Actions of Interleukin-8 and Extracellular Nucleotides on Airway Smooth Muscle from Normal and CF Subjects

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Faculty Regulations concerning the thesis requirements

This thesis has been written according to the guidelines for a manuscript-based thesis issued by the Faculty of Graduate and Post-doctoral Studies of McGill University. The guidelines are as outlined below.

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The thesis must include the following:

1. *a table of contents;*
2. *a brief abstract in both English and French;*
3. *an introduction which clearly states the rational and objectives of the research;*
4. *a comprehensive review of the literature (in addition to that covered in the introduction to each paper);*
5. *a final conclusion and summary;*
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Abstract

Cystic fibrosis (CF) patients have airway neutrophilic inflammation associated with high levels of interleukin-8 (IL-8) and often develop airway hyperresponsiveness (AHR). Aerosolized uridine triphosphate (UTP) has been used to improve mucosal clearance by inducing Cl^- secretion. However, nucleotides such as UTP and ATP (adenine triphosphate) also evoke the release of intracellular Ca^{2+} ($[Ca^{2+}]_i$), which are enhanced by pro-inflammatory mediators, such as IL-1β. Since most of the CF airway epithelium is lost during infection and inflammation, the underlying airway smooth muscle cells (ASMC) are exposed to IL-8 and extracellular nucleotides. Thus, we were particularly interested in evaluating the effects of IL-8, extracellular ATP and UTP on contractile responses of ASMC. For this study, cultured ASM from normal and CF lung specimens were used. We have found that human ASM cells (HASMC) express mRNA and protein for IL-8 receptors (CXCR1 and CXCR2) and IL-8 causes an increase in $[Ca^{2+}]_i$ as well as cellular contraction and migration. Furthermore, changes in the $[Ca^{2+}]_i$ were abrogated on blocking IL-8 receptors with specific neutralizing antibodies, and were also decreased following inhibition of phospholipase C (PLC), indicating that changes in $[Ca^{2+}]_i$ were likely mediated by inositol trisphosphate. The effects of IL-8 on HASMC prompted us to investigate whether IL-8 induced ASMC contraction in CF was different from normal. IL-8 caused larger ASM contractions and greater phosphorylation of myosin light chain (MLC_{20}) compared to normal cells. CF cells expressed more MLC_{20} compared to normal cells. However, the expression of CXCR1, CXCR2, IL-8 induced changes in $[Ca^{2+}]_i$ and ASMC migration in CF cells were similar

to normal cells. We also found that normal HASMC express receptors (subtypes of P_2Y) for ATP and UTP, which stimulated the release of intracellular Ca^{2+}. Brief exposure of normal HASMC with UTP enhanced Ca^{2+} transients in response to UTP, whereas prolonged treatments with UTP reduced the Ca^{2+} transients in response to ATP and UTP. Overall our findings indicate that IL-8 causes ASM contraction by increasing $[Ca^{2+}]_i$ through PLC coupled signaling pathways in HASMC. In CF cells, IL-8 induced contractions are larger than normal cells and accompanied by increased phosphorylation of MLC_{20}, possibly through Ca^{2+} sensitization or through an increase in total MLC_{20}. Furthermore, ASM migrations are mediated by IL-8 in HASM cells. The agonist UTP, envisaged as novel treatment for CF stimulate Ca^{2+} transients in normal ASM cells.

Résumé

Les patients fibrokystiques ont une inflammation neutrophilique dans les voies respiratoires qui est associée à de hauts niveaux d'interleukine-8 (IL-8). Ils développent souvent une hypersensibiltié des voies aériennes. Des aérosols de triphosphate d'uridine (UTP) ont été utilisés pour améliorer la clearance des voies aériennes en stimulant la sécrétion de Cl^-. Cependant, ce nucléotide induit également la libération du Ca^{2+} intracellulaire ($[Ca^{2+}]_i$) qui est potentialisée par les médiateurs pro-inflammatoires tels que IL-1β. Etant donné que l'épithélium des voies aériennes des patients fibrokystiques est détruit par les infections et l'inflammation, les cellules musculaires lisses sous-jacentes (ASMC) sont exposées à l'IL-8 et aux nucléotides extracellulaires. C'est pourquoi nous étions particulièrement intéressés à étudier les effets de l'IL-8 et de l'ATP et UTP extracellulaires sur la réponse contractile des ASMC. Dans cette étude, des cultures cellulaires de muscle lisse des voies respiratoires provenant de poumons normaux et fibrokystiques ont été utilisées. Nous avons trouvé que les cellules musculaire lisses humaines (HASMC) expriment l'ARN messager et la protéine pour les récepteurs IL-8 (CXCR1 and CXCR2) et que IL-8 provoque une augmentation de la $[Ca^{2+}]_i$ ainsi que la contraction et la migration des ASMC. De plus, les changements de $[Ca^{2+}]_i$ étaient nullifiés par le blocage des récepteurs IL-8 par des anticorps neutralisants spécifiques et étaient également diminués suivant l'inhibition de la phospholipase C (PLC), indiquant que les changements dans la $[Ca^{2+}]_i$ étaient probablement engendrés par le triphosphate d'inositol. Les effets de l'IL-8 sur les HASMC nous ont incités à investiguer si la contraction du muscle lisse fibrokystique produite par l'IL-8 était différente de celle du muscle lisse normal. IL-8 produisit des contractions plus grandes

and la phosophorylation de la chaine légère de myosine (MLC_{20}) était augmentée dans les cellules fibrokystiques. Les cellules fibrokystiques expriment également plus de MLC_{20} que les cellules normales. Cependant, l'expression des CXCR1, CXCR2, les changements dans la $[Ca^{2+}]_i$ et la migration des cellules fibrokystiques étaient comparables aux cellules normales. Nous avons également trouvé que les HASMC normales expriment des récepteurs pour l'ATP et l'UTP (sous-types de P_2Y), qui stimulent la libération du Ca^{2+} intracellulaire. Une exposition brève à l'UTP potentialise la libération du Ca^{2+} en réponse à l'UTP alors qu'un traitement prolongé à l'UTP diminue la libération de Ca^{2+} en réponse à l'ATP et l'UTP. Dans l'ensemble, nos découvertes montrent que l'IL-8 produit une contraction du muscle lisse via l'augmentation de la $[Ca^{2+}]_i$ par l'intermédiaire de la cascade engendrée par l'activation de la PLC dans les cellules musculaires lisses. Les contractions induites par l'IL-8 sont plus grandes dans les cellules fibrokystiques que dans les cellules normales et sont accompagnées par une augmentation de la phosphorylation de MLC_{20} possiblement à cause de sensibilisation au Ca^{2+} ou à cause de l'augmentation totale de MLC_{20}. De plus, la migration des cellules musculaires est engendrée par l'IL-8. L'agoniste UTP, envisagé comme nouvel forme de traitement de la fibrose kystique, augmente la libération de Ca^{2+} dans les cellules musculaires lisses.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Airway inflammation and airway hyperresponsiveness (AHR) are the characteristic features of asthma and other chronic airway inflammatory diseases such as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) (87), (178), (145). The term AHR or airway hyperreactivity describes an excessive increase in bronchial smooth muscle tone (contraction) in response to various stimuli including irritants, allergens, inflammatory mediators, smooth muscle constrictive agonists, and cold air. Chronic inflammation leads to thickening of airway walls and results in airway smooth muscle (ASM) remodeling (156). In association with these changes there is increased production of cytokines, chemokines, adhesion molecules, matrix proteins and proteases. These mediators in turn cause airway smooth muscle (ASM) proliferation and migration. The changes in ASM properties may be the cause of AHR, which is observed in asthma, COPD, and CF. Airways of patients with asthma of mild to moderate severity are infiltrated with eosinophils, mast cells and T lymphocytes (87), (82), whereas airways of patients with acute severe asthma, "status asthmaticus", and fatal asthma are infiltrated with neutrophils (155), (185). The airways of patients with COPD are also inflamed and are predominantly infiltrated with neutrophils, macrophages, T lymphocytes (CD8+) and a minor eosinophilic component (311), (248), (284). Furthermore, neutrophilic inflammation is the hallmark of CF airway inflammation (178), (311).

The mechanisms of neutrophil accumulation in the airways are likely several and may vary with the disease. One of the important chemoattrtactant factors for neutrophils is interleukin-8 (IL-8). However, neutrophils themselves produce IL-8, which may have significant biological effects on airway cells, in addition to various proteases and reactive oxygen species, which damage the lung tissues. Even though neutrophils are the major source of IL-8 in the airways, other cells such as macrophages and epithelium also produce IL-8 (86), (235), (311). It has been shown that IL-8 increased acetylcholine-induced contraction of rat intestinal segments, but it is not known whether IL-8 acts on ASM and is thereby involved in bronchoconstriction and AHR. We hypothesize that IL-8 acts on ASM and causes bronchoconstrictiontherby contribute to the airway hyperresponsivenessin in CF and severe asthma. The increased production of IL-8 in CF airways and its known effects will be discussed in greater detail below.

1.2 **Cystic Fibrosis (CF)**

CF is the most common fatal genetic disease in North America and is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR functions as a phosphorylation-regulated chloride channel in the apical membrane of epithelial cells and determines transepithelial salt transport, fluid flow and thereby, airway surface liquid (ASL) ionic composition. The CFTR gene, located on chromosome 7, was first isolated in 1989 by Drs. Lap-Chee and Tsui, John Riordan and

The defects of CFTR mutations can be grouped into five classes (310) (1) defective CFTR protein production (2) defective processing, (3) defective regulation, (4) defective conductance, and (5) defective regulation of other channels. So far, more than 1000 mutations have been identified and the most common mutation worldwide is in defective processing, caused by the deletion of phenylalanine at position 508 (ΔF508, ~ 70% of mutations in North America) of CFTR (277). Any one of the above defects leads to CF disease. Advances in therapy over the past few decades have improved the outlook for the CF patients, enabling more than half of them to survive into their late twenties. Despite the advances in genetics and in the understanding of the molecular basis of CF, there is no clear understanding of the pathogenesis of CF lung disease and none of the approved treatments can correct the cause of the CF disease.

1.2.1 CF Pathogenesis

CF disease primarily involves the lungs, pancreas, intestines, sweat glands and liver. In CF affected lungs, dysfunction of CFTR Cl^- channel disrupts transepithelial ion transport, which causes a reduction of the sol layer of the epithelial lining fluid, and a thickening of the mucus layer that leads to bacterial colonization and airway infection (337). These bacterial infections are mostly caused by *Pseudomonas aeruginosa* and *Staphylococcus aureus.* Chronic bacterial infection progressively destroys the airways causing extensive bronchiectasis, ultimately leading to respiratory failure (178). However, in other organs, CF pathogenesis does not involve bacterial infections and may be based only on the defective transepithelial Cl^- transport. This may explain why the major morbidity and mortality of CF is associated with lung disease (281). Despite

many advances in the understanding of the CFTR gene, we still do not know exactly how mutations in the CFTR gene and defects in CFTR channel ion transport lead to persistent airway infection and inflammation. Furthermore, attempts to link mutations in CFTR to severity of lung disease have not been successful (133).

CFTR may have a pathogenetic role that is unrelated to ion transport. For example, it has been characterized as a receptor for bacteria such as *P. aeruginosa* and one of its functions is to participate in the internalization and killing of the bacterial pathogen (266). ΔF508 CFTR epithelial cells fail to ingest this pathogen, leaving bacteria free to multiply in the airway lumen of the CF patients (266). Several hypotheses link CFTR mutations to the development of bacterial infections. One of the hypotheses suggests that the adherence of *P. aeruginosa* to CF airway epithelial cells is increased and thereby bacteria are difficult to clear from the airway. On the other hand, it is also believed that the bacteria adhere to cell surface receptors such as asialo-GM1, which is up regulated in CF, and not to CFTR (286), (81). There are other hypotheses, which suggest that CF cell organelles are more acidic (268) or alkaline (152) than organelles from normal cells, and thus, altered pH leads to under-sialylated glycoproteins and glycolipids on CF epithelial cell membranes. The increased amount of under-sialylated glycoproteins and glycolipids leads to increased binding of *P. aeruginosa* and S. *aureus* on these cells (268), (152). The salt-defensins hypothesis (303) is based on assumption that defensins, a natural detergent–like peptide, are necessary for the host defense against *P. aeruginosa* infections, but the defensins are inactivated by a salt concentration of greater than 50 mmol/L as in CF. Under these conditions, bacteria can multiply on respiratory epithelial cells, leading to chronic

infection. The isotonic fluid depletion/defective mucociliary clearance hypothesis proposes that defective Cl^- secretion and abnormal Na^+ absorption leads to a water/volume depletion from airway surface liquid (211). Water losses and decreased airway surface liquid (ASL) volume increase mucus viscosity and impair mucociliary clearance, facilitating bacterial colonization and infection.

Although various hypotheses exist, they do not clearly explain the reasons for infections predominantly with certain pathogens. An exaggerated and sustained inflammatory response to bacterial and viral pathogens is associated with neutrophilic infiltration in CF affected lungs. However, it is interesting to note that inflammation has been observed in the airways of the patients with CF even before infection (168). There is evidence that BAL from CF airways contains both inflammatory chemokines and cytokines, such as IL-8, tumor necrosis factor- α (TNF-α), interleukin-1(IL-1) and interleukin-6 (IL-6) (236), (169), (168). Chmiel et al (70) have shown that imbalance in these cytokines leads to severe lung inflammation after challenging mice with *Pseudomonas aeruginosa.* These cytokines are derived from multiple sources including epithelial cells, vascular endothelial cells, lung fibroblasts, smooth muscle cells, and mononuclear cells including neutrophils and macrophages. Among the different cytokines, IL-8 is found in higher concentration in CF airways, which may be of particular significance because it is a potent neutrophil chemoattractant. Furthermore, the neutrophils release multiple reactive oxygen species (ROS), such as $O_2^{\cdot-}$, $OH^{\cdot}$, H_2O_2 etc., proteases (especially elastase) and other mediators that provoke more inflammation and damage the airway wall and other structural proteins (88). As more neutrophils break down and disintegrate, DNA content of the airways is increased, which further

increases the viscosity of the ASL and encourages the mucopurulent plugging of airways. Accumulation of inflammatory products along with airway wall damage leads to an increase in obstruction of the airway lumen (29).

1.2.2 Airway responsiveness in individuals with CF

CF patients, with extensive airway inflammation, have asthma like symptoms such as reversible airway obstruction and airway responsiveness to non-specific irritants (326), (289), (251). Wonne et al (340) have shown that 65% of children with CF tested positively for allergy skin tests and 40% of them exhibited specific airway responsiveness with bronchoconstriction in response to inhaled allergens, despite the absence of asthmatic symptoms. In another study, the bronchial responsiveness to methacholine and histamine were compared between CF and asthma patients (219) and the data showed that more than 50% of CF patients had enhanced bronchial responsiveness to methacholine rather than histamine, whereas all patients with asthma exhibited bronchial hyperresponsiveness to both histamine and methacholine. Methacholine responsiveness in CF patients was correlated with airway obstruction. These findings were also confirmed by another study, where CF patients with severe lung disease had higher frequency of pulmonary exacerbations and increased responsiveness to methacholine (96). Exercise causes bronchodilation and an increase in peak expiratory flow rate in CF patients in contrast to the bronchoconstriction that is often seen in asthmatic patients (302), (148), (326).

1.2.3 Structural changes in CF lung

In patients with CF, histopathological changes are observed to affect the submucosa, airway wall and the supporting structures. In contrast, the alveoli are spared. Dilatation of submucosal glands is followed by changes in epithelial lining, which includes denudation, loss of cilia, and squamous metaplasia (309). Morphological analysis of CF lung by Tiddens et al (320), (318) has shown that about one-third of the basement membrane in central and pheripheral airways was not covered with epithelium. It also revealed the presence of fibrosis in the subepithelial region of central airways and thickening of the inner airway wall (the tissues between the basement membrane and smooth muscle layer). Furthermore, the amount of bronchial smooth muscle in patients with CF has also been found to be increased (31), (94). The magnitude of the thickness of smooth muscle layer is related to the severity of inflammation, airway obstruction and increased bronchial responsiveness in CF patients.

1.2.4 Treatment

Knowledge gained over the years on the CF defects has lead to many new ideas for treatment, but the treatment of the disorder is still palliative rather than curative. At present, prevention of the consequences of bacterial damage to the airways and the maintenance of nutrition are primary aims, even though curative treatment for CF is to restore CFTR function by transfection of the cells with the wild type gene (104). *In vivo* gene therapy using viral vectors and cationic lipids has been tried (177), (9) but without much success. Repeated administrations of adenovirus vectors reduce the efficacy of transfection due to the formation of the specific antibodies, whereas the liposomal gene

transfection due to the formation of the specific antibodies, whereas the liposomal gene therapy does not specifically target CFTR–expressing cells. Alternative strategies using activators of chloride secretion, such as extracellular UTP (uridine triphosphate) (175), (176) and inhibitors of sodium absorption such as amiloride are also used in an attempt to correct the CF defects. Extracellular UTP and other nucleotides such as ATP (adenosine triphosphate) improve mucociliary clearance of airways by stimulating Cl^- secretion through calcium activated Cl^- channels using the specific P2Y receptors present on the airways epithelium (208), (317).

The above mentioned properties of UTP and ATP have led the investigators to propose aerosolized UTP for the treatment of patients with CF so as to improve mucociliary clearance in their airways (175), (176). However, studies have also shown that extracellular ATP and UTP can be released in the airways from the inflammatory cells, damaged epithelium and endothelium. These nucleotides also act as pro-inflammatory mediators by causing aggregation, degranulation and releasing superoxide anion from neutrophils (106), (343). In addition, there are several reports that extracellular ATP enhances the effects of pro-inflammatory mediators such as IL-1β and IL-8 (265). In contrast, Mingyan et al (149) have shown that inflammatory mediators such as IL-1β up-regulate the expression of P2Y receptors and promote the growth and contractility of vascular smooth muscle in response to ATP and UTP. Hence, it is possible that extracellulr ATP and UTP may interact with and enhance the effects of IL-8 or vice versa. Previous work from our laboratory showed that rat ASM expressed receptors for ATP and UTP and their stimulation induced intracellular calcium release (215), (217). Since CF airway mucosa and epithelium are inflamed and

denuded, consideration has to be given to the idea that the extracellular ATP and UTP may act on the human ASM cells and adversely alter the functions of airways.

1.3 Airway Smooth Muscle

Smooth muscle (SM) cells are spindle–shaped, with a diameter ranging from 2 to 10 μm. These cells have single nucleus and are stimulated by many agents to divide continuously throughout the life span of the cell. Based on the electrical characteristics of the plasma membrane, two types of SM tissues are known: (1) Single unit SM, where the whole muscle respond to a stimulus as a single unit because the cells are linked together by adjacent gap junctions e.g. SM from GI tract and small blood vessel, and (2) multiunit SM that has few or no gap junctions between each of the cells, and the contractile response depends on stimulation by neurotransmitters and circulating hormones e.g. SM of large airways (ASM) and large arteries.

Airways have a smooth muscle layer running from trachea to the terminal bronchioles. The cytoplasm of the ASM cell consists of thin filaments containing actin and thick filaments containing myosin, which form the main constituents of the contractile apparatus. The actin filaments are arranged in double helical bundles and are anchored to plasma membranes by dense plaques or to cytoplasmic structure by dense bodies (323). This provides mechanical coupling between the contractile apparatus, the cytoskeleton and the surface of the cell (89), (161). Proteins such as tropomyosin, caldesmon and calponin are also present in actin filaments and are considered to be thin filament-associated regulatory proteins (329), (314). Myosin molecules have two heavy

and four light chains. The heavy chains form a pair of globular heads and rod like structures with functional motor domains, which include nucleotide (ATP) and actin-binding regions. The neck region of each heavy chain has two light chains with molecular weights of 20 kDa and 17 kDa, respectively. The 20 kDa light chain acts as a regulatory protein whereas 17 kDa is termed an essential protein. In SM, the regulation of contraction occurs chiefly *via* myosin, in the presence of Ca^{2+}-calmodulin-myosin light chain kinase although actin-associated regulatory proteins also appear to be involved. The phosphorylation of the regulatory light chain causes repetitive attachment of the myosin head to actin, which undergoes conformational changes resulting in power strokes (333) or cross-bridge cycling. The energy required for the cross bridge cycling is generated by the enzymatic hydrolysis of ATP by the globular heads (275). The unique feature of SM is that once activated by Ca^{2+} stimulated phosphorylation, it continues to generate force despite return of both Ca^{2+} and phosphorylation to normal levels. This process is termed the 'latch state'.

Under normal conditions ASM is a highly differentiated contractile cell but it may undergo phenotypic changes. These have been identified in cells when they are placed in culture. Halayko et al (130) have shown that following plating of ASM cells, the expression of contractile proteins such as actin, myosin and myosin light chain kinase is decreased during the proliferative phase and the cells acquire secretory functions indicating that ASM can undergo phenotypic changes from contractile to proliferative or secretory state. Once the cells are confluent, the expression of contractile proteins does not return to the levels that were present during freshly isolated cells. Even with these phenotypic changes, ASM cells still respond to contractile

agonists by mobilizing Ca^{2+} from intracellular sources and responding to methacholine (254), (316). These results suggest that cultured cells can be used to study the contractile properties of ASM under appropriate conditions.

1.3.1 Functions of airway smooth muscle

The physiological functions of ASM are unknown. However, it may have a role in ventilation distribution and in the maintenance of airway tone. As ASM cells are subjected to the influences of many mediators like neurotransmitters, drugs (331) and environmental irritants, its tone needs to be adjusted on a regular basis. This fine-tuning can be achieved by the contractile and relaxant properties of ASM. However, under diseased conditions such as asthma, CF, COPD and bronchopulmonary dysplasia (BPD), the airways are also subjected to various allergic and inflammatory mediators, which may increase the sensitivity, tone and thickness of the airway SM. The pathological events along with inflammatory edema cause narrowing and reversible obstruction of the airways. During inflammation, ASM cells activate different functions such as bronchoconstriction, proliferation, secretion and migration.

1.3.1.1 Bronchoconstriction

Bronchoconstriction may be caused by either an excessive degree of direct stimulation of the ASM cells or an enhancement of the responses of the ASM to contractile agonists. Usually both mechanisms are operative. Direct stimulation results from the activation of a variety of G-protein coupled receptors through agonists such as histamine, cysteinyl leukotrienes (LTD_4), endothelin-1, thromboxane, PGD_2, $PGF_2\alpha$,

isoprostanes, platelet-activating factor (PDGF), bradykinin (BK), adenosine (ADO) and acetylcholine (Ach). Indirect mechanisms involve an increase in the responses to contractile agonists (Ach, NK, LTD_4, and BK) (300) induced by exposure of the ASM to agents such as allergens and the accompanying multiple inflammatory mediators (eg, cytokines such as TNF-α, IL-1β, IL-4, IL-5, IL-10 and IL-13 and chemokines such as IL-8, eotaxin and RANTES). These mediators are released not only by inflammatory cells but also structural cells (epithelial and ASM cells).

Neurotransmitters (acetylcholine, substance-P, neurokinin A, neuropeptide Y) released from airway nerves can induce either direct or indirect contraction. Many studies have shown that TNF-α, IL-1β, IL-5, IL-10 and IL-13 can increase contractile responses of ASM mediated by Ach (129), (13), (11). Some of the proposed mechanisms for the indirect action of these agents are: (1) altering the common signaling pathways shared by different contractile agonists present during inflammation, (2) increasing the airway smooth muscle contractile proteins and (3) altering the neural control of airways (339). Among the various bronchoconstrictors, cysteinyl leukotrienes (129; 213), thromboxane, endothelin-1 (4), (314), and acetylcholine (206) are the most potent agents.

1.3.1.2 Proliferation

Many studies have shown that the thickness of the airway wall is increased in asthma and CF (60), (95), (319), (48). Even though the mechanisms of proliferation of the smooth muscle are not well characterized, various studies have shown that growth factors, contractile agonists, extracellular matrix, allergic and inflammatory mediators

stimulate the growth of ASM. Platelet–derived growth factor (PDGF) (141), epithelial growth factor (EGF) (308), insulin-like growth factor (IGF-1) (245), histamine (255), thromboxane (246), leukotrienes (267), endothelin-1 and fetal calf serum in cultures (142; 245), are some of the mediators that are potentially involved in proliferation. Inflammatory cytokines such as IL-1β, TGF-α, and IL-6 (84), (12), induce proliferation of ASM in the presence of cyclooxygenase inhibitors. The effects of mitogens are mediated by tyrosine kinase linked receptors (e.g. PDGF, EGF and fibroblast growth factor) and G-protein coupled receptors (eg, histamine, thrombin and endothelin-1). Downstream signaling events activate phosphoinositide 3-kinase (PI3K) which then leads to the activation of p21Ras. The activated Ras binds to GTP and translocates activated raf–1 to the plasma membrane, where it phosphorylates mitogen-activated kinase kinase. Evidence shows that MAP kinases and extracellular regulated protein kinase (ERK) play an important role in ASM cell growth (198). In addition, inflammatory and contractile agents induce proliferation by inducing synergy between tyrosine kinase linked pathways and G-protein coupled receptors (180). Cell-cell interactions (direct contact of immunocytes with ASM) and mechanical stretching of the muscle also serve as mitogenic stimuli. Lazaar et al (188) have studied the effects of T lymphocyte adherence to ASM on DNA synthesis by ASM cells and have shown that activated T cells increase BrdU incorporation approximately 30 fold more than control cells. Furthermore, many other studies (118), (246) also show that activated T cells increase DNA synthesis, indicating that cell contact plays a role in the ASM proliferation. Agents such as PGE_2, transforming growth factor β_1 (TGF-β1), (159), (321), β_2–agonists, and nitric oxide (NO) act as anti-proliferative agents.

1.3.1.3 Synthetic (secretory) functions

ASM cells in asthma respond to inflammation by contracting and proliferating, but in the past few years many studies have shown that ASM cells have an active role in inflammation through the secretion of a wide variety of mediators usually found in allergic and chronic inflammatory diseases (160). Cytokines and chemokines, such as IL-2, IL-5, IL-6, IL-8, IL-12, IL-13, RANTES, eotaxin, monocyte chemoattractant protein (MCP 1,2,3), granulocyte macrophage colony-stimulating factors (GM-CSF), are released from ASM cells in response to either IL-1β, TGF-α, IFN-γ or lipopolysaccharide (LPS) on its own or in combination with the above (158) (128), (290), (111), (270), (125), Furthermore, ASM cells respond to these cytokines by proliferating and expressing adhesion molecules, which permit interactions between ASM and inflammatory cells. Growth factors such as PDGF, IGFs, EGF and adhesion molecules and integrins such as CD44 (surface receptor for hyaluronate), selectins, intercellular adhesion molecules-1 (ICAM-1), vascular cell–adhesion molecule-1 (VCAM-1) are also produced either constitutively (CD44, ICAM-1) or in response to TGF-α (VCAM-1) (245), (84), (189). Some of these growth factors use autocrine mechanisms to regulate growth. The release of cytokines from ASM cells can attract inflammatory cells and sustain inflammatory processes during chronic airway inflammation. ASM cells also secrete thromboxane B2, $PGF_{2\alpha}$, PGD_2, and enzymes involved in 5-lipoxygenase pathways (27), (256), (153), (153), (129). The autocrine and paracrine functions of these eicosanoids could have important consequences for the contractile function of ASM.

ASM cells also release bronchoprotective agents such as PGE_2 in the presence of bradykinin, acetylcholine, β-adrenergic agents, and NO (27), (110), (256), (22). Since PGE_2 and NO inhibit proliferation, they can serve as anti-proliferative agents and furthermore, their release from ASM cells provides feedback mechanisms that limit the inflammatory processes. They are also bronchorelaxant in action and could influence airway tone.

1.3.1.4 Migration

ASM cells are exposed to numerous growth factors and pro-inflammatory cytokines that contribute to SM remodeling in chronic airway inflammatory diseases such as asthma and CF. In these diseases, SM may migrate and proliferate. *In vitro* studies have shown that SM cells can migrate in response to different agents, but it is not known whether ASM migrate *in vivo*. However, *in vivo* migration of vascular SM is well known *in vivo* and it has been shown that vascular SM cells migrate in response to the injury, inflammation and wall stress that are observed in atherosclerosis (293). Furthermore, Mukhina and coworkers have showed that human ASM cells in culture are capable of migration in response to urokinase plasminogen activators (227). Their study also indicates that the migration of ASM resembles the migration that is found in the remodeled vessels of atherosclerosis. Other studies have also shown that ASM migrates in response to PDGF, IL-1β, TGF-β, and fibroblast growth factor (138), (85). However, on testing the effects of LTD_4 on migration, it was found that it augmented the migration induced by PDGF rather than inducing migration by itself (258).

Goncharova et al (117) showed that PGE_2, epinephrine and glucocorticoids inhibit ASM migration but by mechanisms that are as yet unknown.

1.4 Signaling Mechanisms

The airway smooth muscle cell express many receptors, such as G-protein coupled receptors, ion channels-linked receptors and enzyme–linked receptors. Inflammatory mediators and contractile agonists mediate their effects by signaling through G-protein coupled receptors or tyrosine kinase–linked receptors.

1.4.1 Signaling through G-protein coupled receptors

Proteins that are activated by guanosine triphosphate (GTP) binding are called 'G-proteins'. G-proteins exist as monomeric and heteromeric forms. Since heteromeric proteins are involved in contractile signaling, the focus of further discussion is predominantly on this group of proteins and the receptors that couple to them.

G-protein-coupled receptors (GPCR) comprise one of the larger families of cell surface molecules with seven hydrophobic transmembrane domains that span the membrane bilipid layer. These receptors mediate cellular responses to numerous stimuli arising from hormones, neurotransmitters, peptides, odorants, light and various agonists. Activation of GPCRs stimulates specific heteromeric G-proteins, which consist of α, β, and γ subunits encoded by different genes. The α subunit is classified into four groups: $G\alpha s$, $G\alpha i$, $G\alpha q$ and $G\alpha_{12}$ with each class containing different subunits and being activated by different stimuli. The C-terminal sequence of the α-subunit is important for

the interaction of ligand with the receptor, whereas the N-terminal sequence is necessary for binding of βγ-subunits. When the receptor is not activated (See figure 1.1A), GDP is bound to the α-subunit, which is associated with βγ-subunits, whereas in the presence of ligand activation the receptor undergoes conformational changes at the α subunit and GDP is dissociated from the α subunit and then replaced by GTP (figure 1.1B). During this activated GTP-bound state (figure 1.1C), α subunits dissociate from both the receptor and βγ dimeric subunits. The free α and βγ subunits activate specific effector molecules such as phospholipase C (PLC), phospholipase A_2 (PLA_2), phosphoinositide 3-kinase (PI3K) and adenylyl cyclase, thereby producing a variety of second messengers such as diacylglycerol (DAG), inositol 1,4,5-trisphosphate (IP_3) and cAMP in addition to increasing the intracellular Ca^{2+} $[Ca^{2+}]_i$. The activated GTP-bound state is transient because GTP is quickly hydrolyzed to GDP by GTPase. This event is followed by re-association of α and βγ subunits (figure 1.1D). Different G-protein sub-units transmit signals to different effector molecules. For example, Gαq activates phospholipase C and generates IP_3 and DAG and Gαs is stimulatory to adenylyl cyclase (e.g. following β-AR activation) whereas $G\alpha_i$ is inhibitory to adenylyl cyclase (e.g. after muscarinic stimulation). G-protein coupled receptors also signal by cross-talk mechanisms. Selbie et al (297) have described the interactions between the different subtypes of G-proteins and with other receptor classes like tyrosine kinase-linked signaling molecules (MAPK, ERK). Several studies have shown that GPCRs modulate the receptor functions (eg. β_2 adrenergic receptors, muscarinic receptors, dopamine receptors, etc.) by either dimerization or oligomerization (116). This property of GPCR provides a new strategy for the development of novel therapeutic drugs (349), (205),

(116). Desensitization of the receptors following agonist stimulus is a well known property of the GPCR.

1.4.1.1 Desensitization

The decreased response of GPCR generated signals in the continued presence of agonist is termed desensitization. Once the agonist is removed, desensitization is followed by re-sensitization. Desensitization of G-protein mediated signals can be mediated at different levels of the ligand-receptor complexes. Regulators of G-protein signaling (RGS) accelerate the hydrolysis of Gα-bound GTP and promote the deactivation of G-protein (30). Thereby, RGS play an important role in turning-off signals at the G-protein coupling level. Other known mechanisms of desensitization include: (1) rapid phosphorylation and desensitization (uncoupling of the receptor from G-protein), (2) internalization (sequestration) and recycling, and (3) degradation and down-regulation of G-protein, Figure 1.2. GPCR is phosphorylated by a family of cAMP dependent protein kinases (PKA and PKC) and by serine-threonine kinases known as G protein coupled receptor kinases (GRKs). Both protein kinases and GRKs contribute to agonist-dependent desensitization of GPCR. GRK–mediated phosphorylation induces the receptor binding to proteins known as arrestins (arresting agents), which inhibit G-protein coupling and terminate G-protein activation, thereby causing uncoupling of receptors. In addition to uncoupling, GPCRs undergo internalization, which involves the redistribution of receptors from the plasma membrane to the intracellular space (306) (327), and results in down-regulation of receptors. Even though multiple pathways may be involved in the internalization of receptors, so far, only GRK and

arrestin dependent mechanisms are well characterized. Internalization of the receptors is mediated when the arrestins bind to clathrin-coated pits in the presence of adaptor protein AP2 (120) and the internalization mostly affects the desensitized receptors. Following internalization, GPCRs either enter lysosomes or recycle back to the surface of the cell by dephosphorylation (101). Dephosphorylation and subsequent recycling of receptors back to the plasma membrane contributes to re-sensitization. Down-regulation of the receptor occurs as a result of reduced mRNA and protein synthesis as well as increase in the lysosomal degradation of receptors. GRK-mediated phosphorylation coincides with short-term desensitization that lasts for a few minutes to hours, whereas receptor degradation and down-regulation is associated with long-term desensitization over hour to days.

1.4.2 Calcium signaling

Calcium acts as a universal second messenger in a variety of cells by controlling a range of cellular functions such as gene transcription, muscle contraction, secretion, cell proliferation and other biological functions. The contractile stimulus is transduced from the outside to the inside of the cell by changing the levels of intracellular calcium $[Ca^{2+}]_i$. These changes in $[Ca^{2+}]_i$ are effected by allowing the cell to move towards an equilibrium in the level of Ca^{2+} between the extracellular and intracellular spaces through an increase in membrane permeability to Ca^{2+}. An increase in cell membrane permeability allows Ca^{2+} entry whereas an increase in sarcoplasmic reticulum (SR) permeability causes Ca^{2+} release from intracellular stores. These effects

Figure 1.1

The cycle of receptor/G protein interactions in transmembrane signaling.

The components include receptor (R), α subunit, βγ dimer, agonist, and effector. (*A*) The receptor bound to G protein has a greater affinity for the agonist. (*B*) The binding of agonist to the receptor produces a conformational change in the receptor, which results in the release of GDP and binding of GTP to the α subunit. (*C*) The binding of GTP to the α subunit results in dissociation of the α subunit from the receptor and the βγ dimer and association of the α subunit with the effector to stimulate the generation of a second messenger. (*D*) The α subunit-bound GTP is hydrolyzed to GDP by an intrinsic GTPase, and the α subunit becomes dissociated from the effector and re-associates with the βγ dimer/receptor complex to repeat the agonist-coupled cycle. (*From Hakon Hakonarson and Michael M. Grunstein, Am J Respir Crit Care Med, 158, pp S115-S122, 1998*)

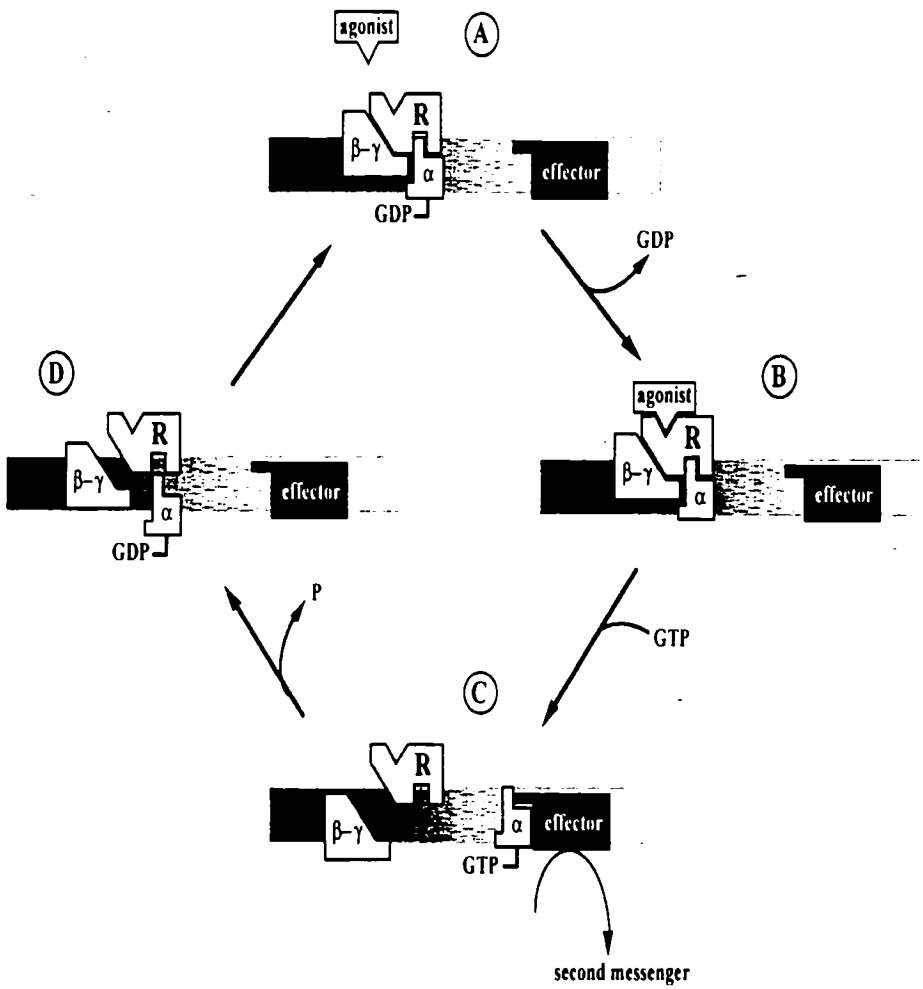
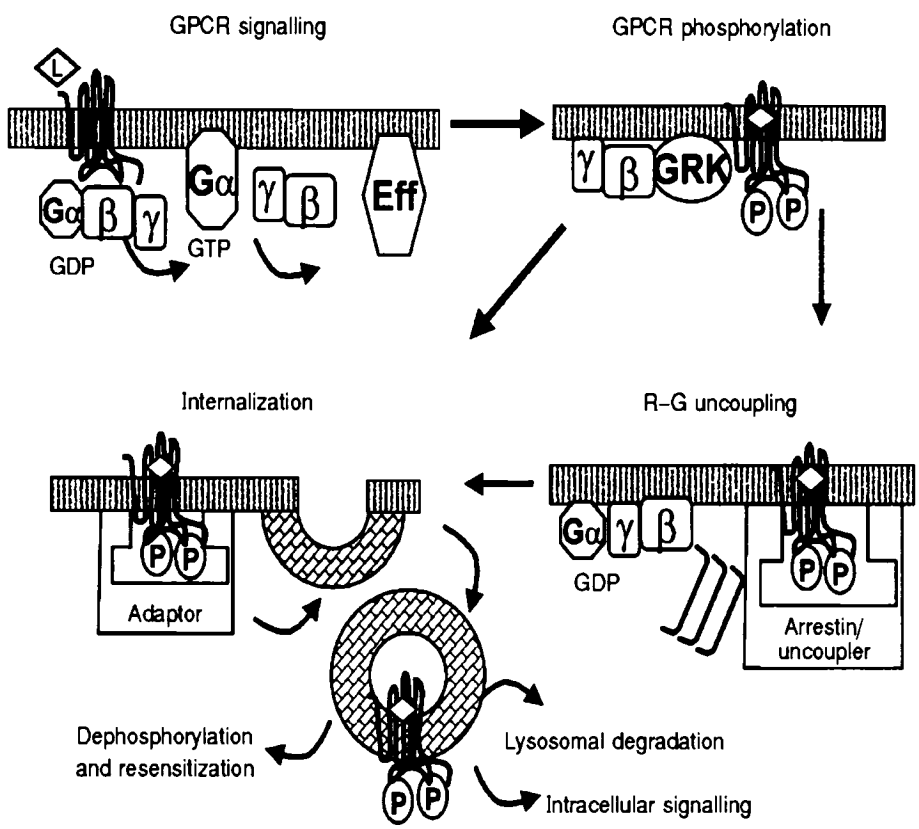

agonist
A
R
β-γ
α
effector
GDP
GDP
B
agonist
R
β-γ
α
effector
GTP
C
R
β-γ
α
effector
GTP
second messenger
P
D
R
β-γ
α
effector
GDP

Figure 1.2

Scheme of GRK-dependent modulation of GPCR-mediated signaling

After activation of GPCRs by ligands (L), the βγ-subunits of G-proteins may recruit GRKs to the membrane to allow for GPCR phosphorylation (P) of activated GPCRs. Phosphorylation by GRKs allows the GPCRs to interact with uncoupling proteins such as arrestins which cause uncoupling of the GPCRs from G-proteins. In addition to the uncoupling reaction, many GPCRs undergo internalization. This can be achieved by targeting of the phosphorylated receptor by an adaptor protein to endocytotic pathways. For some GPCRs, internalization may proceed in a manner that is independent of the uncoupling reaction and may involve yet to be defined adaptors and endocytic machinery. After endocytosis, the GPCRs may undergo lysosomal degradation or recycle to the surface membrane. Eff, effector molecule (*From Moritz Bunemann and Marlene Hosey, J. Physiol, 517, pp 5-23, 1999*)


GPCR signalling
GPCR phosphorylation
L
Gα
β
γ
GDP
GTP
Eff
γ
β
GRK
P
P
Internalization
R–G uncoupling
Adaptor
Gα
γ
β
GDP
Arrestin/
uncoupler
Dephosphorylation
and resensitization
Lysosomal degradation
Intracellular signalling

are mediated by voltage-gated or ion-gated channels, or inositol phosphate receptors. The cytoplasmic concentration of Ca^{2+} can be altered by changes in membrane potential, a phenomenon called electromechanical coupling or by membrane-mediated mechanisms, without any change in the potential difference across the membrane, a process called pharmacomechanical coupling. Both mechanisms occur to a greater or lesser extent in SM cells depending on the type of stimulus.

1.4.2.1 Ca^{2+} channels

In resting cells, Ca^{2+} homeostasis is achieved by maintaining the balance between the Ca^{2+} entry and removal by Ca^{2+} channels or by internal stores. However, in activated cells, the increased levels of $[Ca^{2+}]_i$ are achieved by two different mechanisms: (1) through opening of Ca^{2+}channels located on the plasma membrane and (2) release of Ca^{2+} from intracellular stores by activation of second messenger-activated channels present in the sarcoplasmic reticulum (SR) or endoplasmic reticulum (ER). In addition, other mechanisms such as Na^{+}/ Ca^{2+} exchangers use ion gradients across the membrane to transport Ca^{2+}. Extracellular Ca^{2+} enters the cell through different Ca^{2+}channels: voltage-operated calcium channels (VOCCs), receptor-operated calcium channels (ROCCs), ligand gated non-specific calcium channels (LGCCs) and store-operated calcium channels (SOCCs) (31), (32), (64), (93). VOCCS are employed largely by excitable types of cells (neuronal and vascular smooth muscle cells). Depolarization of the cell membranes by extracellular stimuli activates dihydropyridine (DHP)-sensitive Ca^{2+} channels and generates an action potential. This action potential brings sufficient amounts of Ca^{2+} inside the cells to elicit a contraction (64). In contrast,

during pharmacomechanical coupling, the rise in $[Ca^{2+}]_i$ is caused by a combination of Ca^{2+} release from intracellular stores and entry of Ca^{2+} through non-voltage-operated calcium channels such as ROCCs and SOCCs without membrane depolarization (although minor changes may occur) (39). ROCCs are present in different types of SM cells and are also called receptor-operated cation channels, because their properties resemble the non-selective cation channels. For example in rat ear artery, extracellular ATP activates non-selective cation conductance with significant increase in Ca^{2+} permeability (28). Similar results were also obtained with ATP in rat vas deferens and rabbit portal vein (237), (344). Subsequently it was found that the responses to ATP were similar to those produced by the activation of P2X receptors, which are classified as ligand gated cation channels (167). In addition to ATP, other agonists that bind to GPCR also activate cation current and release IP_3 followed by Ca^{2+}. Protein kinase C (PKC) can activate or inhibit ROCCs and modify non-selective cation currents (331). SOCCs are non-voltage gated channels and are activated during depletion of intracellular Ca^{2+} stores. They play an important role in re-filling the depleted intracellular stores and thereby determine the contractile state of SM cells. In addition to the above-mentioned channels, there are other Ca^{2+} channels that are activated by intracellular lipid messengers such as diacylglycerol, (144) and arachidonic acid (218) to cause Ca^{2+} influx.

1.4.2.2 Ca^{2+} release from intracellular stores

Sarcoplasmic reticulum (SR) and endoplasmic reticulum (ER) are the main physiological intracellular sources of Ca^{2+} and store most of the Ca^{2+} required for cell

signaling (304). The Ca^{2+} binding capacity of the SR is enhanced by the presence of intraluminal Ca^{2+} binding proteins such as calsequestrin and calreticulin. Release of Ca^{2+} from intracellular stores is stimulated by second messenger-activated channels (activators of Ca^{2+}) and by mitochondrial permeability. Among the various second messenger-channels, IP_3 receptors ($InsP_3Rs$) and ryanodine receptors (RYRs) are well characterized. Both $InsP_3Rs$ and RYRs act as focal points for the convergence of many signaling pathways. Following activation of the cell, the released IP_3 binds to $InsP_3Rs$ and changes the conformation of the receptor, which allows the entry of Ca^{2+} from the stores into the cytoplasm. Binding of IP_3 increases the sensitivity of the receptor to Ca^{2+}. RYRs are structural and functional analogues to $InsP_3Rs$. These receptors are mainly present in excitable cells such as neurons and muscle (cardiac). Both IP_3 and Ca^{2+} levels regulate the opening of $InsP_3Rs$. Thereby, $InsP_3Rs$ regulate the Ca^{2+} concentrations inside the cells. The opening of either RYRs or $InsP_3Rs$ initiate a brief burst of Ca^{2+} that is necessary to maintain the cell activation. With the continued presence of a stimulus, the $[Ca^{2+}]_i$ is increased, $InsP_3Rs$ or RYRs receptors exert positive feedback by enhancing the opening of channels (i.e Ca^{2+} - induced Ca^{2+}- release mechanism), but as soon as $[Ca^{2+}]_i$ reaches certain levels, the negative feedback mechanism switches on and closes the receptor channels (42), (35). This mechanism avoids the cytoplasm from being swamped with toxic doses of Ca^{2+}. The opening of these receptors is modulated by many factors such as phosphorylation, adenine nucleotides, pH and Ca^{2+} loads of SR/ER (34). In the case of mitochondria, the $[Ca^{2+}]_i$ levels are maintained by their capacity to buffer cytosolic Ca^{2+} by sequestering Ca^{2+} (222).

Ca^{2+} signals associated with the release of Ca^{2+} from intracellular stores are called by different terms. Ca^{2+} signals produced by $InsP_3Rs$ are called 'blips and puffs' (259) and those produced by RYRs receptors are called 'quarks and sparks' (69), (240). These terms represent whether Ca^{2+} release is associated with the opening of single channels ('blips and quark') or groups of channels ('puff and spark'). Characterization of these events helps us to understand how Ca^{2+} signaling occurs at two levels: (1) elementary Ca^{2+} signaling and (2) global Ca^{2+} signaling. Elementary Ca^{2+} signaling regulates local functions such as activation of ion channels and the release of synaptic and secretory vesicles. These signals have quicker effects at a low energy cost to the cells. In addition, elementary Ca^{2+} signaling also generates global Ca^{2+} signals such as Ca^{2+} waves and oscillations (41), (259). Global Ca^{2+} signals arise following coordinated recruitment of elementary Ca^{2+} release and Ca^{2+} entry from channels. This process can be achieved by setting up Ca^{2+} waves that spread throughout the cells. Global signals are necessary for controlling a diverse range of cellular processes (40). The advantage of understanding this local and global Ca^{2+} signaling is that it helps us to know how Ca^{2+} signals control different processes in the same cells

1.4.3 Signaling mechanisms for contraction

The concentration of intracellular free Ca^{2+} is the major determinant of smooth muscle contraction. Rises and falls in $[Ca^{2+}]_i$ lead to contraction or relaxation of ASM respectively. Activation of the ASM by bronchoconstrictive agents results from binding of the agonists to their relevant GPCR. The ligand-receptor complexes stimulate the associated G-protein (Gq) which further stimulates phosphatidylinositide-specific

phospholipase-C (PI-PLC). Activation of PLC is a critical step in the synthesis of different membrane-derived second messengers. There are two class of PLC which are named according to the type of phospholipids that they hydrolyse (PI- PLC and PC-PLC). PC-PLC cleaves phosphatidyl choline in to choline phosphate and 1,2-DAG, whereas PI-PLC hydrolyse the breakdown of the membrane phospholipid phosphatidyl inositol 4,5 bisphosphate (PIP_2) resulting in the production of the two second messengers, IP_3 and DAG. IP_3 diffuses through the cytosol and binds to the IP_3Rs that are present on the sarcoplasmic reticulum containing the intracellular calcium stores (SR/ER), resulting in the release of Ca^{2+}. Then, IP_3 is broken down into inactive IP_2 by IP_3 kinase and subsequently to inositol, which is re-incorporated into the plasma membrane. IP_3 may also be phosphorylated to IP_4, which may be involved in opening ROCC and refilling of intracellular stores (33). DAG, the other product of the PIP_2 hydrolysis activates protein kinase C (PKC) that is then involved in Ca^{2+} mobilization and Ca^{2+} sensitization. Several isoenzymes of PKC are known (243) but the role of these individual isoenzymes is not understood.

The release of Ca^{2+} initiates a cascade of events that leads to the contraction of ASM cells (see figure1.3). The elevated levels of $[Ca^{2+}]_i$ activate calcium/calmodulin-sensitive myosin light chain kinase (MLCK) and subsequent phosphorylation of the regulatory myosin light chain (MLC_{20}) at serine19 (299). Myosin light chain kinase (MLCK) is the rate-limiting enzyme in the contractile process. Phosphorylation of MLC_{20} allows myosin ATPase to initiate the cross bridge cycling between actin and myosin and contract the ASM (312). Myosin light chain phosphatase (MLCP) causes

the dephosphorylation of MLC_{20} and is involved in the regulation of excitation and contraction.

ASM can contract without changes in $[Ca^{2+}]_i$. Somlyo A.P, et al (305) have show that inhibition of the protein phosphatase that dephosphorylates Ser19 of MLC_{20}. increases MLC_{20} phosphorylation and hence the level of force generated at a given $[Ca^{2+}]_i$ is increased (calcium sensitization). The signal transduction pathways involved in the regulation of Ca^{2+} sensitivity appear to be quite complex and specific to smooth muscle type. Several mechanisms have been proposed: 1) Regulation of phosphatase activity, 2) direct phosphorylation of MLC_{20} by rho-kinase, 3) regulation of free calmodulin concentration and 4) regulation of thin filament-associated proteins such as caldesmon and calponin. Among these, inhibition of myosin phosphatase activity by GTPase RhoA and phosphorylation of phosphatase inhibitor CPI-17 are best studied. Rho is a small GTPase, which exhibits both GDP/GTP binding and GTPase activities (315). Following stimulation, GDP–Rho is converted to GTP –Rho which binds to specific targets and induces Ca^{2+} sensitization. Three putative targets for Rho were identified in bovine brain (p128, p138 and p164) (10), (170). p128 is identified as serine/threonine kinase , p138 as the myosin binding site (MBS) of myosin phosphatase and p164 as a Rho–kinase, which is also known as ROK (200) . In β-escin permeabilized SM strips, agonist induced contraction was increased with GTP and GTPγS at constant $[Ca^{2+}]_i$ levels. Pre-treatment of strips with the C3 exoenzyme of clostridium botulinum, also known as ADP-ribosylate rhofamily protein, completely inhibited GTPγS-mediated sensitization (109). It has also been shown that integrin linked kinase (ILK) activates SM by Ca^{2+} sensitization via phosphorylation of myosin

and activation of CPI-17 and phosphotase inhibitor (PHI) leading to inactivation of MLCP.

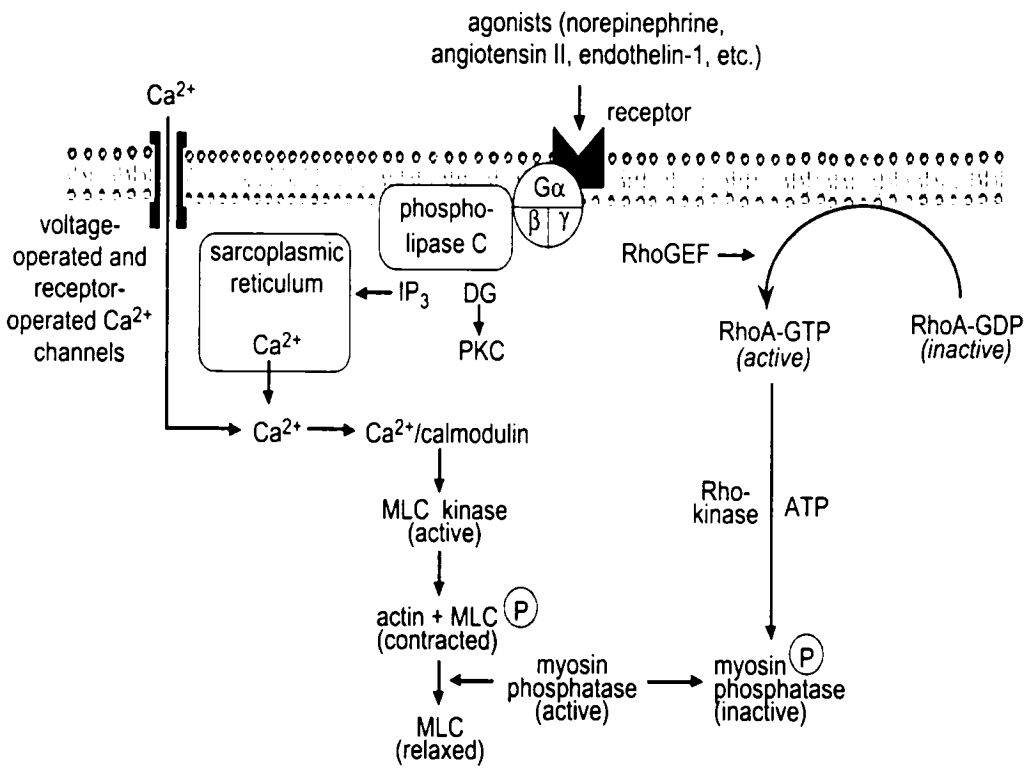
1.4.4 Smooth muscle relaxation

A fall in $[Ca^{2+}]_i$ inactivates MLCK and causes dephosphorylation of MLC_{20} by myosin light-chain phosphatase (MLCP), thereby inactivating the actomyosin ATPase and causing relaxation (90), (119). Relaxation of smooth muscle requires decreased $[Ca^{2+}]_i$ and increase MLC phosphatase activity. Decrease $[Ca^{2+}]_i$ occurs by several mechanisms; removal of Ca^{2+} by plasma membrane Ca^{2+}-ATPase (143) and Na^+ / Ca^{2+} exchanger located on the plasma membrane, hyperpolarization mechanisms, and Ca^{2+} entry blockers. Inhibition of SR Ca,Mg-ATPase activity by pharmacological agents (vanadate, thapsigargin, etc) also contribute to decreased $[Ca^{2+}]_i$. Agonists that cause relaxation initiate the signaling by binding one of the following receptors: β_2-adrenergic (131), VIP, and prostanoids (131), (257). Stimulation of adenylyl cyclase–coupled receptors results in Gsα dependent activation of adenylyl cyclase, which catalyzes the breakdown of adenine triphosphate (ATP) to cyclic-adenine monophosphate (cAMP). Cyclic AMP activates protein kinase A (PKA) leading to the dissociation of a catalytic sub-unit from the regulatory sub-unit (inhibitory). The catalytic sub-unit phosphorylates key targets within the cells and causes relaxation. There are many targets for protein kinase A (PKA) that result in relaxation of ASM. These targets are activated by cyclic-AMP dependent and independent mechanisms. Inhibition of IP_3 hydrolysis, MLCK activity, membrane hyperpolarization by increased reuptake/extrusion and alteration in phosphorylation state of the contractile apparatus are examples of cyclic AMP-

Figure 1.3

Regulation of smooth muscle contraction

Subsequent to agonist binding, the prototypical response of the cell is to increase PLC activity via coupling through a G protein. PLC produces two potent second messengers from the membrane lipid phosphatidylinositol 4,5-bisphosphate: DAG and IP_3. IP_3 binds to specific receptors on the SR, causing release of Ca^{2+}. DAG along with Ca^{2+} activates PKC, which phosphorylates specific target proteins. Activator Ca^{2+} binds to calmodulin, leading to activation of MLC kinase. This kinase phosphorylates the light chain of myosin, and, in conjunction with actin, cross-bridge cycling occurs, initiating shortening of the smooth muscle cell. The contractile response is also maintained by a Ca^{2+}-sensitizing mechanism brought about by the inhibition of myosin phosphatase activity by Rho kinase. (*From Clinton Webb, Adv. Physiol.Educ. 27, pp 201-206, 2003*).


agonists (norepinephrine, angiotensin II, endothelin-1, etc.)
receptor
Ca^{2+}
Gα
β γ
phospho-lipase C
voltage-operated and receptor-operated Ca^{2+} channels
sarcoplasmic reticulum
Ca^{2+}
IP_3
DG
PKC
RhoGEF
RhoA-GTP (active)
RhoA-GDP (inactive)
Ca^{2+}
Ca^{2+}/calmodulin
MLC kinase (active)
Rho-kinase
ATP
actin + MLC (P) (contracted)
myosin phosphatase (active)
myosin (P) phosphatase (inactive)
MLC (relaxed)

dependent mechanisms. Guanylyl cyclase also causes ASM relaxation *via* the formation of cyclic-GMP. Nitric oxide and nitrovasodilators activate guanylyl cyclase (132). K^+ channels play an important role in ASM relaxation following depolarization (36).

1.4.5 Signaling mechanisms of migration

Migration of the cell can be categorized into three forms: 1) random movement, the cells move in the absence of any external stimuli; 2) chemokinesis, random motion in response to a chemical stimulus; 3) chemotaxis, directed movement toward a gradient of a chemical stimulus. Directed movement of the cell along a concentration gradient is a complex process that involves many steps. Even though the signaling mechanisms involved in chemotaxis are well studied in highly motile cells such as *Dictyostelium*, neutrophil and macrophages, they are not well described in non-motile cells such as fibroblasts and myofibroblasts. The signaling pathways involved in motile cells are described below.

G protein coupled receptors, are mainly involved in chemotaxis because most chemoattractants bind to chemokine receptors. However, other groups of receptors such as the tyrosine kinase linked receptors activated by PDGF, nerve growth factor and fibroblast growth factor can also evoke migration of cells (126). The chemotactic ligands bind to a specific GPCR, which results in the dissociation of the G protein into α and $\beta\gamma$ subunits. Depending on the nature of stimulus and the type of cells, different subclasses of $G\alpha i$, $G\alpha 2$, $G\alpha 4$-coupled receptors are activated. It has been shown that the activation of $G\alpha i$ with $\beta\gamma$ subunits are essential for further signaling events (241). $G\alpha i$-coupled receptors along with dissociated $\beta\gamma$ subunits activate target enzymes such

as adenylyl cyclase, PLC, PI3K, and focal adhesion kinase (181), (186) which leads to an increase in levels of $[Ca^{2+}]_i$. The activation of the signaling molecules such as Ca^{2+} and cGMP leads to rearrangement of the actin–myosin cytoskeleton (63). Actin filaments (F actin), associated myosin and surface integrins are necessary for this mechanical movement. In the case of fibroblasts and endothelium, stress fibers are also involved. Actin polymerization at the leading edge results in the protrusion of the edge of the cell towards the stimulus. This process includes cell polarization, formation of pseudopodia or lamellipodia and filipodia and the attachment of cells on the surface, on which the cells are migrating (186), (220). Once the protrusion of the pseudopodia in the leading edge is formed, myosin II, which is present at the back of cell causes retraction of the posterior pole of the cell and retracts them towards the stimulus. Focal adhesions between the cell membrane and matrix transmit the force of the contractile apparatus to pull the cell forward (186). Once the polarity of the cell is stabilized, the extension of the pseudopodia is favored toward the chemoattractant and any change in the direction of the stimulus is sensed and the new leading edge is formed quickly.

1.4.5.1 Regulation of chemotaxis

Data from different studies show that several signal transduction molecules are involved in the regulation of migration. Rho, Rac and Cdc42 regulate the formation of focal adhesions, lamellipodia and filipodia, respectively (276), (179), (244). In addition, activation of ERK via Ras/Raf, MAP kinase and p38 MAPK are also implicated in signaling (174), (36), (283). Among the known signaling molecules, PI3K and phosphorylation of the downstream effector Akt/PKB (protein kinase B) are important regulators of the cellular polarization and motility of neutrophils and *Dictyostelium*

(140), (199). It has been shown that actin polymerization is initiated through the Arp2/3protein complexes along with adaptor proteins such as WASp and Scar. In addition to actin polymerization, many actin binding proteins are involved in pseudopodia formation. Myosin II assembly and motor activity are regulated by phosphorylation of myosin chains by protein kinases (69). Although the signaling pathways required for chemotaxis are known in motile cells, the downstream substrates of kinases and how the signaling pathways couple to changes in the actin cytoskeleton and the regulation of cytoskeletal proteins in non-motile cells are yet to be studied.

1.5 Chemokines and Their Receptors

Chemokines are a group of small (8-14 KD) and structurally related molecules that regulate cell trafficking of various types of leukocytes (352), (16). Two types of chemokines are known: (1) inflammatory chemokines, and (2) homeostatic chemokines. Inflammatory chemokines act mainly on inflammatory cells, such as neutrophils, monocytes, lymphocytes, and eosinophils and are produced during infection or in association with pro-inflammatory stimuli. Several types of cells such as stromal cells (204), endothelial (182), epithelial and smooth muscle cells are able to produce inflammatory cytokines. They play a fundamental role in the development of the immune response (204) by regulating the trafficking of lymphocytes to lymphoid tissues and involving them in immune surveillance. They also function to localize T or B cells with antigen in the lymphatic system. Inflammatory chemokine receptors are associated either with Th1 or Th2 phenotypes (247). CXCR3 and CCR5 are associated

with Th1 phenotypes and CCR3, CCR4 and CCR8 are associated with Th2 sub-types. Depending on the expression of the sub-types of receptors the activation status of the T cells change accordingly.

Homeostatic or developmental chemokines are constitutively produced and secreted within the lymphoid tissues. They are involved in maintaining homeostatic trafficking during non-inflammatory conditions (16) and in controlling the specificity of memory cell subsets in specific tissues (59). These chemokines are further divided into those inducing migration during immune activity and those involved in recirculation of lymphocytes (80), (287). Chemokines also have other effects on different cell types beyond the immune system, including the induction of angiogenesis, thereby involving them in tumor development, autoimmune diseases, and wound healing processes.

1.5.1 Nomenclature and classification of chemokines

So far, more than 50 different human chemokines have been identified (15). Initially, chemokines were named according to their distinctive functions. For example, monocyte chemoattractant protein (MCP-1) or regulated upon activation of normal T cell expressed and secreted (RANTES) protein. Later, additional research showed that some chemokines have more than one function and hence, their original names suggest functions that are more limited than the range of potential biological functions associated with these molecules. Therefore, a new nomenclature was proposed at a recent Keystone Symposium. (Chemokines and Chemokine receptors, Jan. 1999, in Keystone, Co). In this classification, chemokines are divided into four groups based upon their sequence homology and the position of the first two cysteine residues. The

first group of chemokines has an intervening amino acid between the first two cysteines and is called CXC or α-chemokines. The second group of chemokines with cysteines adjacent to each other without intervening amino acids is known as CC or β-chemokines. The third group, defined by three intervening residues between the first two cysteines is called CX3C. The fourth group is the XC chemokine, in which the polypeptide has only two of the four cysteines (352). So far, sixteen CXC, twenty-eight CC, two CX3C, and one XC sub-type of chemokines are known (135). CXC chemokines act mainly on neutrophils and lymphocytes while CC chemokines act on monocytes and lymphocytes, eosinophils, basophils, dendritic cells and NK cells, without affecting neutrophils (207). The CXC sub-family is further divided into two groups based on the functional differences which are dependent on the presence of ELR motif (Glu^4-Leu^5-Arg^6) preceding the first cysteine: (1) ELR-CXC chemokines (e.g CXCL-8; IL-8, GRO; CXCL1, 2 and 3, CXCL5; ENA-78, CXCL-6; GCP-2, Platelet basic protein) and (2) non-ELR-CXC chemokines (e.g CXCL-4; platelet factor-4, CXCL-10; IP-10, CXCL-9; MIG, CXCL-12; SDF-1α, SDF-1β). Like CXC chemokines, CC chemokines are also divided based on the similar functions (280). The receptors that bind to these chemokines are named as CXCR1 to CXCR6, CCR1 to CCR11, XCR1 and CX3CR1 according to specific sub-types of CXC, CC, or CX3C chemokine super family (See Table 1 and 2). A characteristic feature of the chemokines is the redundancy of the system. Redundancy occurs when multiple ligands bind to a single chemokine and multiple receptors to a single ligand leading to similar cellular responses through different pathways (223). Redundancy is specific to the subgroup of the receptors, for example, CXC chemokines bind only to CXC chemokine receptors and

CC chemokines to their receptors, because of the structural differences between the subgroup of the receptors (336).

Table 1. CXC Chemokine receptors and their ligands

Human CXC Chemokine receptors					
Receptor	CXC1	CXC2	CXC3	CXC4	CXC5
	↓	↓	↓	↓	↓
Ligands	IL-8	IL-8	SLC	SDF-1	BCAA-1
	GCP-2	ENA78	I-TAC		
		GCP-2	IP-10		
		GROα	MIG		
		GROβ			
		GROγ			
		NAP-2			

Even though CXCL16 binds to CXCR6 the specific human ligand is not yet known.

Table 2. CC Chemokine receptors and their ligands

Human CC Chemokine receptors							
Receptor	CCR1	CCR2	CCR3	CCR4	CCR5	CCR6	CCR7
	↓	↓	↓	↓	↓	↓	↓
Ligand	MPIF-1	MCP-1	MIP-1	MDC	MCP-2	MIP-3α	SLC
	HCC-1	MCP-2	Eotaxin	TARC	RANTES		MIP-3
	MIP-5	MCP-3	RANTES		MIP-1α		
	MIP-1α	MCP-4	MCP-2		MIP-1β		
	RANTES		MCP-3				
	MCP-3		MCP-4				

Receptor	CCR8	CCR9	CCR10
	↓	↓	↓
Ligand	MIP-α	TECK	CTACK/ILC,
	TARC		MEC
	I-309		

1.5.1.2 Chemokine receptors and signaling

Chemokines are derived from 3 or 4 ancestral genes and are clustered at specific chromosomal loci. CXC chemokine genes and CC chemokine genes are grouped at 4q12-13 and 17q11.2-12 respectively. Their receptors consist of 350 amino acids with molecular weights of around 40 kDa. Three-dimensional structures of chemokine receptors have not been well characterized. The extracellular domain of the receptor consists of a N-terminus and three extracellular loops that bind to the ligand, whereas the intracellular domain has a C-terminus with three intracellular loops, which are also involved in transducing signals (102).

Studies show that chemokine induced signals are initiated by receptor dimerization and activation of JAK/STAT signal pathways (279). This process is mediated by a DRY motif (specific sequence), which is conserved by several members of CXCR and CC receptors. Receptor dimerization leads to phosphorylation of the conserved DRY motif that is necessary for $G\alpha_i$ protein coupling and Ca^{2+} fluxes. Dimerization of receptors was first demonstrated in CCR2 followed by other members of CCR and CXCR families. The biological effects of the chemokines are mediated by a subset of the GPCR super family. On signaling, the main body of the chemokine agonist binds to the specific receptor. This is followed by conformational changes in the chemokine that is presumed to be largely due to the flexible N terminus (79). The conformational change allows the N terminus to make the necessary interactions with the receptor that leads to receptor activation. Following activation of the receptor, the heteromeric $G\alpha\beta\gamma$ protein dissociates into GTP-bound $G\alpha_i$ subunit and $G\beta\gamma$ subunit complex (279) as described above for other GPCRs. The majority of these responses are inhibited by pertussis

(PTX) toxin treatment indicating the involvement of $G\alpha_i$ proteins. In some studies PTX did not completely block the Ca^{2+} response indicating that these responses are also mediated through $G\alpha q$ or G_{16} depending on the type of chemokine receptor (14). Janus kinase association and tyrosine phosphorylation of the receptors recruits $G\alpha_i$ proteins to interact with the intracellular loop regions of the receptors (14) and promotes ligand binding, whereas $G\beta\gamma$ subunits stimulate PLC activation leading to formation of IP_3 and DAG followed by mobilization of $[Ca^{2+}]_i$.

Chemokines also activate different signaling pathways mediated by PI3Kγ and MAP kinase. PI3Kγ is important in both the movement and degranulation of neutrophils. The activation of the above pathways depends on the specific sub-types of the receptor activation (212). The sequence of events leads to leukocyte migration and trafficking, leukocyte degranulation, cell differentiation and angiogenesis (203), (313). Small-molecule antagonists are known for seven of the eighteen chemokine receptors. SB225002 is an inhibitor of ELR+ chemokine receptor interactions. This compound has higher selectivity for CXCR2. Many of the mutated chemokines can also act as partial or complete antagonists.

1.5.2 CXCL8 (Interleukin-8)

The discovery of neutrophil-targeted chemokine IL-8 represents a landmark in immunology, because it was the first leukocyte subtype-selective chemoattractant to be found (347). According to the new nomenclature IL-8 is referred to as CXCL8 (352). CXCL8 is a member of ELR+CXC family and has biological effects primarily on neutrophil recruitment and angiogenesis.

1.5.2.1 Structure of CXCL8

The chromosomal locus of CXCL8 is on 4q12-21 (221). The gene consists of 4 exons and 3 introns. The minimal promoter region is present between –425 and –70 bp of the gene sequence and contains several transcriptional regulatory elements such as nuclear factor kB (NF-kB), NF-IL-6, AP-1 and glucocorticoid regulatory elements (17), (18). CXCL8 protein is basic in nature and contains four cysteines and two disulfide bridges. It is synthesized as a 99 amino acid precursor and after cleavage it is secreted as a protein of 77- or 72- residues. CXCL8 is further processed at the NH_2 terminus and different truncation analogues (77-, 72-, 70-, 69- amino acid forms) are produced. Furthermore, the structure-activity studies indicate that CXCL8 binds at the N-terminal domain of the receptor (72) and causes the activation of G protein. NMR and X-ray crystallography studies have shown that CXCL8 occur as a homo-dimer with two identical sub-units, anchored by two disulfide bonds to a core structure. It contains five domains: a segment of N-terminal to the first cystine; three anti-parallel βsheets; and a α helix C-terminal at the fourth cysteine.

Generally CXCL8 is present in its monomeric form during maximal biological activity (57) but it can exist as a dimer at higher concentrations (271). CXCL8 is resistant to heat, pH changes, degradation by plasma peptidases and other denaturing conditions. However, it is rapidly inactivated by reducing the disulfide bonds.

1.5.2.2 Mechanisms of CXCL8 production

CXCL8 is produced both by leukocytic cells (monocytes, tissue macrophages, T cells, neutrophils, and natural killer cells) and non-leukocytic cells (endothelial cells,

epithelial cells, keratinocytes, synovial cells, chondrocytes, mesangial cells, fibroblastic cells, smooth muscle cells and hepatocytes) (19), (226), (157). The production of CXCL8 is induced by pro-inflammatory mediators such as IL-1, TNF-α (17), (18), TGF–β1 (105) and by other agonists such as PDGF and UDP (332). Bacteria (e.g. *H.pylori, P.aeruginosa*) (6), (86) bacterial products (LPS), viruses (e.g. adenovirus, RSV, cytomegalovirus) (7), (62), (229) viral products (X protein of human hepatitis virus B), environmental factors, hypoxic conditions (183), (345) and high glucose concentrations also induce the production of CXCL8. Neutrophils (24) and hepatoma cell lines (151) constitutively produce CXCL8. The production of CXCL8 is regulated at the level of gene transcription (151). Transcription factors such as NF-kB and activator protein-1 (AP-1) are essential for the induction of CXCL8 gene expression (285). Dexamethasone and other anti-inflammatory cytokines such as IL-4, and IL-10 inhibit the production of CXCL8.

1.5.2.3 Biological activities

CXCL8 is mainly involved in inflammation and host defense processes. It causes trans-migration of neutrophils into tissues such as endothelium (150) pulmonary epithelium and vascular smooth muscle (228). CXCL8 has actions on many different target cells and mediates a wide variety of effects (see Table 3) (225), (250), (61). Elevated levels of CXCL8 in bacterial and viral infections suggest that it plays an important role in the regulation of the host response to these infections (242). CXCL-8 is also involved in the pathogenesis of many inflammatory and autoimmune diseases of skin (psoriasis, atopic and contact dermatitis), bone and joints (arthritis) (49; 188), (296),

blood and synovial fluids (rheumatoid arthritis) (115), kidney (glomerulonephritides), GI tract (ulcerative colitis), central nervous system (meningitis, stroke) and heart (atherosclerosis, and myocardial infarction) (330). In lung, it is involved in CF, bronchitis, COPD (346), (23), acute respiratory distress syndrome (ARDS), persistent asthma (114), occupational asthma (201) and idiopathic pulmonary fibrosis.

1.5.2.4 CXCR1 and CXCR2 receptors

CXCR1 and CXCR2 bind to CXCL8 with high affinity (146), (233) and different specificity. They are the first chemokine receptors that have been identified and are the only known mammalian receptors for CXCL8. These are the major chemokine receptors expressed in neutrophils (232). CXCR1 and CXCR2 are also present on monocytes, lymphocytes, CD8+ T cells, cultured melanocytes, fibroblasts (224) epithelial cells in inflamed skin (292) and smooth muscle cells in burns (238).

Both CXCR1 and CXCR2 consist of 350 and 360 amino acids, respectively and have 80% amino acid sequence homology, except in their NH_2- terminal regions. The differences in the NH_2- terminal regions affect their binding specificities (231). Hebert et al (136) had shown that both CXCR1 and CXCR2 conserve the Glu^4-Leu^5-Arg^6 sequence (ELR motif), which is a characteristic feature for the members of this family. This ELR sequence is necessary for the biological activation of these receptors.

Both CXCR1 and CXCR2 were cloned from rabbit (233). CXCR1 is more selective for CXCL8 than other chemokines such as CXCL6, which has low binding affinity (231) whereas CXCR2 is more selective for CXCL1, CXCL7, CXCL5, than CXCL8 (5) (230), (342).

Table 3. Actions of IL-8 on their target cells

Target cells	Effects
Neutrophils	Chemoattraction, Release of lysosomal enzymes Induction of shape changes, respiratory burst, generation of superoxide, H_2O_2, bioactive lipids (LTB4), ↑ expression of adhesion molecules, ↑ adhesion of endothelial cells, ↑ transendothelial migration Phagocytic capacity
T cell	Chemoattraction
Eosinophils	Chemoattraction
Basophils	Histamine release, Leukotriene release
B cells	decrease in IgE production
Fibroblast	Inhibition of collagen expression
Endothelial cells	Chemoattraction (angiogenesis)

Reference (282)

Human tyrosyl tRNA synthetase, a non-chemokine ligand also acts on CXCR1 (328). Both CXCR1 and CXCR2 undergo homologous and heterologous desensitization (8). The internalization mechanisms are different for CXCR1 and CXCR2 receptors. The internalization of CXCR1 is dependent on phosphorylation by GRK-2 (21) whereas the internalization of CXCR2 is independent of phosphorylation by GRK-2 (348). Since desensitization and internalization of CXCR2 occur faster than desensitization of CXCR1 at lower concentration of CXCL8, CXCR1 plays an important role in inflammation where higher levels of CXCL8 are present (71).

1.5.2.5 Signaling mechanisms for CXCL8

The signals for CXCL8 are mediated by GPCR through CXCR1 and/or CXCR2. It has been shown that CXCR1 and CXCR2 couple to G_i2, G_i3, Go, G15 and G16 subunits (341). The receptor-ligand complexes activate PLC, which catalyzes the synthesis of IP_3 and DAG followed by an increase in $[Ca^{2+}]_i$. Although some Ca^{2+} independent CXCR activation occurs, Ca^{2+} signals are the major intracellular events (103). CXCL8 activates PI3K-γ (298) which in turn generates PIP_3. PIP_3 activates protein kinase B (Akt) as well as GTPase, resulting in cell migration. Other signaling molecules such as phospholipase-D (PLD), small GTPase Rho/Ras, and tyrosine kinase linked MAPK, ERK (174) are also activated by CXCL8. Activation of small GTPase Rho/Ras regulates cytoskeletal rearrangements and induction of the respiratory burst in neutrophils (38). Druey et al (334) have shown that RGS protein reduces CXCL8-mediated migration and adherence indicating that desensitization of CXCRs occurs.

Following CXCL8 binding, the receptors are internalized, recycle and reappear on the cell surface within 60 minutes (288).

1.6 Purinergic receptors

Adenosine 5'- triphosphate (ATP) nucleotide plays a key role in cellular metabolism by acting as an enzyme cofactor. It is mainly used as a source of cellular energy during the biosynthesis of proteins, carbohydrate, nucleic acids, phospholipids and metabolism of protein and phosphorylation reactions. ATP is synthesized by the addition of a phosphate group to adenosine diphosphate (ADP) during oxidative phosphorylation, glycolysis, and photosynthesis. Furthermore, it was generally believed that the release of ATP into the extracellular milieu rarely occurred, that cells stored their ATP intracellularly and did not, at all costs, allow ATP to cross the plasma membrane of the viable cells. Subsequently, it was shown that ATP was present in variable amounts in the extracellular space and that it could influence many biological processes at low micromolar concentrations. There are many sources for extracellular nucleotides: cell lysis (122), transmembrane transport *via* specific transporter proteins (294), discharge of the contents from vascular endothelium and smooth muscle cells in culture (264), and degranulation from platelets, neurons, and cells of the adrenal medulla. In addition, hypoxia / ischemia, shear stress forces, stretching, osmotic swelling and mechanical stimulation (change of cell culture medium) also trigger the release of ATP (124), (192). Adrenal chromaffin granules, platelets, mast cells, erythrocytes, basophilic leukocytes, cardiomyocytes, fibroblasts, endothelial cells and

epithelial cells (65), (44), (98), (107), (123), (274), (252), (107) are other important sources of ATP that can be released under various physiological and pathological conditions. Several mechanisms have been proposed for the release of the cytosolic contents of ATP that include ATP binding cassette (ABC) transporters and gap junction–type hemi-channels. The concentration of the pericellular ATP easily can go up to the micromolar range and a significant portion can be released from the cells without loss of viability (187).

1.6.1 Effects of extracellular ATP

The extracellular actions of purine nucleotides were first recognized about seventy years ago in mammalian heart.. After that, until the 1960s, research was mainly focused on the actions of adenosine nucleotides on the cardiovascular system. In 1959, Holton (147) showed that during antidromic stimulation of sensory nerves, ATP was released in sufficient quantities and its degradation products to produce vasodilatation of rabbit ear arteries. In the 1970s Burnstock (50), (54) proposed that ATP is released from autonomic nervous system and called this branch of the nervous system 'purinergic'. Further experimental evidence strengthened this concept and demonstrated the role of ATP as a neurotransmitter or co-transmitter with norepinephrine, acetylcholine, glutamate, and neuropeptide Y. Even though initial studies showed the effects of extracellular nucleotides on the cardiovascular system, later studies showed its effects in a wide range of biological systems from single cells to whole organisms. These include endothelial dependent vasodilatation (83) smooth muscle contraction, proliferation and migration (216), (214), (67), (163). It is also involved in exocrine and

endocrine secretions (239), immune responses (291), (58), mast cell activation, inflammation, metastasis formation, platelet aggregation (43), pain (53), pulmonary function and neurotransmission in peripheral and central nervous systems (52).

1.6.2 Extracellular UTP

Uridine triphosphate (UTP) is a pyrimidine nucleotide and is synthesized by two different pathways in mammalian cells; (1) the *de novo* pathways, where, uridine mono phosphate (UMP) is synthesized from glutamine and aspartate in the presence of enzymes such as dihydro orotase (DHO) synthase and UMP synthase followed by conversion of UMP to UTP. Some cells use orotate for UTP synthesis; (2) in salvage pathways, UTP is synthesized by either deamination or phosphorylation of cytidine and uridine in the presence of monophosphate kinase. Although the measurement of UTP in free plasma was difficult due to hydrolysis of nucleotides, the measurement of micromolar concentrations of uridine as an indirect measurement of UTP in normal human plasma indicates that the release of UTP may occur physiologically. Later on development of methods such as high pressure liquid chromatography (HPLC) to purify nucleotides and their analogues allowed the measurement of UTP concentrations in extracellular fluids (191), (196). UTP is released into the bloodstream under various physiological and pathological conditions such as exercise, tissue hypoxia, inflammation, hemolytic anemia, mechanical stimulation or biological activation of astrocytoma cells, epithelium and endothelium (190), (197), (97). UTP has many known functions. The effects of UTP on the cardiovascular system have shown that it mediates endothelium-dependent relaxation of vascular beds, enhances vascular smooth muscle

cell growth (45), (324), (100). It also stimulates sympathetic neurons, enhances mucociliary clearance of the airways through the stimulation of Cl^- channels and mobilizes intracellular Ca^{2+} from different types of cells (249), (295).

1.6.3 Metabolism of the extracellular triphosphate nucleotides

Extracellular ATP is degraded into adenine diphosphate (ADP), adenine mono phosphate (AMP), and adenosine by a family of ectonucleotidases (351), which limits the actions of extracellular nucleotides by enhancing their removal. Ectonucleotidases are classified into two families based on their molecular characterization and distinct properties (350). The first family is called E-NTPDase or CD39, exhibits ectopyrase or ectonucleotide diphosphatase activity and hydrolyzes either nucleotide 5'-triphosphate or both nucleotide 5'-tri- and diphosphates. These enzymes are divided into many groups according to their phylogenetic relationships. Enzymes from the second family are called as E-PDNP (ecto-phosphodiesterase/nucleotide pyrophosphatase) and catalyze the cleavage of ATP into AMP and pyrophosphate as well as several other reactions (ATP- ADP, ADP-AMP and AMP-ADO).

An important difference between purine and pyrimidine metabolism in mammals is that pyrimidines are recycled from nucleosides, mainly from uridine, whereas purines are recycled from their bases (78). Inter-conversion of adenine and uridine nucleotide has been shown in 1321N human astrocytoma cells. Nucleoside diphosphokinase catalyses the transfer of phosphate from nucleoside diphosphate to nucleoside triphosphate and *vice versa*, e.g in the presence of ATP, nucleoside

diphosphokinase catalyses the conversion of uridine diphosphate (UDP) to UTP (194) or ATP to ADP;

$$ATP + UDP \xrightarrow{\text{Nucleoside diphosphokinase}} ADP + UTP.$$

1.6.4 Extracellular nucleotide receptors and their classification

Purine and pyrimidine nucleotides mediate their effects by interacting with the distinct cell surface receptors called purinergic receptors. In 1980, Burnstock proposed a classification of purinergic receptors (51). These receptors are divided into two main families: the P1 receptors and the P2 receptors.

1.6.4.1 P1 receptors

P1 or adenosine receptors are classified according to their distinct molecular structure, pharmacological profiles, and tissue distribution. These receptors have been cloned from different species and are characterized into five sub-types, A_1, A_{2A}, A_{2B}, A_3 and A_4. Adenosine receptors are G-protein coupled receptors. These receptors are distinct from each other, lacking consistent shared amino acid sequence homology (e.g. the homology between A_1 and A_{2b} is only 45% (307). Even though adenosine is a potent agonist for these receptors, ATP and metabolically stable ATP derivatives (adenosine 5'-O-(3-thiotriphosphate, ATPγS and β,γ–meATP) can act directly as agonists on P1 receptors in some tissues such as rat colon smooth muscle (20). Xanthines and xanthine derivatives including natural derivatives such as theophylline and caffeine act as non-selective adenosine receptor antagonists (269).

A_1 receptors are sub-divided into $A1_a$ and $A1_b$ receptors (127). A_1 receptors mediate a broad range of signaling responses. The most widely recognized signaling pathway for the A_1 receptor is the inhibition of adenylate cyclase causing a decrease in the intracellular concentrations of the second messenger cAMP (325) coupling through $G_{i/o}$ protein α-subunits. Other pathways include the activation of PLC (154), PKC, PLD (113) opening of K^+ channels (26) and inhibition of Ca^{2+} currents (108). CCPA (2-chloro-N6-cyclopentyladenosine) is the most potent selective agonist for the A1 subtype. Modifications of methylxanthines, at certain positions can make them potent antagonists of A1 receptors (322).

A2 receptors are sub-divided into $A2_a$ and $A2_b$. The most commonly recognized signal transduction mechanism at the A2 receptor is the activation of adenylyl cyclase by coupling through Gs. A2a receptors are restricted to tissues such as immune cells, platelets, CNS, vascular smooth muscle and endothelium. A2b receptors are present on all cells in most species and have a wide variety of biological functions. These receptors couple to $G_{q/11}$ and mainly regulate PLC activity. NECA (5'-N-ethylcarboxamido-adenosine) is a potent agonist at A2 receptors.

A3 receptors have been cloned from rat. They are distributed in all tissues but the highest levels are found in lungs and liver. These receptors couple to Giα, 2-Gi α3 and to lesser extent, to $Gq_{/11}$ and stimulate PLC, thereby elevating IP_3 levels and intracellular Ca^{2+}. CI-IB- MECA is a potent agonist at A3 receptor.

1.6.4.2 P2 receptors

P2 receptors bind to ATP, ADP, AMP, UTP, UDP and their analogues. Burnstock suggested the sub-classification of P2 receptors into P2X and P2Y (55). P2X are ligand–gated ion channels (134), (28) and P2Y are G-protein coupled receptors (91), (3). P2 receptors were initially classified on the basis of the differences in rank-order potency of nucleotides and their analogues (pharmacological nomenclature). Subsequently, pharmacological nomenclature was replaced by the current nomenclature that is based on molecular structure and signal transduction mechanisms.

1.6.4.3 P2X receptors (Ionotropic ligand-gated channels)

Currently there are 7 sub-types of P2X receptors (P2XRs) exist and they have been cloned from human and rat. P2XRs have intrinsic ion channels opened by micromolar concentrations of extracellular nucleotides. These receptors consist of 379-472 amino acids that are inserted into the membrane to form a pore comprising two hydrophobic domains with short intracellular N- and C-termini and an extensive extracellular loop (see figure 1.4).

$P2X_1R$ is a selective cation channel and was first cloned from rat, human and mouse in 1994 (47). It is activated by 2MeSATP ≥ ATP>, α,β–met-ATP > ADP, UTP and GTP. This receptor desensitizes very rapidly. $P2X_2R$ was cloned from rat and displays only 40% homology with $P2X_1R$ (46). Two other splice variants are known for this receptor (301). α,β–met-ATP is the most potent agonist followed by other agonists such as ATP, ATPγS and 2–MESATP. This receptor undergoes little or no desensitization. $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$ receptors were also cloned from rat

and respond to a 2MeSATP, ATP and α,β–met-ATP. Many selective P2X sub-type antagonists are present. These antagonists are analogues of either suramine or pyridoxal phosphate-6-azophenyl 2', 4'-disulphonic acid (PPADS). Coomassie brilliant blue-G is the most potent antagonist for $P2X_7$ and $P2X_4$ subtypes.

P2XRs mediate rapid non-selective transport or diffusion (within seconds) of cations (Na^+, K^+ and Ca^{2+}) and increases Ca^{2+} influx followed by depolarization (25), (92). The direct Ca^{2+} influx following secondary activation of voltage-dependent Ca^{2+} channels in the presence of depolarization does not involve any second–messengers. Hence, the response time is very rapid and is appropriate for fast neuronal signaling. P2XRs are distributed in central and peripheral nervous system and are mainly involved in activity of neurons. P2XR are also expressed in vascular smooth muscle and are involved in the opening of the voltage-dependent Ca^{2+} channels, Ca^{2+} entry and contraction of smooth muscle.

1.6.4.4 P2Y receptor (G-Protein coupled receptors)

At present, 8 subtypes of P2YRs ($P2Y_{1, 2, 4, 6, 11, 13,}$ and $_{14}$) are known (173), (1). These include selective purinergic receptors ($P2Y_1$, $P2Y_{11}$), selective pyrimidinergic receptors ($P2Y_6$ and $P2Y_{14}$), mixed selective ($P2Y_2$ and $P2Y_4$, although species-dependent) and selective ADP activated ($P2Y_{12}$) receptor. $P2Y_1$Rs were cloned from different species such as chicken, turkey, mice, rat, bovine, and human (261). $P2Y_2$R, $P2Y_4$ R and $P2Y_6$ Rs were cloned from human and rat (68), (260), (77), (37), (66), (76). Other subtypes such as $P2Y_3$R, $P2Y_5$R, $P2Y_7$R, $P2Y_9$R, and $P2Y_{10}$R have been cloned from non-mammalian vertebrates. They do not have any known functional role in the

presence of the nucleotides. $P2Y_3R$, represents a species homologue of the human $P2Y_6$ R. $P2Y_7R$, is a leukotriene B4 receptor whereas $P2Y_5R$ and $P2Y_9R$ and $P2Y_{10}R$ are considered as orphan receptors (171). Alignment of the amino acid sequences of these cloned P_2YRs show 20-57% of identity among the members of the family.

1.6.4.5 Structure of the P2Y receptors

P2Y receptors (P2YRs) vary in length ranging from 308-379 amino acids, with molecular weight of 41-53 kDa in glycosylated form. They belong to the seven trans-membrane-spanning α-helical hydrophobic regions of the GPCR. The NH_2 terminal of the receptor is on the extracellular side of the plasma membrane, whereas the COOH terminus lies on the cytosolic side of the membrane (see figure 1.5). The three dimensional orientation of the trans-membrane domain creates the pocket at which the positively charged amino acids interact with the phosphate groups of the ligands (ATP/ADP/UTP/diadenosine polyphosphate). The binding sites have been identified on the 6th and 7th trans-membrane domains.

1.6.4.6 Ligands for the P2YR subtypes

The identification of the P2YRs agonists and characterization of their rank order of potency is complicated by factors such as cross contamination of nucleotides preparation, degradation by ectonucleotidesase and inter-conversion between adenosine and uridine nucleotides (193). Therefore, ligands to the specific receptors were confirmed by the studies performed with HPLC- purified nucleotides, by adding hexokinase to the medium in order to consume extracellular ATP. ADP is a selective

physiological agonist to $P2Y_1R$ (137), and ATP is partial agonist, the intrinsic activity depends on the size of the receptor reserve (253). In platelets, ATP antagonizes the action of ADP and these actions are mediated by competitive inhibition of ADP receptors (121). ATP, UTP and UTPγS are equipotent (195) on $P2Y_2$ R (human, rat and mouse) whereas ATPγS is less potent than ATP and UTP (202). 2Me-SATP and α,β-meATP are weak or inactive agonists. $P2Y_4R$ is activated by UTP and not by UDP. ATP is a full agonist on rat and partial agonist on human $P2Y_4R$ (165), (139). UDP is most potent to $P2Y_6R$ followed by UTP. $P2Y_{11}R$ is mainly selective for ATP >2MeSATP> ADP where as UDP and UTP are inactive (75). $P2Y_{12}$ R is selective for ADP. UDP-glucose acts on $P2Y_{14}$ R (2).

1.6.4.7 Antagonists for P2Y receptors

Non-selective inhibitors such as suramine, reactive blue and PPADS can be used to antagonize either or both P2YRs and P2XRs. Only few sub-types of the P2YR have specific antagonists. The most potent $P2Y_1R$ antagonists are structural analogues of ADP (e.g. MRS2179 and MRS2279) (172) and $P2Y_{12}R$ has a structural analogue of ATP (AR-C67085 and AR-C69931) as an antagonist. All other sub-types do not have any potent and selective antagonists, although suramine ($P2Y_2R$, and $P2Y_{11}R$) reactive blue 2 ($P2Y_4R$ and $P2Y_6R$) and PPADS ($P2Y_4R$, $P2Y_6R$, and $P2Y_{11}R$) act as partial antagonists. ATP itself is considered to be a potent antagonist at rat $P2Y_4R$ and competitive antagonist with moderate potency at human $P2Y_4R$ (164).

Figure 1.4

Schematic diagram of $P2X_2$ receptor showing both N and C terminals

Diagram of proposed transmembrane topology for $P2X_2$ receptor showing both N- and C-terminals in the cytoplasm. Two putative membrane spanning segments (M1 and M2) traverse the lipid bilayer of the plasma membrane and are connected by a hydrophilic segment of 270 amino acids. This putative extracellular domain is shown containing two disulphide-bonded loops (S-S) and three *N*-linked glycosyl chains (triangles). (*From Burnstock G. Neuropharmacology, vol 36, pp 1127-1139, 1997.*)

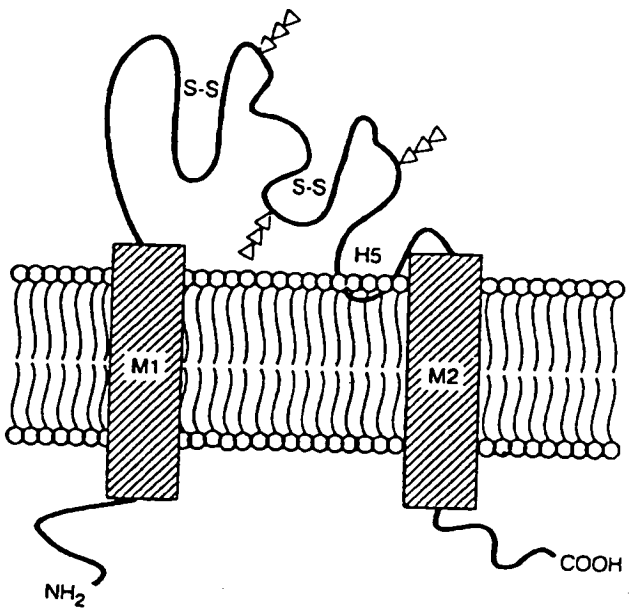
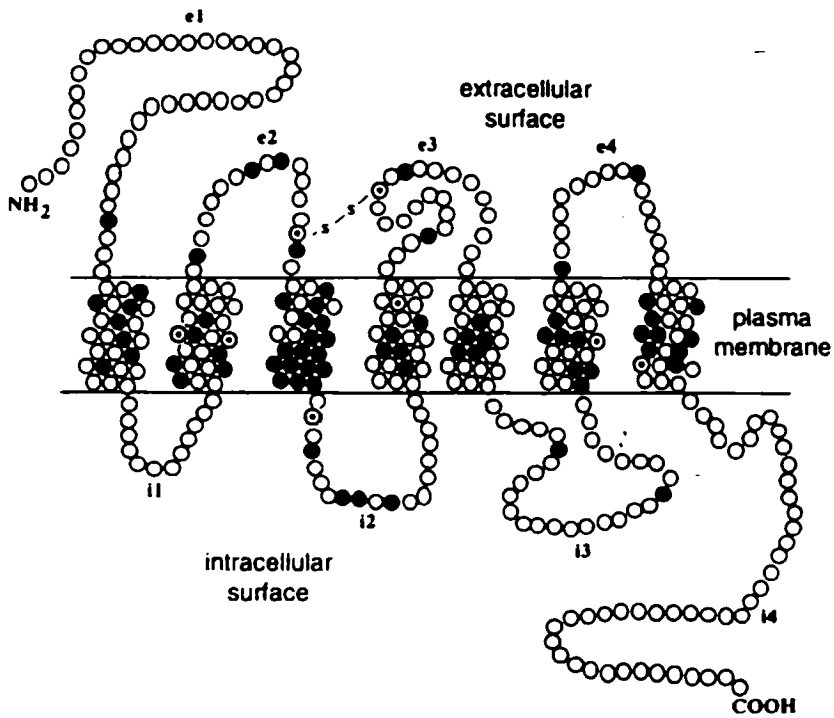

S-S
S-S
H5
M1
M2
NH2
COOH

Figure 1.5

Schematic diagram of the sequence of the P2Y 1 receptor

Diagram of the sequence of the P2Y1 receptor showing seven transmembrane domains with an extracellular N-treminus and an intracellular C-terminus. (*From Burnstock G. Neuropharmacology, vol 36, pp 1127-1139, 1997.*)


e1
extracellular
surface
e2
e3
e4
NH2
s
s
plasma
membrane
i1
i2
i3
i4
intracellular
surface
COOH

1.6.4.8 Desensitization

P2Y receptors do not desensitize rapidly. However, maximum desensitization of the $P2Y_2R$ was observed at 5 to 10 minutes in different cell types after UTP exposure and the receptor responses recovered after removal of the agonist (112). The mechanism for the desensitization is not well understood, but it may involve receptor phosphorylation by protein kinase and uncoupling of receptors from the associated G-protein.

1.6.4.9 Signaling pathways mediated through P2YRs

P2YRs are coupled to GPCR and different sub-types interact with different sub-units of the G protein through their intracellular loops. On signaling, G-proteins activate phosphoinositol specific PLC, resulting in mobilization of Ca^{2+} from calcium stores. The sensitivity to and the degree of inhibition by pertussis toxin (PTX) are variable from one sub-type to another. $P2Y_1R$, $P2Y_6R$ and $P2Y_{11}R$ are not inhibited by PTX treatment (335), (74) whereas $P2Y_2R$ and $P2Y_4R$ are partially inhibited (99). In gastric smooth muscle cells, the ATP/UTP induced activation of PLC was partially inhibited by antibodies against $G\alpha q/_{11}$ or $G\beta$, while the combination of the two produced complete inhibition (234). Similarly a complete inhibition was obtained by combining the $G\alpha q/_{11}$ antibody and PTX treatment. Combining antibodies against specific G proteins and PLC led the conclusion that $P2Y_2R$ coupled to the PLC $\beta 1$ via $G\alpha q/_{11}$ and to PLCβ-3 via $G\beta\gamma_{i3}$.

Depending on the sub-type of the receptors and the cells that express the receptors, several intracellular signaling pathways are activated. These include PLC,

PLA_2, PLD and PKC (56), (273). In addition, ATP also activates and/or inhibits adenylyl cyclase through direct or indirect mechanisms. The direct activation of adenylyl cyclase was shown by its action on human $P2Y_{11}$ receptors in platelets (73). The indirect stimulation of adenylyl cyclase occurs following the activation of A2 receptors by adenosine formed during ectonucleotidases activity.

Extracellular ATP is also involved in the activation of other signaling pathways such as Rho-dependent kinase (ROCK), ERK1 and ERK2 MAP kinases. In platelets, ADP induced shape changes were inhibited by selective Rho-dependent kinase inhibitor Y-27632 (263). In vascular endothelial cells (262), (338) and in vascular smooth muscle cells, ATP/UTP induced the tyrosine phosphorylation and the activation of ERK1 and ERK2, MAP kinases through its action $P2Y_2$.

1.6.4.10 Biological effects of P2YRs

$P2Y_1R$ are distributed in heart, immune and neural tissues and are co-expressed with $P2Y_{12}$ receptors in platelets. Activation of $P2Y_1R$ regulates the vascular tone either by contraction or relaxation. $P2Y_1R$ present on the endothelium and SM mediate vasodilatation (162), by Ca^{2+} dependent activation of endothelial derived relaxing factors (ERDF) from endothelium. These receptors also mediate insulin and renin secretion, platelet shape changes, aggregation and raise $[Ca^{2+}]_i$ in cells such as epithelium, smooth muscle cells and hepatocytes (209), (217). $P2Y_2R$ are present in wide variety of tissues. In endothelium, activation stimulates the release of prostacyclin and NO, leading to vasodilatation (272) whereas in smooth muscle, it causes contraction by mobilizing the $[Ca^{2+}]_i$. In neutrophils, it causes degranulation and induces superoxide

production (184). In human nasal mucosa, it activates Ca^{2+} dependent Cl^- channels and increases the fluid secretion (210). Uridine nucleotide-specific receptors, $P2Y_4R$ and $P2Y_6R$ mediate metabolic effects, contractile responses of vascular smooth muscle and hemodynamic effects. They also enhance smooth muscle cell growth.

Differential expression of purinoreceptor sub-types and ligand—receptor signaling allows the generation of very specific physiological responses in particular cells or tissues. It is for these reasons that nucleotide and nucleoside receptor have now become therapeutic targets for the treatment of conditions such as CF, pain, urinary incontinence, stroke and depression.

1.7 Summary

CF patients have airway hyperresponsiveness (AHR) and higher levels of IL-8 in their airways (178). In addition, CF airways are denuded of epithelium due to chronic infection and inflammation. This exposes ASM to IL-8. So, we hypothesize that IL-8 may act on ASM and cause or contribute to AHR in CF airways. Furthermore, CF patients are treated with aerosolized ATP and UTP to improve their airway mucociliary clearance. These nucleotides are also shown to induce $[Ca^{2+}]_i$ in rat ASM through P2Y receptors and cause contraction. Since IL-1β has been shown to up-regulate P2Y receptors in vascular smooth muscle cells, it is also possible that IL-8 may up-regulate P2Y receptors in CF ASM and enhance the contraction in response to ATP and UTP. Therefore, we investigated the expression of CXCRs and the effects of IL-8 on $[Ca^{2+}]_I$ release, contraction and migration of HASM compared the effects IL-8

between normal and CF ASM cells, studied the expression of P2YRs in normal HASM and the effects of ATP and UTP on $[Ca^{2+}]_I$ release.

Chapter 2, describes the study on the expression of CXCRs and the effects of IL-8 on normal HASM cells. Chapter 3 compares the effects of IL-8 induced contraction between CF and normal ASM cells. Chapter 4 explore the effects of Ca^{2+} release in response to ATP and UTP and the expression of various subtypes of the P2YR in normal HASM cells. Chapter 5 concludes with the general summary and discussion.

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CHAPTER 2

INTERLEUKIN-8: NOVEL ROLES IN HUMAN AIRWAY SMOOTH MUSCLE CELL CONTRACTION AND MIGRATION

2.1 Prologue

Airway hyperresponsiveness (AHR) is observed in patients with chronic asthma, CF and COPD. These patients have elevated levels of interleukin-8 in their airways due to chronic infections and neutrophilic inflammation of the airways. In addition, pro-inflammatory agents such as TNF-α and IL-1β have been shown to stimulate the production of IL-8 from ASM cells, raising the question of possible autocrine and paracrine actions of IL-8. However, it is not known whether IL-8 has effects on ASM cells. We reasoned that it was likely that IL-8 acted on these cells and that characterization of IL-8's actions on normal ASM cells would help us to better understand its potential role in dysfunction of diseased airways. We were particularly interested in effects of relevance to contraction of ASM and remodeling of ASM tissue through migration of these cells from sites of proliferation. We have therefore investigated the expression of the CXCR receptors in ASM cells from normal subjects and the effects of IL-8 on $[Ca^{2+}]_i$ as well as contraction and migration of ASM cells.

2.2 Abstract

Patients with cystic fibrosis (CF) and severe asthma suffer from airway hyperresponsiveness and elevated levels of interleukin-8 (IL-8) are found in their airways. IL-8 is a CXC chemokine that is chemoattractant for neutrophils through CXCR1 and CXCR2, G-protein coupled receptors. We hypothesized that IL-8 may affects airway smooth muscle cells (ASMC) directly to cause contraction and migration. The aim of this study was to determine if human ASM (HASMC) express functional IL-8 receptors (CXCR1 and CXCR2) linked to contraction and migration. Experiments were conducted on cells harvested from human lung specimens. Real time PCR and FACS analysis showed that HASM cells expressed mRNA and protein for both CXCR1 and CXCR2. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) increased from 93.3 nM to 273 nM in response to IL-8 (100 nM) and this response decreased following inhibition of phospholipase C (PLC) with U-73122. On blocking the receptors with specific neutralizing antibodies, changes in $[Ca^{2+}]_i$ were abrogated. IL-8 also contracted the HASM cells, decreasing the length of cells by 15% and induced a 2.5 fold increase in migration. These results indicate that HASMC constitutively express functional CXCR1 and CXCR2 that mediate IL-8 triggered $Ca^{2+}{}_i$ release, contraction and migration. PLC mediated signaling pathways are involved in Ca^{2+} signaling. Both subtypes of receptor contribute to the intracellular signaling that increases the $[Ca^{2+}]_i$.

2.3 Introduction

Interleukin-8 (IL-8) is a member of the CXC chemokine sub-family of cytokines and acts as a key mediator in the migration of neutrophils, monocytes (47), and eosinophils (49) to the site of inflammation, injury and infection. In neutrophils, IL-8 induces shape changes, exocytosis of stored proteins and the respiratory burst, resulting in the release of superoxide anion and hydrogen peroxide (6). IL-8 is also involved in wide variety of physiological and pathological processes, including host defense against bacterial infection, angiogenesis, arteriosclerosis and autoimmune disorders of skin, bones, and joints (18), (20). In addition, elevated concentrations of IL-8 are found in pulmonary diseases such as severe asthma, occupational asthma (19), (29), cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), (60), (8), bronchitis (1), acute respiratory distress syndrome (ARDS) and idiopathic pulmonary fibrosis. The potential sources of IL-8 in the airways, include neutrophils, airway epithelial cells (30), fibroblasts (44), mast cells (32), and macrophages (52).

IL-8 mediates its effects through the activation of specific G protein-coupled receptors (27). Two sub-types of receptors have been described, CXCR1 (23) and CXCR2 (37). CXCR1 principally binds IL-8 with high affinity, whereas CXCR2 binds to IL-8 and other CXC chemokines, such as GRO-α, GRO-β, GRO-γ, NAP-2 and ENA-78 (33), (17), (2). In neutrophils, IL-8 activates the phospholipases C (PLC) (58), phospholipases D (PLD) (28) and mobilizes intracellular calcium. IL-8 has actions on different cells and tissues (45). Similar actions in airway smooth muscle (ASM) would be expected to result in contraction.

CF and chronic asthmatic patients have narrowing of the airways and reversible airway obstruction in response to nonspecific irritants and inflammatory mediators (13), (26), (5), (54). Chronic infections and inflammation of the airways, damage the airway epithelium and increase the thickness of the ASM (14), (53). ASM has the ability to contract in response to many contractile agents (e.g: acetylcholine, histamine, leukotriene D_4, etc) and this response is enhanced by cytokines such as IL-1β, IL-13, IL-5, IL-10, TNFα and granulocyte-macrophage colony stimulating factor leading to reduction in airway caliber (3), (4), (34), (48). Recently, the property of cellular migration in response to agonists has been proposed as one of the mechanisms of the increase in ASM mass observed in asthma and other diseases associated with ASM hyperplasia (25), (35). Given the signal transduction pathways activated by IL-8, an excess of IL-8 in the airways might be expected to have stimulatory effects on ASM functions, including contraction and migration. We hypothesized that IL-8 has pro-asthmatic effects by increasing the tone of ASM and has potential to promotes airway remodeling through effects on ASM cell migration.

2.4 Materials and Methods

Cell cultures

Bronchial specimens were obtained from both donors and recipients lung transplants. Specimens were transferred in the Hanks' balanced salt solution (HBSS). Primary cultures of HASM cells were prepared from surgical specimens as previously described (38). Tissue digestion was performed by incubating the samples for 90 minutes at 37° C in 10 ml of HBSS to which 640 U collagenase type IV, 10 mg soybean trypsin inhibitor and 100 U elastase (type IV) had been added. The digested tissue was then filtered through a 125 μm Nytex mesh and the resulting cell suspension centrifuged. The pellet was then reconstituted in growth medium (Clonetics, California, USA) and plated in 25-cm^2 flask. Once the cells were grown to confluence, they were detached with 0.025% trypsin -0.02 % ethylenediaminetetraacetic acid (EDTA). The cells were plated at the density of 5000 cells/ml and grown on 25mm glass coverslips for single cell imaging of calcium transients, contraction studies and on 6 well culture dishes for RNA extraction, flow cytometry (FACS) and chemotaxis assays. Confluents cells from 1^{st} to 4^{th} passage were used.

Detection of mRNA expression of CXCR1 and CXCR2 by real time PCR

HASM cells were grown for 90 to 95% confluence and total RNA was extracted from by TRIzol reagent (Gibco BRL) according to the manufacturer's instructions. To analyze messenger RNA (mRNA), single strand cDNA was synthesized in a 20 μl reaction using 2 μg of total RNA as a template, oligo $(dT)_{12-18}$ primer and superscript™

II enzyme in the presence of acetylated BSA (Gibco BRL, Burlington, ON, Canada) and RNAguard ribonuclease inhibitor (Pharmacia Biotech, Quebec, Canada). PCR reactions were performed (Light Cycler: Roche Molecular Biochemicals) with 20μl of reaction mixture containing 2μl of cDNA, 4μl of both sense and antisense primers for CXCR-1, CXCR-2, (Sequences, CXCR-1: sense-5' ATGTCAAATATTACAGATCC, antisense-3'AGATTCATAGACAGTCCCCA and CXCR-2: sense-5'GAGGACCCAGGTGATCC AGG, antisense-3'GAGAGTAGTGGAAGTGTGCC) or house keeping gene (β-actin), 1.6 μl $MgCl_2$ (3 mM) and 2μl of fast start DNA SYBR green with Taq polymerase. The following PCR cycling regime was performed: 45 cycles of 95°C for 10 min of denaturation followed by PCR at 95° C for 15 sec, annealing temperature (CXCR-1: 57°C, CXCR-2: 58°C and β-actin: 62°C) and extension at 72°C for 24 sec. This reaction was followed by melting curve analysis, parameters: 1 cycle of 95° C followed by 70° C for 30 sec and ended by 95°C and cooling.

FACS analysis

Harvested HASM cells (0.5million cells/ml) were washed with PBS and then incubated with 0.3 % bovine serum albumin and 0.2% naïve rat serum on ice to block nonspecific binding. Fluorescein isothiocyanate (FITC) or Phycoerythrin (PE) conjugated monoclonal antibodies (Abs) to CXCR-1 and CXCR-2 (BD Bioscience, Mississauga, ON, Canada) or control isotype mouse IgG Ab were incubated with cells at 4°C for 30 minutes. After extensive washing, cells were fixed with 1%

paraformaldehyde in PBS at 4°C. Cell-associated immunofluorescence was analyzed with a FACScan flow cytometer (Becton Dickinson Bioscience, San Jose, CA) and Cell Quest software to determine the levels of surface expression of CXCR-1 and CXCR-2 sub-types. The results are presented as overlaid histograms and the percentages of positive cells were calculated.

Measurement of intracellular Ca^{2+}

HASM cells grown on 25 mm diameter coverslips were used from 10-14 days post plating (90 to 95% confluent). Cells were incubated for 30 min at 37° C with Hanks' buffer (in mM: NaCl 137, $NaHCO_3$ 4.2, glucose 10, Na_2HPO_4 3, KCl 5.4, KH_2PO_4 0.4, $CaCl_2$ 1.3, $MgCl_2$ 0.5, $MgSO_4$ 0.8, *N*-2-hydroxyethylpiperazine-*N'*-ethane sulfonic acid [Hepes] 5) containing 5μM Fura–acetoxymethylester (Fura-2-AM) and 0.02% pluronic F-127. The loaded cells were then washed and the coverslips were placed in a Leiden chamber (Medical Systems Corp, Greenville, NY) containing 450 μl of Hanks' buffer on the stage of an inverted microscope equipped for cell imaging with 40x oil objective (Nikon. Montreal, QC, Canada). The cells were imaged using an intensified camera (Videoscope IC 200) and PTI software (Photon Technology International Inc, Princeton, NJ) at a single emission wavelength (510 nm) with double excitatory wavelengths (345 and 380 nm). The fluorescence ratio (345/380) was measured in individual cells (n=8 per slide) and the free $[Ca^{2+}]_i$ was calculated using a calculated Kd of Ca^{2+} to Fura-2 of 224 nM . R_{max} was determined in cells exposed to ionomycin 10^{-5} M in the presence of 1.3 mM $CaCl_2$ and R_{min} in Ca^{2+} free buffer to which

EGTA 10^{-3} M and ionomycin 10^{-5} M had been added. Background fluorescence and autofluorescence were subtracted.

All test drugs were diluted in Hanks' buffer from frozen stock solutions. They were pre-warmed to 37°C before being added in the appropriate concentrations in a 450µl volume.

Cell preparation for receptor blocking antibody and PLC inhibitor experiments

HASM cells were cultured on six well plates. Once the cells reached aprroximatly 90 to 95% confluence, the cells were loaded with Fura -2- AM for the measurement of $[Ca^{2+}]_i$. After washings, the cells were incubated with either CXCR1 or CXCR2 receptor blocking antibodies (20ug/ml, Research Diagnostic, Flanders, NJ, USA) or with vehicle for 10 minutes at room temperature and $[Ca^{2+}]_i$ was measured in response to IL-8 (100 nM). HASM cells were also incubated with either the phospholipase C (PLC) inhibitor U-73122 (Biomol, Plymouth Meeting, PA, USA) or the inactive control U-73345 for 5 minutes and then $[Ca^{2+}]_i$ levels were measured in response to IL-8 (100 nM).

Contraction studies

HASM cells were grown on 25 mm diameter glass cover slips for 4 days (50% confluence). For the study of cell contraction, images were taken using an inverted microscope with 20x magnification using Nomarski optics. A CCD camera (Hamamatsu C 2400) was used to acquire the images and recorded with image master (Photon Technology International Inc, Princeton, NJ). To measure the contraction of the

cells, images were taken once before and up to10 minutes after the addition of IL-8. Images were analyzed with the Scion software (National Institutes of Health, Bethesda, MD).The length of the cells were measured by measuring the distance between the ends of each cells. The length of the cell was measured once before and at different time points (0.5, 1, 2, 5, 7 and 10 minutes) after the addition of IL-8. The averages of cells were calculated and the contraction was expressed as the percentage decrease in cell length from the initial value. Four independent experiments were performed and 10 to 15 cells were analysed from each slide.

Chemotaxis assay

Cell migration assays were performed using a modified Boyden chamber (Neuroprobe, Cabin John, MD) (22). Cells were harvested with trypsin (0.025%) and EDTA (0.02%) and were re-suspended (80,000 cells /ml) in serum-free growth medium. The cells were plated on the upper side of the chamber. A polycarbonate membrane (8.0 μM pore) was treated with Type I collagen overnight at 4° C and placed between two chambers. Chemoattractants or vehicle (IL-8, PDGF or PBS in 0.1% BAS) were added to the lower well. After 4 hours of incubation at 37 °C, the membranes were removed after the cells on the upper face of the membranes had been cleared and the cells that migrated to the lower side of the membrane were fixed and stained with Diff-Quick (Dade Behring Inc, Newark, DE). The number of cells was counted in five random fields under 40x magnification. Four assays were done in triplicate using cells from three different individuals.

Data analysis

Data are represented as mean ± SEM. Comparison of means was performed with paired or unpaired Student-t tests as appropriate. A difference was considered to be statistically significant when the *P* value was less than 0.05.

2.5 Results

Expression of mRNA transcripts for the CXCR1 and CXCR2

Real time PCR was performed to determine if mRNA transcripts for CXCR1 and CXCR2 were expressed in HASM cells. Total RNA was extracted from cells harvested from 3 different individuals and cDNA was obtained by RT. PCR products were analyzed with 2% agarose gel electrophoresis and the resulting bands corresponded to the expected base pair sequences (CXCR1:499 bp, CXCR2:249 bp). As shown in figure 2.1, HASM cells constitutively express mRNA transcripts for both CXCR1 and CXCR2. β-actin (299 bp) was used as a house keeping gene.

Surface expression of the CXCR1 and CXCR2

FACS analysis was done to detect the surface expression of CXCR1 and CXCR2 on HASM cells. Five independent experiments were done with cells from 3 different individuals. Approximately 10,000 cells were used for acquisition for each condition (unstained, isotype and positive staning for CXCR1 or CXCR2). The difference between the cells stained for CXCR1 or CXCR2 to that stained for the respective isotype controls were used to calculate the % of positive cells. The results were express as % of positive cells that expressed CXCR1 or CXCR2. Figures 2.2A and 2.2B show that 23.8 ± 2 % of cells expressed CXCR1 and 21.2 ± 4% (p-NS) of cells expressed CXCR2 respectively. Human neutrophils were used as positive controls. Approximately 95 to 98% cells express both CXCR1 and CXCR2.

Functionality of IL-8 receptors in HASM cells

Functional activities of CXCR1 and CXCR2 were confirmed by measuring the changes in the $[Ca^{2+}]_i$, contraction and migration of HASM cells in response to IL-8

Effects of IL-8 on changes in $[Ca^{2+}]_i$

The $[Ca^{2+}]_i$ was measured by single cell fluorescence imaging and the calcium measurements, representing the mean response for cells from 3 different individuals, are shown in figure 2.3. Stimulation of cells with IL-8 (100 nM) induced a rapid transient increase in the $[Ca^{2+}]_i$ to a peak value of 273 ± 12 nM compared to a baseline value of 128 ± 8 nM. The peak changes in $[Ca^{2+}]_i$ were seen between 10-20 seconds after the administration of IL-8 and subsequently returned towards baseline values. $[Ca^{2+}]_i$ was also measured in the presence of calcium-rich and calcium-free medium. As indicated in figure 2.4, the presence of calcium in the extracellular medium did not change the magnitude of the change in $[Ca^{2+}]_i$ (Ca^{2+} rich medium 209 ± 9 nM, Ca^{2+} free medium 173 ± 11 nM), in response to IL-8 (100 nM) indicating that intracellular stores of calcium were responsible for the increase.

Effects of neutralizing antibodies to CXCR1 and CXCR2 on IL-8-induced increases in $[Ca^{2+}]_i$

HASM cells were incubated with either CXRC1 or CXCR2 specific neutralizing antibodies and the $[Ca^{2+}]_i$ were measured to determine which of these receptors was involved in the mobilization of calcium. Figures 2.5C and 2.5E, show that incubation of

cells with either CXCR1 or CXCR2 neutralizing antibodies completely abrogated the changes in $[Ca^{2+}]_i$; values of 104 ± 6 nM (anti-CXCR1), 110 ± 3 nM (anti-CXCR2) were recorded compared to vehicle treated cells where induced peak values of 246 ± 13 nM were observed (figure 2.5A). Histamine-induced $[Ca^{2+}]_i$ following IL-8 stimulations were used as a positive control to confirm the cellular responses. The baseline calcium levels were comparable in both antibody and vehicle treated conditions.

Effects of a PLC inhibitor (U-73122) on IL-8-induced $[Ca^{2+}]_i$

To determine the role of phospholipase C (PLC) in mediating the IL-8-induced changes in $[Ca^{2+}]_i$, cells were incubated with U- 73122 (100 nM) or U-73343 (100 nM) and $[Ca^{2+}]_i$ in response to IL-8 (100 nM) was measured. U- 73122 completely blocked the IL-8-induced $[Ca^{2+}]_i$ (figure 2.6), whereas the control compound U-73343 did not have any effect.

Effects of IL-8 on contraction of HASM cells

IL-8-induced contraction of HASM was measured by stimulating cells with IL-8 (100 nM) or vehicle. As a measure of contraction the lengths of the cells were measured before and at different times points (30 seconds and 1,2,5,7 and 10 minutes), after the addition of IL-8 or vehicle. Figures 2.7A and 2.7B show the changes in the lengths of the cells with and without addition of IL-8. Cells from 4 different individuals and 15 to 20 cells/slide were analyzed.

Figure 2.1

Expression of CXCR1 and CXCR2 in HASM cells

Total RNA was extracted from smooth muscle cells harvested from bronchial biopsies of 3 different individuals (1 - 3). β-actin as a house keeping gene. M: DNA molecular weight markers. P: Positive control (human neutrophils)

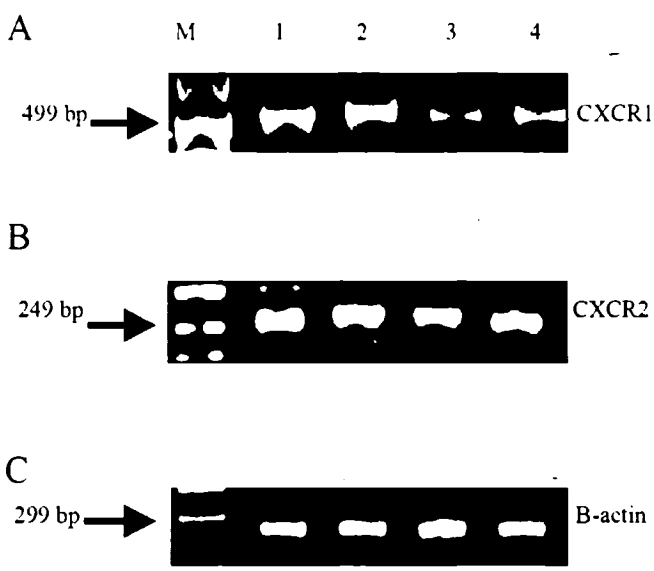
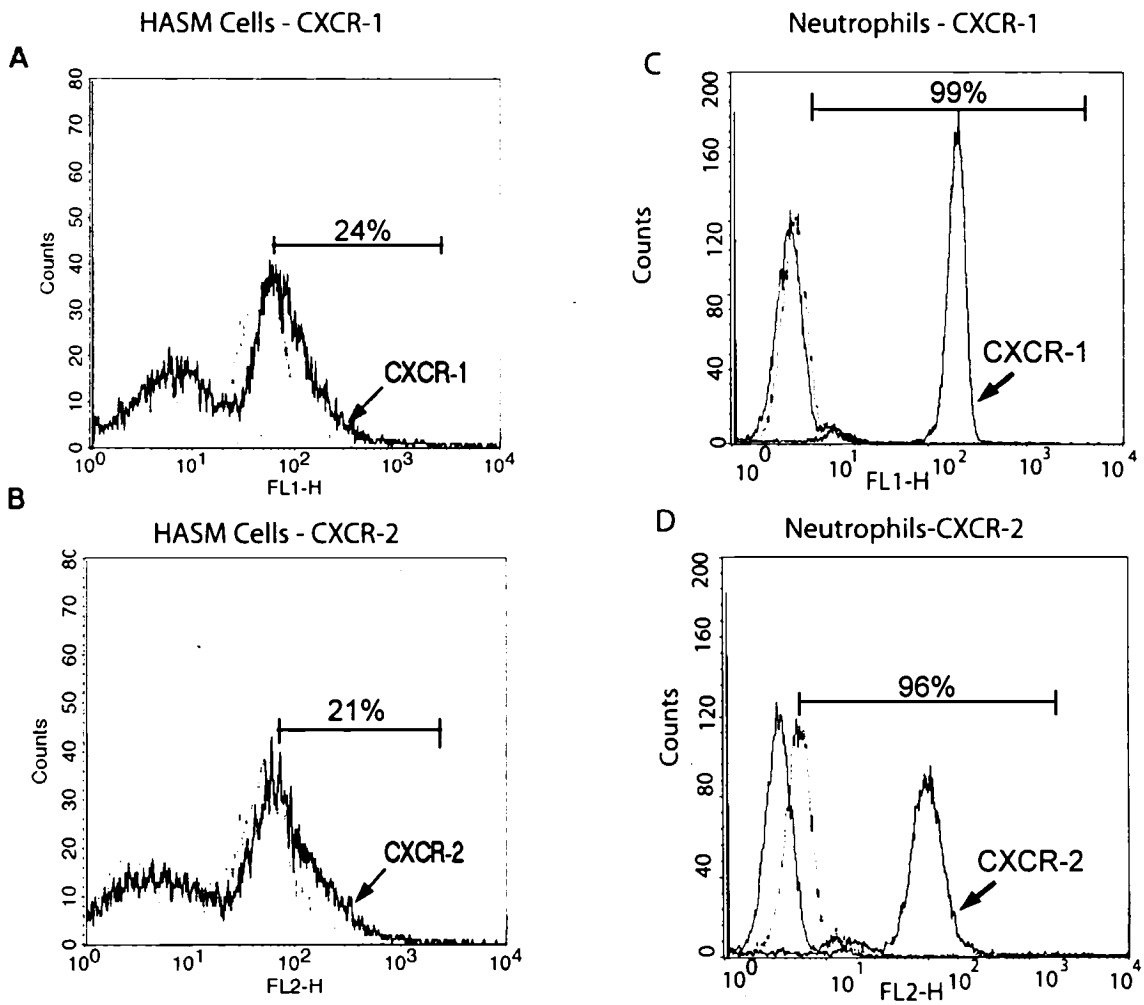

A
M
1
2
3
4
499 bp
CXCR1
B
249 bp
CXCR2
C
299 bp
B-actin

Figure 2.2

Flow cytometric analysis of the surface expression of CXCR1 and CXCR2 on HASM cells

Cells from three different individuals were stained for the identification of the receptors. Panels A and B show representative example of the expression of CXCR1 and CXCR2 respectively. Grey shaded area in the graph for unstained cells, black line for stained cells for receptors and gray line for isotype controls. Panels C and D are representative histogram for the expression of CXCR1 and CXCR2 respectively. Cells from 5 different cell preparations were used ($p < 0.05$).


A
HASM Cells - CXCR-1
Counts
24%
CXCR-1
FL1-H
B
HASM Cells - CXCR-2
Counts
21%
CXCR-2
FL2-H
C
Neutrophils - CXCR-1
Counts
99%
CXCR-1
FL1-H
D
Neutrophils-CXCR-2
Counts
96%
CXCR-2
FL2-H

The addition of IL-8 significantly decreased the length of the cells by 15 ± 1 % compared to the vehicle-treated cells which decreased the length by 5 ± 1 % (Fig 2.7C). The t ½ for changes in length was calculated to be 2 minutes, approximately.

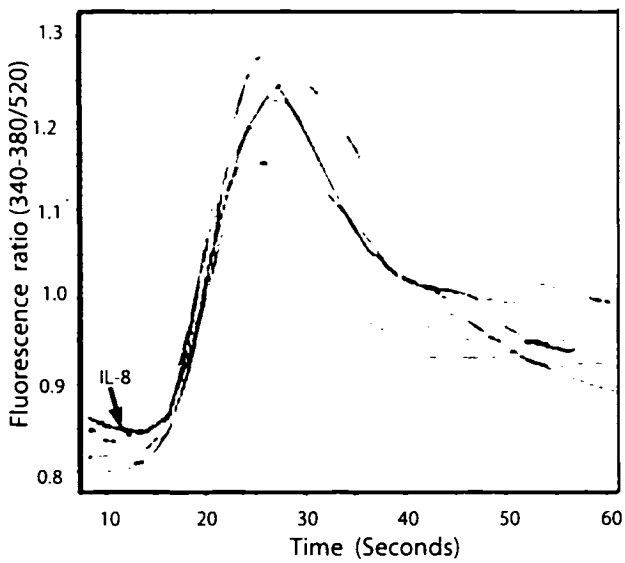
Effects of IL-8 on migration of cells

Chemotaxis assays were performed to study whether IL-8 induced migration of HASM cells. Figure 2.8 shows the number of cells migrating after IL-8 exposure (10 and 100 nM) in the Boyden chamber and the results are expressed as fold difference compared to vehicle treated cells. IL-8 increased the migration of cells by 2.9 fold at a concentration of 100 nM, 1.5 fold at 10 nM whereas PDGF (0.5ng/ml) caused a 2.8 fold change in cell migration and served as a positive control.

Figure 2.3

Effects of IL-8 on $[Ca^{2+}]_i$

Cultured HASM cells were stimulated with IL-8 (100 nM). Panel A shows, IL-8 induced release of intracellular Ca^{2+} and represent fluorescence ratios of 8 different cells from one slide. Panel B shows the mean of the peak $[Ca^{2+}]_i$ values from 48 cells. The data presented are means ± SEM.


Fluorescence ratio (340-380/520)
1.3
1.2
1.1
1.0
0.9
0.8
IL-8
10
20
30
40
50
60
Time (Seconds)

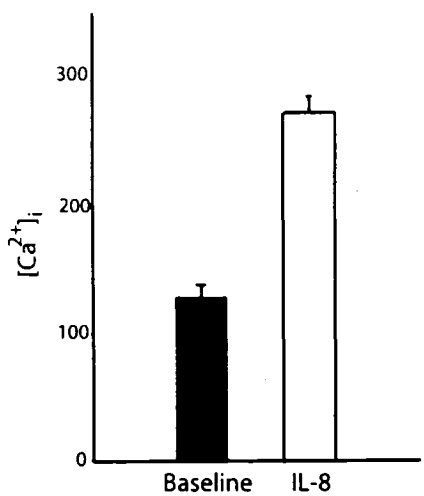

300
200
100
0
$[Ca^{2+}]_i$
Baseline
IL-8

Figure 2.4

Effects of IL-8 on $[Ca^{2+}]_i$ in the presence and absence of calcium in extracellular medium (Hanks' buffer)

IL-8 induced intracellular Ca^{2+} were measured in the presence and absence of Ca^{2+} in extracellular medium. Solid bars represent the resting Ca^{2+} levels and the open hatched bars represent the peak $[Ca^{2+}]_i$ levels in response to IL-8 (100 nM). The data presented are means ± SEM. NS: not significant.

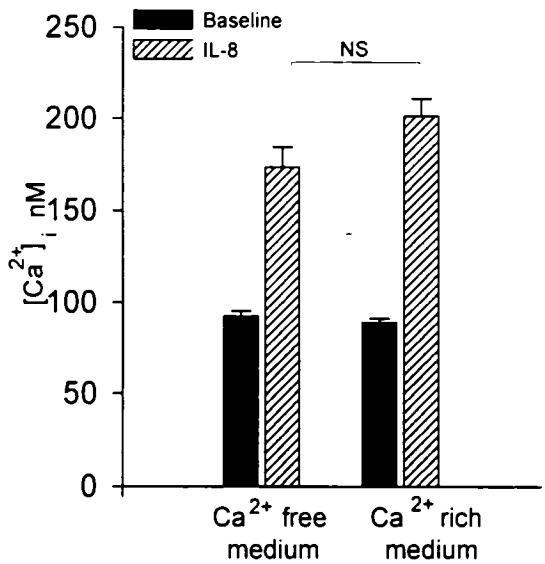

Baseline
IL-8
NS
250
200
150
100
50
0
$[Ca^{2+}]_i$ nM
Ca^{2+} free medium
Ca^{2+} rich medium

Figure 2.5

Effects of neutralizing antibodies to CXCR1 and CXCR2 on $[Ca^{2+}]_i$

Cells were incubated with either CXCR1 or CXCR2 neutralizing antibodies and $[Ca^{2+}]_i$ levels were measured in response to IL-8 (100 nM) and histamine (100 μM). Panel A represent the fluorescence ratios of the cells in response to IL-8 and histamine in untreated (control) cells. Panel B represent the mean peak $[Ca^{2+}]_i$ levels with IL-8 and histamine from control cells (n=48). Panel C and E represent the fluorescence ratios of the cells and panel D and F represent the mean peak $[Ca^{2+}]_i$ levels in response to IL-8 and histamine in cells (n = 40) treated with CXCR1 or CXCR2 neutralizing antibodies respectively. The data presented are the means ± SEM

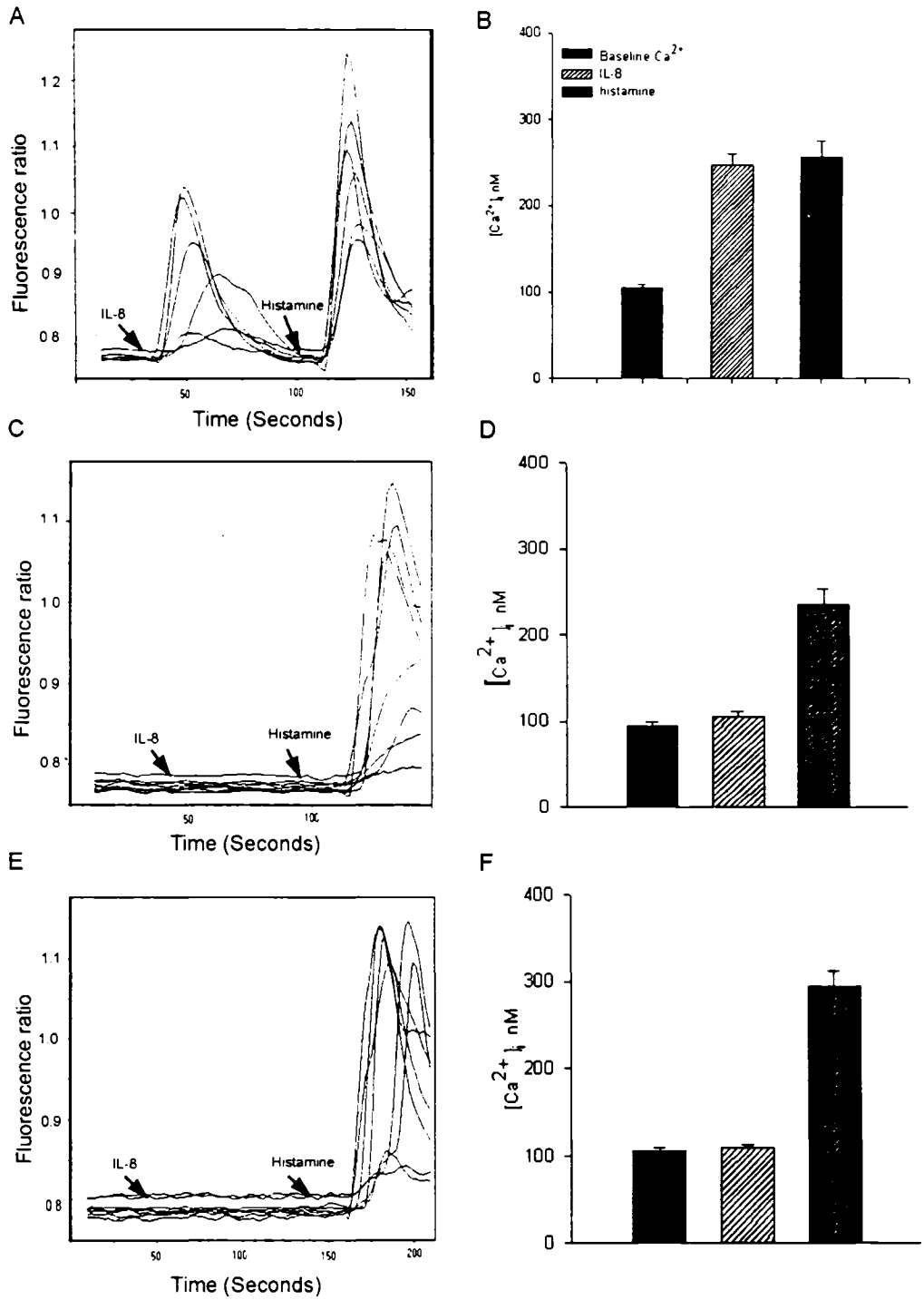

A
Fluorescence ratio
1.2
1.1
1.0
0.9
0.8
IL-8
Histamine
50
100
150
Time (Seconds)
B
400
300
200
100
0
Baseline Ca^{2+}
IL-8
histamine
$[Ca^{2+}]_i$ nM
C
Fluorescence ratio
1.1
1.0
0.9
0.8
IL-8
Histamine
50
100
Time (Seconds)
D
400
300
200
100
0
$[Ca^{2+}]_i$ nM
E
Fluorescence ratio
1.1
1.0
0.9
0.8
IL-8
Histamine
50
100
150
200
Time (Seconds)
F
400
300
200
100
0
$[Ca^{2+}]_i$ nM

Figure 2.6.

Effects of PLC inhibitor (U-73122) and inactive PLC inhibitor (U-73343) on $[Ca^{2+}]_i$

Solid bar represent the baseline calcium and gray and black hatch bars indicate the $[Ca^{2+}]_i$ levels in response to IL-8 following incubation with U-73343 and U-73122 respectively. The data presented are the means ± SEM

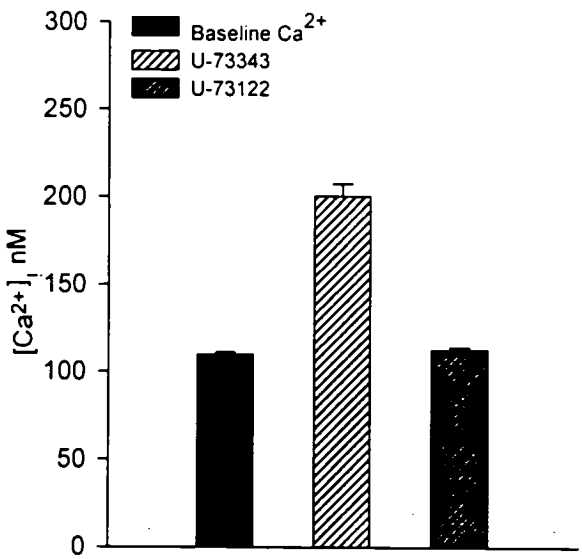
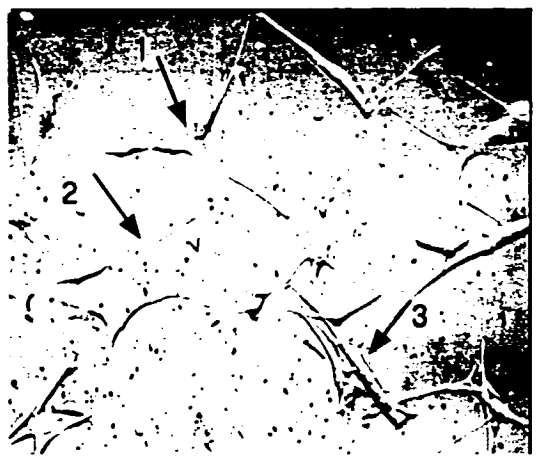

300
250
200
150
100
50
0
$[Ca^{2+}]_i$ nM
Baseline Ca^{2+}
U-73343
U-73122

Figure 2.7

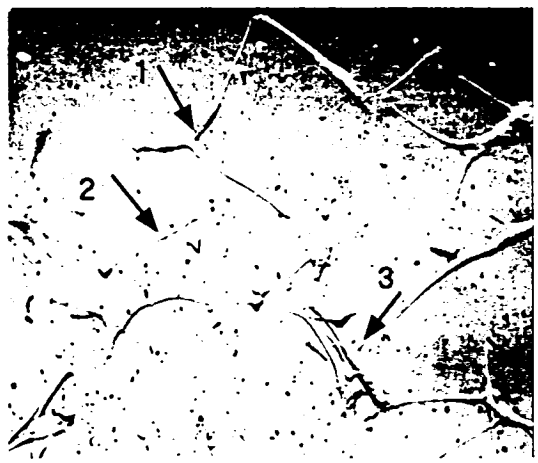
Contraction of HASM cells with IL-8

Imaging studies were done and the contraction of the cells with IL-8 (100 nM) was measured. Panel A and B represent the images captured before and 10 minutes after the addition of the IL-8. Number 1, 2 and 3 indicates the contracted cells with IL-8 treatment. In panel B, the length of the cells were decreased compared to the same cells in panel A. Panel C represent the % decrease in the cell length with IL-8 and is compared with Hanks' treated cells.

A



B



C

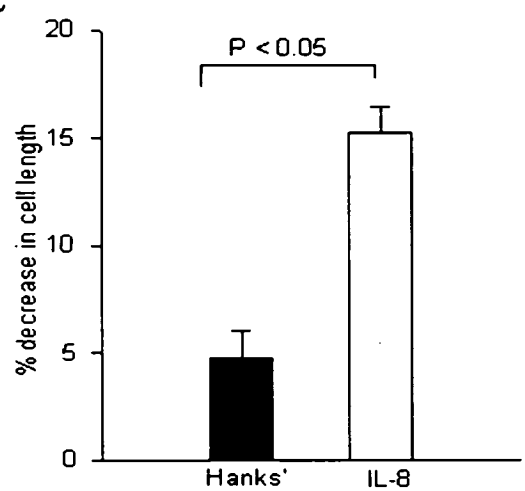
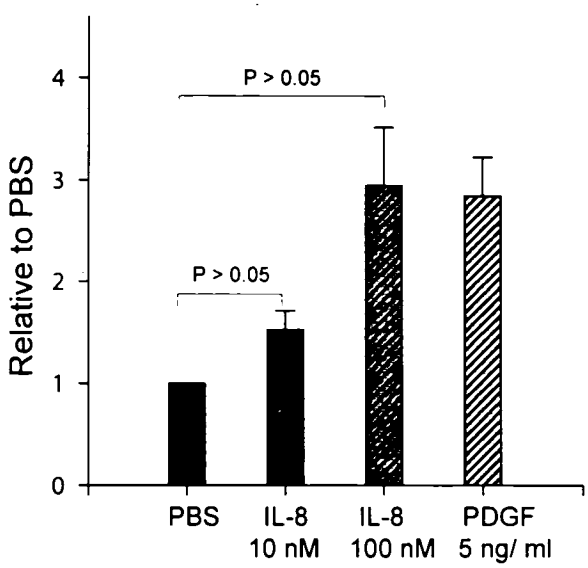


Figure 2.8

The effects of IL-8 on HASM cell migration

IL-8 causes the migration of cells at the concentration of 10 and 100 nM. PDGF was used as positive controls. The data is represented as the fold difference and compared to the vehicle treated cells (PBS).


Relative to PBS
4
3
2
1
0
P > 0.05
P > 0.05
PBS
IL-8
10 nM
IL-8
100 nM
PDGF
5 ng/ ml

2.6 Discussion

IL-8 is produced by airway structural cells, including epithelial cells and smooth muscle cells, in response to various inflammatory mediators. IL-8 acts on specific receptors on neutrophils to cause a number of effects, including changes in intracellular calcium, chemotaxis, cell morphology, actin polymerization and degranulation (6), that are mediated by two G-protein coupled receptor subtypes, CXCR1 and CXCR2 (23), (37). Other cell types including monocytes (51), epithelial (50) and endothelial cells (57), express CXCR1 and CXCR2. Although HASM cells have been shown to synthesize IL-8 in response to the proinflammatory substances TNF-α, IL-1 β (24) and bradykinin (39), it is not known whether these cells constitutively express CXCR-1 and/or CXCR-2 receptors and could also therefore react to IL-8. In the current study we demonstrate that HASM cell constitutively express mRNA and protein for CXCR1 and CXCR2. Furthermore, we show that both of these receptors are functional and mediate substantial biological effects.

IL-8 is a chemokine that is emerging as an important contributor to airway inflammation in several airway diseases associated with neutrophilic inflammation. Increased levels of IL-8 have been observed in epithelial cells and epithelial lining fluid from CF and asthmatic patients (12), (30), (31), and is postulated to contribute to the airway neutrophilia associated with these diseases (12), (43). CF and asthmatic patients also suffer from airway hyperresponsiveness, a phenomenon that results from contraction of airway smooth muscle. Although there are many candidate mediators for bronchoconstriction in these diseases, we hypothesized that IL-8 might be involved in

airway constriction through a direct effect on HASM cells. The receptors for IL-8, CXCR1 and 2, are coupled to phospholipase C and inositol phosphate hydrolysis leading to mobilization of Ca^{2+} (28). In support of the plausibility of the hypothesis that IL-8 may have bronchoconstrictive properties we first demonstrated the expression of IL-8 receptors by HASM cells; mRNA transcripts for both CXCR1 and CXCR2 were shown by real-time PCR and CXCR1 and CXCR2 receptor protein on the surface of cells was confirmed by flow cytometry. Both receptors were expressed to a comparable extent; flow cytometry showed that 24% of the cells expressed CXCR1 and 21% of cells expressed CXCR2.

We wished to test whether the receptors were functional. To do this we chose a number of outcome measures. The release of $Ca^{2+}{}_i$ is one of the hallmarks of the activation of smooth muscle contractile processes and is an important second messenger triggering bronchoconstriction. IL-8 has been previously shown to induce Ca^{2+} release in neutrophils and monocytes (58). Our data show that exposure to IL-8 caused a transient increase in $[Ca^{2+}]_i$ in the HASM cells up two fold the values in resting cells. These transients were unaffected by removing Ca^{2+} from the extracellular medium, indicating that IL-8 was released Ca^{2+} from intracellular sources. Our data are consistent with the results obtained with human neutrophils (11), showing that IL-8 induces release of calcium from intracellular sources and not from extracellular sources, whereas GROα, another chemokine that binds to CXCR2, induces significant influx of calcium from extracellular sources. The blocking effects of a PLC inhibitor on IL-8 induced changes in $[Ca^{2+}]_i$, indicate that second messengers such as inositol

trisphosphate is responsible for mediating the signals leading to a Ca^{2+} transient. Our data with IL-8 also indicate that the changes in calcium are coupled to contraction.

IL-8 activates both CXCR1 and CXCR2 but binds CXCR1 with high affinity whereas CXCR2 also binds other ligands such as GROα (33), (17), (2). Selective blocking of CXCR1 and CXCR2 with neutralizing antibodies abrogated the release of Ca^{2+}, confirming that both the CXCR1 and CXCR2 receptors were involved in calcium signaling. Even though the functional form of IL-8 exists as monomer (42) (58) under different concentrated solutions and in crystallized state it occurs as a homodimer (7). The interaction between IL-8 and its receptor seems to be complex and a number of functional domains on the IL-8 molecule have been identified as essential for this interaction (9), (10), (21). Wu et al (59) have shown that in neutrophils, binding of IL-8 with and signaling of IL-8 through its receptors occur in at least two discrete steps involving distinct domains of the receptor and also suggest that conformational changes of the receptor secondary to ligand binding are necessary to trigger various biological responses. Since the binding of IL-8 to its receptors seems to be complex, and it is possible that following signaling with IL-8 there might be the interaction between CXCR1 and CXCR2 for calcium signaling. This could be responsible for the abrogated calcium signals that we observed with the receptor neutralization experiments that suggest that coordinated effects or differential interaction of both subtypes of receptors are necessary for Ca^{2+}_i release.

M. Fujimura et al (16) have shown that IL-8 inhalation causes bronchoconstriction partly through the release of histamine without cellular infiltration in guinea pig airways. Plattner et al (41) have also shown that IL-8 increases

acetylcholine-induced contraction in rat intestinal segments. The above experiments involve preparations that include many cell types. Indeed degranulation of mast cells seems to be involved in the induction of bronchoconstriction in the guinea pig. So far, it is not known whether IL-8 is directly involved in contraction of ASM cell. We used phase contrast microscopy to measure IL-8 induced contraction. The observed shortening of the cells indicates that IL-8 not only causes calcium signals but that these signals are coupled to contraction. The number cells that contracted in a given population is consistent with the results of flow cytometry, which indicate that 20 to 25% of cells express surface receptor protein in detectable levels.

IL-8 has potent chemotactic properties for neutrophils but its effects are not restricted to this cell type. It also causes the migration of hepatocytes (56) and in the current study ASM cells. Migration of smooth muscle cells is important for repair following vascular damage (36), (15) and it has been shown that IL-8 causes migration of rat aortic smooth muscle cells (61). It is postulated that migration of ASM cells may be one of the events involved in the airway remodeling that is observed in asthma and cystic fibrosis (25), (35). Parameswaran. K et al (40) have shown that ASM cells show a chemotactic response to PDGF and that this response is augmented by cysteinyl leukoterines, mediators that have been shown to be important for airway remodeling in animal models of allergic asthma (55), (46). Our results showed a migration rate that was 2.5 fold higher than that of vehicle treated cells and comparable to that induced by PDGF.

In conclusion, we have demonstrated that IL-8 induces a change in the $[Ca^{2+}]_i$ and causes the contraction and migration of HASM cells. These cells express both

CXCR1 and CXCR2 mRNA transcripts and surface receptor proteins. Both receptor subtypes appear to be important in mediating the effects of IL-8 on the cells. Based on these findings we speculate that IL-8 could contribute to airway narrowing, bronchial hyper-responsiveness and airway remodeling in airway diseases such as asthma, CF and COPD by mechanisms that are independent of the inflammatory response that it also engenders.

2.7 Reference List

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CHAPTER 3

THE EFFECTS OF INTERLEUKIN-8 ON AIRWAY SMOOTH MUSCLE CONTRACTION IN CYSTIC FIBROSIS

3.1 Prologue

We have demonstrated in the previous chapter that normal ASM cells express CXCRs and that interleukin-8 causes contraction of ASM cells by releasing intracellular Ca^{2+}. IL-8 also stimulates the contraction and migration of ASM cells. Since IL-8 is present in substantial amounts in the airways of CF patients, we wondered if ASM cells from CF-affected subjects responded normally to IL-8. We hypothesized that IL-8 may enhance the contraction of CF ASM cells and thereby contribute to AHR and bronchospasm in CF patients. In this study, we compared the effects of IL-8 induced $[Ca^{2+}]_i$, contraction and migration ASM cells between CF and normal subjects.

3.2 Abstract

Cystic fibrosis (CF) patients develop chronic bacterial colonization with severe neutrophilic inflammation and high levels of interleukin-8 (IL-8) in their airways. Many CF patients also have airway hyperresponsiveness and reversible airways obstruction. IL-8 binds to G-protein coupled receptors (CXCRs) linked to phospholipase C and signals through calcium release intracellularly. We hypothesized that IL-8 might alter the functions of airway smooth muscle (ASM) cells and contribute to bronchospasm in CF patients. Experiments were conducted with ASM cells harvested from lung transplant specimens from normal donors and subjects with CF. We examined the expression of CXCRs in ASM cells by flow cytometry and the effects of IL-8 on ASM by measuring intracellular calcium ($[Ca^{2+}]_i$), contraction and migration. We found that ASM from both normal and CF subjects expressed CXCR1 and CXCR2 to comparable extents. In normal cells, IL-8 (100 nM) evoked higher levels of peak $[Ca^{2+}]_i$ compared to CF cells ($p < 0.05$). However, the delta Ca^{2+} levels in response to IL-8 were not different. IL-8 caused greater contractions of CF cells compared to normal cells. Furthermore, IL-8 exposure resulted in higher phosphorylation of myosin light chain (MLC_{20}) in CF cells compared to normal cells. In addition the expression of total MLC_{20} was also increased in CF cells. In contrast, IL-8 induced migration of ASM cells was not different between CF and normal cells. These results indicate that IL-8 causes greater contractions of CF ASM cells through increased levels of phosphorylated MLC_{20} and higher expression of MLC_{20} by CF cells.

3.3 Introduction

Cystic fibrosis (CF) is a genetic disease caused by defective Cl^- secretion and enhanced Na^+ absorption across the airway epithelia (34), The airways become predominantly infected with *P. aeruginosa* (37), (21) and to a lesser extent with other organisms such as *S. aureus*, *H. influenzae*, and respiratory syncytial virus (2), (20), (36). Chronic bacterial infections and inflammation of the lung are the main causes of morbidity and mortality in CF patients, 1990, (6). With increasing age most CF patients develop airway obstruction and many of these patients (approximately 50 %) also suffer from airway hyperresponsiveness and asthma-like symptoms (28), (26), (8), (43). It has also been shown that children with CF have higher (at least 25%) prevalence of asthma than those observed in the general population (39), (19), (20). Furthermore, Tiddens et al (42) have shown that airway remodeling reminiscent of asthma affects CF airways; most of the CF airways examined were not covered with epithelium and the smooth muscle area of the peripheral airways was increased. They also showed that both baseline airway resistance and maximal airway resistance in response to bronchoconstrictors were increased in CF airways compared to patients with chronic obstructive pulmonary disease. In addition, i*n vivo* studies with inhalation of bronchodilators improved the symptoms associated with bronchial responsiveness in CF patients indicating the presence of an asthma-like syndrome (31), (43), (3). These findings suggest that the bronchial responsiveness observed in CF may be related to an increase in airway smooth muscle (ASM) contraction, as has been suggested for asthma (24) (16).

Since CF is a chronic inflammatory disease, many inflammatory cytokines are produced in the airways. Several studies have documented increased levels of interleukin-8 (IL-8) in BAL, sputum and bronchial glands of patients with CF (30), (33), (40). It has also been reported that increased production of IL-8 is observed in CF patients even in the absence of infection (5). In CF lung, IL-8 is produced by neutrophils, airway epithelial cells, macrophages, and monocytes (23). IL-8 is a CXC chemokine and binds to the G-protein coupled receptors CXCR1 and CXCR2 (14). It acts as a chemotactic agent for neutrophils, T lymphocytes (4), basophils (25), NK cells and melanocytes (44). It has also been shown to have other functions such as enhancement of angiogenesis (22), increase in leukocyte adhesion between fibrinogen and endothelial cells by upregulating the expression of CD11b/CD18 (7). In addition, IL-8 stimulates the proliferation and migration of rat vascular smooth muscle (46), (45). IL-8 inhalation provokes bronchoconstriction in guinea pigs *in vivo* (9). As IL-8 is increased in CF and has functions distinct from its role in inflammation, it is possible that IL-8 may be involved in the airway hyperresponsiveness of CF by increasing smooth muscle contraction. Recently, we have demonstrated that ASM from healthy individuals expresses CXCR1 and CXCR2 and IL-8 induces contraction of these cells by increasing the concentration of intracellular calcium ($[Ca^{2+}]_i$) (10). Therefore, we hypothesized that IL-8 may cause the contraction of ASM cells and may contribute to airway narrowing in CF patients. Thus, we investigated the effects of IL-8 on the release of intracellular Ca^{2+} and contraction of ASM from CF-affected subjects and compared our findings to those of cells from normal subjects. We also examined the

expression of CXCRs and the effects of IL-8 on cellular migration in both normal and CF-affected subjects.

3.4 Materials and Methods

Cell cultures

Primary cultures of ASM cells from CF patients and normal individuals were prepared from lung transplant specimens using a modification of the method published by Randell et al (32). Specimens from CF patients were kept for 1-8 hours in Hanks' balanced salt solution (HBSS) containing the following antibiotics: ceftazidime 100 μg/ml, ciprofloxacin 20 μg/ml, colistin 5 μg/ml, tobramycin 80 5 μg/ml, gentamycin 50 μg/ml, penicillin 10000 unit/ ml, streptomycin 10 mg/ml, and amphotericin 25 μg/ml. The tissues were cut into small pieces of about 5mm x 5mm and incubated for 20 minutes at 4°C in HBSS in the presences of DTT (0.5mg/ml) and Dnase type I (10 μg/ml). Then tissue digestion (both normal and CF) was performed by incubating the tissues in HBSS containing collagenase type IV (0.4 mg/ml), elastase (0.38 mg/ml) and soybean trypsin inhibitor (1 mg/ml) at 37°C for 90 min with gentle shaking. The dissociated cells were collected by filtration through 125 μm Nytex mesh followed by centrifugation. The pellet was then reconstituted in growth medium (DMEM-Ham's F12 medium supplemented with 10% fetal bovine serum, Penicillin 10000 unit/ ml, streptomycin 10 mg/ml, and amphotericin 25 μg/ml)) and plated in 25-cm^2 flask. Confluent cells were detached with 0.025% trypsin solution containing 0.02 % ethylenediaminetetraacetic acid (EDTA) and were plated with the density of 5000 cells/ml for different experiments. Equal density of cells from CF and normal subjects were grown on 25mm diameter glass coverslips for single cell imaging of calcium transients, contraction studies and on 6 well culture dishes for flow cytometry, protein

extraction, and chemotaxis assays. Cells from passage 1 to 4 were used for all the experiments. The morphology of normal and CF cells appear similar. However ASM cells from CF grow faster and were confluent by 10 to 11 days compared to normal cells which was about 12 to 13 days.

Contraction studies

ASM cells from CF and normal individuals were grown for 4 days on glass slides covered with homologous cell substrate as previously described (41). The glass slides were placed in a Leiden chamber where the temperature was maintained at 37 ± 0.5°C using a temperature controller (model TC-102; Medical System Corp). The cells were visualized using an inverted microscope with 20x magnification using Nomarski optics. A CCD camera (Hamamatsu C2400) was used to acquire and record images (Photon Technology International Inc, Princeton, NJ). Images were taken just before and at intervals up to 10 minutes after the addition of IL-8. Images were digitized and analyzed with the Scion software (National Institutes of Health, Bethesda, MD). The length of the cells was measured in order to study the SM cell contraction. The difference in the length before and after the addition of IL-8 is expressed as a measure of contraction. The lengths of 6 to 8 cells per slide (four experiments) were measured at different time points (0, 1 and 10 minutes) and the contraction was expressed as the percentage decrease in cell length from the initial value.

Flow Cytometry

Confluent ASM cells from both CF patients and normal individuals were detached with cell dissociation buffer (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. The cell suspensions were incubated with 0.03 % bovine serum albumin and 0.2% naïve rat serum on ice to block nonspecific binding. This was followed by incubation of cells (0.5 million cells/ml) with Fluorescein isothiocyanate (FITC) conjugated monoclonal antibody to CXCR1 or Phycoerythrin (PE) conjugated monoclonal antibody to CXCR2 respectively (BD Bioscience, Mississauga, ON, Canada) at 4°C for 30 minutes in the dark. As a control, ASM cells were also incubated with isotype mouse IgG antibody. After several washes, cells were fixed with 1% paraformaldehyde and were analyzed with a flow cytometer (FACScan, Becton Dickinson Bioscience, San Jose, CA) and Cell Quest software was used to determine the levels of surface expression of CXCR1and CXCR2.

Measurement of intracellular Ca^{2+}

Cytosolic calcium measurements were performed as previously described (27). ASM cells grown on glass slides and incubated for 30 min at 37° C in Hanks' buffer (in mM: NaCl 137, $NaHCO_3$ 4.2, glucose 10, Na_2HPO_4 3, KCl 5.4, KH_2PO_4 0.4, $CaCl_2$ 1.3, $MgCl_2$ 0.5, $MgSO_4$ 0.8, *N*-2-hydroxyethylpiperazine-*N*'-ethane sulfonic acid [Hepes] 5) in the presence of 5 μM Fura–2-acetoxymethylester (Fura-2-AM). The loaded cells were then washed and the glass slides were placed in a Leiden chamber (Medical Systems Corp, Greenville, NY) containing 450 μl of Hanks' buffer on the stage of an inverted microscope equipped for cell imaging with 40x oil objective

(Nikon. Montreal, QC, Canada). The images of the cells were captured using an intensified camera (Videoscope IC 200) and PTI software (Photon Technology International Inc, Princeton, NJ) at a single emission wavelength (510 nm) with double excitatory wavelengths (345 and 380 nm). The fluorescence ratio (345/380nm) was measured in individual cells (n=8 per slide) and the free $[Ca^{2+}]_i$ was calculated using Kd values as indicated previously in section 2.4. The agonists were dissolved in the appropriate buffers and were pre-warmed to 37°C before being added to the chamber. Background fluorescence and autofluorescence were automatically subtracted.

Protein extraction and Immunoblotting

Myosin light chain (MLC_{20}) expression and phosphorylation were quantified by immunoblotting in cells from normal and CF patients. Confluent cells were washed with Hanks' buffer and were rested for 10 minutes at room temperature. Cells were stimulated with IL-8 or vehicle for different time periods (1 and 5 minutes) and were washed with ice cold PBS. The reaction was stopped immediately with lysis buffer (Tris HCL 62.5mM with pH 6.8, sodium dodecyl sulphate (SDS) 2%, Glycerol 10%, DTT 50 mM, and bromophenol blue 0.01%). Cells were lysed with lysis buffer on ice. Lysates were centrifuged and supernatants were stored at -80°C for protein analysis. Bradford assays were performed to measure the protein contents and appropriate volumes of loading buffers were mixed with extracted samples. Equal amount of total proteins from both CF and normal cells were loaded in the same gel in order to control the variations in loading. Samples were heated at 95°C for 5 minutes and then loaded for protein separation. Electrophoresis was carried out using 12% SDS-PAGE gel and proteins

were transferred to nitrocellulose membranes. Membranes were blocked for 2 hours at room temperature with 5% dried milk in Tris-HCl containing Tween 20 buffer solution (TTBS). Primary antibodies (rabbit polyclonal IgG, Cell Signalling, Santa Cruz, CA) were diluted in TTBS with the dilutions of 1:1000 for phophorylated MLC_{20} and 1:500 for expression of MLC_{20} respectively. The membranes were incubated overnight at 4°C followed by incubation with secondary antibodies (goat anti-rabbit IgG HRP conjugated, Lake Placid, NY) at the dilution of 1:2500 at room temperature for 1 hr. Blots were developed by chemiluminescence and the signals were developed with Fluoro^{TM} 800 Advanced Fluorescence Imager (Alpha Innotech Corporation, Montreal, QC, Canada) and densitometry was done with Fluorochem^{TM} software. Resting phosphorylation was measured with vehicle-treated cells. Myosin from chicken gizzard was used as a positive control.

Chemotaxis assay

Chemotaxis assays were simultaneously performed for the cells from both CF-affected and normal individuals using a modified Boyden chamber (Neuroprobe, Cabin John, MD) (12). Harvested ASM cells were re-suspended (80,000 cells /ml) in serum free growth medium (DMEM-Ham's F12 medium). The cells were placed in different wells (duplicates for each agonist) on the upper side of the chamber. Chemoattractants (IL-8, PDGF) or vehicle (PBS containing 0.1% BSA) were added to the lower well. A polycarbonate membrane (8.0 μM pore) treated with Type I collagen overnight at 4°C was placed between the two chambers. The chamber was then incubated at 37°C for 4 hours under humidified air and 5% CO_2. The membranes were removed and the cells

that migrated towards the agonists were fixed and stained with Diff-Quick (Dade Behring Inc, Newark, DE). The cells were counted in five random fields per well under 40x magnification and average of duplicates was calculated. The results are expressed as fold difference by comparing IL-8 induced experiments to vehicle treated ones.

Data analysis

Data are represented as mean ± SEM. Comparison of means was performed with paired Student-t tests. One-way ANOVA followed by Student's t-test was used for chemotaxis assay. A difference was considered to be statistically significant when the *P* value was less than 0.05.

3.5 Results

Effects of IL-8 on contraction of ASM from CF individuals

Contraction of ASM from CF and normal individuals was measured by stimulating cells with IL-8 (100 nM) or vehicle. Cells were plated on homologous cell substrate and the temperature was maintained at 37°C to simulate a physiological milieu. As a measure of contraction, the lengths of the cells were measured before and at 10 minutes after the addition of IL-8. Six to 8 cells from each slide were randomly selected and analyzed. Figures 3.1A, 3.1C and 3.1B, 3.1D show the changes in the lengths of the cells before and 10 minutes after the addition of IL-8 to CF and normal cells respectively. In panel B and D, the lengths of cells were decreased compared to length of same cells in panel A and C. The values are expressed in % decrease in the length of the cell following IL-8 stimulation and cells from 4 different individuals from each group were studied. IL-8 significantly ($p < 0.05$) decreased the length of the cells by 19 ± 3 % in CF cells compared to 8 ± 2 % in normal cells (Figure 3.1E) whereas the changes in length of normal and CF cells treated with vehicle (1.5 ± 1 % and 3.7 ± 3 %, respectively) did not differ significantly.

Expression and quantification of CXCR1 and CXCR2

The surface expression of CXCR1 and CXCR2 protein on ASM cells from both normal and CF subjects was studied by flow cytometry. The results were presented as overlaid histograms and the percentages of positive cells were calculated. Figure 3.2 shows that 37 ± 2% of CF cells (panel A) and 34 ± 2 % of normal cells (panel B)

expressed CXCR1, whereas 16 ± 0.8 % CF cells (panel C) and 22 ± 2% of normal cells (panel D) expressed CXCR2. Panel E shows % of positive cells stained for CXCR1 and CXCR2 in CF and normal groups. There is no significant difference in the expression of CXCR1 and CXCR2 by normal and CF ASM cells.

Effects of IL-8 on $[Ca^{2+}]_i$

IL-8 induced calcium transients were measured in cells from normal and CF-affected individuals. Figure 3.3 shows that IL-8 (100 nM) induced a rapid increase in the $[Ca^{2+}]_i$, which subsequently returned towards baseline values. IL-8 increased the $[Ca^{2+}]_i$ up to 228.03 ± 7 nM in normal cells compared to 198.03 ± 10 nM in CF cells ($p<0.05$). The baseline value $[Ca^{2+}]_i$ of was 87 ± 2 nM in normal cells compared to 72 ± 2 nM in CF cells ($p<0.05$). However, the difference between the baseline $[Ca^{2+}]_i$ and the peak evoked by IL-8 did not differ significantly between CF (126 ± 11 nM) and normal cells (135 ± 10 nM). The $[Ca^{2+}]_i$ levels were measured from a total of 48 cells from four different experiments.

IL-8 induced phosphorylation of myosin light chain$_{20}$ (MLC_{20})

Immunoblots were done to study the IL-8 induced phosphorylation of MLC_{20} in CF and normal cells. Figure 3.4 shows the extent of MLC_{20} phosphorylation in CF (panel A) and normal (panel B) cells under control conditions and stimulation by IL-8 for 1 and 5 minutes and the corresponding quantification by densitometry (panel C and D). The densitometry results were expressed as the relative units compared to control cells. As shown in panel 4E, the phosphorylation of MLC_{20} was increased at 1 minute after treatment with IL-8 consistent with activation of contractile signaling. At 1 minute

MLC_{20} was significantly greater in CF cells (1.5 fold) compared to normal (1.2 fold). At 5 minutes, there was a further slight increase in phosphorylation, but the differences were not quiet statistically significant between CF and normal cells.

Expression of myosin light chain$_{20}$

Proteins were extracted from unstimulated CF and normal cells and the expression of total MLC_{20} was determined by immunoblotting. Figure 3.5A shows the Western analysis for the expression of MLC_{20} protein in CF and normal cells. Quantitative assessment with densitometry shows that the content of MLC_{20} was higher (Figure 3.5B, $p<0.05$) in CF (28 ± 2 arbitrary units) than normal cells (21 ± 2 arbitrary units).

Effects of IL-8 on migration of cells

A chemotaxis assay to IL-8 was performed and the results are shown in Figure 3.6 for the migration of CF and normal cells two concentrations of IL-8 (10 and 100 nM). The results are expressed as relative units compared to vehicle treated cells. IL-8 induced the migration of both normal and CF cells at concentrations of 10nM and 100nM compared to vehicle-treated cells. However, there was no difference in the migration of cells between CF and normal cells. PDGF (0.5nM) was used as a positive control.

Figure 3.1

Contraction of CF and normal ASM cells treated with IL-8

Contraction of the cells with IL-8 (100 nM) was measured with imaging studies. Panel A and B represent the images captured before and 10 minutes after the addition of the IL-8 in CF cells. Arrows with number 1, 2 and 3 indicates the contracted cells. Panel C and D show the images of normal cells before and after the addition of IL-8. Panel E represents the histogram of % decrease in the CF cell length on IL-8 treatment compared to control cells (C) ($p < 0.05$). 40 CF cells and 36 normal cells from four different individuals per group were used.

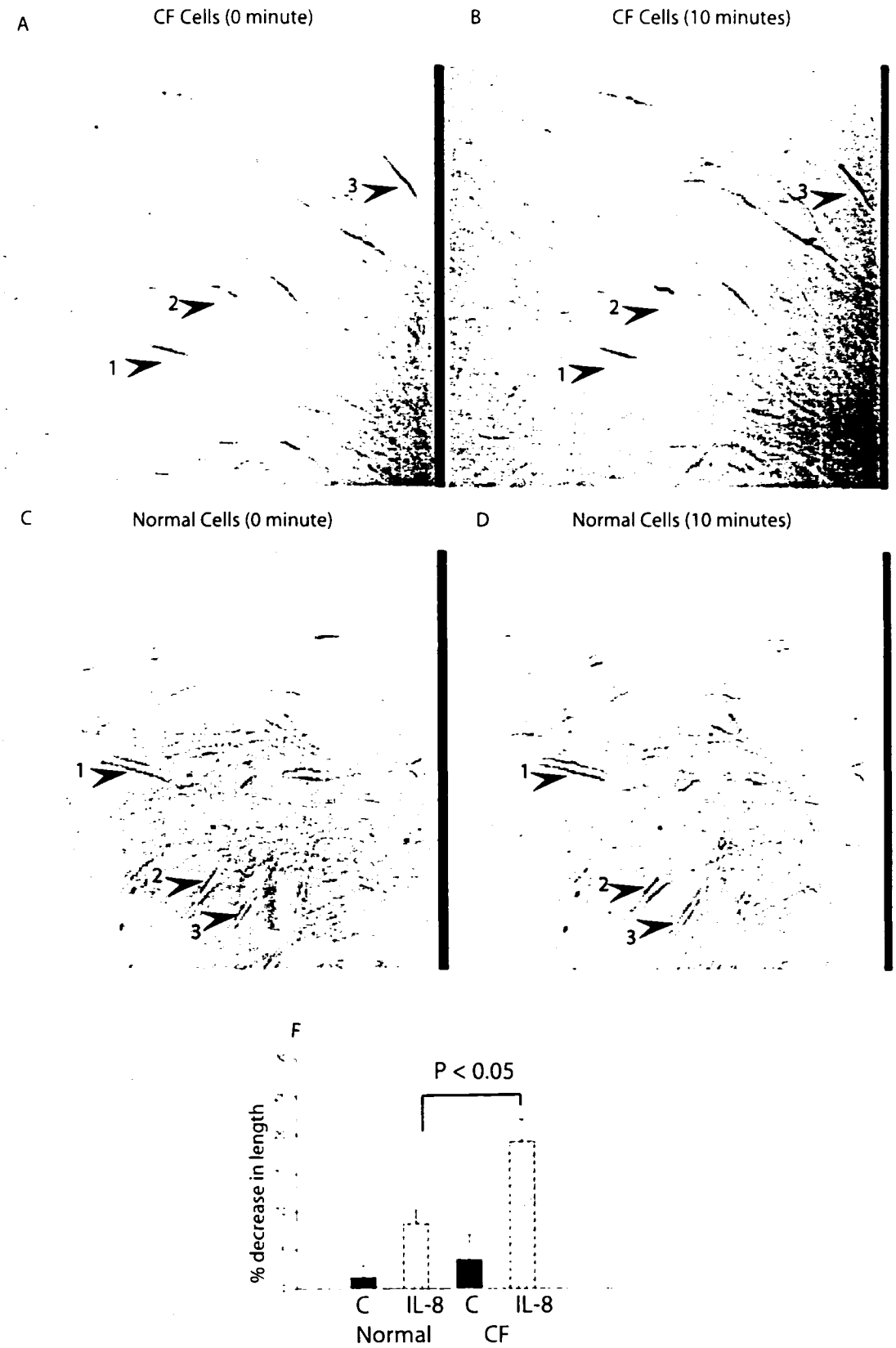

A
CF Cells (0 minute)
B
CF Cells (10 minutes)
1
2
3
C
Normal Cells (0 minute)
D
Normal Cells (10 minutes)
F
P < 0.05
% decrease in length
C
IL-8
C
IL-8
Normal
CF

Figure 3.2

Flow cytometric analysis of the surface expression of CXCR1 and CXCR2 on ASM cells from CF and normal patients.

Representative histograms shows the expression of CXCR1 and CXCR2 from CF and normal patients were shown in panels A, B and C, D respectively. Grey shaded area shows the unstained cells. Block line and grey line represents positively stained cells for receptors and isotype control cells respectively. Panel E show the percentage of positive cells stained for CXCR1 and CXCR2 from 4 different cell preparations of CF and normal patients ($p < 0.05$).

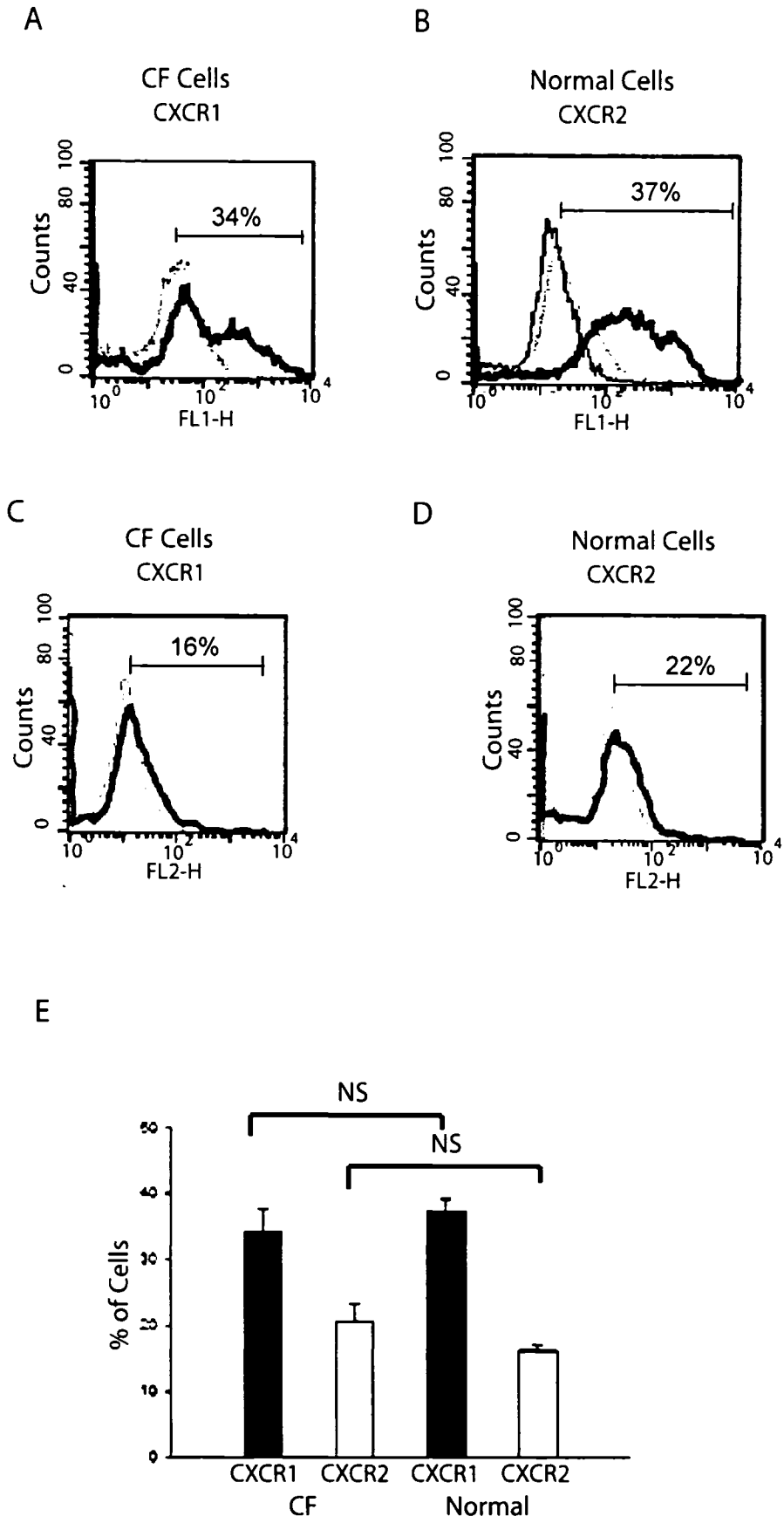

A
CF Cells
CXCR1
34%
Counts
FL1-H
B
Normal Cells
CXCR2
37%
Counts
FL1-H
C
CF Cells
CXCR1
16%
Counts
FL2-H
D
Normal Cells
CXCR2
22%
Counts
FL2-H
E
NS
NS
% of Cells
CXCR1
CXCR2
CXCR1
CXCR2
CF
Normal

Figure 3.3

Effects of IL-8 on $[Ca^{2+}]_i$ in CF and normal cells.

Cultured ASM cells from CF and normal patients were stimulated with IL-8 (100 nM). The baseline Ca^{2+} (dark columns) and the IL-8 induced release of $[Ca^{2+}]_i$ (white columns) from CF and normal group are illustrated. The mean of the peak $[Ca^{2+}]_i$ values were obtained from 48 cells per each group. The data presented are mean ± SEM.

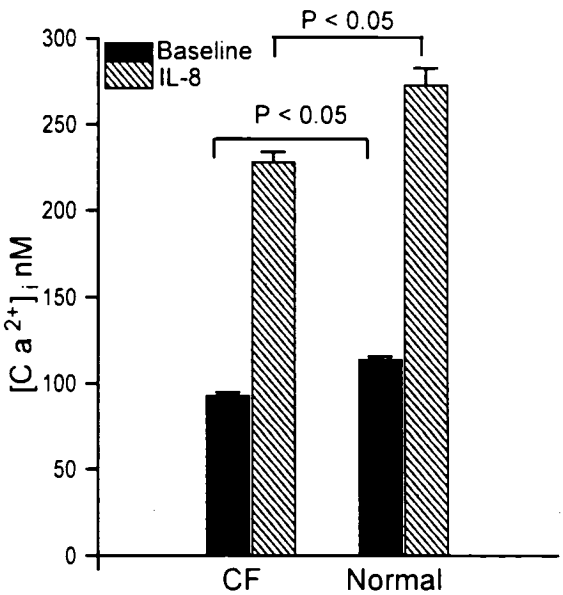

P < 0.05
Baseline
IL-8
P < 0.05
300
250
200
150
100
50
0
[C a 2+]i nM
CF
Normal

Figure 3.4

IL-8 induced phosphorylation of MLC_{20} from CF and normal cells

Panel A and B is a representative blot of myosin light chain (MLC_{20}) phosphorylation from CF and normal cells. Bands correspond to control, IL-8 stimulation at 1 and 5 minutes are illustrated. Myosin from chicken gizzard was used as a positive control (PC). Panel C and D are the densitometry histograms of MLC_{20} phosphorylation of CF and normal cells under control and IL-8 (100 nM) stimulated conditions at 1 and 5 minutes. Bars are presented as fold difference compared to controls (n=6 per group). Panel E illustrate the comparative histogram of MLC_{20} phosphorylation from CF and normal cells ($P < 0.05$). The data presented are mean ± SEM.

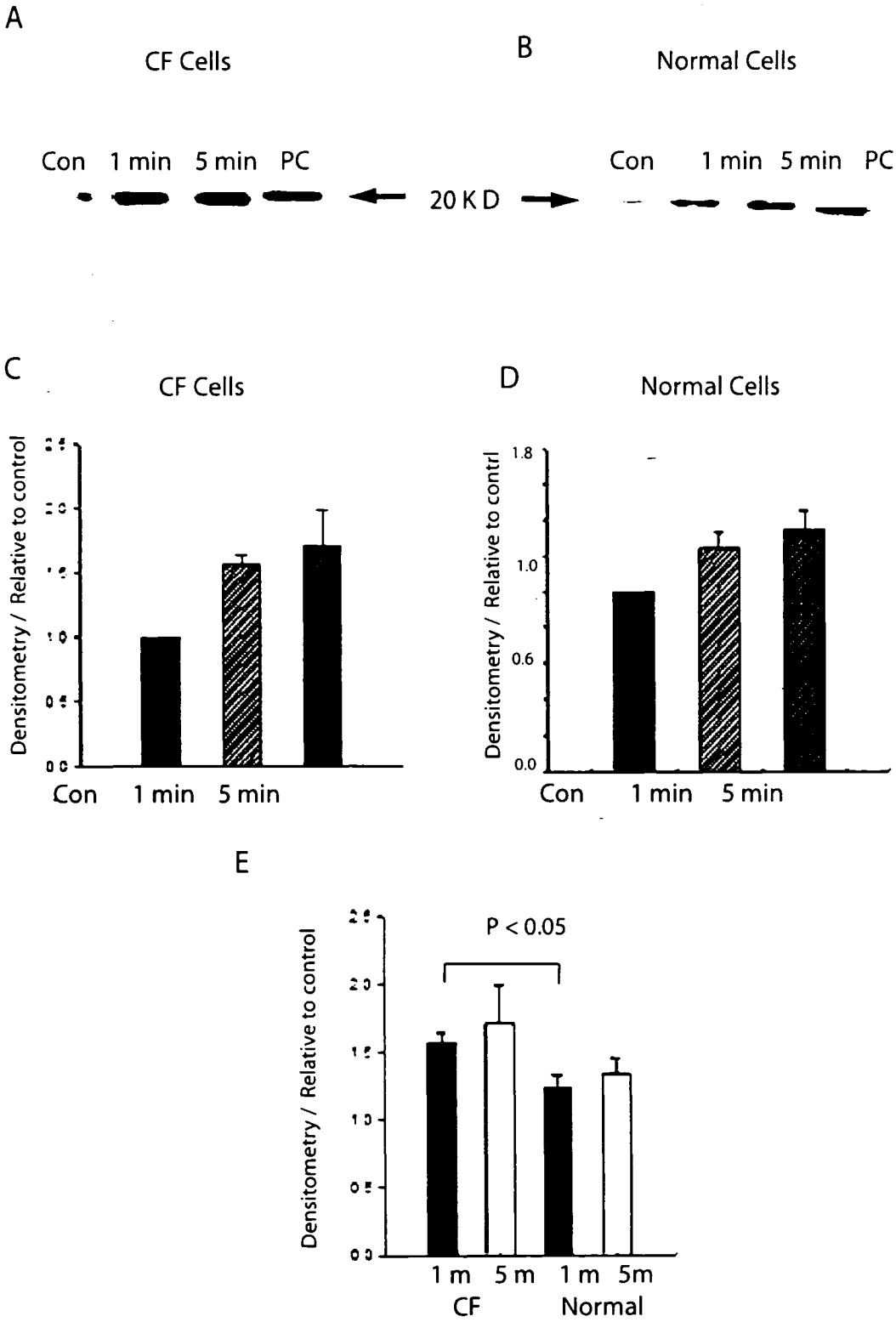
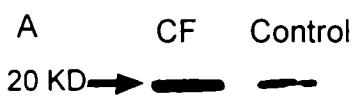

A
CF Cells
B
Normal Cells
Con
1 min
5 min
PC
20 K D
Con
1 min
5 min
PC
C
CF Cells
Densitometry / Relative to control
Con
1 min
5 min
D
Normal Cells
Densitometry / Relative to contrl
1.8
1.0
0.6
0.0
Con
1 min
5 min
E
P < 0.05
Densitometry / Relative to control
1 m
5 m
1 m
5m
CF
Normal

Figure 3.5

Expression of total MLC_{20} in CF and normal cells

Panel A is the representative blot for the expression of MLC_{20} in CF and normal cells. Mean densitometry value of MLC_{20} expression in CF cells is higher than normal cells (panel B, P = 0.05). n=4 experiments per group.


A
CF
Control
20 KD

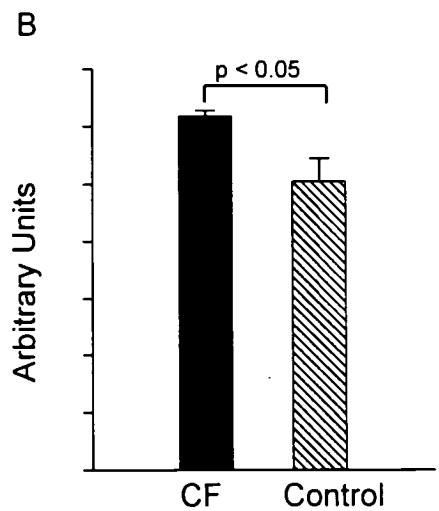
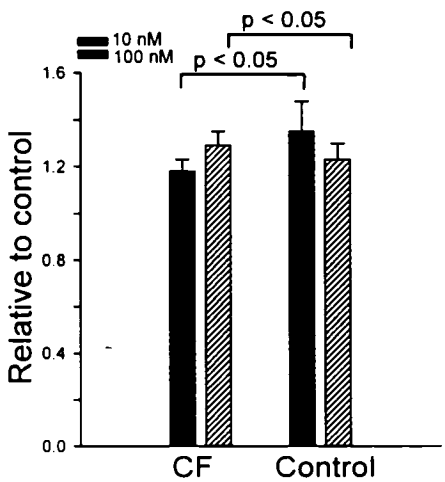

B
p < 0.05
Arbitrary Units
CF
Control

Figure 3.6

The effects of IL-8 on ASM cell migration in CF and normal cells

Histogram illustrates the IL-8 induced migration of cells in CF and normal cells at the concentration of 10 and 100 nM. The data is represented as the fold difference compared to vehicle treated cells


p < 0.05
10 nM
100 nM
p < 0.05
1.6
1.2
0.8
0.4
0.0
Relative to control
CF
Control

3.6 Discussion

The results of this study demonstrate that IL-8 induces a greater contraction of ASM cells from CF patients compared to normal individuals. The increase in ASM contraction correlates with a greater level of phosphorylation of MLC_{20} with IL-8 and higher expression of MLC_{20} in CF cells. However, there was no difference in the expression of CXCRs or in the increase in $[Ca^{2+}]_i$ evoked by IL-8 in both CF and normal cells, even though $[Ca^{2+}]_i$ were low in CF cells. These results indicate that there is a hyperresponsiveness to IL-8 that is mediated by mechanisms downstream of the regulation of calcium.

Chronic infection and inflammation leads to loss of more than one third of the epithelium from the basement membrane of central and peripheral airways of CF patients (42). As a result, the ASM cells are exposed to various inflammatory mediators such as TNF-α, IL-1β and IL-8. Some of these mediators may adversely affect the phenotype of the ASM to accentuate bronchoconstriction of CF airways. It has been shown that cytokines such as TNF-α, IL-1β, IL-5 and IL-13 modulate the contraction of ASM by changing the cellular phenotype (1), (35), (11). Since IL-8 is a chemokine and CXCR is a G-protein coupled receptor linked to phospholipase C, we reasoned that IL-8 was likely to induce contraction of ASM cells by releasing Ca^{2+} from the sarcoplasmic reticulum. Until recently, IL-8 had not been explored for its potential to alter ASM contractile function. To do so we used imaging techniques to investigate the effects of IL-8 on ASM contraction using cells from CF and normal subjects. The average length

of CF cells decreased twice as much as that of normal cells in response to IL-8 indicating that CF ASM cells are hyperresponsive to IL-8.

We tested the possible signaling mechanisms for the enhancement of the contraction in response to IL-8 by measuring the expression of CXCRs and the effects of IL-8 on $[Ca^{2+}]_i$. Flow cytometry demonstrated that there was no difference in the expression of CXCR1 and CXCR2 between CF and normal cells. This finding excluded the possibility that the regulation of receptor expression might be involved in the greater responsiveness of CF cells to IL-8. Therefore, we explored next the possibility that the enhanced ASM contraction in CF might be related to $[Ca^{2+}]_i$ release. However, the fluorescence imaging of intracellular Ca^{2+} showed that the IL-8 evoked higher Ca^{2+} transients in normal cells compared to CF cells. Interestingly, absolute values of baseline $[Ca^{2+}]_i$ and IL-8 stimulated peak $[Ca^{2+}]_i$ were lower in CF compared to normal cells, but the significance of these findings is somewhat uncertain. Since the peak intracellular Ca^{2+} release in response to IL-8 in CF cells was lower than normal cells, we explored the involvement of other mechanism such as Ca^{2+}sensitization in the increased contraction of CF ASM cells.

It is known that smooth muscle contraction is mainly regulated by a Ca^{2+} dependent increase in MLC_{20} phosphorylation. However, higher phosphorylation of MLC_{20} at any given $[Ca^{2+}]$i can also result in contraction of ASM cells. Himpens et al (13), have shown that contraction of rabbit pulmonary artery are regulated by MLC_{20} phosphorylation without accompanying changes in $[Ca^{2+}]_i$, indicating altered Ca^{2+} sensitivity of the contractile apparatus. It has also been suggested that MLC_{20} phosphorylation is regulated by $[Ca^{2+}]$- independent changes in the activities of myosin

light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) (reviewed in 38). In the present study, the observation of enhancement of MLC_{20} phosphorylation at 1 minute in response to IL-8 suggest that Ca^{2+} sensitization pathways may be involved in the enhancement of IL-8 induced contraction in CF ASM cells. Stephens et al (17) have shown that ASM from the trachea of an allergic dog model of asthma had increased phosphorylation of MLC_{20} and higher content of MLCK. Our measurement of higher phosphorylation of MLC_{20} in CF cells is consistent with such a mechanism for enhanced contraction of ASM to IL-8. However, we also measured the expression of MLC_{20} in unstimulated CF and normal cells and found that CF ASM cells expressed higher levels of MLC_{20}. This provides an alternate explanation for greater phosphorylation of MLC_{20}.

Apart from its role in contraction, ASM is also known to respond to various stimuli in other ways such as by proliferation and migration (29). Migration of ASM cells occurs in response to plasminogen activators and PDGF (29). Furthermore, it has been suggested that inflammatory mediators may also induce migration of ASM and is considered to be one of the causes of airway remodeling in asthmatic patients (18). In the present study, we expected that ASM cells might migrate in response to IL-8 and result in airway thickening due to remodeling. However, chemotaxis data indicate that IL-8 induced migration of CF ASM cells did not differ from that of normal cells and this is consistent with our data showing relatively comparable degrees of release of $[Ca^{2+}]_i$ in response to IL-8 in CF and normal cells.

Various biochemical mechanisms may underlie airway hyperresponsiveness in CF. Based on their responses to histamine and methacholine, two types of airway

responsiveness have been described in CF patients (28). CF patients with concomitant asthma have similar airway reactivity to both methacholine and histamine, whereas those without concomitant asthma respond less to histamine compared to methacholine. Responses to exercise are also different in both groups and seem to be mediated by cholinergic nerves (vagal fibers) and the products of airway damage (15), (43). In conclusion, our finding on IL-8 induced enhancement of bronchoconstriction may be an additional mechanism for airway hyperresponsiveness in CF patients. Our data show the mechanism of increased responsiveness to IL-8 lies downstream of Ca^{2+} regulation and may involve either increase Ca^{2+} sensitivity or higher total MLC20expression. The greater expression of total MLC20 in CF ASM cells suggests that these cells may have more contractile phenotype but the link between defective CFTR and contractile properties requires elucidation

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CHAPTER 4

ATP AND UTP INCREASE INTRACELLULAR Ca^{2+} IN HUMAN AIRWAY SMOOTH MUSCLE CELLS THROUGH P2Y RECEPTORS

4.1 Prologue

Use of extracellular nucleotides has been proposed for the treatment of impaired mucociliary clearance in CF airways. It is also known that these nucleotides are released in the airway milieu from various inflammatory cells and damaged structural cells such as endothelium and epithelium. Thus, CF ASM cells are exposed to extracellular nucleotides during infection and inflammation and also, during the treatment with nucleotides. We therefore hypothesized that these extracellular nucleotides may have direct effects on ASM cells and/or may enhance the effects of other inflammatory agents such as IL-8. In this study, we examined the expression of various subtypes of P2YRs and explored the effects of different extracellular nucleotides on $[Ca^{2}_{+}]_i$ in ASM cells from normal subjects. We also exposed the ASM cells to UTP for various time periods and studied the effects of ATP and UTP induced changes in $[Ca^{2}_{+}]_i$ to explore the possibility of modification of the effects of these agonists based on their prior exposure history.

4.2 Abstract

Extracellular ATP and UTP modulate the function of many cell types through the activation of specific P2 or purinergic receptors. The aims of this study were to examine human airway smooth muscle cells for the presence of P2 receptors and their subtypes and to determine the effects of P2 receptor stimulation on smooth muscle cell intracellular Ca^{2+}. RT-PCR and Western blot analysis of cultured cells showed the expression of the $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor subtypes. At concentrations ranging from 10^{-6} to 10^{-4}M, extracellular ATP, UTP, ADP and UDP produced significant increases in intracellular Ca^{2+} which peaked to 491± 51 nM ($p<0.001$) with ATP at 10^{-5}M. Pretreatment of the cells with UTP (10^{-4}M) for short periods of time (10 and 30 minutes) enhanced the peak Ca^{+2} release to UTP. Peak Ca^{+2} release in response to ATP and UTP was reduced by repeated and prolonged pretreatments with UTP. These results indicate that several subtypes of P2Y receptors are present in human airway smooth muscle cells and that their stimulation enhances peak Ca^{+2} release. These data also show that the sensitivity of the receptors is increased following short periods of exposure to UTP but prolonged and repeated exposure causes desensitization.

Abbreviations

ATP: Adenosine 5'- triphosphate,

UTP: Uridine 5'- triphosphate,

ADP: Adenosine 5'-diphosphate,

ADO: Adenosine,

UDP: Uridine 5'-diphosphate,

2- MeSATP: 2-(methylthio)-adenosine-5' triphosphate,

ATP-γ -S: Adenosine 5'-(3-thio) triphosphate,

EDTA: ethylenediaminetetraacetic acid

EGTA: ethyleneglycol-bis-(-β-aminoethyl ether)-*N*, *N*'-tetraacetic acid.

4.3 Introduction

Extracellular triphosphate nucleotides such as ATP, UTP and related compounds have significant biological effects on many tissues and cell types, including vascular and airway smooth muscle cells (9), (37), (43). In the respiratory system, ATP and UTP stimulate bronchial gland secretions and enhance the production of surfactant from alveolar type II cells (13), (22), (26), (42). It has also been reported that airway epithelial cells are a significant source of ATP and UTP, which can be released following simple mechanical stimuli (34) and cellular swelling (11), (45). In addition, Lazarowski et al (33) have reported that the concentration of ATP in the extracellular fluid overlying cultured human bronchial epithelial cells remains stable, despite continuous ATP hydrolysis indicating the presence of constant sources of the ATP. Extracellular ATP and UTP also stimulate chloride secretion and facilitate fluid transport from airway epithelial cells (6), (14), (29). These observations have prompted the consideration of the therapeutic potential of P2Y receptor stimulation for the improvement of mucociliary clearance in subjects with cystic fibrosis (7), (30). Kreda et al have also described another therapeutically oriented use of the $P2Y_2$ receptor and its ligand UTP as a gene transfer vector (31).

The effects of ATP and UTP are mediated by the activation of specific receptors, namely P2 receptors, present on the cell surface membrane. P2 receptors are classified into two broad categories: the P2X receptors and P2Y receptors (20) (43). The P2X -receptors are ligand-gated ion channels and 7 subtypes ($P2X_{1-7}$) have been identified on the basis of their molecular structure(19). The P2Y-receptors are G-protein coupled receptors and 8 subtypes are widely recognized ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$,

$P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) (27), (1). The missing subtypes in the $P2Y_{1\text{-}N}$ sequence represent the receptors cloned from non-mammalian vertebrates or receptors that are currently under functional characterization. P2 receptors are present on various smooth muscle cell types including gastrointestinal and vascular smooth muscle cells. ATP causes contraction of both gastrointestinal and vascular smooth muscle cells and proliferation and migration of vascular smooth muscle cells (3), (24), (28), (43). The effects of ATP and UTP on airway smooth muscle cells are, on the other hand, not well characterized. In rat airway smooth muscle cells, extracellular ATP and UTP trigger a substantial increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$), one of the main determinants of smooth muscle cell contraction (38). Recently, we have shown that ATP and UTP enhance the proliferation of rat airway smooth muscle cells through the stimulation of various subtypes of P2 receptors (37).

The effects of ATP and UTP on human airway smooth muscle cells have not been, to our knowledge, described nor is it known whether purinergic receptors are present. As cystic fibrosis patients frequently have inflamed and ulcerated airways, therapies based on the inhalation of P2Y receptor ligands to stimulate airway clearance could also affect the underlying airway smooth muscle cells. Thus, the aim of this work was to determine if P2 receptors are present in human airway smooth muscle cells and to characterize further the direct and indirect effects of ATP and UTP and their metabolites on the cells. In an attempt to mimic the *in vivo* conditions that would prevail if cystic fibrosis patients were to be treated with inhaled P2Y receptor ligands, the modifying effects of short and prolonged periods of exposure to UTP on smooth muscle cell responses were also measured.

4.4 Materials and Methods

Cell cultures

Primary cultures of human airway smooth muscle cells (HASM cells) were prepared from surgical and lung transplant specimens as described by Panettieri et al (42). Segments of lobar or main bronchus measuring 5x2mm were incubated for 90 minutes at 37° C in 10 ml Hanks' balanced salt solution (in mM: KCl 5, KH_2PO_4 0.3, NaCl 138, $NaHCO_3$ 4, Na_2HPO_4 5.6) to which 640 U collagenase type IV, 10 mg soybean trypsin inhibitor and 100 U elastase (type IV) had been added. The digested tissue was then filtered through a 125 μm Nytex mesh and the resulting cell suspension centrifuged. The pellet was reconstituted in culture medium containing FBS 5%, insulin, hFGF, GA-1000, hEGF (Clonetics, California, USA) and plated in a 25 cm^2 culture flask. Confluent cells were detached with a 0.025% trypsin and 0.02% EDTA solution and grown on 25mm diameter glass cover-slips for Ca^{2+} imaging and on 150 X 25 mm culture dishes for protein and total RNA extraction. Confluents cells from 1^{st} to 4^{th} passage were used. They were identified as smooth muscle cells by positive immunohistochemical staining for smooth muscle specific α-actin and positive identification of myosin light chain kinase and calponin by Western blotting. The project was approved by the local ethics committees.

Measurement of intracellular Ca^{2+}.

Confluent cells grown on 25 mm diameter coverslips in 35 mm diameter wells were used 10-14 days post plating. As described previously (38), cells were incubated for 20 to 30 min at 37° C with Hanks' buffer (in mM: NaCl 137, $NaHCO_3$ 4.2, glucose

10, Na_2HPO_4 3, KCl 5.4, KH_2PO_4 0.4, $CaCl_2$ 1.3, $MgCl_2$ 0.5, $MgSO_4$ 0.8, *N*-2-hydroxyethylpiperazine-*N*'-ethane sulfonic acid [Hepes] 5) containing 5μM Fura–acetoxymethylester (Fura-2-AM) and 0.02% pluronic F-127. The loaded cells were then washed and the coverslips placed in a Leiden chamber (Medical Systems Corp, Greenville, NY) containing 450 μl of Hanks' buffer on the stage of an inverted microscope equipped for cell imaging with 40x oil objective (Nikon. Montreal, QC,Canada). The cells were imaged using an intensified camera (Videoscope IC 200) and PTI software (Photon Technology International Inc, Princeton, NJ) at a single emission wavelength (510 nm) with double excitatory wavelengths (340 and 380 nm). The fluorescence ratio (340/380) was measured in individual cells (n=8 per slide) and the intracellular free Ca^{2+} ($[Ca^{2+}]_i$) calculated using a dissociation constant of Ca^{2+} to Fura-2 of 224 nM (47). Maximum ratio was determined in cells exposed to 10^{-5} M ionomycin in the presence of 1.3 mM $CaCl_2$ and minimum ratio in Ca^{2+} free Hanks' buffer to which EGTA (10^{-3} M) and ionomycin (10^{-5} M) had been added. Background fluorescence and autofluorescence were automatically subtracted.

Detection of P_2-receptor subtype mRNA expression.

Total RNA was extracted from confluent cells (10^{th} day after passage) by TRIzol reagent (Gibco BRL) according to the manufacturer's instructions. To analyze messenger RNA (mRNA), single strand cDNA was synthesized in a 20 μl reaction using 2 μg of total RNA as a template, oligo $(dT)_{12-18}$ primer and superscript™ II enzyme in the presence of acetylated BSA (Gibco BRL, Burlington, ON, Canada) and RNAguard ribonuclease inhibitor (Pharmacia Biotech, Quebec, Canada). Conventional

PCR was done for the detection of $P2Y_1$, $P2Y_4$, $P2Y_6$ subtypes and real time PCR for $P2Y_2$. The PCR mixture consisted of 1.5mM $MgCl_2$, 1X PCR buffer, 0.2mM dNTP mixture, 2.5 units of Platinum Taq polymerase (Gibco BRL), and 20 pmol of the 5' and 3' primers. mRNA from $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor and from cyclophilin (a house-keeping gene) was amplified from the resulting cDNA with the appropriate oligonucleotide primers (Table 1) by PCR. For the amplification of $P2Y_4$ and $P2Y_6$, the PCR conditions were as follows: initial denaturation for 5 min at 94° C followed by 35 cycles with each cycle consisting of denaturation at 94° C for 1 min, annealing at specific temperature for 1 min and extension at 72°C for 3 min. For $P2Y_1$, 25 cycles for the 1st reaction followed by 20 cycles for the 2nd reaction were performed. Real time PCR was performed using a fluorescence temperature cycler (Light Cycler: Roche Molecular Biochemicals). 20µl of reaction mix contained 2µl of cDNA and 4µl of both sense and antisense primers for $P2Y_2$, $MgCl_2$ (3 mM) and 2µl of fast start DNA SYBR green with Taq polymerase and H_2O. PCR cycling parameters: initial denaturation step at 95°C for 10 min was followed by PCR (95° C for 15 sec, and extension at 72°C for 24 sec) was performed for 40 cycles. After completion of the cycling process, samples were subjected to melting curve analysis for 1 cycle (denaturation at 95° C followed by 66°C, 95°C and ended by cooling). The amplified products were visualized by ethidium bromide staining after 2% agarose-gel electrophoresis and the size of the bands was determined by comparison with the DNA molecular markers (Roche Molecular Biochemicals, Montreal, QC, Canada.). To confirm the presence of $P2Y_2$ and $P2Y_4$ mRNA, PCR products were purified with GFX™ PCR and gel purification kit

(Amersham Pharmacia Biotech, NJ, U.S.A) and sequenced at the Institute de Recherche en Sciences de la Vie et de la Santé, (University of Laval, QC, Canada). Samples for RNA extraction were obtained from 4 different subjects. In all experiments, positive controls were included and human placenta (Clontech, California, USA) was used as positive controls. PCR primers were synthesized at the Sheldon Biotechnology Centre (Montreal, QC, Canada).

Experimental protocol

Pharmacological P2 receptor identification: Peaks in $[Ca^{2+}]_i$ were determined following stimulation with a range of concentrations (10^{-6} M to 10^{-4} M) of ATP, ADP, ADO, UTP, UDP and 2-MeSATP. All test drugs were diluted in Hanks' buffer from frozen stock solutions. They were prewarmed to 37°C before being added in the appropriate concentrations in 50µl volume

Short term exposure of HASMC to UTP: Cells were cultured on glass coverslips in six well plates. On the 10^{th} day post plating, UTP (10^{-4} M) or vehicle (Hanks' buffer) was added for 5, 10, 30 or 60 minutes to the culture medium. Following exposure to UTP, the cells were washed and loaded with FURA-2AM for measurement of $[Ca^{2+}]_i$.

Prolonged and repeated exposures of HASMC to UTP: Cells were cultured on glass coverslips in six well plates. On the 7^{th} day post plating, either vehicle or UTP (10^{-4} M) was added once a day for three days and $[Ca^{2+}]_i$ was measured in response to

various agonists on the 10^{th} day. The experiments on short and long term exposure to UTP were carried out on cells originating from 4 different patients.

Chemicals

Elastase and collagenase were purchased from Sigma (St.Louis,MO), Fura-2AM and pluronic F-127 were from Molecular Probes (Eugene,OR). ATP, ADP, UTP and 2-MeSATP and were purchased from Sigma. UDP was from Boehringer, Mannheim. EGTA was from Calbiochem (San Diego, CA, USA).

Statistical Analysis.

The data are expressed as mean ± standard error of the mean (SEM), n= number of cells. In pharmacological experiments the cells were obtained from 4 different subjects. Comparison of means was performed using paired or unpaired Student t-tests as appropriate and when multiple comparisons were made the Bonferroni correction was applied. ANOVA followed by Student's t-test was used for acute UTP exposure studies. $P< 0.05$ was considered to be statistically significant.

Table 1. Summary of PCR primers sequences for P2Y receptor.

Gene	Primer Sequences (5'-3')	Target size (bp)	Annealing (°C)	Cycle
$P2Y_1$	F: CCGCCGCCTAAGTCGAG	640	52	25
	R: GGTGTCGTAACAGGTGATGG			followed by 20
$P2Y_2$	F: CCAGGCCCCCGTGCTCTACTTTG	367	56	40
	R: CATGTTGATGGCGTTGAGGGTGTG			
$P2Y_4$	F: CCACCTGGCATTGTCAGACACC	425	58	35
	R: GAGTGACCAGGCAGGCACGC			
$P2Y_6$	F: CGCTTCCTCTTCTATGCCAACC3	365	60	35
	R: CCATCCTGGCGGCACAGGCGGC			
Cycl	F: GGTCAACCCCACCGTGTTCTTCG	566	60	25
	R: GTGCTCTCCTGAGCTACAG			

4.5 Results

Pharmacological identification of P_2 receptors.

Concentration-response curves for ATP, ADP and adenosine are shown in figure 4.1A. ATP and ADP induced significant increases in $[Ca^{2+}]_i$ at all concentrations tested (10^{-6}M to 10^{-4} M). Following ATP stimulation at a concentration of 10^{-5} M, peak $[Ca^{2+}]_i$ was 491 ± 51 nM ($p<0.001$, n=27), while a concentration of 10^{-4} M elicited a smaller release of Ca^{2+} with peak $[Ca^{2+}]_i$ reaching 389.0±34.2 nM, indicating that a plateau had been reached. ADP also induced a significant Ca^{2+} release which peaked at 10^{-5} M with a $[Ca^{2+}]_i$ of 349±27 nM ($p<0.001$, n=32). Exposure to adenosine, the metabolite of ATP produced very small, albeit significant increases in $[Ca^{2+}]_i$ at 10^{-5} and 10^{-4} M, indicating the presence of functional adenosine receptors.

Figure 4.1B, shows the effects of UTP and UDP. Exposure to UTP produced an increase in $[Ca^{2+}]_i$ reaching a maximum at 10^{-4} M: 321±30 nM ($p<0.005$, n=58). Exposure to UDP also increased maximal $[Ca^{2+}]_i$ release at 10^{-4} M: 255±41 nM ($p<0.005$, n=57). The approximate half maximal concentration of these agonist were ATP:6 μM, ADP:2 μM, UTP:1 μM and UDP:0.7 μM. Fig 4.2A shows the increase in $[Ca^{2+}]_i$ produced by 2-MeSATP, an agonist for $P2Y_1$ and $P2X_1$ and $P2X_3$ receptors, in the presence and in the absence of Ca^{2+} in the extracellular fluid. In the presence of Ca^{2+}, the agonist caused significant increases in $[Ca^{2+}]_i$: 25.8±6.5 nM ($p<0.05$, n= 24) at 10^{-5} M and 133.9±19.8 nM ($p<0.005$, n=43) at a concentration of 10^{-4} M. In Ca^{2+} free medium, peak Ca^{2+} signals were not statistically different from those observed in the Ca^{2+} containing Hanks', indicating that the increase in $[Ca^{2+}]_i$ is due to the release of Ca^{2+} from intracellular stores rather than influx from extracellular sources. Thus, these

data indicate that the response is mediated by the $P2Y_1$ receptor subtype, a G-protein coupled receptor, rather than the $P2X_1$ or $P2X_3$ receptors which are ligand-gated ion channels. Figure 4.2B shows the release of Ca^{2+} produced by ATP 10^{-5} M in Ca^{2+} containing and Ca^{2+} free extracellular medium. The increase in $[Ca^{2+}]_i$ was comparable in both conditions: 111.8±15.0 nM (n=32) in presence of Ca^{2+} versus 106.0±16.3 nM (n=24) in the absence of Ca^{2+} indicating that Ca^{2+} release was not dependent on extracellular Ca^{2+} influx and confirming that the agonist is acting on a G-protein coupled receptor, possibly P2Y, rather than on ligand-gated ion channels.

Expression of mRNA for P2Y receptor subtypes

Conventional and real time PCR analysis using different sets of primers for P2Y receptors (Figure 4.3) showed the expression of mRNA coding for $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ in HASM cells. mRNA expression for $P2Y_1$, $P2Y_4$ and $P2Y_6$ receptors was detected with conventional PCR. $P2Y_2$ receptor mRNA was detected with real time-PCR. As we had difficulty in obtaining the signals from positive controls (human placenta) with $P2Y_2$ receptor primers, the PCR products from $P2Y_2$ receptor reactions were sent for sequencing. The sequences were matched with the reported gene sequences for the $P2Y_2$ gene (Gene bank # NM_002564.2), confirming the expression of the $P2Y_2$ receptor. In the case of $P2Y_4$, two bands were detected. Hence, the PCR products from the most prominent band were purified and sequenced. The sequenced products matched the published $P2Y_4$ gene sequence (Gene bank # NM-002565). We did not explore the other weakly expressed band which might be a splice variant.

Effects of short term pretreatment of HASM cells with UTP on responses to P2Y receptor stimulation

Peak $[Ca^{2+}]_i$ was measured in response to UTP 10^{-4} M in cells that were previously incubated for 5, 10, 30, and 60 min with UTP (10^{-4} M) and the results are shown in figure 4. The resting $[Ca^{2+}]_i$ values were comparable between the vehicle and the UTP pretreated group (131 ± 3 nM, n= 148 and 141 ± 8 nM, n= 152) Exposure to UTP 10^{-4} M elicited significant increases in $[Ca^{2+}]_i$ in both vehicle and UTP exposed cells, but peak $[Ca^{2+}]_i$ was significantly greater in the cells previously incubated with UTP for 10 and 30 min (476 ± 41 nM n=36 and 458 ± 42 nM, n=38) compared to cells incubated with vehicle alone (350 ± 39 nM n=37 and 228 ± 35, n=40, $p < 0.05$). Following 5 and 60 minute incubations with UTP, peak $[Ca^{2+}]_i$ was comparable to that measured in vehicle treated cells: 228 ± 23 nM (n=37) and 221 ± 14 nM (n=37) versus 290 ± 40 nM (n= 35) and 245 ± 25nM (n=37, p=0.35)

Effects of prolonged periods of pretreatment of HASM cells with UTP on responses to P2Y receptor stimulation

Following prolonged and repeated exposures of HASM cells to UTP 10^{-4} M (72 hours, 3 doses, each at the intervals of 24 hours), $[Ca^{2+}]_i$ was measured in response to UTP 10^{-4} M, ATP 10^{-4} M and histamine 10^{-4} M and the results are shown in figure 5. Resting $[Ca^{2+}]_i$ levels were not significantly affected by incubation with UTP. Peak $[Ca^{2+}]_i$ levels were significantly decreased in response to UTP 10^{-4} M: 152 ± 12 nM (n=35) in the cells pretreated with UTP versus 349 ± 24 nM (n= 36)($p < 0.001$) in the cells exposed to vehicle alone. Peak Ca^{2+} release in response to ATP 10^{-4} M was also

decreased in cells exposed to UTP for prolonged periods compared to those exposed to vehicle alone: 148 ± 7 nM (n= 38) versus 340 ± 20 nM (n= 38) ($p<0.001$). In contrast, the peak $[Ca^{2+}]_i$ measured in response to histamine 10^{-4} M was comparable in both vehicle (413 ± 45 nM, n=31) and UTP pretreated cells (368 ± 36 nM, n=34).

Figure 4.1

Effects of nucleotides on $[Ca^{2+}]_i$

Effects of increasing concentration of ATP, ADP and ADO (panel A) and UTP and UDP (panel B) on peak $[Ca^{2+}]_i$ release. R represents the resting Ca^{2+} levels. The data presented are means ± SEM. *P< 0.001,**P< 0.01.

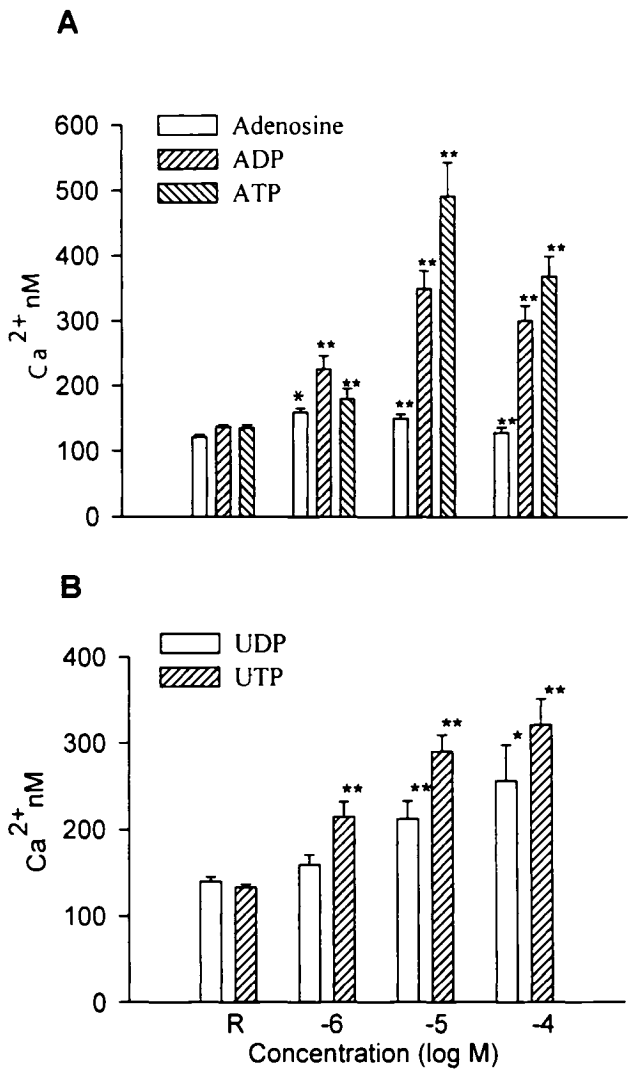

A
600
500
400
300
200
100
0
Adenosine
ADP
ATP
Ca2+ nM
B
400
300
200
100
0
UDP
UTP
Ca2+ nM
R
-6
-5
-4
Concentration (log M)

Figure 4.2

Effects of 2-MeSATP and ATP in Ca^{2+} free extracellular medium

Increase in Ca^{2+} in response to 2-MeSATP (Panel A) and ATP (Panel B) in Ca^{2+} rich (white columns) and Ca^{2+} free medium (striped columns). The data presented are means ± SEM.

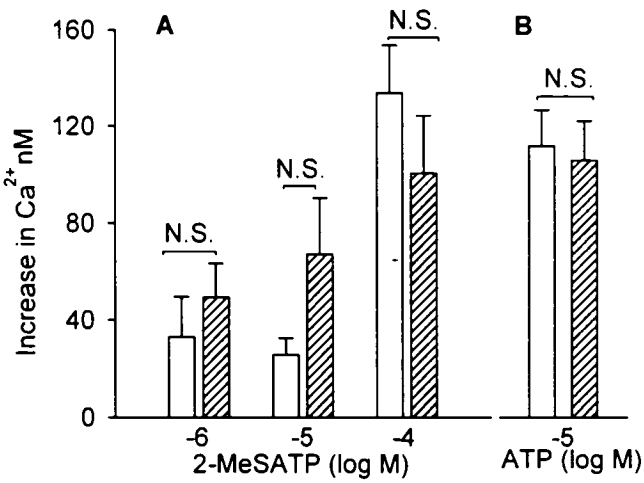

160
120
80
40
0
Increase in Ca^{2+} nM
A
B
N.S.
N.S.
N.S.
N.S.
-6
-5
-4
-5
2-MeSATP (log M)
ATP (log M)

Figure 4.3

Expression of mRNA for P2Y receptor subtypes

mRNA expression of $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors by PCR. Lane 1-4 represents mRNA from four different individuals. M: DNA molecular marker. Cyclophilin : positive control.

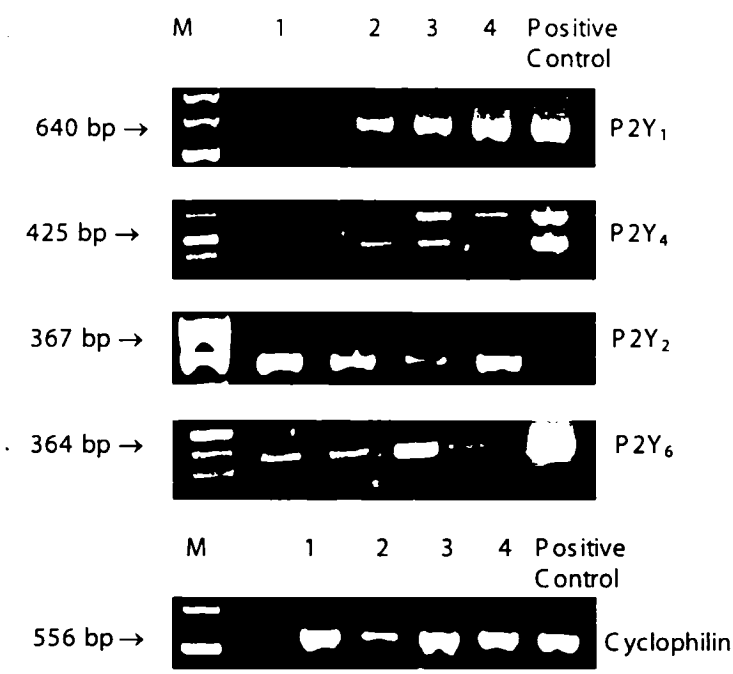

M
1
2
3
4
Positive Control
640 bp →
P2Y1
425 bp →
P2Y4
367 bp →
P2Y2
364 bp →
P2Y6
M
1
2
3
4
Positive Control
556 bp →
Cyclophilin

Figure 4. 4

Effects of UTP on peak $[Ca^{2+}]_i$ following short time exposure of ASM cells to UTP

Increase in Ca^{2+} in response to UTP 10^{-4} M following cell incubation in Hanks' buffer (white column) or in UTP 10^{-4} M (striped column). R represents the resting Ca^{2+} levels from all experiments done at different time periods. The data presented are means ± SEM. * $p<0.05$.

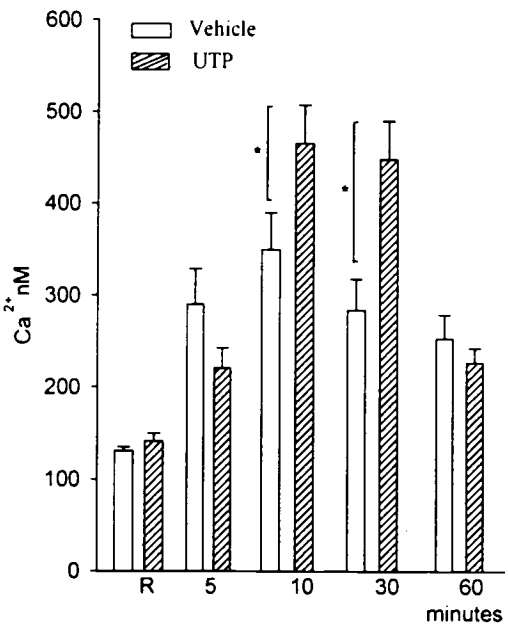
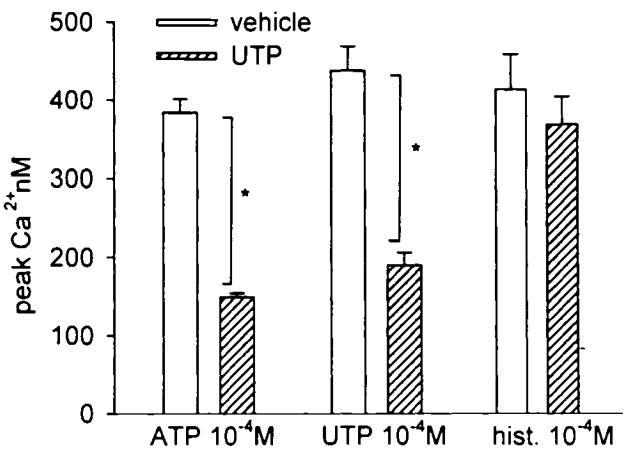

600
500
400
300
200
100
0
Vehicle
UTP
Ca^{2+} nM
R
5
10
30
60
minutes
*
*

Figure 4.5

Effects of UTP and ATP and histamine on peak $[Ca^{2+}]_i$ following Prolonged exposure of ASM cells to UTP

Peak Ca^{2+} release in response to UTP, ATP and histamine (10^{-4} M) following prolonged and repeated exposure of HASM cells to UTP10^{-4}M. Cells were treated with UTP10^{-4}M (striped columns) or vehicle (white columns) once a day for 3 days. Values presented are means ± SEM. * $p<0.001$


vehicle
UTP
500
400
300
200
100
0
peak Ca 2+ nM
*
*
ATP 10-4M
UTP 10-4M
hist. 10-4M

4.6 Discussion

Extracellular adenine and uridine nucleotides and their metabolites have a range of effects on a wide variety of cells through the activation of specific P_1 and P_2-receptors (10), (15), (32), (48). Biological responses to ATP are numerous and include the stimulation or inhibition of contractility in vascular and visceral smooth muscle, the stimulation or modulation of the release of neurotransmitters and hormones in the central and peripheral nervous systems, the stimulation of gland secretions, the proliferation of various cell types including vascular smooth muscle, the release of inflammatory mediators by mast cells and the priming of the functional response of leukocytes to inflammatory stimuli (15).

Although the effects of extracellular ATP and other triphosphate nucleotides have been studied in various lung cell types including alveolar macrophages, mast cells, alveolar type II cells and pulmonary vascular smooth muscle cells, the effects of these compounds on airway smooth muscle (ASM) cells have not been studied as extensively. *In vivo,* it has been reported that intratracheal instillations of ATP produce an increase in pulmonary resistance in rats(19). *In vitro* measurements have produced conflicting results, some authors reporting that ATP induces contraction of tracheal preparations while others observe an inhibitory effect (2), (16), (18). This inhibitory effect appears to be dependent on the presence of the epithelium (4). Previous work from our laboratory has shown that in cultured rat airway smooth muscle cells, extracellular ATP and UTP produce an increase in $[Ca^{2+}]_i$ and promote airway smooth muscle cell proliferation (37), (38) confirming the presence of functional receptors to these agonists on rat airway smooth muscle cells. To our knowledge, the possible effects of these

triphosphate nucleotides on HASM cells have not been described. The results of the current study show that exposure to extracellular ATP, UTP and their metabolites, produces a release of intracellular Ca^{2+} in HASM cells. The data also show that adenosine has very little effect on $[Ca^{2+}]_i$ confirming that the increase in $[Ca^{2+}]_i$ observed following ATP exposure is due to a direct effect of ATP rather than to an effect of its metabolite adenosine.

The effects of adenine and uridine nucleotides are mediated by a number of specific P2 receptors. The $P2Y_1$ receptor responds to ADP more than ATP but not to UTP while the $P2Y_2$ responds to both ATP and UTP. The $P2Y_4$ response to UTP>ATP in humans (25). The $P2Y_6$ subtype responds preferentially to UDP and has little affinity for ATP and the $P2Y_{11}$ responds to ATP but not to UTP. The P2Y receptors are coupled to a $G_{q/11}$ protein, and some may also couple to a Gi ($P2Y_2$, $P2Y_4$) or to a G_s protein ($P2Y_{11}$) (39), (43). The results we obtained showing that ATP, ADP, UTP and UDP produce an increase in $[Ca^{2+}]_i$, suggest that $P2Y_{1,2,4}$ and $_6$ receptors are likely present on HASM cells. The response to ADP, comparable in amplitude to that of ATP suggests that the $P2Y_1$ subtype which responds more to ADP>ATP is present. To confirm this latter observation, a dose- response curve to 2-MeSATP, a selective agonist of $P2Y_1$, $P2X_1$ and $P2X_3$ receptors was obtained. Exposure to this agonist produced an increase in $[Ca^{2+}]_i$ that was independent of the presence of Ca^{2+} in the extracellular medium, indicating that the peak $[Ca^{2+}]_i$ was due to Ca^{2+} release from intracellular stores rather than from extracellular sources, which is consistent with the response being mediated by a P2Y (G protein-coupled receptor) rather than by a P2X (ion gated channel) receptor subtype. UDP, a specific agonist of the $P2Y_6$ receptor (18) also

produced an increase in $[Ca^{2+}]_i$ indicating that this receptor must be present on HASM cells. This increase in $[Ca^{2+}]_i$ occurred within 30 seconds after the addition of the agonist, and thus is attributable to UDP itself and not to a possible transphosphorylation of UDP into UTP; this reaction is slow to occur (25), (35).

Even though selective antagonists are available for $P2Y_1$ and $P2Y_{12}$, highly selective antagonists are not available for other subtypes of P2Y receptors (24), (28). This makes further pharmacological characterization of the receptors difficult, hence we used RT-PCR analysis to confirm the presence of P2Y receptor mRNA transcripts. As our pharmacological data indicated the presence of G protein coupled P2Y subtypes of the receptors rather than P2X receptors, identification of mRNA transcripts of P2X receptors was not explored. The RT-PCR analysis of mRNA expression confirms that HASM cells express $P2Y_1$. $P2Y_2$. $P2Y_4$ and $P2Y_6$ receptors.

P2 receptors can be stimulated by extracellular ATP and/or UTP through endogenous as well as exogenous sources. An endogenous source of extracellular ATP and UTP is the airway epithelium. Indeed, airway epithelial cells constitutively secrete ATP and UTP and can also release ATP and/or UTP following various biological and mechanical stimuli (14), (33, (34), (41) Considering that airway epithelium and smooth muscle cells are in close proximity, it is likely that the ATP and/or UTP released by epithelial cells can have a direct effect on HASM cells, particularly in pathological conditions such as asthma and cystic fibrosis, in which the airway epithelium is disrupted. Also, aerosolized UTP has been proposed as a therapeutic agent to enhance mucociliary clearance in cystic fibrosis patients as the stimulation of $P2Y_2$ receptors and to a lesser extent of $P2Y_4$ and of $P2Y_6$ (via UDP, the metabolic product of UTP) (32)

on human epithelial cells produces an increase in chloride ion secretion and ciliary beat frequency (40). The potential limitation of this therapy is that G protein-coupled receptors including $P2Y_2$ receptors undergo desensitization upon repeated agonist exposure (8), (44), (49). Although the mechanism of homologous desensitization has been well established for adrenergic receptors (23), the pathways for the desensitization of P2Y receptors are not well characterized. Brown et al (8), have shown that incubation of airway epithelial cells with UTP for 5 minutes or longer decreases the total inositol phosphate turnover, suggesting that agonist induced desensitization is a characteristic of its receptor. In the present study, we have determined the effects of short and prolonged, repeated exposures to UTP on the increase in intracellular Ca^{2+} in response to *de novo* UTP stimulation. UTP mediated increases in $[Ca^{2+}]_i$ were enhanced when the cells were exposed to UTP for 10 and 30 minutes suggesting that the sensitivity of the receptors was increased by short periods of exposure to UTP. However, the data also show Ca^{2+} release was decreased in response to both UTP and ATP following longer and repeated exposure to UTP (once a day/3days). The response to histamine, which is also mediated by a G protein-coupled receptor was not affected by pre-exposure to UTP, suggesting that desensitization was confined to the P2 receptors. These data are consistent with studies by Clarke et al (12) who showed that in epithelial cells, the desensitization of $P2Y_2$ receptors depends on the duration of exposure to UTP. Although we have not addressed the mechanism of desensitization of the receptors, work from other authors suggests that uncoupling and sequestration of the $P2Y_2$ receptors, caused by phosphorylation of the carboxy terminus of the receptor by a G protein-coupled receptor kinase (21), and agonist promoted internalization are likely mechanisms (44).

In conclusion, these data show that several subtypes of P2Y receptors are present in human airway smooth muscle cells and that their stimulation produces an increase in $[Ca^{2+}]_i$, an important determinant of cell contractility. The physiological role(s) of these receptors merits further investigation. In addition, considering that patients with cystic fibrosis often have airway hyperresponsiveness as well as an increase in airway smooth muscle mass (5), (36), (46) the effects of UTP aerosol therapy on airway smooth muscle contractility and proliferation

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CHAPTER 5

DISCUSSION AND CONCLUSIONS

5.1 Discussion

AHR is a characteristic feature of asthma, but it is not considered to be diagnostic. Other chronic airway inflammatory diseases such as CF, COPD and chronic bronchitis also display AHR. Although AHR in CF patients is not as common as in asthmatic patients, a significant number of CF patients suffer from bronchospasm and their airways are infiltrated with neutrophils. Furthermore, there is abundant evidence indicating that a non-eosinophilic form of asthma is present in patients with acute severe asthma and fatal asthma (19), (20), (18), (15) and these patients have elevated neutrophils associated with higher levels of IL-8 in their airways (6) (17). Pro-inflammatory mediators such as IL-1β, TNF-α and contractile agonists like bradykinin induce ASM cells to produce IL-8 (10), (13) but it is not known whether HASM cells respond to IL-8 by expressing the pertinent receptors. Therefore, we explored the effects of IL-8 and the expression of the pertinent chemokine receptors, CXCRs, in ASM from normal subjects. The findings of this thesis may have relevance for both asthma and CF which are airway diseases associated with elevated concentrations of IL-8 and may help to explain bronchoconstriction in these conditions.

Experiments were conducted on cultured ASM cells which provides the advantage of studying many functions of ASM, such as the measurement of calcium transients in single cell, contraction of cells on different surfaces (glass and homologous cell substrate) and migration of cells to different agonist (IL-8, and PDGF) in homogeneous cell populations. The homogeneous culture system also helps us to study mRNA expression and the percentage quantification of CXCRs protein in pure ASM cells. We confirmed our cultured cells as ASM cells by studying the expression of

smooth muscle specific alpha-actin and other smooth muscle markers such as myosin heavy chain, and calponin. Although ASM cells undergo phenotypical variations during cultured conditions, they still retain their contractile properties (12), (16) indicating that cultured ASM cells can be used for studying the above mentioned functions.

The data presented in chapter 2, demonstrate that approximately twenty percent of normal HASM cells expressed both CXCRs constitutively. The functions of CXCRs were confirmed by measuring the effect of IL-8 induced increase in $[Ca^{2+}]_i$ and by inhibiting their response with specific neutralizing antibodies to CXCRs. Furthermore, our data indicate that IL-8 evoked Ca^{2+} transients are mediated through PLC coupled signaling pathways, which lead to contraction of ASM cells. The observation of IL-8 induced contraction of ASM cells is perhaps the most significant finding of this thesis. The number of the ASM cells contracted in response to IL-8 is approximately correlated with the percentage of cells that expressed CXCRs in normal ASM cells. Since the amount of bronchial smooth muscle is increased in CF and asthma, it is possible that higher numbers of ASM may respond to IL-8 and thereby, contribute to enhanced bronchoconstriction and/or hyperresponsiveness. In other words IL-8, and indeed other chemokines such as eotaxin that are also produced by ASM may be non-classical bronchoconstrictors that cause airway narrowing. It is also possible that following inflammation, the expression of CXCRs may be up-regulated or the sensitivity of receptors may be increased, which may lead to enhanced contraction in response to IL-8.

Apart from AHR, patients with chronic inflammatory diseases (especially asthma) also have airway remodeling, i.e. changes in the architecture of the airways, due to shedding of epithelium, thickening of the sub-epithelial basement membrane,

fibrosis, ASM hypertrophy and hyperplasia. Recently, it has also been suggested (1), (2) that ASM cells migration might contribute to the progression of airway remodeling. It is suggested that new ASM cells may arise from subepithelial myofibroblasts and migrate to join ASM bundles in the submucosal region. Our data on IL-8 induced migration of normal HASM cells are consistent with this suggestion although it is not known whether the gradient for IL-8 is more marked towards the lumen of the airway or towards the smooth muscle bundles. Both epithelial cells and ASM synthesize IL-8 so that there is potentially a gradient of IL-8 in both directions from the subepithelial area where myofibroblasts are located.

As described in chapter 3, IL-8 enhanced cell contraction (2 fold) in CF ASM compared to normal ASM cells. IL-8 released intracellular Ca^{2+} in both normal and CF cells, Interestingly, the absolute values of baseline calcium and IL-8 stimulated peak $[Ca^{2+}]_i$ were lower in CF compared to normal cells. A similar pattern was also observed with histamine induced Ca^{2+} release in CF ASM cells. Studies with different Cl^- channel blockers (11) that Cl^- channels modulate resting $[Ca^{2+}]_i$ and Ca^{2+} release in response to histamine in the ASM cells. Since CFTR is defective in CF cells, both resting $[Ca^{2+}]_i$ and peak Ca^{2+} release are expected to be lower than normal ASM cells. The significance of these findings for organ dysfunction is uncertain but it is interesting that CF patients suffer from intermittent bowel obstruction that is usually attributed to viscid intestinal secretions but it is possible that it is related to reduced bowel motility. The role of these channels and the significance of the altered responses of CF ASM cells require to be further explored.

Although smooth muscle contraction is mainly regulated by a Ca^{2+} dependent increase in MLC_{20} phosphorylation, other mechanisms have also been described for the regulation of smooth muscle contraction through MLC_{20} phosphorylation without an increment in $[Ca^{2+}]_i$ (7), (14). MLCK and RhoA-associated Rho-kinase (ROK) are the major cellular targets that regulate the degree of ASM contraction for a given level of intracellular calcium or so-called Ca^{2+} sensitivity (9), (4). Rho-kinase phosphorylates the regulatory subunit (MYPT-1) of the smooth muscle myosin light chain phosphate (MLCP) (5) either directly or through myosin phosphatase associated kinases (3). These phosphorylation reactions inhibit the phosphatase activity of MLCP leading to increased accumulation of MLC_{20} and a subsequent increase in the contraction. In our studies, higher MLC_{20} phosphorylation was observed in response to IL-8 and we consider this as a possible mechanism for the IL-8 induced enhancement of contraction in CF ASM cells. However, the mechanism for the increased phosphorylation of MLC_{20} in response to IL-8 is not known. Furthermore, the higher expression of MLC_{20} in unstimulated CF cells shows that CF cells have higher contractile proteins than normal cells, which may be another reason for higher contraction.

As reported in chapter 4, we studied the effects of changes in $[Ca^{2+}]_i$ in response to ADO, ADP, ATP, UDP and UTP and found that these nucleotides modulate $[Ca^{2+}]_i$. Furthermore, our experiments with calcium-free extracellular medium have confirmed that these nucleotides released Ca^{2+} from intracellular sources rather than from extracellular sources. This finding also suggests that the above nucleotides mediate intracellular Ca^{2+} release through G protein coupled receptors (P2YR). This is consistent with the expression of different subtypes of P2YRs ($P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$

receptors) in normal ASM cells. The main finding of this study is that ATP and UTP release intracellular Ca^{2+} in ASM cells through $P2Y_2$ and/or $P2Y_4$Rs.

Although UTP has been proposed for treatment of CF patients to enhance the mucosal clearance of their airways, the effects of long-term treatment with UTP on ASM function are not known. We studied the effects of UTP modulated $[Ca^{2+}]_i$ in ASM cells by exposing them to UTP for different time. When ASM cells were treated with UTP for shorter times, peak $[Ca^{2+}]_i$ were increased in response to the UTP stimulus. In contrast, repeated treatments of cells with UTP for longer times decreased the peak $[Ca^{2+}]_i$ following the UTP stimulus, indicating the possible desensitization of receptors in ASM cells. Interestingly the $[Ca^{2+}]_i$ in response to histamine in UTP pretreated cells did not alter the peak Ca^{2+} release suggesting that desensitization occurs specifically to P2YRs and not to histamine receptors. Both histamine and P2Y receptors are G-protein coupled receptors that mediate the intracellular Ca^{2+} release through PLC-coupled signal transduction mechanisms. Hence, we compared both signaling pathways to find out the mechanism responsible for lower Ca^{2+} response to UTP in ASM cells pre-treated with UTP for long time.

Pro-inflammatory mediators like IL-1β enhance contractile effects of ATP and UTP in vascular smooth muscle by up-regulating $P2Y_2$R expression (8). Based on these observations, we hypothesized that IL-8 may up-regulate P2YRs and thereby, enhance the effects of ATP and UTP on intracellular Ca^{2+} release in ASM cells. To test this hypothesis, we incubated normal ASM with IL-8 (10 ng/ml) for 24 hours and measured intracellular Ca^{2+} in response to UTP and ATP. UTP evoked a greater peak $[Ca^{2+}]_i$ up to 358 ± 29 nM after IL-8 pre-treatment compared to the control value of 265 ± 12 nM (p

< 0.005) and there were no significant changes in the resting $[Ca^{2+}]_i$. ATP also evoked an intracellular peak $[Ca^{2+}]_i$ up to 421 ± 26 nM after IL-8 pre-treatment compared to control 342 ± 26 nM ($p < 0.05$) (data is shown in appendix- figureAP-1 and figure AP2). These results indicate that there is an interaction between IL-8 and extracellular UTP or ATP, possibly by increasing the sensitivity P2YRs stimulation in normal ASM cells. Further studies are needed to identify the mechanisms for the enhanced sensitivity of the P2YRs.

5.2 Conclusions

We can draw several conclusions from the work presented in this thesis. First, IL-8 triggers the contraction and migration of normal ASM cells by releasing intracellular Ca^{2+} through PLC-coupled signal transduction mechanisms. Second, IL-8 enhances the contractions of CF ASM cells due to higher MLC_{20} phosphorylation and thereby, may contribute to bronchospasm and AHR in CF patients. Third, extracellular ATP and UTP evoke Ca^{2+} transients in normal ASM cells by actions on $P2Y_2R$ or $P2Y_4R$ that are expressed on ASM. Fourth, prolonged exposure of normal ASM cells to UTP causes desensitization of P2YRs and short time exposure causes increased sensitivity of P2YRs. Finally, exposure of normal ASM cells to IL-8 enhances UTP and ATP induced Ca^{2+} transients, possibly by increasing the sensitivity of P2Y receptors or related downstream signal transduction pathways. This thesis has therefore demonstrated the potential for IL-8 and purine nucleotides to affect airway smooth muscle contractile properties and may account for some of the abnormal ASM function observed in inflammatory airway diseases such as asthma, COPD and cystic fibrosis.

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Statement of Originality

This thesis contributes several novel findings on the actions of IL-8, extracellular ATP and UTP on ASM cells. To start with, this work has shown for the first time that normal ASM cells express receptors for IL-8, namely CXCRs and that these receptors mediate IL-8 evoked Ca^{2+} transients. IL-8 induces intracellular Ca^{2+} release by phospholipase C (PLC) coupled signal transduction mechanisms. Other investigators have reported that ASM cells secrete IL-8 following exposure to pro-inflammatory mediators, but none has shown that ASM cell respond to IL-8 by releasing Ca^{2+} intracellularly. Next, the finding that IL-8 caused contraction and migration of normal ASM is a novel finding in the field of ASM biology and immunology. Furthermore, the observation of enhanced IL-8 induced contractions of ASM and an increase in regulatory myosin light chain (MLC_{20}) phosphorylation possibly through Ca^{2+} sensitization or more likely through an increase in total MLC_{20} in cystic fibrosis (CF) cells are also novel findings. These findings suggest that IL-8 may be among the inflammatory agents that causes the contraction of CF ASM cells and may contribute to bronchospasm and AHR in CF.

The studies of the effects of extracellular ATP and UTP on $[Ca^{2+}]_i$ and the expression of subtypes of the P2Y receptors in human ASM cells are the first description of this important class of receptors in human ASM and extend findings already published for rat ASM cells. However, we are the first laboratory to describe the expression of different P2YR subtypes in human ASM cells. Furthermore, other observations of decreased Ca^{2+} transients in response to UTP in ASM cells pretreated with UTP for longer times and the possible desensitization of P2YRs in ASM cells are

additional novel findings to the desensitization of P2YRs in airway epithelium, which has already been shown.

Finally, our observation on IL-8 enhancement of ATP and UTP stimulated Ca^{2+} transients in normal ASM cells is an interesting original observation, which needs to be studied further to better understand the important interactions between IL-8 and extracellular nucleotides. These studies were not completed for this thesis but provide preliminary data for subsequent studies.

APPENDIX

Figure AP-1

Effects of UTP on $[Ca^{2+}]_i$ in HASM cells incubated with IL-8 for 24 hours

Cultured ASM cells from HASM cells were incubated with IL-8 (10 ng/ml) for 24 hours and cells were stimulated with UTP (10^{-4} M). The baseline Ca^{2+} (dark columns) and the IL-8 induced release of $[Ca^{2+}]_i$ (white columns) from control and IL-8 exposed cells are illustrated. The mean of the peak $[Ca^{2+}]_i$ values obtained from approximately 75 cells per each group. The data presented are mean ± SEM.

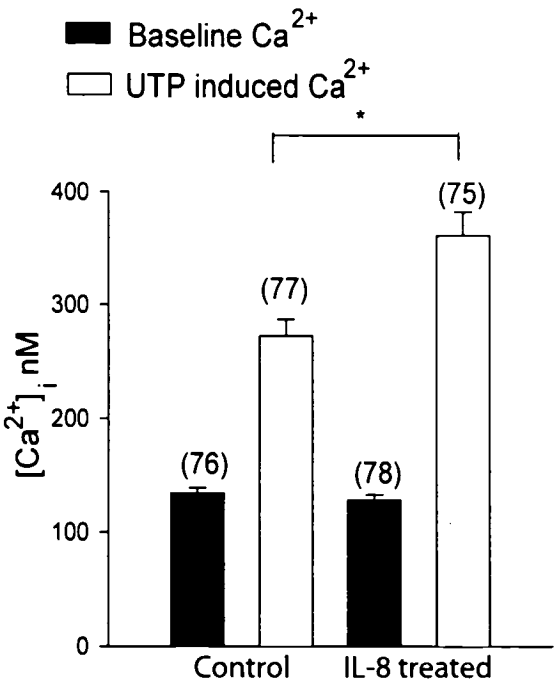
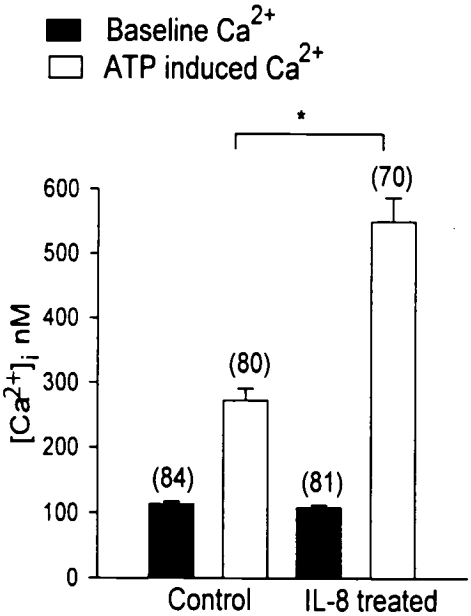

Baseline Ca2+
UTP induced Ca2+
*
400
300
200
100
0
[Ca2+]i nM
(76)
(77)
(78)
(75)
Control
IL-8 treated

Figure AP-2

Effects of ATP on $[Ca^{2+}]_i$ in HASM cells incubated with IL-8 for 24 hours

Cultured ASM cells from HASM cells were incubated with IL-8 (10 ng/ml) for 24 hours and cells were stimulated with ATP (10^{-4} M). The baseline Ca^{2+} (dark columns) and the IL-8 induced release of $[Ca^{2+}]_i$ (white columns) from control and IL-8 exposed cells are illustrated. The mean of the peak $[Ca^{2+}]_i$ values obtained from approximately 80 cells per each group. The data presented are mean ± SEM.


Baseline Ca2+
ATP induced Ca2+
*
600
500
400
300
200
100
0
[Ca2+]i nM
(84)
(80)
(81)
(70)
Control
IL-8 treated