult to stimulate using IgE crosslinking. However, HMC-1 are stimulated by PMA to release significant levels of MMP-9. Our results show that C5a, another activator of HMC-1, does not release MMP-9.

To determine the role of CFTR in MMP-9 release, HMC-1 were treated with various blockers of Cl^- channels. Unfortunately, there are no known peptide blockers of CFTR and no known organic blockers exhibit a high affinity and exclusive selectivity for CFTR. Most Cl^- channels can be blocked by three classes of compounds; the disulfonic stilbenes (e.g. DIDS), the arylaminobenzoates (e.g. DPC) and sulfonylurea (e.g. glibenclamide) compounds. CFTR Cl^- channel activity (as measured by patch clamp) in epithelial cells is blocked with DPC and glibenclamide, but not with the broad anion exchanger inhibitor DIDS (19).

PMA induced release of MMP-9 from HMC-1 is sensitive to glibenclamide, a blocker of CFTR, but not to DIDS or DPC. Therefore, a glibenclamide-sensitive ion channel (perhaps CFTR) may be involved in PMA-induced MMP-9 release. Pharmacologic inhibition of CFTR with DPC and glibenclamide is measured after short incubation times (0 to 10 min) and these blockers are often unstable in aqueous solution. Therefore, DIDS and DPC may not have any effect on MMP-9 release because of the lengthy (24 hr) incubation required in this assay.

However, DIDS, DPC and glibenclamide are not specific inhibitors of CFTR. Both DIDS and DPC can block the Cl^-/HCO_3^- exchanger, which is an alkalinizing mechanism responsible for maintaining internal pH in MC (19,20). Glibenclamide can also inhibit ATP-activated K^+ channels (21). Therefore, glibenclamide sensitivity does not always show CFTR involvement, although it remains a possibility.

Preliminary experiments with BMMC derived from CFTR (-/-) and CFTR (+/+) mice show that the absence of CFTR expression has no effect on MC differentiation and proliferation in culture in the presence of recombinant mouse IL-3. In addition, BMMC derived from CFTR (-/-) mice respond to FcεR stimulation and release β-hex. These are preliminary experiments from two independent cultures and must be repeated in order to reach any further conlusions.

In summary, these preliminary results show that ODN complementary to CFTR mRNA can be used to inhibit CFTR protein expression in MC. Antisense ODN have no effect on PMA-induced MMP-9 release from MC. However, antisense ODN may have effects on the release of other mediators such as cytokines (e.g. IL-3 and TNF) or chemotaxis in response to C5a or C3a. The observation that T lymphocytes from CF patients produce 45% less IL-10 (2) suggesting that CFTR may be involved in cytokine production. We hypothesize that MC that lack expression of CFTR may also have altered cytokine production and the antisense technology optimized in this study would be a powerful tool to test this hypothesis. In addition, BMMC from CFTR (-/-) mice, stimulated via FcεR, may also prove useful in testing this hypothesis.

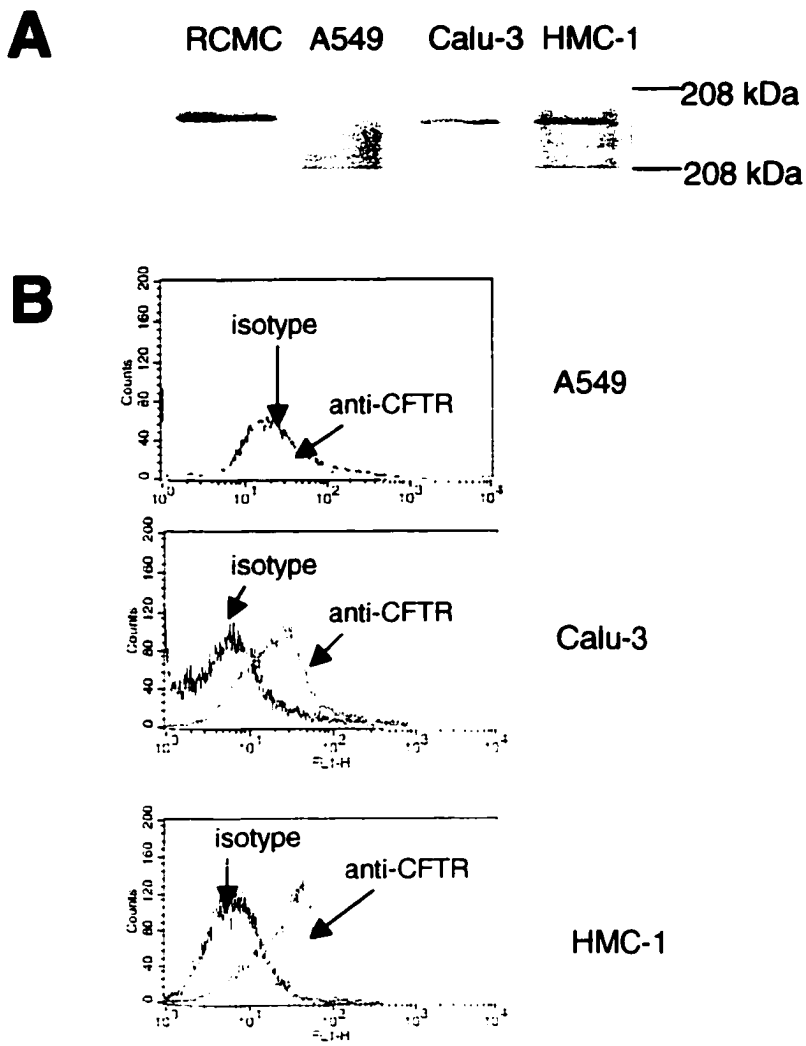


Figure 7.1. A human mast cell line express CFTR. (A) human mast cells (HMC-1), rat mast cells (RCMC), human bronchial epithelial cells (Calu-3), and human epithelial cells (A549) cell lysates were resolved on an 8% polyacrylamide gel, transferred to a nitrocellulose membrane and blotted with an anti-CFTR mAb. (B) Flow cytometry analysis of A549, Calu-3 and HMC-1 cells showing staining with isotype control and anti-CFTR mAb. (n=5)

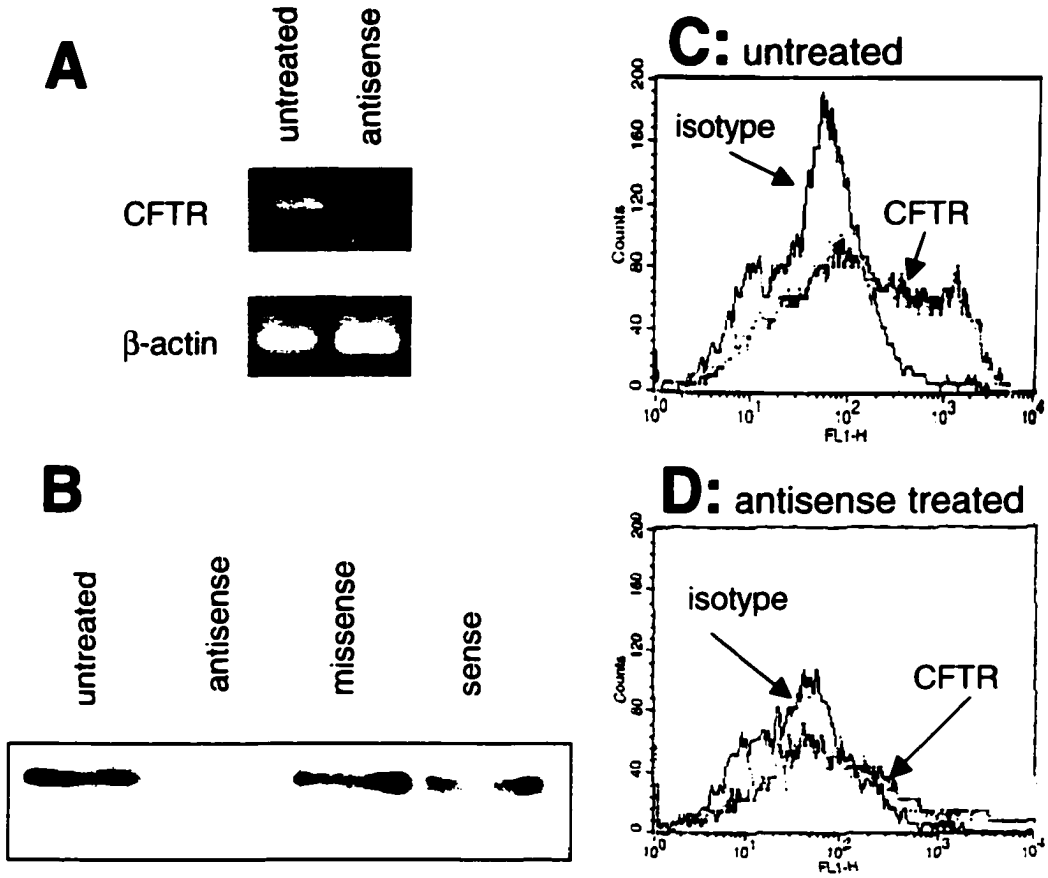


Figure 7.2. Antisense inhibits CFTR mRNA (A) and protein (B) expression in T84 cells. RNA was isolated from antisense treated (20 mM for 12 h) and untreated T84 epithelial cells and RT-PCR was used to detect CFTR mRNA expression. β-actin is shown as control. Cell lysates from antisense treated and untreated T84 were resolved on an 8% polyacrylamide gel and blotted with anti-CFTR mAb (B). Antisense treated (D) and untreated T84 (C) were incubated with anti-CFTR mAb and surface expression of CFTR was analyzed using flow cytometry. Representative experiment of n=2.

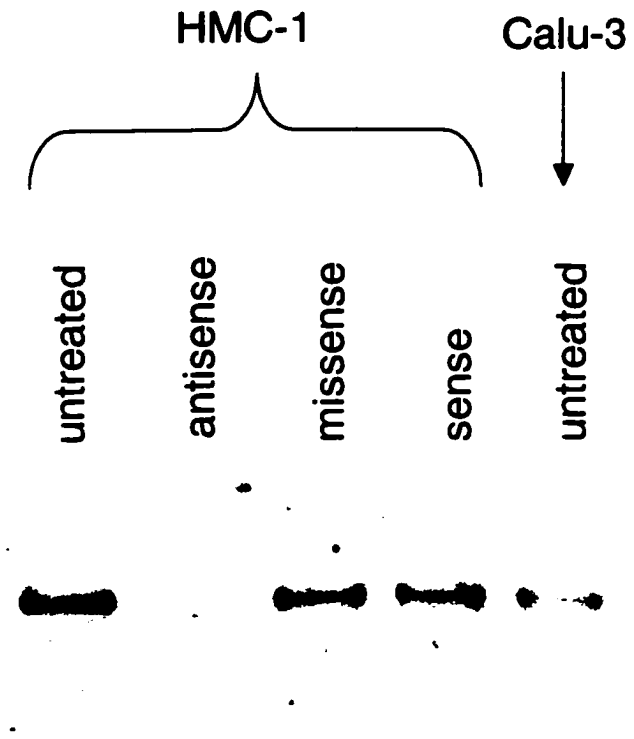


Figure 7.3. Antisense inhibits CFTR protein expression in HMC-1. HMC-1 were treated (20 μM for 72 h), with antisense, missense and sense oligonucleotides complementary to huCFTR mRNA or untreated. Total cell lysates were resolved on an 8 % polyacrylamide gel and immunobloted with an anti-CFTR mAb.

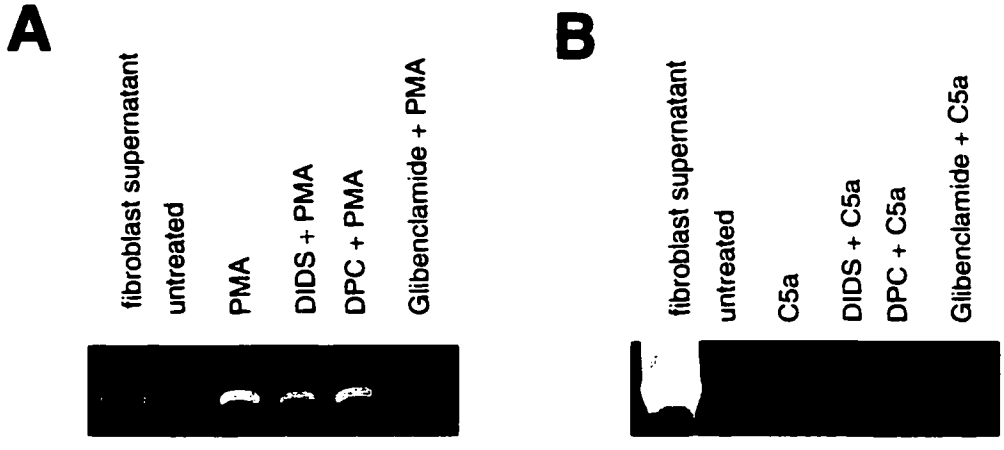


Figure 7.4. PMA induces mast cells to produce matrixmetalloprotease-9 activity. (A) Zymography was conducted on supernatants from mast cells (HMC-1) treated with PMA (50 ng/mL) or PMA+ human IFN-γ (500 U/mL) or PMA + DPC (10 μM) or PMA + glibenclamide (30 μM). HMC-1 cells were cultured for 24 hr in serum free medium and supernatants were collected, resolved on SDS-PAGE and stained with Coomassie brilliant blue as described in methods. Fibroblast supernatant (also stimulated with 50 ng/mL PMA) was used as a positive control for MMP-9 production. (B) HMC-1 were also treated with C5a, DIDS + C5a, DPC + C5a, and glibenclamide + C5a. HMC-1 were cultured for 24 hr in serum free medium and supernatants were collected and analyzed by zymography as before.

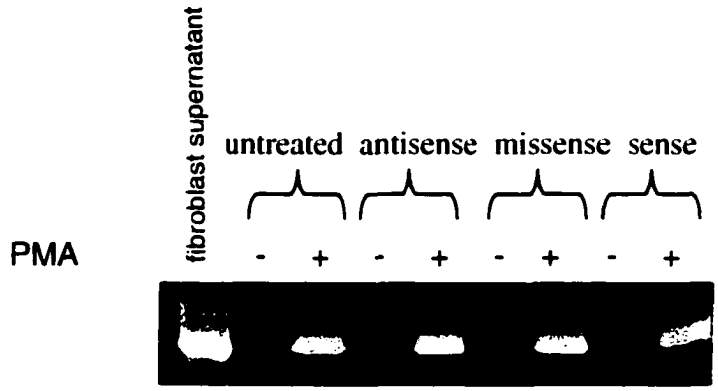


Figure 7.5. Antisense oligodeoxynucleotides (ODN) complementary to CFTR mRNA have no effect on PMA-induced release of matrixmetalloprotease-9 activity. Mast cells (HMC-1) were treated with CFTR antisense (20μM), missense (20μM), and sense (20μM) ODN for 72 hr. Mast cells were treated with PMA (50 ng/mL) for 24 hr in serum free medium and supernatants were collected, resolved on SDS-PAGE and stained with Coomassie brilliant blue as described in methods. Fibroblast supernatant (also stimulated with 50 ng/mL PMA) was used as a positive control for MMP-9 production.

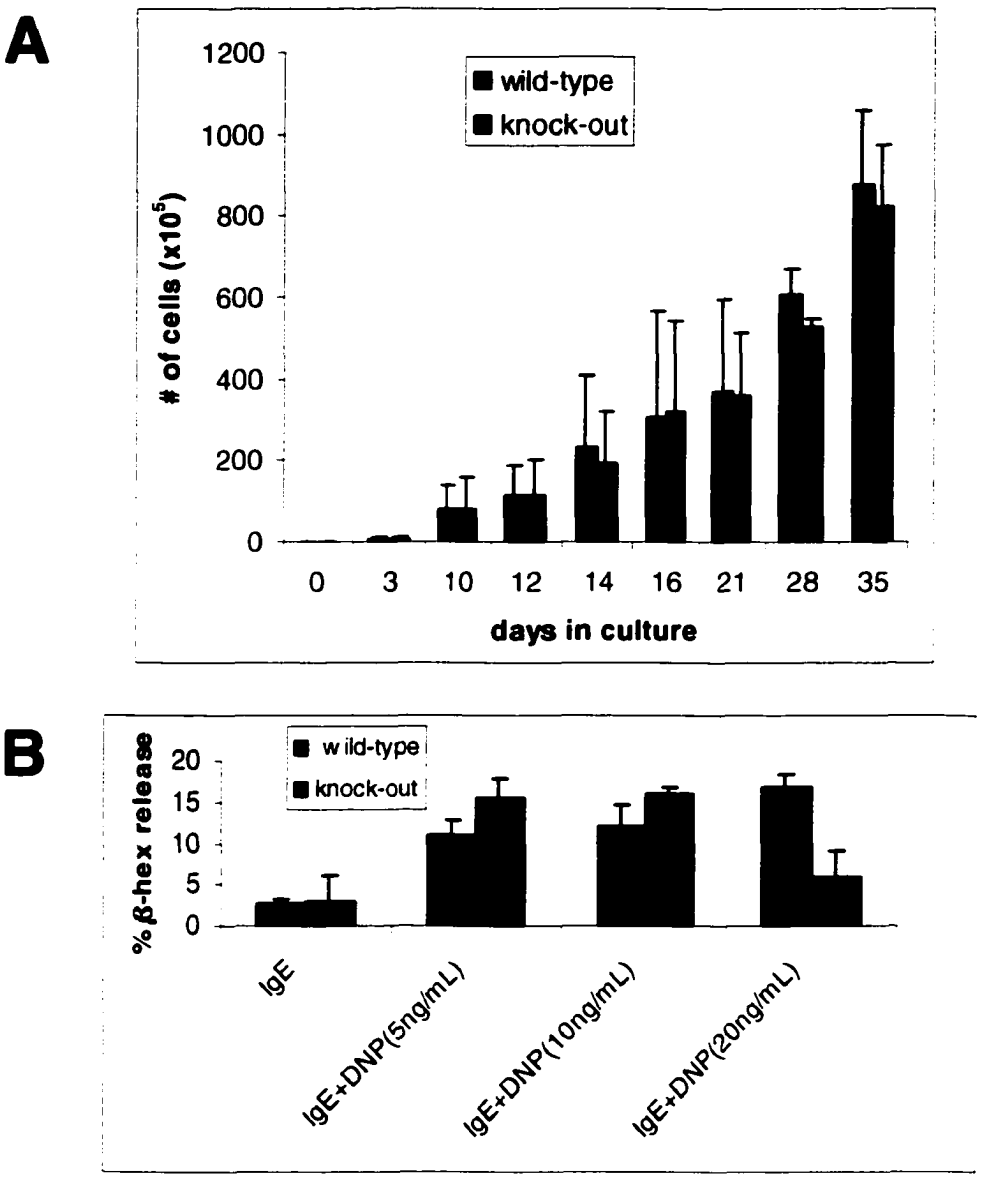


Figure 7.6. Mast cells derived from wild-type and knock-out mouse bone marrow proliferate normally (A) and release β-hexosaminidase in response to FcεR crosslinking (B). Bone-marrow derived mast cells (BMMC) were isolated and grown in IL-3 (10 ng/mL) and at each time point cells were stained with toluidine blue and counted. (B) BMMC were sensitized with 10 μg/mL of mouse IgE (anti-DNP) for 2 hr and stimulated with DNP-human serum albumin for 30 min. Each data point represents experiments performed on two independently grown cultures.

References

1. Quinton, P. M. 1999. Physiological basis of cystic fibrosis: a historical perspective. *Physiol. Rev. 79*:S3.

2. Moss, R. B., R. C. Bocian, Y. P. Hsu, Y. J. Dong, M. Kemna, T. Wei, and P. Gardner. 1996. Reduced IL-10 secretion by CD4+ T lymphocytes expressing mutant cystic fibrosis transmembrane conductance regulator (CFTR). *Clin. Exp. Immunol. 106*:374.

3. Kulka, M., M. Gilchrist, M. Duszyk, and A. D. Befus. 2002. Expression and functional characterization of CFTR in mast cells. *J. Leukoc. Biol.* (In press)

4. Furukawa, T., T. Ogura, Y. Katayama, and M. Hiraoka. 1998. Characteristics of rabbit ClC-2 current expressed in Xenopus oocytes and its contribution to volume regulation. *Am. J. Physiol. 274*:C500.

5. Mo, L., H. L. Hellmich, P. Fong, T. Wood, J. Embesi, and N. K. Wills. 1999. Comparison of amphibian and human ClC-5: similarity of functional properties and inhibition by external pH. *J. Membr. Biol. 168*:253.

6. Wagner, J. A., T. V. McDonald, P. T. Nghiem, A. W. Lowe, H. Schulman, D. C. Gruenert, L. Stryer, and P. Gardner. 1992. Antisense oligodeoxynucleotides to the cystic fibrosis transmembrane conductance regulator inhibit cAMP-activated but not calcium-activated chloride currents. *Proc. Natl. Acad. Sci. U. S. A. 89*:6785.

7. Krauss, R. D., G. Berta, T. A. Rado, and J. K. Bubien. 1992. Antisense oligonucleotides to CFTR confer a cystic fibrosis phenotype on B lymphocytes. *Am. J. Physiol. 263*:C1147.

8. Kumar, B. V., G. Medoff, G. Kobayashi, and D. Schlessinger. 1974. Uptake of Escherichia coli DNA into HeLa cells enhanced by amphotericin B. *Nature 250*:323.

9. Yovandich, J., B. O'Malley,Jr., M. Sikes, and F. D. Ledley. 1995. Gene transfer to synovial cells by intra-articular administration of plasmid DNA. *Hum. Gene Ther. 6*:603.

10. Stein, C. A. and Y. C. Cheng. 1993. Antisense oligonucleotides as therapeutic agents--is the bullet really magical? *Science 261*:1004.

11. Chan, B. M., C. S. Ra, W. N. Hu, K. McNeill, H. R. Miller, D. Befus, J. P. Kinet, and A. Froese. 1990. Characterization of rat tissue cultured mast cells. *Eur. J. Immunol. 20*:185.

12. Iwagaki, H., M. Marutaka, T. Yoshino, L. Cao, I. Sakuma, M. Uomoto, Y. Takeuchi, and N. Tanaka. 1996. Gamma-interferon upregulates transferrin receptors and decreases membrane microviscosity in K562 cells. *Res. Commun. Mol. Pathol. Pharmacol. 92*:191.

13. Kanbe, N., A. Tanaka, M. Kanbe, A. Itakura, M. Kurosawa, and H. Matsuda. 1999. Human mast cells produce matrix metalloproteinase 9. *Eur. J. Immunol. 29*:2645.

14. Hofmann, A., S. Laue, A. K. Rost, W. A. Scherbaum, and G. Aust. 1998. mRNA levels of membrane-type 1 matrix metalloproteinase (MT1-MMP), MMP-2, and MMP-9 and of their inhibitors TIMP-2 and TIMP-3 in normal thyrocytes and thyroid carcinoma cell lines. *Thyroid. 8*:203.

15. Snouwaert, J. N., K. K. Brigman, A. M. Latour, N. N. Malouf, R. C. Boucher, O. Smithies, and B. H. Koller. 1992. An animal model for cystic fibrosis made by gene targeting. *Science 257*:1083.

16. Gosselin, D., M. M. Stevenson, E. A. Cowley, U. Griesenbach, D. H. Eidelman, M. Boule, M. F. Tam, G. Kent, E. Skamene, L. C. Tsui, and D. Radzioch. 1998. Impaired ability of Cftr knockout mice to control lung infection with Pseudomonas aeruginosa. *Am. J Respir. Crit. Care Med. 157*:1253.

17. MacDonald, A. J., J. Pick, E. Y. Bissonnette, and A. D. Befus. 1998. Rat mucosal mast cells: the cultured bone marrow-derived mast cell is biochemically and functionally analogous to its counterpart in vivo. *Immunology 93*:533.

18. Hartmann, K., B. M. Henz, S. Kruger-Krasagakes, J. Kohl, R. Burger, S. Guhl, I. Haase, U. Lippert, and T. Zuberbier. 1997. C3a and C5a stimulate chemotaxis of human mast cells. *Blood 89*:2863.

19. Ballard, S. T., L. Trout, Z. Bebok, E. J. Sorscher, and A. Crews. 1999. CFTR involvement in chloride, bicarbonate, and liquid secretion by airway submucosal glands. *Am. J. Physiol. 277*:L694.

20. Vilarino, N., M. R. Vieytes, J. M. Vieites, and L. M. Botana. 1998. Role of HCO3- ions in cytosolic pH regulation in rat mast cells: evidence for a new Na+-independent, HCO3--dependent alkalinizing mechanism. *Biochem. Biophys. Res. Commun. 253*:320.

21. Virag, L., T. Furukawa, and M. Hiraoka. 1993. Modulation of the effect of glibenclamide on KATP channels by ATP and ADP. *Mol. Cell Biochem. 119*:209.

Chapter 8

General Discussion and Conclusions

Mast cells (MC) are relatively large leukocytes that are widely distributed and important effector cells in innate immunity and inflammatory responses. Activation of MC results in the release of mediators that can initiate immune responses and stimulate smooth muscle, lymphocytes, eosinophils and submucosal glands (1). Many MC functions rely on influx of Ca^{2+} and Cl^- ions and blocking these ion fluxes inhibits MC mediator release (Chap 1). Interferon-gamma (IFN-γ), a pleotropic cytokine produced by T lymphocytes and several other cell types, is an inhibitor of MC proliferation, differentiation, mediator release and adhesion (2-4) yet the mechanism of IFN-γ-mediated inhibition of MC functions is poorly understood. One possible mechanism of IFN-γ-mediated downregulation of MC function may involve changes in Cl^- flux.

Preliminary experiments in our laboratory had used differential display analysis of mRNA from IFN-γ and sham treated MC to determine differences in gene expression. These studies revealed several bands that appeared to be upregulated in IFN-γ-treated MC. One of these bands, a 218 bp fragment, had 72% homology over a 51 bp portion to chloride channel-3 (ClC3), a ubiquitously expressed chloride channel. Our initial hypothesis was that IFN-γ treatment increased ClC3 mRNA transcription in mast cells. However, reverse transcriptase-polymerase chain reaction (RT-PCR) using a primer for ClC3 was unable to confirm differences in expression between IFN-γ-treated and untreated mast cells (Chap 3). Subsequently, the effect of IFN-γ on the expression of other Cl^- channels was analyzed.

The central hypothesis of this thesis is that IFN-γ effects on MC may involve changes in Cl^- ion flux and/or modulation of Cl^- channel expression. Yet, Cl^- channel expression in MC is unknown and a great deal of controversy exists about the exact role of Cl^- channels in MC function. This project has attempted to (1) identify some of the Cl^- channels expressed in MC, (2) use pharmacologic studies to determine the possible role of these Cl^- channels in MC functions and (3) determine the effect of IFN-γ on expression of these Cl^- channels. Initially, it was postulated that IFN-γ may effect net changes in Cl^- flux and that these changes could be measured using fluorochromes sensitive to $[Cl^-]$.

I. Summary of Results

A. Interferon-γ alters mast cell chloride flux

IFN-γ is an important immunomodulatory cytokine that has several inhibitory effects on MC function. Data presented in this thesis show that IFN-γ inhibits β-hexosaminidase release (β-hex; Chap 2). This observation supports previous studies that have shown that IFN-γ inhibits MC release of preformed mediators such as serotonin and histamine and newly synthesized mediators such as arachidonic acid and tumor necrosis factor (TNF) (5-8). IFN-γ has suppressive actions on several MC functions such as clonal proliferation, differentiation, TNF-mediated cytotoxicity, adhesion and mediator release (2,3,5,8,9). IFN-γ also modulates the expression of several proteins in MC such as major histocompatibility complex-II (MHC class II), Fc-gamma receptor-I (FcγRI) and Fc-gamma receptor –III (FcγRIII) (10-12).

Ion flux is an important part of MC differentiation and homeostasis (13) and there is some evidence to suggest that Cl^- may also be important in MC functions such as mediator release (14-18). For example, resting MC display a Cl^- current which is increased when MC are activated by Fc-epsilon receptor-I (FcεRI) crosslinking (14) (Chap 1). However, the effect of IFN-γ on resting MC Cl^- was unknown and the first aim of this thesis was to determine the effect of IFN-γ on MC Cl^- flux. Fluorescent dye measurements suggest that IFN-γ alters Cl^- flux in MC. However, it is difficult to interpret these results since fluorescent dye measurements have at least three disadvantages. (1) The amount of fluorochrome loaded into each cell is not necessarily the same. Therefore these are crude measurements of Cl^- flux in a heterogeneously labelled cell population. (2) Since MC are loaded with dye in hypotonic buffer containing low [Cl^-] and placed in a buffer containing high [Cl^-], this assay measures Cl^- influx, not efflux. (3) The fluorochrome can attach to lipid membranes and quenching may occur on the cell surface as well as in the cytoplasm. Therefore, these studies measure

fluorochrome quenching in a heterogeneously labelled cell population under a large osmotic gradient. This assay is unlikely to detect minute changes in Cl^- channel function.

Studies using ^{36}Cl show that resting MC take up ^{36}Cl and that IFN-γ treated MC take up less ^{36}Cl over a 30 min period. These results suggest that IFN-γ alters Cl^- flux such that ^{36}Cl uptake is inhibited. This assay measures ^{36}Cl fluxes in one direction (into the cell) and final $[Cl^-]$ is dependent upon fluxes into and out of the cell. Previous studies have suggested that such Cl^- fluxes are present in resting MC and that $[Cl^-]$ is near equilibrium (E_{Cl}) (15,19). Therefore, it isn't surprising that resting MC placed in ^{36}Cl-containing buffer take up ^{36}Cl. However, our studies also show that antigen stimulation decreases net ^{36}Cl uptake after 30 min. Antigen stimulation decreases MC volume and changes cell shape and granule content (20) and decreases in cell size may ultimately decrease the amount of Cl^- ions present in the cytoplasm. Therefore, decreased $^{36}Cl^-$ uptake may not be a consequence of secretion but the result of cell volume changes.

Although the exact mechanism by which IFN-γ may be affecting Cl^- uptake is unknown, IFN-γ ligation of the IFN-γ receptor (IFN-γR) regulates gene transcription via the Janus kinase(JAK) and signal transducers and activators of transcription-1 (STAT1) pathway. In addition to cotransporters and as yet unidentified mechanisms, Cl^- channels are responsible for mediating Cl^- fluxes. Therefore a possible mechanism of IFN-γ alterations in Cl^- flux may be mediated by changes in gene transcription of Cl^- channels. Certainly, IFN-γ inhibition of Cl^- flux occurs at 2 hr and is appropriate length of time for a gene transcription-dependent event.

B. Mast cells express chloride channels

Although Cl^- conductances in resting and activated MC have been measured (18,21), the specific Cl^- channels responsible for these conductances are unknown. Therefore, the next aim of this project was to characterize some of these Cl^- channels in MC and pinpoint some of the possible targets of IFN-γ (Fig 8.1). At the start of this project, few antibodies were available to these Cl^- channels (especially the ClC family). In many cases, this project has optimized and re-developed assays (originally used in

other cell types) to characterize Cl^- channel expression and function in MC. The S1 nuclease assay, for example, is a quantitative measure of mRNA expression originally designed to characterize γ-aminobutyric acid type A receptor ($GABA_AR$) mRNA in rat neurons. Fluorochromes sensitive to changes in [Cl^-], originally used in epithelial cells, were used to measure minute changes in Cl^- uptake between IFN-γ-treated and untreated MC. A ^{36}Cl release assay, optimized for adherent epithelial cells, was used to determine cystic fibrosis transmembrane conductance regulator (CFTR) activity in MC stimulated with cAMP analogues (Chap 5). Carefully controlled RT-PCR analysis was used to determine expression of Cl^- channels and the effect of IFN-γ on their expression.

There are at least three major families of Cl^- channels expressed in mammalian cells. These include the $GABA_AR$, voltage-gated ClC and CFTR. Hybridization analysis using radiolabelled oligonucleotides show that MC express several subunits that contribute to the pentameric $GABA_AR$ and immunoblot analysis shows that MC express the $GABA_AR\alpha 1$ subunit, commonly expressed in neurons (Table 8.1).

$GABA_AR$ is a ligand-gated Cl^- channel and binds a number of ligands such as GABA, benzodiazepine (BDZ), muscimol and ethanol (22). BZD binding sites have been reported and characterized on rat peritoneal mast cells (PMC), although little is known regarding the biological effect of BDZ on MC function (23) (Fig 8.2). The BDZ Ro5-4864 (10^{-6} M) has no effect on histamine or serotonin release in either unstimulated PMC or those stimulated by compound 48/80 or substance P (23). However, midzolam and diazepam (10^{-6} to 10^{-4} M) decrease proliferation, β-hex release, tumor necrosis factor (TNF) release and nitrite production from bone marrow-derived MC (BMMC) (24). Ligation of BDZ binding sites on rat PMC inhibits calcium influx generated by concanavalin A (25). However, there is no evidence to suggest that these BDZ binding sites are $GABA_AR$. In fact, radioligand binding assays (Chap 4) show that GABA and muscimol bind to MC non-specifically and may not involve a specific receptor.

RT-PCR analysis of MC RNA also shows that RCMC express several Cl^- channels, ClC2, 3, 4, 5 and 7 and PMC express ClC 7 (Table 8.1, Chap 3). Some mAb have recently become available to some of the ClC members, which may prove useful in further characterizing their expression in MC. However, patch clamp studies using

antisense oligodeoxynucleotides to block ClC expression may prove much more useful in delineating ClC function in MC. Certainly, patch clamp studies have had little luck in matching specific Cl^- currents with specific signalling pathways. Carefully controlled RT-PCR analysis shows that IFN-γ does not modulate expression of these ClC at the mRNA level. It is possible that a more quantitative technique such as the S1 nuclease assay could be applied to determine relative expression of ClC mRNA before and after IFN-γ.

Another possible mechanism of IFN-γ modulation of Cl^- flux in MC may be via protein phosphorylation (Fig 8.3). JAK2 activation in mammary epithelial cells regulates Na^+-K^+-$2Cl^-$ function via tyrosine phosphorylation (26). Although CFTR was unaffected by JAK2 activation (26) in this study, it is possible that phosphorylation events through other phosphotases and/or kinases activated by IFN-γ may regulate CFTR via phosphorylation and dephosphorylation. RT-PCR analysis indicates that MC express CFTR mRNA and protein and that this channel is activated by cAMP. Also, IFN-γ upregulates CFTR in MC in a dose-depdendent manner (Chap 6). The R domain contains consensus sequences for phosphorylation by protein kinase A (PKA) and by protein kinase C (PKC) (27). PKC phosphorylation of CFTR is diacylglycerol-dependent but Ca^{2+}-independent (28) and in intestinal epithelium, membrane targeting of cGMP-dependent protein kinase (cGK) type II induces CFTR Cl^- channel activity (27). Therefore, CFTR acitivty is controlled by phosphorylation events but, unlike $GABA_AR$, phosphorylation opens the CFTR Cl^- channel.

Some ClC may also be phosphorylated by JAK2. However, ClC-2 is the only ClC shown to be phosphorylated and it is unlikely that ClC-2 is the target of phosphorylation-mediated regulation since PKA phosphorylation has no effect on ClC-2 function (29).

Phosphorylation plays an important role in the regulation of ligand-gated channels. The $GABA_AR$ complex is phosphorylated by several protein kinases *in vitro* and the addition of Mg^{2+}-ATP to the internal pipette solution prevents the time-dependent decrease of GABA-activated currents suggesting that phosphorylation confers an activated (open) conformation to the channel (30). PKC phosphorylation sites are located

in the intracellular domains of the β (Fig 1.5) and γ2 subunits, and increased PKC phosphorylation causes a reduction of GABA-activated currents in hippocampal neurons and those expressed in oocytes injected with mRNA (31). Also, increases in cAMP-dependent protein kinase activity decreases $GABA_AR$ mediated $^{36}Cl^-$ uptake by brain microsacs (32) showing that $GABA_AR$ are no longer conducting a Cl^- current when phosphorylated. These findings show that, unlike CFTR and ClC, $GABA_AR$ are inhibited by phosphorylation.

C. Role of $GABA_AR$ in mast cell function and inflammation

Communication between the immune and neuroendocrine system has been demonstrated in various ways, including regulation of MC functions. Therefore, our observation that $GABA_AR$ are expressed on MC is extremely interesting from a neuroimmune point of view. The $GABA_AR$ belong to a large family of ligand-gated channels that are responsible for the majority of inhibitory neurotransmitter activity in the brain and CNS. $GABA_AR$ are mainly expressed on neurons although recent evidence has also demonstrated expression on T lymphocytes (33). Ligation of T cell $GABA_AR$ inhibits T cell proliferation and cytotoxicity (33,34). These observations suggest that GABA and $GABA_AR$ may modulate immune responses.

One of the ligands for $GABA_AR$ are the benzodiazepines (BDZ) that interact allosterically with the GABA agonist site or the receptor channel (22). BDZ also bind other receptors, located mainly in peripheral tissues and glial cells in the brain, which are macromolecular complex composed of an isoquinoline carboxamide-binding protein (IBP), a voltage-dependent anion channel (VDAC) and an adenine nucleotide carrier (ADC) (35). Peripheral BZD receptors are expressed on monocytes (36), lymphocytes (37-41), platelets (39), neutrophils (42) and mast cells (23). Interestingly, peripheral BDZ receptor ligands have an anti-inflammatory effect on mouse models of inflammation. BDZ derivatives inhibit prostaglandin E_2 and IL-6 production and leukocyte recruitment in a mouse air-pouch model of local inflammation (43). PK11195 and Ro5-4864 inhibit mouse paw edema induced by IL-13 and IL-6 (44). Although Ro5-

4864 (10^{-6} M) has no effect on histamine or serotonin release in either unstimulated PMC or those stimulated by compound 48/80 or substance P (23), MC activated by pro-inflammatory cytokines may be sensitive to Ro5-4864 inhibition in this paw edema model. GABA inhibition of MC mediator release may similarly regulate inflammatory responses.

Could $GABA_AR$ expression on MC be a signalling link that ultimately results in downregulation of inflammatory responses? Certainly, we have shown that GABA inhibits MC β-hex release. Similarly, BDZ decrease MC proliferation, β-hexosaminidase release, tumor necrosis factor (TNF) release and nitrite production (24). If BDZ and GABA inhibit MC mediator release, $GABA_AR$ antagonists would be expected to potentiate pro-inflammatory cytokine release. This hypothesis is supported by studies in human monocytes where inhibition of BDZ receptor (and $GABA_AR$?) signalling, can potentiate inflammatory responses. Triakontatetraneuropeptide (TTN), an endogenous peptide inhibitor of diazepam binding sites, potentiates the release of IL-1β, granulocyte/macrophage colony-stimulating factor (GM-CSF) and TNF from human monocytes possibly by inducing PGE_2 production (45).

GABA is a normal constituent of the mammalian central nervous system (CNS) and no other mammalian tissue, with the exception of the retina, contains more than a mere trace of this compound (22). Therefore, GABA inhibition of MC mediator release most likey occurs in the CNS. MC are often aggregated around nerves and the brain, like other tissues, contains resident MC. Brain MC are found intracranially, in the dura mater, leptomeninges, choroid plexus and the parenchyma of the brain (particularly the thalamus) (46-49). In the rat brain, there is a correlation between MC numbers and content of histamine (50) and isolated rat brain MC release 5-hydroxytryptamine (5-HT) and histamine in response to neuropeptides and neurotransmitters released from nearby neurons (51). The balance between histamine and GABA release in the brain may be important in regulating neuron activity. Activation of the $GABA_AR$ (by muscimol) in the CNS suppresses hyperglycemia induced by histamine stimulation of histaminergic neurons (52) and inhibits histamine-induced [^{3}H]inositol monophosphate accumulation of rat cerebral cortex (53). It is also possible that release of histamine can modulate

$GABA_AR$-mediated responses in neurons. For example, oxidation of histamine in the brain produced imidazoleacetic acid, an agonist of $GABA_AR$, and provides a link between the histaminergic and GABAergic systems in the brain (54).

Therefore, the observation that MC express $GABA_AR$ creates several hypotheses which have profound implications for MC-neuron communication. Further studies will need to confirm expression of a functional $GABA_AR$ chloride channel receptor on MC. These studies should also characterize the ligand-binding affinities of MC $GABA_AR$ for GABA and BDZ. If MC express a $GABA_AR$ capable of binding GABA and BDZ, this could change current models of inflammatory responses in the CNS and other tissues.

D. Role of CFTR chloride channels in mast cell function-pharmacologic studies

Studies using pharmacologic inhibitors suggest that Cl^- channel function may be important in β-hex release from MC. MC treated with 4,4'-diisothiocyano-2,2'-stilbenedisulphonate (DIDS) and diphenylamine-2-carboxylate (DPC) show a marked inhibition of β-hex release. The use of DIDS as an inhibitor of Cl^- is problematic since DIDS inhibits a variety of anion channels and can potentially modify amino acids in plasma membrane proteins. In fact, SITS (a DIDS-related compound) was originally designed as general fluorescent, impermeant covalent modifier of membrane amino groups (55). In addition, the quality of commercially available DIDS is not uniform because companies offer them as free acids or sodium salts with varying degrees of *cis/trans*-isomerization and different ratios of NCS and NH_2 groups. DIDS has hydroscopic properties and the NCS group and the NH_2 groups have a tendency to form polymers such as DIDS-DIDS or DIDS-4,4'-diaminostilbene-2,2'-disulfonic acid (DADS) (56). Therefore, interpretation of data obtained using DIDS must always take into account its other, "non-specific," effects. However, there are no specific protein or pharmacologic inhibitors to CFTR or ClC members, and DIDS is one of the pharmacologic inhibitors available to study Cl^- channels in a variety of cell types.

Although DIDS does not directly block CFTR from the extracellular side, it may indirectly influence CFTR-mediated Cl^- secretion. For example, DIDS has been reported

to increase short-circuit current and serosal-to-mucosal Cl^- fluxes across stripped rabbit colonic mucosa at concentrations between 10 to 200 μM (57). This temporary increase was also observed for whole cell Cl^- current in T84 and Calu-3 cells and was attributed to the elevation of cytosolic Ca^{2+} levels (58). Although the exact mechanism by which DIDS increases Ca^{2+} levels is unknown, the fact that DIDS can temporarily elevate intracellular Ca^{2+} levels complicates its role as an inhibitor of MC mediator release. Given that increases in intracellular Ca^{2+} have been shown to be involved in MC mediator release, DIDS-mediated elevations in intracellular Ca^{2+} would be expected to potentiate MC mediator release. However, the opposite is true, suggesting that either DIDS is unable to elevate Ca^{2+} in MC or that DIDS-mediated block of Cl^- channels is more important in MC mediator release.

However, the DIDS data also suggests that other ion channels may be just as important in MC mediator release. In addition to blocking Cl^- channels, DIDS is among the most potent inhibitors of band 3- or AE1 (anion exchanger)-mediated anion exchange (59). Anion exchangers are present in a number of tissues and may also be present in MC. As well, DIDS has been shown to effect various epithelial K^+ channels such as I_{SK} (60), K_{ATP} (60), the Ca^{2+}-activated K^+ channel in smooth muscle cells (61), and outwardly rectifying Cl^- channels (ORCC) that were originally thought to be the PKA-regulated Cl^- channel defective in CF (62). Any one of these channels could be expressed in MC and could modulate membrane conductance such that MC mediator release is inhibited.

DPC inhibits CFTR in a voltage-dependent manner (currents at positive potentials are not affected, but currents at negative potentials are blocked), reversibly and is thought to bind to a transmembrane region inside the channel pore (63). Like DIDS, but with a higher IC_{50}, DPC also blocked β-hex release from MC. However, like DIDS, DPC use is replete with complicating factors. Firstly, DPC is not a specific inhibitor of CFTR since it can also block ORCC in the human carcinoma cell line HT-20, rat colonic enterocytes, and cultured human respiratory cells (63). DPC can also block a Ca^{2+} dependent Cl^- channel from sheep airway epithelium and a voltage-sensitive outwardly rectifying Cl^- channels from various epithelia (63). DPC and NPPB can inhibit Na^+-K^+-$2Cl^-$ cotransporters with an IC_{50} of 100 and 30 μM respectively (64), but this may only be a

consequence of CFTR blockade since CFTR and Na^+-K^+-$2Cl^-$ are intimately linked (Fig 1.9).

Secondly, DPC has also been reported to inhibit important signal transduction pathways in epithelial cells. DPC inhibits forskolin-stimulated cAMP production by 28 % (65). Increases in intracellular cAMP activate CFTR Cl^- current, suggesting that DPC might inhibit CFTR function by two mechanisms; (1) binding to the channel pore and (2) lowering intracellular cAMP. However, lowering intracellular cAMP might also have profound effects on other MC functions such as mediator release (66,67). DPC also inhibits prostaglandin D_2 synthesis from arachidonic acid by inhibition of cyclooxygenase (68). Therefore, DPC appears to inhibit both eicosanoid and cAMP signal transduction pathways and data suggesting that DPC blocks MC mediator release must be interpreted with caution regarding the mechanism of action.

Glibenclamide, a sulfanomide, reduces the open probability of CFTR in a structure-dependent and concentration-dependent manner by direct interaction with the channel as recorded in cell-free membrane patches with an effective concentration of IC_{50} 2 to 30 μM (63). In MC, glibenclamide (30 μM) inhibited matrix metalloproteinase-9 (MMP-9) release (Chap 7) but did not affect (3 to 300 μM) β-hex release (Chap 5). Glibenclamide may not effect β-hex release because (1) glibenclamide activates/inhibits other channels and enzymes and these are involved in MC mediator release or (2) glibenclamide is unable to enter the cell and block the CFTR pore. Glibenclamide has a K_D for the sulfonylurea receptor, a member of the ABC transporter superfamily and has been shown to inhibit a variety of K^+ channels (69), numerous enzymes including PKA (70) and other Cl^- channels (63). Alternatively, it has been suggested that the glibenclamide binding site on K^+ channels resides on or near the cytoplasmic face of the channel (63). Recent evidence shows specific binding of [^{125}I]iodoglibenclamide to CFTR in a structure-dependent way showing that there is a similar binding site present on CFTR (63). If the glibenclamide binding site on CFTR is also cytoplasmic, then inhibition is dependent upon glibenclamide permeation through the plasma membrane. The inability of glibenclamide to modify mediator release from MC could be because the drug cannot enter the MC.

As outlined above, experimentation using pharmacologic inhibitors of Cl^- is complicated by the fact that these inhibitors are not specific to Cl^- channels and have the potential to modify a number of other ion channels. These inhibitors are often used in patch clamp experiments where they are often added to bath or pipette solutions directly and results are recorded instantaneously. However, measuring the effect of these inhibitors on mediator release from MC requires lengthy incubation in aqueous solution (30 min for β-hex release and 24 hr for MMP-9 release). Although the specificity and potency of DIDS, DPC and glibenclamide is unknown in these systems, they inhibit mediator release and suggest a role for ion channels in this process.

E. Role of other ion channels in mast cell functions

MC express a variety of other ion channels which likely play a role in MC functions such as mediator release. For example, MMC grown from bone-marrow, express two K^+ conductances that are absent in progenitor cells (13). However, upon ligation of the P2 receptor, a G coupled receptor able to potentiate mediator release (71), an outwardly rectifying K^+ conductance is initiated (13,72). However, K^+ current activation appears to be dependent on the type of stimuli, since FcεRI crosslinking increases the open probability of an inwardly rectifying K^+ channel that affects Ca^{2+} uptake in RBL cells and may also potentiate mediator release (73).

MC express a non-voltage activated Na^+ channel that undergoes voltage-dependent transitions (74). Although Ca^{2+} is the main cation used in intracellular signalling, changes in the cytoplasmic free Na^+ can also regulate several cellular processes. Increases in intracellular Na^+ levels have been implicated in cell volume regulation (75), modulation of excitability through changes in activities of the Na^+/Ca^{2+} exchanger, ATPases (76) and regulation of Na^+-activated K^+channels (77). Activation of cell-surface receptors in lymphocytes or vasopressin receptors in fibroblasts have been found to elevate intracellular Na^+ concentration (78) and in platelets, changes in intracellular Na^+ alter the affinity of α_2-adrenergic receptors for adrenaline (79). Voltage-gated Na^+ are rare in non-excitable cells and the precise role of non-voltage activated Na^+

conductance in MC is unknown. However, it is possible that FcεRI crosslinking elicits Na^+ flux, facilitating mediator release.

Our data suggests that MC mediator release is inhibited in Cl^- -reduced buffer (Chap 5). Although this decrease in mediator release may be mediated by Cl^- channels, it may also be the result of alkalinization of intracellular cytosol via the bicarbonate exchanger. The HCO_3^- -dependent transporter contributes to the regulation of pH_i , cell volume, cell growth and intracellular Na^+ (80). In the presence of Hepes/HCO_3^-, resting intracellular pH of PMC is 6.67 $\pm$ 0.015 and placing cells in a Cl^- -free medium (substituted with gluconate) results in a marked increase in intracellular pH to 6.80 $\pm$ 0.020 that is independent of Na^+ (81). The alkalinization in Cl^- -free medium is not affected by 50 μM DIDS, although the recovery of pH to physiological levels is completely blocked by DIDS (81). 48/80 or antigen-stimulated PMC release approximately 10 to 20 % less histamine in Hepes/HCO_3^- buffer than cells stimulated in HCO_3^- -free Hepes buffer yet this effect is not due to differences in intracellular pH (81). Therefore, the role of HCO_3^- in histamine release is not dependent on regulation of intracellular pH but is involved in some other process. These experiments also point out that the electrochemical equilibrium for Cl^- is into the cell and that the extrusion of HCO_3^- out of the cell by the cotransporter can be driven by the movement of Cl^- ions into the cell. This further supports our hypothesis that when MC Cl^- channels are opened, one would expect an influx of Cl^- ions as is seen with the fluorescent probe experiments and the $^{36}Cl^-$ influx studies in Chap 2.

II. The potential significance of mast cells in cystic fibrosis

A. Role of epithelial cells in CF

One of the major clinical manifestations of CF is excessive airway inflammation, suggesting that CFTR affects the function of airway epithelial cells in inflammatory responses (82-84). Defective CFTR expression in lung epithelial cells not only causes abnormal electrolyte secretion but may be responsible for abnormal bacterial clearance. The lung epithelium comprises a variety of cell types, including ciliated cells and goblet

cells. Ciliated epithelial cells are the major cell type and responsible for propelling the tracheobronchial secretions toward the pharynx (85) and defects in their function may cause abnormal bacterial clearance. Bacteria persist in the respiratory tract of CF patients due to decreased antimicrobial activity in airway surface fluid and impaired bacterial clearance due to mucus plugging (86). Cells with CFTR mutations have increased numbers of asialoglycolipid receptors for common bacterial pathogens (87). Human respiratory epithelial cells growing from CF nasal polyp are unable to inhibit *Pseudomonas aeruginosa* growth (88). However, when these cells are transfected with wild-type CFTR, bacterial killing is restored (88). Epithelial expression of IL-8 after bacterial stimulation is increased in cells with CFTR dysfunction, a finding demonstrated in both cell lines (89) and in CFTR-deficient mice (compared with normal control mice, 90). These findings suggest that there is an underlying immune dysfunction in CF that may be mediated by immune cells resident in the lung.

B. Potential role of mast cells in CF lung

Little data is available on MC number, morphology and function in CF. However, one small histological study suggests that nasal polyps from CF patients contain greater numbers of MC, endothelial cells, lymphocytes and plasma cells then from non-CF specimens (91). In a similar study of nasal polyps, a predominance of macrophages, T cells, B cells and moderate number of neutrophils were found in CF polyps (92). However, in the same study, polyps from non-CF patients also contained lymphocytes and eosinophils but there was no mention of MC numbers. Therefore, it is difficult to determine if MC are important in CF. To begin to address this lack of knowledge, a complete, systematic study of MC numbers, morphology and functions in CFTR knock-out mice would be valuable. Studies of MC in CF patients would also be valuable and should include histological characterization of MC numbers in the lung and their ability to release mediators upon stimulation with bacteria.

MC mediators regulate several epithelial cell functions (Fig 8.4). Histamine, for example, can reversibly and significantly depolarize the membrane voltage and increase the whole cell Cl^- conductance in a bronchial epithelial cell line that expresses high levels

of CFTR (93). Histamine effects are probably not mediated through general alternations in cell permeability since apically applied histamine has no effect on human bronchial epithelial cell permeability or tight junction integrity (94). These studies suggests that MC release of histamine activates epithelial Cl^- transport and abnormally low histamine release (in CF, for example) may result in low epithelial Cl^- transport. Histamine is also an important activator of goblet cell secretion (95) in the airway but does not effect goblet cells expressed in the pancreas (96). Abnormal histamine release from MC may ultimately effect goblet cell mucus secretion and affect mucociliary clearance in the lung. MC release of TNF could also modulate epithelial cell Cl^- currents. For example, TNF downregulates CFTR expression in lung epithelial cells and decreases Cl^- currents (97,98). Therefore, abnormal production of TNF could also create abnormal secretion from epithelial cells.

III. Implications of IFN-γ-mediated upregulation of CFTR expression

A. Increased CFTR expression may alter Cl^- current

One of the most surprising observations in this project is that IFN-γ upregulates CFTR expression in MC. This is opposite to the effect of IFN-γ on CFTR in epithelial cells, where IFN-γ downregulates CFTR expression (98). Both epithelial cells and MC are considered to be important regulators of the innate immune response in the lung. Western immunoblot and flow cytometry studies show that IFN-γ upregulates CFTR expression in MC. At first glance it may appear that increases in CFTR expression might result in increases in Cl^- secretion. In epithelial cells, IFN-γ downregulates CFTR expression and ^{36}Cl efflux and whole cell current studies indicate that CFTR function is impaired in IFN-γ treated cells (98). Using epithelial cells as a model, increases in MC CFTR might result in increases in Cl^- current.

However, level of CFTR expression does not always correlate with level of CFTR activity. For example, sodium 4-phenylbutyrate (PBA), a short-chain fatty acid that functions as an ammonia scavenger and is used to treat patients with urea cycle enzyme deficiency, increases CFTR expression in Calu-3 cells, but inhibits Cl^- secretion (99).

PBA also increases expression of Na^+-K^+-ATPase, which would be expected to stimulate, not inhibit, Cl^- secretion (99). The Na^+-K^+-ATPase contributes to the driving force for Cl^- secretion via CFTR in normal lung epithelial cells (Fig 1.10) and an increase in the activity of this cotransporter should increase the driving force for Cl^- secretion. However, the opposite is true since Ussing chamber studies reveal that PBA reduces apical Cl^- secretion (99). The mechanism for inhibition of Cl^- current by increased expression of CFTR is unknown, although other ion channels may be involved. Studies showing increased CFTR expression and reduced Cl^- flux show that expression of other transport proteins is also altered. For example, PBA treatment increases Na^+-K^+-ATPase and reduces Na^+-K^+-$2Cl^-$ expression (99). Although Na^+-K^+-$2Cl^-$ transporter contributes only nominally to cAMP-stimulated Cl^- secretion by epithelial cells, increased expression of the Na+-K+-ATPase may alter the Cl^- gradient and inhibit Cl^- flux.

In this project, IFN-γ-mediated increase of CFTR expression in MC inhibited Cl^- influx and mediator release. Experiments using Cl^- reduced buffers show that β-hex release from MC is inhibited in low [Cl^-] suggesting that Cl^- is necessary for maximal mediator release. Other studies have shown IgE activated MC uptake of Cl^- during mediator release. If Cl^- is necessary for MC mediator release, then IFN-γ upregulation of CFTR expression might decrease net Cl^- influx and inhibit MC mediator release.

B. Upregulation of CFTR may alter mast cell gene transcription

Several lines of evidence suggest that CFTR expression is required for normal gene transcription in epithelial cells. Comparison of endogenous activation of NF-κB in CFTR mutant cell lines and a cell line expressing a functional CFTR showed that cells containing the mutant form of CFTR display elevated activation of NF-κB and elevated IL-8 expression (89) (Fig 8.5). Northern blot analysis and ELISA analysis of bronchial gland cells isolated from CF patients shows increased IL-8 expression that is sensitive to dexamethasone and genistein (100,101). Exactly how CFTR dysfunction contributes to the activation and nuclear localization of NF-κB is unclear. However, there is some evidence suggesting that cell stress caused by accumulation of CFTR protein in the ER

(102) may induce increases in $[Ca^{2+}]_i$, stimulating NF-κB (89) or that there is an absence of inhibitor of κB (IκB) expression in mutant cells (101).

NF-κB is a transcription factor required for the expression of many genes important in inflammation. It is complexed in the cell cytoplasm with IκB-α and -β, which inactivates the transcription factor (103). However, in response to stimuli such as Fc receptor ligation, IκB proteins are selectively phosphorylated, ubiquitinated and degraded in the proteasome in a Ca^{2+}-dependent manner, releasing NF-κB to translocate the nuclear membrane and initiate the transcription of several pro-inflammatory cytokines (103). In MC, NF-κB activation is an important process in cytokine production. Coactivation of MC with IgE-antigen and lipopolysaccharide (LPS), for example, leads to activation in NF-κB and production of IL-9 (104). Therefore, aberrations in CFTR may also effect NF-κB-mediated production of pro-inflammatory cytokines by MC such as IL-9. CFTR regulation of NF-κB is probably a complex process, perhaps through indirect mechanisms. However, if the absence of functional CFTR activates endogenous NF-κB, then perhaps upregulation of CFTR by IFN-γ may inhibit endogenous NF-κB (Fig 8.5). Therefore, CFTR upregulation in MC could inhibit the production of several pro-inflammatory cytokines including IL-9.

C. Other possible IFN-γ effects on CFTR function

Although it appears that all IFN-γ effects on cell phenotype are mediated through changes in gene transcription via the JAK/STAT1 pathway, there is some evidence that some of the kinases involved in IFN-γ signalling may modulate ion transport without gene transcription. JAK2, for example, regulates prolactin-mediated Cl^- transport in mouse mammary epithelial cells through tyrosine phosphorylation of Na^+-K^+-$2Cl^-$ cotransporter (26). Prolactin is a lactogenic hormone that, much like IFN-γ, signals through the JAK/STAT pathway. Prolactin binding triggers dimerization of the receptor and activation of JAK2, which mediates phorphorylation of STAT5 (26). JAK2 also mediates phosphorylation of the Na^+-K^+-$2Cl^-$ cotransporter, increasing Cl^- transport (26). However, JAK2 does not phosphorylate CFTR expressed in these cells (26) suggesting that CFTR is incapable of JAK2 phosphorylation in this cell line.

IV. Implications for nitric oxide regulation of chloride channels in mast cells

Nitric oxide (NO) is an important messenger in cellular functions. IFN-γ upregulates expression of nitric oxide synthase (NOS) and several of the regulatory proteins that are involved in NO production (105). IFN-γ mediated upregulation of CFTR is independent of endogenous NO since LNMMA does not block IFN-γ action. However, some preliminary experiments suggest that RCMC do not express some isoforms of NOS and may not be capable of producing NO following IFN-γ treatment (unpublished).

However, exogenous NO (in the form of GSNO) upregulates CFTR expression. NO initiation of CFTR transcription may the result of nitrosylation of cysteine residues on transcription factors. NO has been found to inhibit the DNA binding activities of NF-κB (106), AP-1 (107) and c-Myb (108) via modification of their DNA-binding regions which contain a redox-regulated cysteine residue. NO can activate the transcription of genes such as TNF by inhibiting DNA binding of repressors of transcription (109). Therefore, GSNO may activate CFTR transcription indirectly, possibly via initiation of cytokines such as TNF that can activate the CFTR promoter. In epithelial cells, TNF and IFN-γ synergistically decrease CFTR expression and Cl^- current (97,98). If the opposite is true in MC (TNF upregulates CFTR expression like IFN-γ), then NO induction of TNF production may work in an autocrine fashion to upregulate CFTR expression.

A. Nitric oxide modulation of mast cell channel activity

Although many of the studies of MC ion channels have involved indirect pharmacological characterization or minute measurements of electronic current, there is a wealth of information suggesting that ion channels are integral to MC physiology and important targets in the modulation of MC function. Electrophysiological measurements indicate that MC in the resting state express currents indicative of inwardly rectifying and outwardly rectifying K^+ channels, several types of Ca^{2+} channels, GTP-activated Na^+ channels and Cl^- channels. (14,110,111). Previous studies have demonstrated that MC

produce NO when stimulated. Given that NO can be a potent and precise regulator of ion channels in most cell types, it is reasonable to assume that MC NO might regulate various MC ion channels.

Exogenous NO can activate the cystic fibrosis Cl^- channel or cystic fibrosis transmembrane conductance regulator (CFTR). Patch clamp recordings show that in epithelial cells and normal T cell clones, NO can activate a cGMP-dependent CFTR-mediated Cl^- current which is absent in cells derived from CF patients (112,113). NO may also regulate MC CFTR and ultimately MC mediator release since MC CFTR may regulate mediator release (16). Cl^- channel expression and presumably Cl^- flux is also necessary for NO production. Loss of CFTR activity by overexpressing its regulatory domain, reduces NOS mRNA expression and decreases overall NO production in epithelial cells (114).

In endothelial cells, NO production is triggered by an influx of Ca^{2+} but is independent of intracellular Ca^{2+} concentration (115). Release of Ca^{2+} from intracellular stores by histamine in the presence of an inhibitor of receptor-activated Ca^{2+} influx does not induce the production of NO (115). Thus, NO production appears to require influx of Ca^{2+} through Ca^{2+} channels. It has been shown that NOS resides in calveoli, which also contain calmodulin and various ion channels including Ca^{2+} and K^+ channels (116). When activated by an agonist, Ca^{2+} would open, facilitating the entry of extracellular Ca^{2+} and increasing the local concentration of Ca^{2+} on the internal side of the plasma membrane. Ca^{2+} ions would bind calmodulin and induce the formation of the NOS-Ca-calmodulin complex, thereby dissociating the enzyme from the plasma membrane and activating its catalytic activity.

B. Nitric oxide regulation of ion channel expression in MC

Very little data is available on MC ion channels and even less on NO modulation of MC ion channels. It is possible, however, the deduce a few key points from the data above. Firstly, NO regulation of MC ion channels is most likely mediated by alterations in SGC, which can have profound affects on channel conductance and electrophysical characteristics. Secondly, it is possible that NO directly oxidizes or nitrosylates key

amino acid residues on MC ion channels. Candidates for this type of regulation include various K^+ channels (including those expressed differentially throughout MC differentiation), Ca^{2+} and Cl^- channels whose pore contain thiol groups susceptible to NO nitrosylation. All of these events are most probably under the tight control of calmodulin which senses changes in intracellular Ca^{2+} and alters NOS activity accordingly.

V. Future Directions

This study has shown that MC express a variety of Cl^- channels some of which are regulated by IFN-γ, yet several questions remain. Are all of these Cl^- channels capable of conducting a Cl^- current? Currently, only CFTR has been shown to transduce a Cl^- current when activated with cAMP. To fully characterize the role of ClC in MC function, it will be necessary to (1) determine the expression of the different ClC members at the protein level, (2) determine if the ClC are functional Cl^- channels in rat MC and (3) determine the regulatory mechanisms that modulate the opening of these channels.

The role of Cl^- channels in MC functions such as mediator release remains poorly understood. Studies using pharmacological inhibitors suggests that DIDS-sensitive ion channels and DPC-sensitive channels (perhaps CFTR) might be necessary for maximal β-hex release from antigen and 48/80 stimulated MC. Preliminary experiments using antisense oligonucleotides to CFTR mRNA indicate that this approach inhibits CFTR expression and would be a more effective tool to study CFTR regulation of MC mediator release. CFTR knock-out mice might also offer insights into the role of CFTR in MC differentiation, proliferation, adhesion, cytokine production, degranulation and modulation of hypersensitivity disease. My preliminary results suggest that bone marrow-derived mast cells (BMMC) can be successfully grown from CFTR knock-out mouse bone marrow and that FcεR crosslinking induces β-hex release from these MC (Chap 7). Further experimentation may reveal differences in cytokine expression between CFTR knock-out BMMC and wild-type BMMC. The effect of IFN-γ on these cell populations may also be of interest.

The precise mechanisms of IFN-γ downregulation of MC release is still unknown. Although Cl^- channels might be important in this process, it is more likely that IFN-γ

modulates the expression of a number of genes and these alter MC response to antigen. These genes may include the subunits that comprise the FcεR, PKC or various molecules involved in granule fusion with the membrane.

V. Conclusion

It is clear that a number of distinct ion channels and tranporters are necessary for a variety of MC functions. We have shown that MC express several types of Cl^- channels and that IFN-γ modulates the expression of mRNA and protein of CFTR. Although it is possible that IFN-γ modulation of CFTR expression may be one of the mechanisms by which IFN-γ downregulates MC mediator release, further experiments will need to be performed using the antisense technology developed during the course of this study to address this posibility.

Table 8.1. Chloride channel expression in MC and their regulation by IFN-γ.

	$GABA_AR$ subunits	ClC	CFTR
RCMC	α (1-5), β (1 and 3) and γ (1-3)	2, 3, 4, 5 and 7	CFTR
IFN-γ-treated RCMC	upregulated expression of α1, α3 α4, α5, β1, β2, β3, γ1, γ2 and γ3	no change in expression	upregulated expression
PMC	α1	7	CFTR
IFN-γ-treated PMC	?	no change	upregulated expression
HMC-1	?	3 and 4/5	CFTR

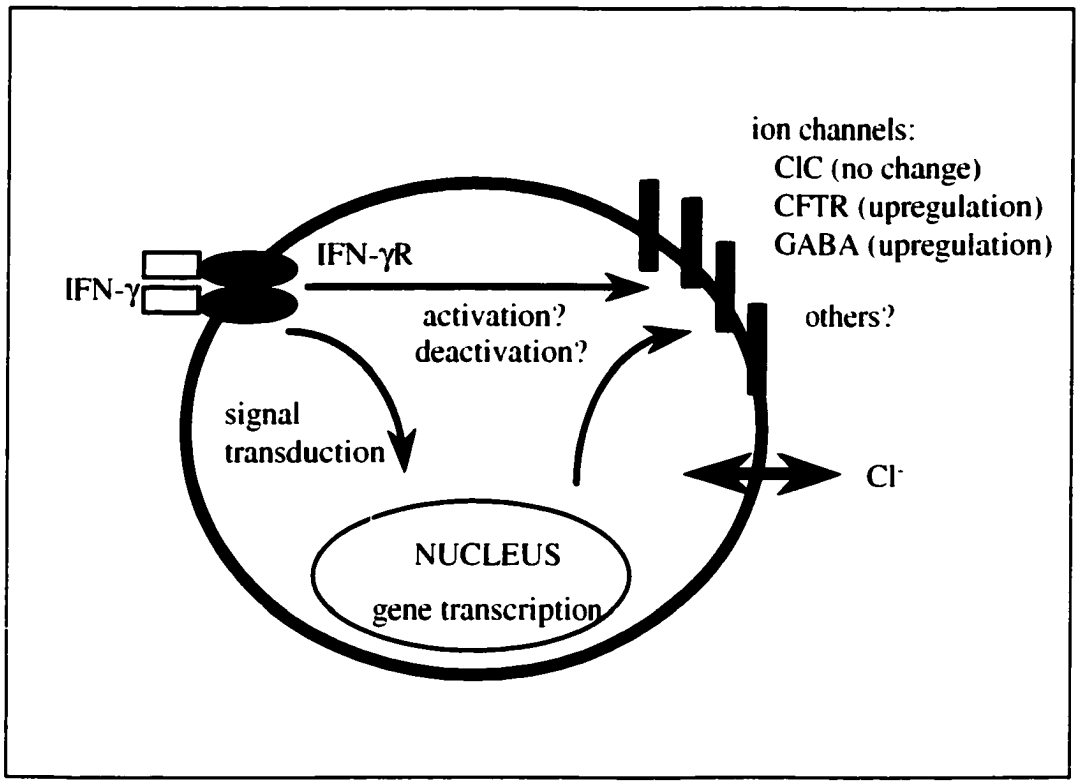


Figure 8.1. Conceptual model of IFN-γ effect on chloride channel expression and function in mast cells.

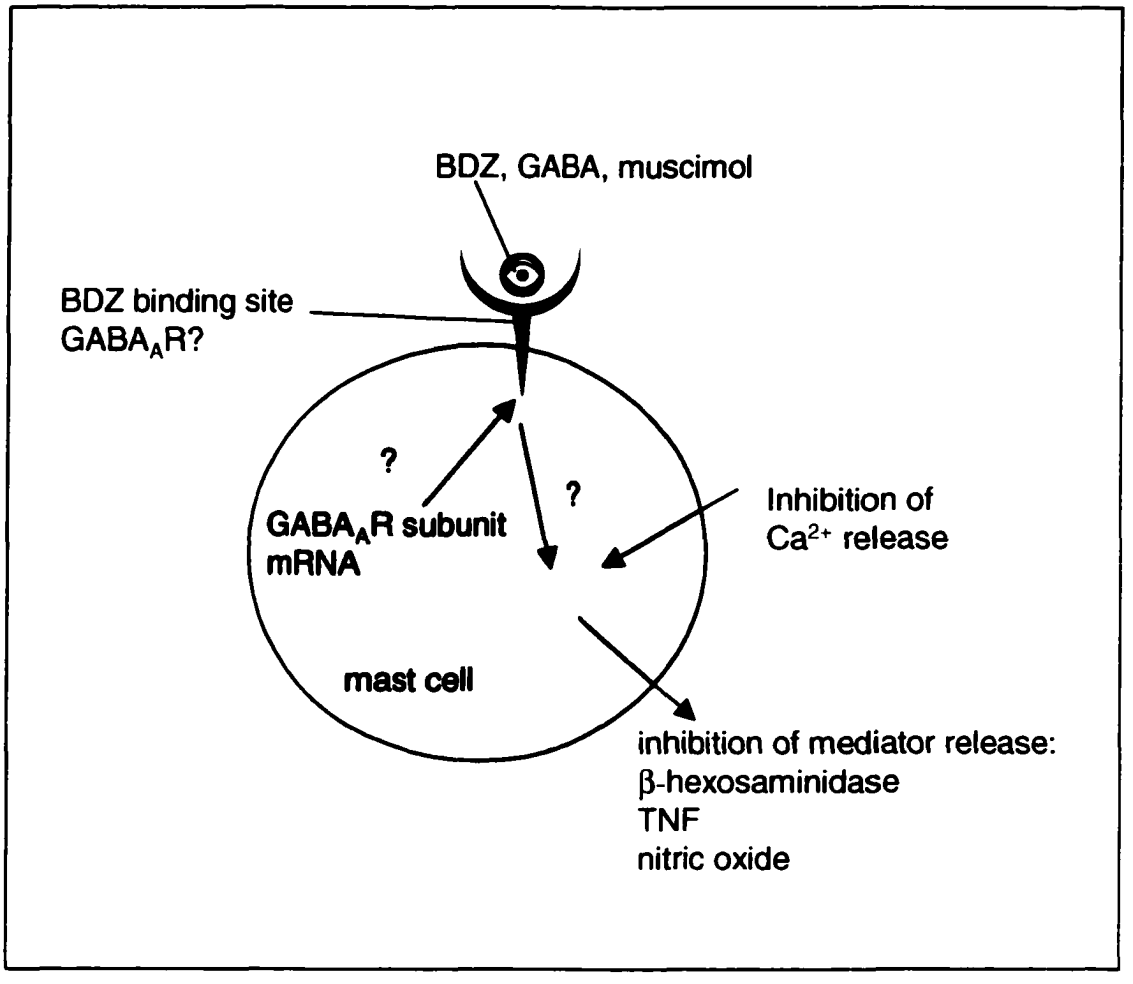


Figure 8.2. Model of benzodiazepine (BDZ), γ-aminobutyric acid (GABA) and muscimol binding to mast cells and their effects on mediator release. Mast cells express $GABA_AR$ subunit mRNA and express $GABA_AR$ α1protein. GABA, BDZ and muscimol may bind $GABA_AR$ or other sites and inhibit various mast cell functions including release of β-hexosaminidase, tumor necrosis factor (TNF) and nitric oxide.

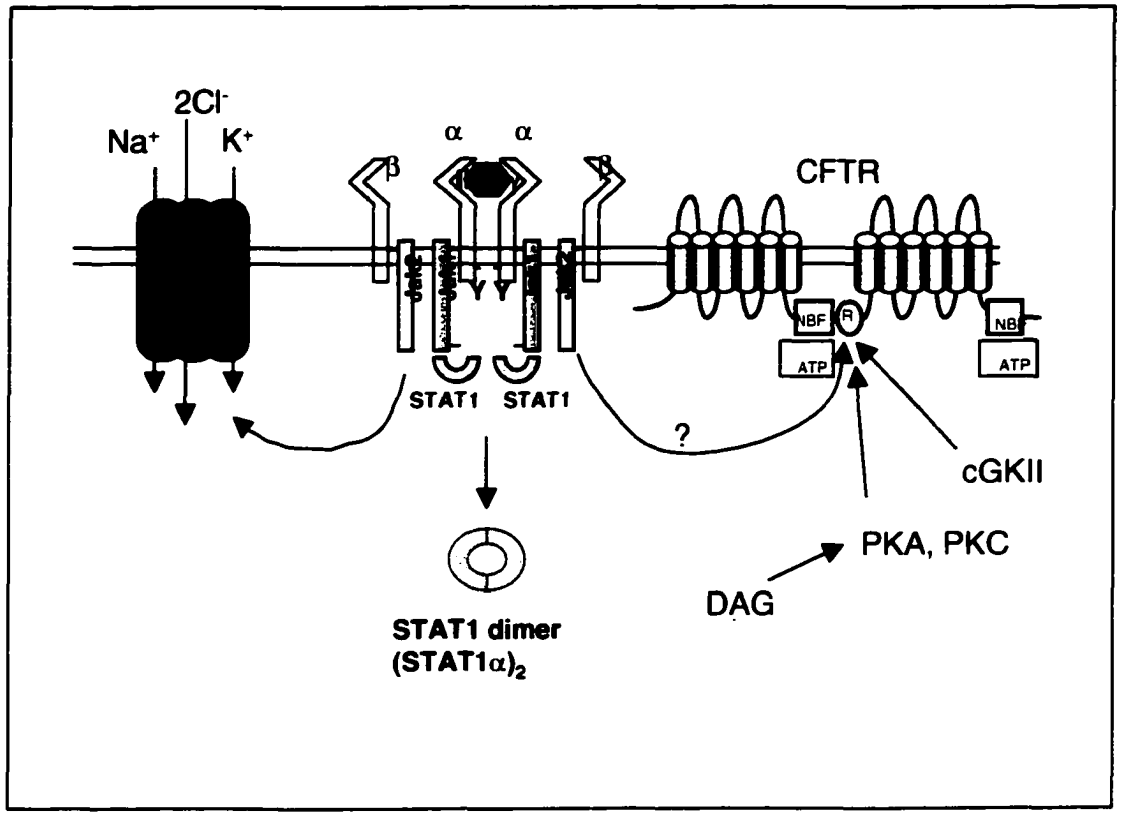


Figure 8.3. CFTR regulation by phosphorylation. IFN-γR ligation activates the Janus kinases (JAK). JAK2 activation in mammary epithelial cells regulates Na^+-K^+-$2Cl^-$ function via tyrosine phosphorylation. JAK activation may potentially phosphorylate CFTR. The regulator (R) domain contains consensus sequences for phosphorylation by protein kinase A (PKA) and by protein kinase C (PKC). PKC phosphorylation of CFTR is diacylglycerol-dependent but Ca^{2+}-independent and in intestinal epithelium, membrane targeting of cGMP-dependent protein kinase (cGK) type II induces CFTR Cl^- channel activity.

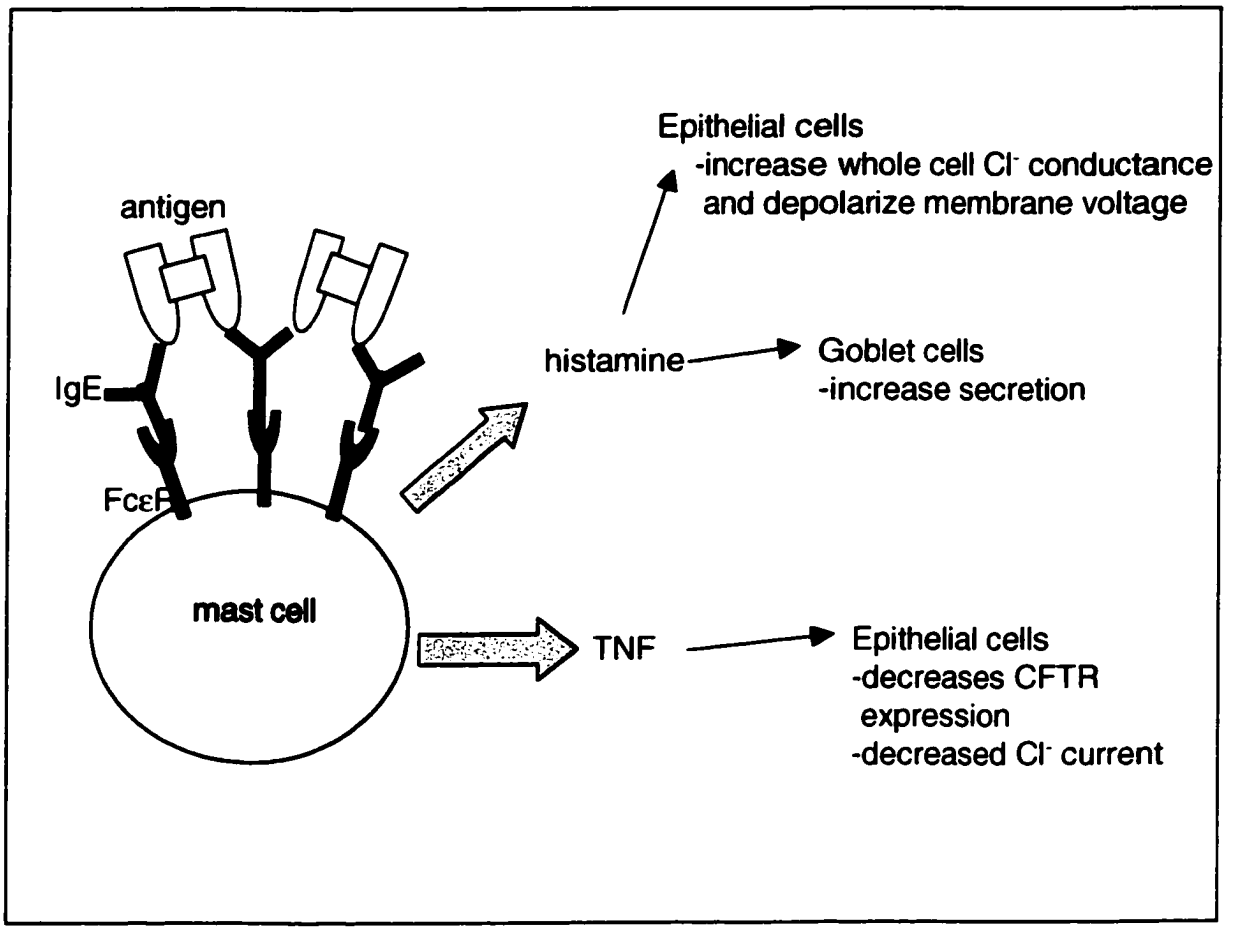


Figure 8.4. Model of mast cell functions in the lung and their effect on cells important in cystic fibrosis. Mast cells release histamine that can modulate epithelial cell cell voltage and Cl^- conductance. Mast cell tumor necrosis factor (TNF) can also downregulate CFTR expression in epithelial cells, resulting in decreases Cl^- currents.

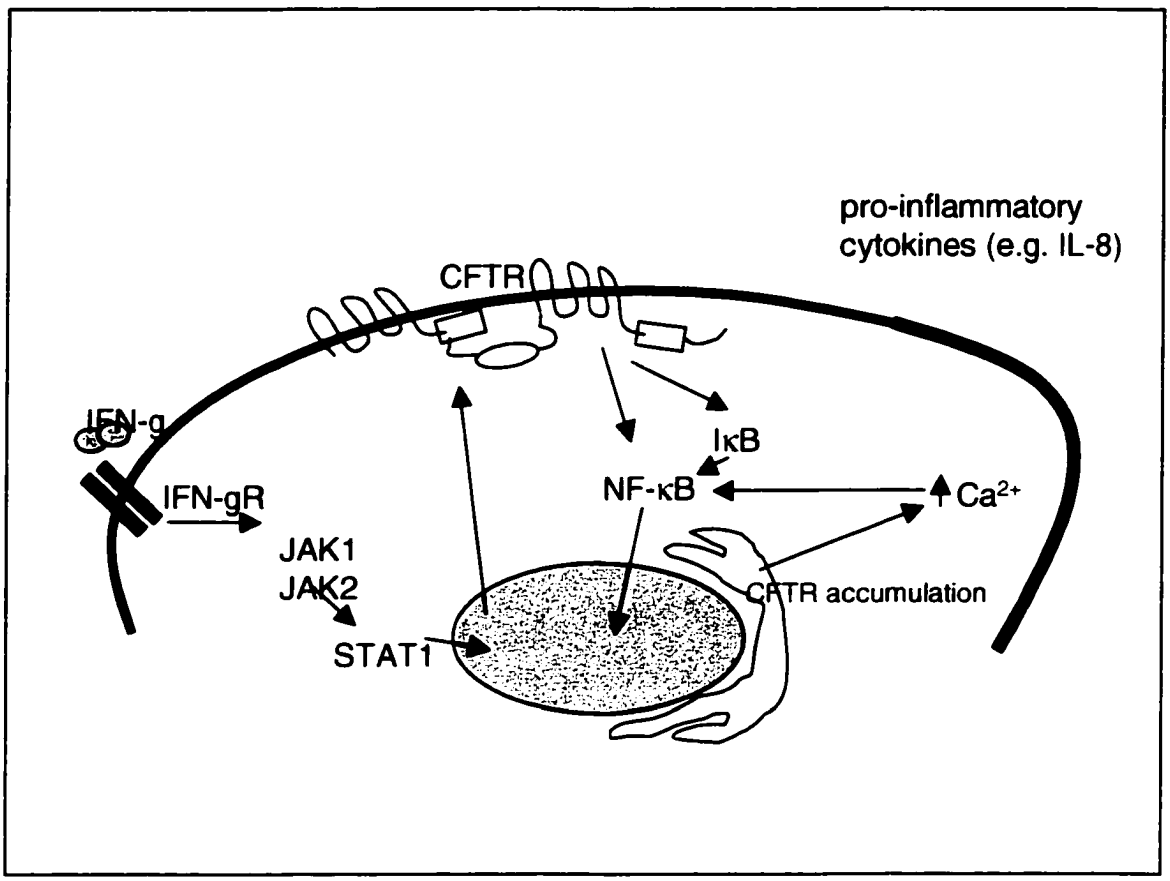


Figure 8.5. Possible mechanism of CFTR-mediated regulation of NF-κB gene transcription in mast cells. Exactly how CFTR dysfunction contributes to the activation and nuclear localization of NF-κB is unclear. However, there is some evidence suggesting that cell stress caused by accumulation of CFTR protein in the ER may induce increases in $[Ca^{2+}]_i$, stimulating NF-κB or that there is an absence of inhibitor of κB (IκB) expression in mutant cells.

References

1. Kaplan, A. P. 2001. Chemokines, chemokine receptors and allergy. *Int. Arch. Allergy Immunol. 124*:423.

2. Kirshenbaum, A. S., A. S. Worobec, T. A. Davis, J. P. Goff, T. Semere, and D. D. Metcalfe. 1998. Inhibition of human mast cell growth and differentiation by interferon gamma-1b. *Exp. Hematol. 26*:245.

3. Wills, F. L., M. Gilchrist, and A. D. Befus. 1999. Interferon-gamma regulates the interaction of RBL-2H3 cells with fibronectin through production of nitric oxide. *Immunology 97*:481.

4. MacDonald, A. J., J. Pick, E. Y. Bissonnette, and A. D. Befus. 1998. Rat mucosal mast cells: the cultured bone marrow-derived mast cell is biochemically and functionally analogous to its counterpart in vivo. *Immunology 93*:533.

5. Bissonnette, E. Y., B. Chin, and A. D. Befus. 1995. Interferons differentially regulate histamine and TNF-alpha in rat intestinal mucosal mast cells. *Immunology 86*:12.

6. Bissonnette, E. Y., J. A. Enciso, and A. D. Befus. 1995. Interferon and antiallergic drug regulation of histamine and tumor necrosis factor-alpha in rat mast cell subsets. *Int. Arch. Allergy Immunol. 107*:156.

7. Holliday, M. R., E. M. Banks, R. J. Dearman, I. Kimber, and J. W. Coleman. 1994. Interactions of IFN-gamma with IL-3 and IL-4 in the regulation of serotonin and arachidonate release from mouse peritoneal mast cells. *Immunology 82*:70.

8. Coleman, J. W., M. G. Buckley, M. R. Holliday, and A. G. Morris. 1991. Interferon-gamma inhibits serotonin release from mouse peritoneal mast cells. *Eur. J. Immunol. 21*:2559.

9. Nafziger, J., M. Arock, J. J. Guillosson, and J. Wietzerbin. 1990. Specific high-affinity receptors for interferon-gamma on mouse bone marrow-derived mast cells: inhibitory effect of interferon-gamma on mast cell precursors. *Eur. J. Immunol. 20*:113.

10. Frandji, P., C. Tkaczyk, C. Oskeritzian, J. Lapeyre, R. Peronet, B. David, J. G. Guillet, and S. Mecheri. 1995. Presentation of soluble antigens by mast cells: upregulation by interleukin-4 and granulocyte/macrophage colony-stimulating factor and downregulation by interferon-gamma. *Cell Immunol. 163*:37.

11. Poncet, P., M. Arock, and B. David. 1999. MHC class II-dependent activation of CD4+ T cell hybridomas by human mast cells through superantigen presentation. *J. Leukoc. Biol. 66*:105.

12. Okayama, Y., D. D. Hagaman, M. Woolhiser, and D. D. Metcalfe. 2001. Further characterization of FcgammaRII and FcgammaRIII expression by cultured human mast cells. *Int. Arch. Allergy Immunol. 124*:155.

13. McCloskey, M. A. and Y. X. Qian. 1994. Selective expression of potassium channels during mast cell differentiation. *J. Biol. Chem. 269*:14813.

14. Friis, U. G., T. Johansen, N. A. Hayes, and J. C. Foreman. 1994. IgE-receptor activated chloride uptake in relation to histamine secretion from rat mast cells. *Br. J. Pharmacol. 111*:1179.

15. Matthews, G., E. Neher, and R. Penner. 1989. Chloride conductance activated by external agonists and internal messengers in rat peritoneal mast cells. *J. Physiol. 418:131-44*.131.

16. Kulka, M., M. Gilchrist, M. Duszyk, and A. D. Befus. 2002. Expression and functional characterization of CFTR in mast cells. *J. Leukoc. Biol.* (In press)

17. Lau, H. Y. and S. P. Wan. 2000. Inhibition of compound 48/80 induced histamine release from mast cells by chloride channel blockers is affected by methods of drug preincubation. *Inflamm. Res. 49*:S21.

18. Redrup, A. C., J. C. Foreman, N. A. Hayes, and F. L. Pearce. 1997. Fc epsilon RI-mediated chloride uptake by rat mast cells: modulation by chloride transport inhibitors in relation to histamine secretion. *Br. J. Pharmacol. 122*:1188.

19. Romanin, C., M. Reinsprecht, I. Pecht, and H. Schindler. 1991. Immunologically activated chloride channels involved in degranulation of rat mucosal mast cells. *EMBO J.10*:3603.

20. Perretti, M., S. Nuti, and L. Parente. 1989. Investigation of rat mast cell degranulation using flow cytometry. *J. Pharmacol. Methods 23*:187.

21. Duffy, S. M., M. L. Leyland, E. C. Conley, and P. Bradding. 2001. Voltage-dependent and calcium-activated ion channels in the human mast cell line HMC-1. J.*Leukoc. Biol. 70*:233.

22. Kardos, J. 1999. Recent advances in GABA research. *Neurochem. Int. 34*:353.

23. Miller, L. G., A. Lee-Paritz, D. J. Greenblatt, and T. C. Theoharides. 1988. High-affinity benzodiazepine binding sites on rat peritoneal mast cells and RBL-1 cells: binding characteristics and effects on granule secretion. *Pharmacology 36*:52.

24. Bidri, M., B. Royer, G. Averlant, G. Bismuth, J. J. Guillosson, and M. Arock. 1999. Inhibition of mouse mast cell proliferation and proinflammatory mediator release by benzodiazepines. *Immunopharmacology 43*:75.

25. Suzuki-Nishimura, T., T. Sano, and M. K. Uchida. 1989. Effects of benzodiazepines on serotonin release from rat mast cells. *Eur J.Pharmacol. 167*:75.

26. Selvaraj, N. G., E. Omi, G. Gibori, and M. Rao. 2000. Janus kinase 2 (JAK2) regulates prolactin-mediated chloride transport in mouse mammary epithelial cells through tyrosine phosphorylation of Na^+-K^+-$2Cl^-$. *Mol. Endocrin. 14*:2054.

27. Gadsby, D. C. and A. C. Nairn. 1999. Control of CFTR channel gating by phosphorylation and nucleotide hydrolysis. *Physiol. Rev. 79*:S77.

28. Liedtke, C. M. and T. S. Cole. 1998. Antisense oligonucleotide to PKC-ϵ alters cAMP-dependent stimulation of CFTR in Calu-3 cells. *Am. J. Physiol. 275*:C1357.

29. Park, K., T. Begenisich, and J. E. Melvin. 2001. Protein kinase A activation phosphorylates the rat ClC-2 Cl^- channel but does not change activity. *J. Membr. Biol. 182*:31.

30. Gyenes, M., Q. Wang, T. T. Gibbs, and D. H. Farb. 1994. Phosphorylation factors control neurotransmitter and neuromodulator actions at the gamma-aminobutyric acid type A receptor. *Mol. Pharmacol. 46*:542.

31. Leidenheimer, N. J., S. J. McQuilkin, L. D. Hahner, P. Whiting, and R. A. Harris. 1992. Activation of protein kinase C selectivity inhibits the gamma-aminobutyric $acid_A$ receptor, role of desensitization. *Mol. Pharmacol. 41*:1116.

32. Leidenheimer, N. J., T. K. Machu, S. Endo, R. W. Olsen, R. A. Harris, and M. D. Browning. 1991. Cyclic cAMP-dependent protein kinase decreases gamma-aminobutyric $acid_A$ receptor-mediated $^{36}Cl^-$ uptake by brain microsacs. *J. Neurochem. 57*:722.

33. Tian, J., C. Chau, T. G. Hales, and D. L. Kaufman. 1999. GABA(A) receptors mediate inhibition of T cell responses. *J. Neuroimmunol. 96*:21.

34. Bergeret, M., M. Khrestchatisky, E. Tremblay, A. Bernard, A. Gregoire, and C. Chany. 1998. GABA modulates cytotoxicity of immunocompetent cells expressing GABAA receptor subunits. *Biomed. Pharmacother. 52*:214.

35. Gavish, M., I. Bachman, R. Shoukrun, Y. Katz, L. Veenman, G. Weisinger, and A. Weizman. 1999. Enigma of the peripheral benzodiazepine receptor. *Pharmacol. Rev. 51*:629.

36. Rocca, P., A. M. Beoni, C. Eva, P. Ferrero, E. Zanalda, and L. Ravizza. 1998. Peripheral benzodiazepine receptor messenger RNA is decreased in lymphocytes of generalized anxiety disorder patients. *Biol. Psychiatry 43*:767.

37. Rocca, P., A. M. Beoni, C. Eva, P. Ferrero, G. Maina, F. Bogetto, and L. Ravizza. 2000. Lymphocyte peripheral benzodiazepine receptor mRNA decreases in obsessive-compulsive disorder. *Eur Neuropsychopharmacol. 10*:337.

38. Maeda, S., T. Miyawaki, T. Nakanishi, M. Takigawa, and M. Shimada. 1998. Peripheral type benzodiazepine receptor in T lymphocyte rich preparation. *Life Sci. 63*:1423.

39. Pavese, N., G. Giannaccini, L. Betti, S. Ferrari, E. Bonanni, U. Bonuccelli, L. Murri, and A. Lucacchini. 2000. Peripheral-type benzodiazepine receptors in human blood cells of patients affected by migraine without aura. *Neurochem. Int. 37*:363.

40. Cahard, D., X. Canat, P. Carayon, C. Roque, P. Casellas, and G. Le Fur. 1994. Subcellular localization of peripheral benzodiazepine receptors on human leukocytes. *Lab. Invest. 70*:23.

41. Alexander, B. E., E. Roller, and U. Klotz. 1992. Characterization of peripheral-type benzodiazepine binding sites on human lymphocytes and lymphoma cell lines and their role in cell growth. *Biochem. Pharmacol. 44*:269.

42. Caldiroli, E., F. Marino, M. Cosentino, F. De Ponti, A. M. Fietta, A. Mazzone, A. Zibetti, S. Lecchini, and G. M. Frigo. 1998. Peripheral benzodiazepine receptor expression on leukocytes and neutrophil function during anticonvulsant monotherapy. *Pharmacology 57*:215.

43. Fruscella, P., M. Sottocorno, M. Di Braccio, L. Diomede, N. Piccardi, A. Cagnotto, G. Grossi, M. Romano, T. Mennini, and G. Roma. 2001. 1,5-Benzodiazepine tricyclic derivatives exerting anti-inflammatory effects in mice by inhibiting interleukin-6 and prostaglandinE(2) production. *Pharmacol. Res. 43*:445.

44. Torres, S. R., T. S. Frode, G. M. Nardi, N. Vita, R. Reeb, P. Ferrara, R. M. Ribeiro-do-Valle, and R. C. Farges. 2000. Anti-inflammatory effects of peripheral benzodiazepine receptor ligands in two mouse models of inflammation. *Eur J. Pharmacol. 408*:199.

45. Taupin, V., J. Gogusev, B. Descamps-Latscha, and F. Zavala. 1993. Modulation of tumor necrosis factor-alpha, interleukin-1 beta, interleukin-6, interleukin-8, and granulocyte/macrophage colony-stimulating factor expression in human monocytes by an endogenous anxiogenic benzodiazepine ligand, triakontatetraneuropeptide: evidence for a role of prostaglandins. *Mol. Pharmacol. 43*:64.

46. Michaloudi, H. C. and G. C. Papadopoulos. 1999. Mast cells in the sheep, hedgehog and rat forebrain. *J. Anat. 195*:577.

47. Zhuang, X., A. J. Silverman, and R. Silver. 1999. Distribution and local differentiation of mast cells in the parenchyma of the forebrain. *J. Comp. Neurol. 408*:477.

48. Zhuang, X., A. J. Silverman, and R. Silver. 1997. Mast cell number and maturation in the central nervous system: influence of tissue type, location and exposure to steroid hormones. *Neuroscience 80*:1237.

49. Dimitriadou, V., A. Rouleau, M. D. Trung Tuong, G. J. Newlands, H. R. Miller, G. Luffau, J. C. Schwartz, and M. Garbarg. 1997. Functional relationships between sensory nerve fibers and mast cells of dura mater in normal and inflammatory conditions. *Neuroscience 77*:829.

50. Hough, L. B., S. Jackowski, N. Eberle, K. R. Gogas, N. A. Camarota, and D. Cue. 1988. Actions of the brain-penetrating H2-antagonist zolantidine on histamine dynamics and metabolism in rat brain. *Biochem. Pharmacol. 37*:4707.

51. Roznieckl, J. J., R. Letourneau, M. Sugiultzoglu, C. Spanos, J. Gorbach, and T. C. Theoharides. 1999. Differential effect of histamine 3 receptor-active agents on brain, but not peritoneal, mast cell activation. *J. Pharmacol. Exp. Ther. 290*:1427.

52. Nonogaki, K., X. Li, K. Ozawa, H. Miura, G. Watanabe, Y. Hiyoshi, T. Tamagawa, N. Sakamoto, and A. Iguchi. 1993. Activation of GABAA receptor in the brain suppresses neostigmine or histamine-induced central nervous system-mediated hyperglycemia. *Life Sci. 53*:PL159.

53. Crawford, M. L. and J. M. Young. 1988. GABAB receptor-mediated inhibition of histamine H1-receptor-induced inositol phosphate formation in slices of rat cerebral cortex. *J. Neurochem. 51*:1441.

54. Prell, G. D., A. M. Morrishow, E. Duoyon, and W. S. Lee. 1997. Inhibitors of histamine methylation in brain promote formation of imidazoleacetic acid, which interacts with GABA receptors. *J. Neurochem. 68*:142.

55. Maddy, A. H. A. 1964. A fluorescent label for the outer components of the plasma membrane. *Biochem. Biophys. Acta 88*:390.

56. Racker, E. 1985. *Transporters of plasma membranes*. Academic, Orlando,FL,

57. Smith, P. L., S. K. Sullivan, and R. D. McCabe. 1986. Concentration-dependent effects of disulfonic stilbenes on colonic chloride transport. *Am. J. Physiol. 250*:G44.

58. Singh, M., M. Krouse, S. Moon, and J. J. Wine. 1997. Most basal I(SC) in Calu-3 human airway cells is bicarbonate-dependent Cl- secretion. *Am. J.Physiol. 272*:L690.

59. Alper, S. L. 1991. The band 3-related anion exchanger (AE) gene family. *Annu. Rev. Physiol. 53*:549.

60. Shen, Z., J. Liu, D. C. Marcus, N. Shiga, and P. Wangemann. 1995. DIDS increases K+ secretion through an IsK channel in apical membrane of vestibular dark cell epithelium of gerbil. *J. Membr. Biol. 146*:283.

61. Hogg, R. C., Q. Wang, and W. A. Large. 1994. Effects of Cl channel blockers on Ca-activated chloride and potassium currents in smooth muscle cells from rabbit portal vein. *Br. J. Pharmacol. 111*:1333.

62. Schwiebert, E. M., D. J. Benos, M. E. Egan, M. J. Stutts, and W. B. Guggino. 1999. CFTR is a conductance regulator as well as a chloride channel. *Physiol. Rev. 79*:S145.

63. Schultz, B. D., A. K. Singh, D. C. Devor, and R. J. Bridges. 1999. Pharmacology of CFTR chloride channel activity. *Physiol. Rev. 79*:S109.

64. Wangemann, P. M., M. Wittner, A. Di Stefano, H. C. Englert, H. J. Lang, E. Schlatter, and R. Greger. 1986. Cl^- channel blockers in the thick ascending limb of the loop of Henle. Structure activity relationship. *Pflugers Arch. 407*:S128.

65. Heisler, S. 1991. Antagonists of epithelial chloride channels inhibit cAMP synthesis. *Can. J. Physiol. Pharmacol. 69*:501.

66. Sullivan, T. J., K. L. Parker, S. A. Eisen, and C. W. Parker. 1975. Modulation of cyclic AMP in purified rat mast cells. II. Studies on the relationship between intracellular cyclic AMP concentrations and histamine release. *J. Immunol. 114*:1480.

67. Bidri, M., P. A. Becherel, L. Le Goff, L. Pieroni, J. J. Guillosson, P. Debre, and M. Arock. 1995. Involvement of cyclic nucleotides in the immunomodulatory effects of nitric oxide on murine mast cells. *Biochem. Biophys. Res. Commun. 210*:507.

68. Stutts, M. J., D. C. Henke, and R. C. Boucher. 2001. Diphenylamine-2-carboxylate (DPC) inhibits both Cl^- conductance and cyclooxygenase of canine tracheal epithelium. *Pflugers Arch. 415*:611.

69. Edwards, G. and A. H. Weston. 1993. Induction of a glibenclamide-sensitive K^+ current by modification of a delayed recitifier channel in rat portal vien and insulinoma cells. *Br. J. Pharmacol. 110*:1280.

70. Caro, J. F. 1990. Effects of glyburide on carbohydrate metabolism and insulin action in the liver. *Am. J. Med. 89*:51S.

71. McCloskey, M. A. and M. D. Cahalan. 1990. G protein control of potassium channel activity in a mast cell line. *J. Gen. Physiol. 95*:205.

72. Qian, Y. X. and M. A. McCloskey. 1993. Activation of mast cell K+ channels through multiple G protein-linked receptors. *Proc. Natl. Acad. Sci. U. S. A. 90*:7844.

73. Gericke, M., O. Dar, G. Droogmans, I. Pecht, and B. Nilius. 1995. Immunological stimulation of single rat basophilic leukemia RBL-2H3 cells co-activates Ca(2+)-entry and K(+)-channels. *Cell Calcium 17*:71.

74. Parekh, A. B. 1998. Voltage-dependent conductance changes in a nonvoltage-activated sodium current from a mast cell line. *J. Membr. Biol. 165*:145.

75. Kaplan, M. R., D. B. Mount, and E. Delpire. 1996. Molecular mechanisms of NaCl cotransport. *Annu. Rev. Physiol. 58*:649.

76. Hancox, J. C. and A. J. Levi. 1995. Na-Ca exchange tail current indicates voltage dependence of the Cai transient in rabbit ventricular myocytes. *J. Cardiovasc. Electrophysiol. 6*:455.

77. Kameyama, M., M. Kakei, R. Sato, T. Shibasaki, H. Matsuda, and H. Irisawa. 1984. Intracellular Na^+ activates a K^+ channel in mammalian cardiac cells. *Nature 309*:354.

78. Harootunian, A. T., J. P. Kao, B. K. Eckert, and R. Y. Tsien. 1989. Fluorescence ratio imaging of cytosolic free Na^+ in individual fibroblasts and lymphocytes. *J. Biol. Chem. 264*:19458.

79. Motulsky, H. J. and P. A. Insel. 1983. Influence of sodium on the alpha2-adrenergic receptor system of human platelets. Role for intraplatelet sodium in receptor binding. *J. Biol. Chem. 258*:3913.

80. Redron, J. and D. Batlle. 1994. Regulation of intracellular pH in the spontaneously hypertensive rat. Role of bicarbonate-dependent transporters. *Hypertension 23*:503.

81. Jensen, T. B., U. G. Friis, and T. Johansen. 1998. Role of physiological HCO3-buffer on intracellular pH and histamine release in rat peritoneal mast cells. *Pflugers Arch. 436*:357.

82. Khan, T. Z., M. M. K. Gillespie, P. E. Parson, J. S. Waegener, D. W. H. Riches, and F. J. Accurso. 1995. Airway inflammation in infants with cystic fibrosis can occur in the absence of detectable endotoxin in bronchoalveolar lavage fluid. *Am. J. Respir. Crit. Care Med. 151*:1075.

83. Konstan, M. W., K. A. Hilliard, T. M. Norvell, and M. Berger. 1994. Bronchoalveolar lavage findings in cystic fibrosis patients with stable, clinically mild lung disease suggest ongoing infection and inflammation. *Am. J. Respir. Crit. Care Med. 150*:448.

84. Muhlebach, M. S., P. W. Stewart, M. W. Leigh, and T. L. Noah. 1999. Quantification of inflammatory responses to bacteria in young cystic fibrosis and control patients. *Am. J. Respir. Crit. Care Med. 160*:638.

85. Renauld, J. C. 2001. New insights into the role of cytokines in asthma. *J. Clin. Pathol. 54*:577.

86. Smith, J. J., S. M. Travis, E. P. Greenberg, and M. J. Welsh. 1996. Cystic fibrosis airway epithelia fail to kill bacteria becase of abnormal airway surface fluid. *Cell 85*:229.

87. Saiman, L. and A. Prince. 1993. *P. aeruginosa* pili bind to asialoGM1 which is increased on the surface of cystic fibrosis epithelial cells. *J. Clin. Invest. 92*:1875.

88. Biffi, A., G. Sersale, A. Cassetti, A. Villa, C. Bordignon, B. M. Assael, and M. Conese. 1999. Restoration of bacterial killing activity of human respiratory cystic fibrosis cells through cationic vector-mediated cystic fibrosis transmembrane conductance regulator gene transfer. *Hum. Gene Ther. 10*:1923.

89. Weber, A. J., G. Soong, R. Bryan, S. Saba, and A. Prince. 2001. Activation of NF-kB in airway epithelial cells is dependent on CFTR trafficking and Cl^- channel function. *Am. J. Physiol. Lung Cell Mol. Physiol. 281*:L71.

90. van Heeckeren, A., R. Walenga, M. W. Konstan, T. Bonfield, P. B. Davis, and T. Ferkol. 1997. Excessive inflammatory response of cystic fibrosis mice to bronchopulmonary infection with *Pseudomonas aeruginosa*. *J. Clin. Invest. 100*:2810.

91. Henderson, W. R. and E. Y. Chi. 1992. Degranulation of cystic fibrosis nasal polyp mast cells. *J. Pathol. 166*:395.

92. Lesprit, E., E. Escudier, G. Roger, V. Pruliere, G. Lenoir, P. Reinert, and A. Coste. 2000. Characterization of inflammatory reaction in upper airways of cystic fibrosis patients. *Histol. Histopathol 15*:395.

93. Kunzelmann, K., T. Koslowsky, T. Hug, D. C. Gruenert, and R. Greger. 1994. cAMP-dependent activation of ion conductances in bronchial epithelial cells. *Pflugers Arch. 428*:590.

94. Devalia, J. L., R. W. Godfrey, R. J. Sapsford, N. J. Severs, P. K. Jeffery, and R. J. Davies. 1994. No effect of histamine on human bronchial epithelial cell permeability and tight junctional integrity in vitro. *Eur Respir. J. 7*:1958.

95. Tamaoki, J., J. Nakata, K. Takeyama, A. Chiyotani, and K. Konno. 1997. Histamine H2 receptor-mediated airway goblet cell secretion and its modulation by histamine-degrading enzymes. *J. Allergy Clin. Immunol 99*:233.

96. Hootman, S. R. and J. de Ondarza. 1995. Regulation of goblet cell degranulation in isolated pancreatic ducts. *Am. J. Physiol. 268*:G24.

97. Fish, S. M., R. Proujansky, and W. W. Reenstra. 1999. Synergistic effects of interferon gamma and tumour necrosis factor alpha on T84 cell function. *Gut 45*:191.

98. Besancon, F., G. Przewlocki, I. Baro, A. S. Hongre, D. Escande, and A. Edelman. 1994. Interferon-gamma downregulates CFTR gene expression in epithelial cells. *Am. J. Physiol. 267*:C1398.

99. Loffing, J., B. D. Moyer, D. Reynolds, and B. A. Stanton. 1999. PBA increases CFTR expression but at high doses inhibits Cl(-) secretion in Calu-3 airway epithelial cells. *Am. J. Physiol. 277*:L700.

100. Tabary, O., J. M. Zahm, J. Hinnrasky, J. P. Couetil, P. Cornillet, M. Guenounou, D. Gaillard, E. Puchelle, and J. Jacquot. 1998. Selective up-regulation of chemokine IL-8 expression in cystic fibrosis gland cells *in vivo* and *in vitro*. *Am. J. Pathol. 153*:921.

101. Tabary, O., S. Escotte, J. P. Couetil, D. Hubert, D. Dusser, E. Puchelle, and J. Jacquot. 1999. Genistein inhibits constitutive and inducible NFkappaB activation and decreases IL-8 production by human cystic fibrosis bronchial gland cells. *Am. J. Pathol. 155*:473.

102. Cheng, S. H., R. J. Gregory, J. Marshall, S. Paul, D. W. Souza, G. A. White, C. R. O'Riordan, and A. E. Smith. 1990. Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell 63*:827.

103. Baeuerle, P. A. and D. Baltimore. 1995. NF-kB: ten years after. *Cell 87*:13.

104. Stassen, M., C. Muller, M. Arnold, L. Hultner, S. Klein-Hessling, C. Neudorfl, T. Reineke, E. Serfling, and E. Schmitt. 2001. IL-9 and IL-13 production by activated mast cells is strongly enhanced in the presence of lipopolysaccharide: NF-kappa B is decisively involved in the expression of IL-9. *J. Immunol. 166*:4391.

105. Gilchrist, M., M. Savoie, N. S. Hirji, O. Nohara, F. L. Wills, J. L. Wallace, and A. D. Befus. 2002. Nitric oxide synthase and nitric oxide production in in-vivo derived mast cells. *J. Leukoc. Biol.* (In press)

106. Moormann, A. M., R. J. Koenig, and S. R. Meshnik. 1996. Effects of hydrogen peroxide, nitric oxide and antioxidants on NF-kB. *Redox. Rep. 2*:249.

107. Nikitovic, D., A. Holmgren, and G. Spyrou. 1998. Inhibition of AP-1 DNA binding by nitric oxide involving conserved cysteine residues in Jun and Fox. *Biochem. Biophys. Res. Commun. 242*:109.

108. Brendeford, E. M., K. B. Andersson, and O. S. Gabrielsen. 1998. Nitric oxide (NO) disrupts DNA binding of the transcription factor c-Myb in vitro. *FEBS Lett. 425*:52.

109. Wang, S., W. Wang, R. A. Wesley, and R. L. Danner. 1999. A Sp1 binding site of the tumor necrosis factor α promoter functions as a nitric oxide response element. *J. Biol. Chem. 274*:33190.

110. Hill, P. B., R. J. Martin, and H. R. Miller. 1996. Characterization of whole-cell currents in mucosal and connective tissue rat mast cells using amphotericin-B-perforated patches and temperature control. *Pflugers Arch. 432*:986.

111. Meyer, G., S. Doppierio, P. Vallin, and L. Daffonchio. 1996. Effect of frusemide on Cl- channel in rat peritoneal mast cells. *Eur. Respir. J. 9*:2461.

112. Dong, Y. J., A. C. Chao, K. Kouyama, Y. P. Hsu, R. C. Bocian, R. B. Moss, and P. Gardner. 1995. Activation of CFTR chloride current by nitric oxide in human T lymphocytes. *EMBO J. 14*:2700.

113. Thethi, K., P. Jurasz, A. J. MacDonald, A. D. Befus, S. F. Man, and M. Duszyk. 1997. Determination of cell surface charge by photometric titration. *J. Biochem. Biophys. Methods 34*:137.

114. Jilling, T., I. Y. Haddad, S. H. Cheng, and S. Matalon. 1999. Nitric oxide inhibits heterologous CFTR expression in polarized epithelial cells. *Am. J. Physiol. 277*:L89.

115. Jin, J. S., D. W. Wilde, R. C. Webb, and L. G. D'Alecy. 1996. Calcium channel activity increased by plasma from ischemic hindlimbs of rats: role of an endogenous NO synthase inhibitor. *Am. J. Physiol. 270*:H1484.

116. Martens, J. R., R. Navarro-Polanco, E. A. Coppock, A. Nishiyama, L. Parshley, T. D. Grobaski, and M. M. Tamkun. 2000. Differential targeting of Shaker-like potassium channels to lipid rafts. *J. Biol. Chem. 275*:7443.

Appendix A

I. Calculating $[Cl^-]_i$ using fluorescent indicators for chloride

N-substituted quinoline compounds such as MQAE and SPQ have been used to measure Cl^- flux and measure $[Cl]_i$ by measuring the quenching of fluorescence emission. Physiological pH levels and the concentration of non-chloride ions do not significantly affect the fluorescence of SPQ (1) and SPQ is not cytotoxic even when used in high concentration (20 mM) (2). SPQ is water soluble and therefore is loaded into cells by a hypotonic method (3). SPQ, however, has a half-life of only 8 to 9 minutes at 37 to 38°C and quenching of SPQ fluorescence by Cl^- is less effective inside cells (K_{sv}=18 M^{-1}) than in aqueous solution (K_{sv}=118 M^{-1}) (4).

MQAE is much more sensitive to intracellular Cl^- than SPQ (K_{sv}=64 M^{-1}), making it about five times more sensitive (4). MQAE is also insensitive to changes in pH or bicarbonate, borate, nitrate or sulfate anions, although quenching by $H_2PO_4^-$ and carboxylates has been reported (4,5). The ester group of MQAE may slowly hydrolyse inside cells, resulting in a change in its fluorescence response (6). In addition, MQAE undergoes slow leakage from liposomes and cells (7).

The relationship between fluorescence intensity of the chloride indicator and chloride concentration is given by the Stern-Volmer equation:

$$(F_o/F) - 1 = K_{sv}\,[Cl]_i$$

where F_o is the fluorescence intensity without halide or other quenching ions, F is the fluorescence intensity in the presence of quencher, $[Cl]_i$ is the concentration of intracellular Cl^- and K_{sv} is the Stern-Volmer constant.

II. Calculating $^{36}Cl^-$ influx

Measurement of the Cl^- influx in unstimulated peritoneal mast cells was performed using $Na^{36}Cl$ (ICN; stock = 1.07 M, 0.867 mCi/mL or 12.0 mCi/g). Stock ^{36}Cl was diluted 1/123 to give a final concentration of 8.7 mM in flux buffer (137 mM NaCl, 4 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 20 mM HEPES, 1 mg/mL BSA, 1 mg/ml glucose). Cells were incubated in flux buffer for 30 min at 37°C. The incubation was terminated by transferring 100 µL of the cell suspension onto 120 µl of silicone oil (density: 1.0274 g/mL) in long, thin Eppendorf tubes. The tubes were centrifuged in a microfuge at 18,000 *g* for 30 sec and then placed into a freezing methanol bath until frozen. The bottom of each tube was cut off and placed into a scintillation vial with 48 mM NaOH. Each scintillation vial was vortexed for 1 min after which 5 mL of scintillation fluid was added and the vial was counted on a Beckman scintillation counter. $^{36}Cl^-$ uptake was calculated based on the specific activity of ^{36}Cl in the extracellular medium. The specific activity was calculated as follows:

$$\frac{[\text{extracellular } Cl^- \text{ added}] + [{}^{36}Cl^- \text{ added}]}{\text{radioactivity of added } {}^{36}Cl^-} = \text{specific activity (nmole/cpm)}$$

All values of ^{36}Cl uptake were corrected for ^{36}Cl trapped in the extracellular space. ^{36}Cl trapped in the extracellular space was determined by measuring cpm at time zero, immediately after the addition of ^{36}Cl (50 $\pm$ 10.2 cpm). Intracellular Cl^- concentration was calculated under the assumption that intracellular water is 1.3 µL/10^6 cells (8).

References

1. Engblom, A. C. and K. E. Akerman. 1993. Determination of the intracellular free chloride concentration in rat brain synaptoneurosomes using a chloride-sensitive fluorescent indicator. *Biochim. Biophys. Acta 1153*:262.

2. Geddes, C. D., K. Apperson, J. Karolin, and D. J. Birch. 2001. Chloride-sensitive fluorescent indicators. *Anal. Biochem. 293*:60.

3. Krapf, R., N. P. Illsley, H. C. Tseng, and A. S. Verkman. 1988. Structure-activity relationships of chloride-sensitive fluorescent indicators for biological application. *Anal. Biochem. 169*:142.

4. Verkman, A. S., M. C. Sellers, A. C. Chao, T. Leung, and R. Ketcham. 1989. Synthesis and characterization of improved chloride-sensitive fluorescent indicators for biological applications. *Anal. Biochem. 178*:355.

5. Kaneko, H., T. Nakamura, and B. Lindemann. 2001. Noninvasive measurement of chloride concentration in rat olfactory receptor cells with use of a fluorescent dye. *Am. J Physiol. Cell Physiol. 280*:C1387.

6. Koncz, C. and J. T. Daugirdas. 1994. Use of MQAE for measurement of intracellular [Cl-] in cultured aortic smooth muscle cells. *Am. J Physiol. 267*:H2114.

7. Paula, S., A. G. Volkov, and D. W. Deamer. 1998. Permeation of halide anions through phospholipid bilayers occurs by the solubility-diffusion mechanism. *Biophys. J 74*:319.

8. Jensen, T. B., U. G. Friis, and T. Johansen. 1998. Role of physiological HCO3-buffer on intracellular pH and histamine release in rat peritoneal mast cells. *Pflugers Arch. 436*:357.

Appendix B

Optimization of surface charge measurements

Determination of CFC charge

Surface charge measurements are often done using the polycation Cat-Floc (CFC) and toluidine blue (TB) as a color indicator. TB binds to the cell surface producing a color change from blue to purple but CFC displaced TB from the cell surface and reverses the color back to blue. Cell surface charge is determined by calculating the amount of CFC necessary to displace the TB. In order to accurately determine surface charge, the charge of CFC must known. However, CFC is often available as a heterogenous mixture and the charge is often variable. Therefore, the charge of CFC was determined using TB as a color indicator and titrating with an anion, dextran sulfate (DS).

Materials and methods

The charge of CFC was determined by adding 10 μL of 2.5 μM DS (charge density 5.74 μeq/mg, (1)) to 10 μL of 1 mM TB dissolved in 800 μL of 0.25 M sucrose. The TB/DS solution was placed in the spectrophotometer and the mixture was titrated with CFC. The changes in relative absorbance at 630 nm and the corresponding Gran plot were calculated using the equation:

$$\frac{V_e C_t}{V} = [SC\text{-}TB] + [SC^-] \qquad \text{(equation \#1)}$$

or $v[TB^+] = K_a (V_e + v)$ at the equivalence point. (equation #2)

where V is the total volume of the reaction, C_t is the concentration of CFC, v is the volume of CFC added and V_e is the volume of titrant added at equivalence point. The plot of $v[TB^+]$ versus v is a straight line, where K_a is the slope and V_e is the intercept with the X-axis.

Results and Discussion

Addition of CFC to the TB/DS solution produced a change in color from purple to blue and resulted in a shift in absorption from A_{535} to A_{630}. In a typical experiment, the $A_{630}/(A_{630}+A_{525})$ ratio was calculated and a Gran plot was used to calculate the charge of CFC as described above. The charge concentration was calculated to be 34.3 $\pm$ 5.1 μeq/mg (n=3).

To validate photometric measurements using this CFC (and a charge concentration value of 34.3 μeq/mg), CFC was titrated with two compounds of known charge concentration. Heparin isolated from porcine intestinal mucosal (Sigma) has a charge concentration of 4.28 $\pm$ 0.15 μeq/mg (1). A 1 mL aliquot of 170 U/mL (58.8 μg/mL) heparin solution (in 0.25 sucrose) was titrated with CFC in the presence of 0.1 mM TB. The resulting change in $A_{630}/(A_{630}+A_{525})$ ratio was plotted against the amount of CFC added (Fig B.1A).

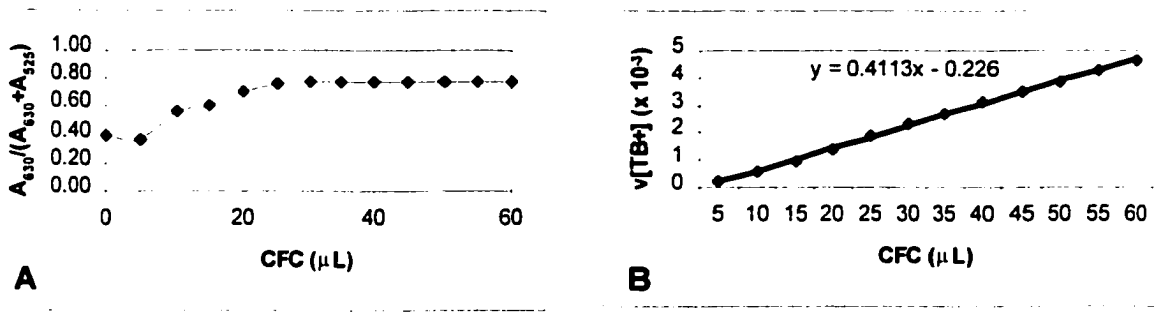


Figure B.1. Typical titration curves for 58.8 μg of porcine heparin titrated with a 25 μM solution of Cat-floc (CFC) in the presence of 0.1 mM toluidine blue (TB). (A) Changes in the relative absorption of TB as a function of CFC added. (B) Gran plot corresponding to the data shown in panel A. Solid line represents best fit of equation #2 above. (n=3)

Gran plot analysis (Fig B.1B) shows that the heparin charge concentration is 3.2 μeq/mg (n=3) which is slightly lower than the published value.

To further validate the use of DS solution and the photometric technique, the charge concentration of the cationic compound 48/80 was determined using DS. The charge of 48/80 was determined by adding 10 μL of 2.5 μM DS (charge density 5.74 μeq/mg, (1)) to 10 μL of 1 mM TB dissolved in 800 μL of 0.25 M sucrose. The TB/DS solution was placed in the spectrophotometer and the mixture was titrated with aliquots of a15 μg/mL solution of 48/80. The resulting change in $A_{630}/(A_{630}+A_{525})$ ratio was plotted against the amount of 48/80 added (Fig B.2).

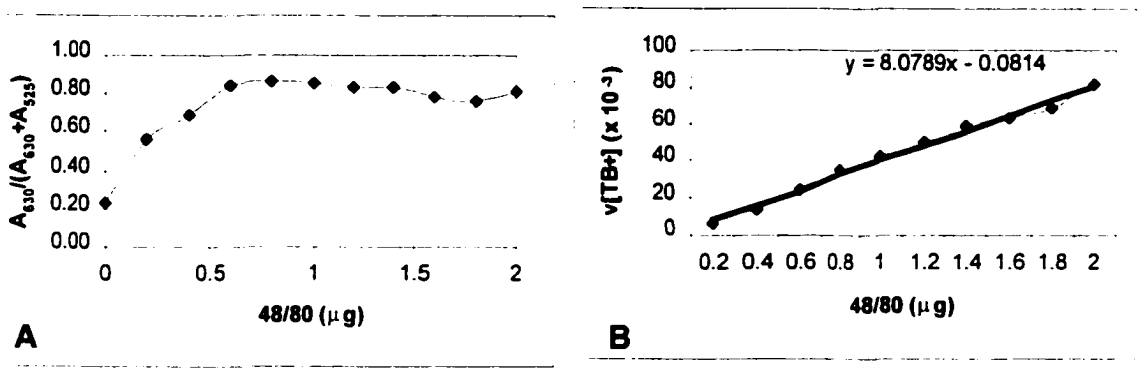


Figure B.2. Typical titration curves for 0.012 μg of dextran sulfate (DS) titrated with a 15 μg/mL solution of 48/80 in the presence of 1 mM toluidine blue (TB). (A) Changes in the relative absorption of TB as a function of 48/80 added. (B) Gran plot corresponding to the data shown in panel A. Solid line represents best fit of equation #2 above. (n=3)

Gran plot analysis (Fig B.2B) shows that the charge of 48/80 is 7.4 ± 0.75 μeq/mg (n=3) and this value corresponds to the published value of 7.6 ± 0.98 μeq/mg (2).

Conclusion

Titration with DS shows that the charge concentration of CFC is 34.3 μeq/mg. The colloid titration procedure using this CFC solution as a titrant is an effective method of determining surface concentration of a common anionic molecule, heparin.

References

1. Van Damme, M.-P. I., S. T. Blackwell, W. H. Murphy, and B. N. Preston. 1992. The measurement of negative charge content in cartilage using a colloid titration technique. *Anal. Biochem. 204*:250.

2. Thethi, K., P. Jurasz, A. J. MacDonald, A. D. Befus, S. F. Man, and M. Duszyk. 1997. Determination of cell surface charge by photometric titration. *J. Biochem. Biophys. Methods 34*:137.