ROLE OF EOSINOPHILS AND MUSCARINIC RECEPTORS IN PARADOXICAL AIRWAY HYPERREACTIVITY ASSOCIATED WITH ANTIGEN CHALLENGE

by

Norah G. Verbout

A DISSERTATION

Presented to the Department of Physiology & Pharmacology

and the Oregon Health & Science University

School of Medicine

in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

June 2008

School of Medicine
Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. dissertation of

Norah G. Verbout

has been approved

_____
Mentor/Advisor

_____
Member

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ACKNOWLEDGEMENTS

I would like to thank the individuals on my thesis committee, Dr. David Farrell, Dr. Pamela Lein, Dr. David Jacoby, Dr. Owen McCarty and Dr. Virginia Brooks. In particular, I appreciate their patience, flexibility and encouragement during this process.

I am especially grateful to Dr. Allison Fryer for her mentorship; I have thrived in her presence and owe all of my success to her exceptional training. With great care and patient guidance, Dr. Fryer has shown me all things important to success in science: write a fundable grant, give a noteworthy talk, publish a controversial paper and possibly most important to me, find the silver lining on an experiment gone awry. Dr. Fryer is an outstanding educator and by far, the most dedicated advocate of graduate students. I will always remember her drawing out models and schemes on the dry erase board. Lastly, I would like to recognize Dr. Fryer for being an amazing personal role model and a talented scientist.

I would equally like to thank Dr. David Jacoby, who is a brilliant scientist and generous person. His fantastical stories and quick wit will not be forgotten. My gratitude to both Dr. Fryer and Dr. Jacoby for creating a sense of family and welcome in all environments in and outside of the lab.

To all within the lab, I have been so fortunate to have such good-natured friends to share my time and labors. My utmost appreciation goes to Jesse Lorton for working with me on portions of this project; his grace and humble nature are rare qualities. Not to mention, without Jesse's "Jack of All Trades" ability to solve all problems, the lab might unravel. To my very wise and good friends Kirsten Blensly and Kalmia Buels—I could never have asked for more caring and lovely people to share graduate life with. Your support and friendship have meant so much to me.

Lastly, I would like to express my most sincere appreciation for my family. They are truly the source of my joy and success; I am so fortunate for their presence in my life. I am grateful to my parents for believing in my abilities and inspiring me to accomplish whatever I set out to do. I will miss our weekly family dinners together. Without any doubt, I would like to acknowledge that I could never have made it through this process without the support and dedication of my wonderful husband, Mike. His generosity and compassion are unsurpassed. I cherish my precious son Chase, who has enriched my life in infinite ways. I am amazed by his insight and intelligence.

Thank you all.

PUBLICATIONS

Manuscripts:

1.**Verbout NG,** Lorton JK, Jacoby DB, Fryer AD. Atropine Pretreatment Enhances Airway Hyperreactivity in Antigen Challenged Guinea Pigs Through an Eosinophil Dependent Mechanism. *Am J Physiol Lung Cell Mol Physiol* 292 (5): L1126-L1135, 2007.

2. **Verbout NG,** Jacoby DB, Fryer AD. Atropine-Enhanced, Antigen Challenge-Induced Airway Hyperreactivity in Guinea Pigs is Mediated by Nerve Growth Factor. [Submitted: *Am J Physiol Lung Cell Mol Physiol*, July 2008].

3. Moreno L, **Verbout NG,** Fryer AD, Jacoby DB. In revision: Retinoic Acid Prevents Virus Induced Airway Hyperreactivity and M_2 Receptor Dysfunction via Anti-Inflammatory and Antiviral Effects. [In revision: *Am J Physiol Lung Cell Mol Physiol*, May 2008]. *The work done for this publication is not presented as part of this dissertation.

Abstracts:

1. **Verbout NG**, Jacoby DB, Fryer AD. (2008) Atropine-Induced Potentiation of Airway Hyperreactivity in Antigen Challenged Guinea Pigs is Mediated by Nerve Growth Factor. FASEB J. 22:670.16.

2. **Verbout NG**, Jacoby DB, Fryer AD. (2008) Muscarinic Receptors on Eosinophils Inhibit PAF-induced Eosinophil Activation. American Society for Pharmacology and Experimental Therapeutics Meeting; Recent Advances in Muscarinic Receptor Pharmacology and Therapeutics.

3. **Verbout NG**, Lorton JK, Jacoby DB, Fryer AD. (2007) Atropine Pretreatment Enhances Eosinophil Activation in Airways of Antigen Challenged Guinea Pigs. 5th International Eosinophil Symposium.

4. **Verbout NG**, Lorton JK, Jacoby DB, Fryer AD. (2007) Atropine Pretreatment Enhances Eosinophil Activation in Airways of Antigen Challenged Guinea Pigs. Proceedings of the American Thoracic Society 175:A666.

5. **Verbout NG**, Lorton JK, Jacoby DB, Fryer AD. (2006) Atropine Pretreatment Enhances Airway Hyperreactivity in Antigen Challenged Guinea Pigs. 2006 British Pharmacological Society Meeting, 4th James Black Conference.

6. **Verbout NG,** Lorton JK, Jacoby DB, Fryer AD. (2006) A Functional Role for Muscarinic Receptors on Eosinophils in the Airways. Proceedings of the American Thoracic Society 3:A587.

7. Nelson CS**, Verbout NG,** Fryer AD, Jacoby DB. (2006) Neuronal M2 Receptor Expression is Inhibited by Tumor Necrosis Factor-alpha and by Retinoic Acid.

Proceedings of the American Thoracic Society 3:A464. *The work done for this publication is not presented as part of this dissertation.

ABSTRACT

The novel results presented in this thesis demonstrate that human and guinea pig eosinophils express muscarinic receptors, which inhibit eosinophil activation in vitro (Chapter V). Furthermore, I have demonstrated that muscarinic blockade during antigen challenge significantly increases eosinophil activation and subsequent vagally mediated hyperreactivity in guinea pigs (Chapter III and IV) via a mechanism involving eosinophils (Chapter III) and nerve growth factor (NGF) (Chapter IV). These data suggest that the inhibitory muscarinic receptors I have identified in vitro are functional in vivo.

Previous work has demonstrated that antigen-induced airway hyperreactivity in guinea pigs is mediated by eosinophil major basic protein (MBP) and neuronal M_2 receptor dysfunction. In this thesis, I have identified an alternative mechanism by which eosinophils mediate hyperreactivity in antigen challenged guinea pigs that is not mediated by loss of neuronal M_2 function. In this newly identified pathway, both eosinophils and NGF play a critical role since anti-IL-5 and anti-NGF antibodies prevent antigen-induced airway hyperreactivity. NGF may be acting upstream of eosinophil activation, since anti-NGF antibodies prevent increased eosinophil activation.

One possible explanation is that NGF induces eosinophils to release a mediator that alters nerve function, and release of this factor is inhibited by acetylcholine. Eosinophils produce a number of mediators that may alter nerve function, including NGF. One key effect of neurotrophins is that they can alter neuronal neurotransmitter content, a

phenomenon called neural plasticity. Thus, eosinophils mediate airway hyperreactivity via multiple mechanisms, one involving MBP blockade of neuronal M_2 receptors and the other involving NGF and possibly neural plasticity.

The implications of these findings are clinically relevant, considering the poor performance of anticholinergics in management of chronic asthma. Anticholinergic drugs are effective bronchodilators when given during an asthma exacerbation. However, these drugs are not as effective as predicted from animal studies and are not recommended for chronic treatment of asthma. The findings in this thesis provide an explanation for this paradox, and suggest that timing of anticholinergic administration is very important. Anticholinergics after antigen challenge are effective bronchodilators, but if administered prophylactically, as would be the case if given chronically, may make hyperreactivity worse.

In summary, I have discovered a novel interaction between the parasympathetic nerves and eosinophils. While it has been known that eosinophils can affect parasympathetic nerve function, I have identified a potential mechanism by which nerves may inhibit eosinophil function. This potential mechanism may be a negative feedback loop in which acetylcholine release from nerves dampens the negative effects of eosinophils on neuronal M_2 receptor function in allergic asthma. These observations are clinically important because they may explain why anticholinergics treatments that should be effective in managing asthma are not, despite the current body of knowledge in this field and may provide a rationale for new approaches to pharmacological control of the lungs.

These findings are significant because they contribute to our understanding of the complex interactions between the immune and nervous systems and because they will influence future therapeutics for treating diseases characterized by increased eosinophil activation, such as asthma.

CHAPTER I.

INTRODUCTION

INTRODUCTION

This thesis focuses on the interaction between eosinophil inflammatory cells and parasympathetic nerves in the lungs and how the complex interactions between these cells may contribute to asthma.

A. AIRWAY STRUCTURE

1. Anatomy of human airways

The airways consist of a series of branching tubes that become narrower, shorter and more numerous as they penetrate deeper into the lung. Inspired air passes through the mouth and nasal passages, proceeding distally through the nasopharynx and larynx to the trachea. These airways are outside of the lung, thus they are *extrapulmonary* (Figure 1.1). The trachea then bifurcates at the *carina* into the right and left main bronchi, which enter the lungs and continue branching. These *intrapulmonary* airways are the secondary and tertiary bronchi, bronchioles and terminal bronchioles. All of these bronchi make up the *conducting airways*. Their function is to carry inspired air to sites of gas exchange. From there, terminal bronchioles continue to divide into respiratory bronchioles, which have alveolar sacs and alveolar ducts budding from their walls. This alveolated region is the site of gas exchange, and is called the *respiratory zone* (West, 2005). A description of the airways and major structural differences are summarized in Figure 1.1.

2. Structural components of human airways

The airways are supported by cartilage, which decreases with successive generations, terminating prior to the bronchioles (Figure 1.1). The trachea is supported by C-shaped cartilage rings with gaps facing the posterior. In addition to cartilage, the airways also contain smooth muscle. The posterior wall of the trachea is held together by interlacing bundles of connective tissue and muscle. There are two layers of non-striated muscle in the trachea, longitudinal and transverse. Further down, circular bands of smooth muscle surround the main bronchi, smaller bronchi and bronchioles (Jeffery, 1995). The arrangement of the smooth muscle around the airways is such that when the muscle contracts, the airway both shortens and constricts. Thus, airway diameter is regulated by smooth muscle contraction. The thickness of airway smooth muscle is greatest in the trachea and decreases with successive generations, ending at the respiratory bronchioles and alveolar ducts, which have only very sparse smooth muscle (Tyler, 1983) (Figure 1.1).

The lumen of conducting airways is lined with a layer of epithelial cells connected to the basement membrane. As the airways decrease in size, the height and degree of ciliation of epithelial cells also decreases. In addition, the epithelium of the trachea, main bronchi and secondary bronchi are interspersed with mucous-secreting glands and mucin-producing goblet cells (Jeffery, 1995). Goblet cells are also present in the larger bronchioles, but decrease in density as bronchioles become smaller (Jeffery, 1995). The small bronchioles have Clara cells interspersed within the epithelium, which secrete surfactants to reduce alveolar surface tension and prevent collapse (Jeffery, 1995).

3. Blood supply to human lungs

Two circulations supply the lung, the *bronchial* and the *pulmonary*. The bronchial circulation is a high-pressure system that provides oxygenated blood to conducting airways and supporting tissues (Figure 1.1). Bronchial circulation is supplied by the bronchial artery, which originates from the aorta (Charan, 1992). In contrast, the pulmonary circulation is a low-pressure vascular system that provides respiratory airways with deoxygenated blood. It is supplied by right and left pulmonary arteries, which originate from the right cardiac ventricle. In the respiratory bronchioles, the vasculature becomes a dense network of capillary beds within the alveolar walls. The alveolar walls are very thin, permitting oxygen and carbon dioxide diffusion to and from the pulmonary circulation, respectively. Oxygenated blood is then returned to systemic circulation via the pulmonary veins, which empty into the left atrium (Vandam, 1952).

4. Anatomical and structural differences among humans and non-human animals

The anatomy and physiology of the guinea pig lung closely resembles that of humans (Canning, 2003; Canning *et al.*, 2008; Richardson, 1979; Richardson *et al.*, 1979). Guinea pigs have very similar distribution of epithelium (Dalen, 1983; Jeffery, 2001), goblet cells (Rogers, 2001), mucus glands (Poblete *et al.*, 1993), and smooth muscle (Canning *et al.*, 2008) compared to humans. The parasympathetic nerves supplying the airway smooth muscle in guinea pigs are also very similar to humans (Canning, 2006), and will be discussed in greater detail in section C of this chapter. These anatomical features are very different from mice, which have very few glands, sparser smooth muscle and major differences in muscle innervation compared to humans (Choi *et al.*,

2000; Persson *et al.*, 1997b; Widdicombe *et al.*, 2001). Another species difference is bronchial artery distribution, which varies depending on the types of lung tissues requiring systemic blood. For example, the bronchial vasculature extends from the bifurcation of the main bronchi to the terminal bronchioles in most species (Charan, 1992; Deffebach *et al.*, 1987), however mice do not have a functional bronchial vasculature beyond the main bronchi (Mitzner *et al.*, 2004).

FIGURE 1.1. Structural features of the airways.

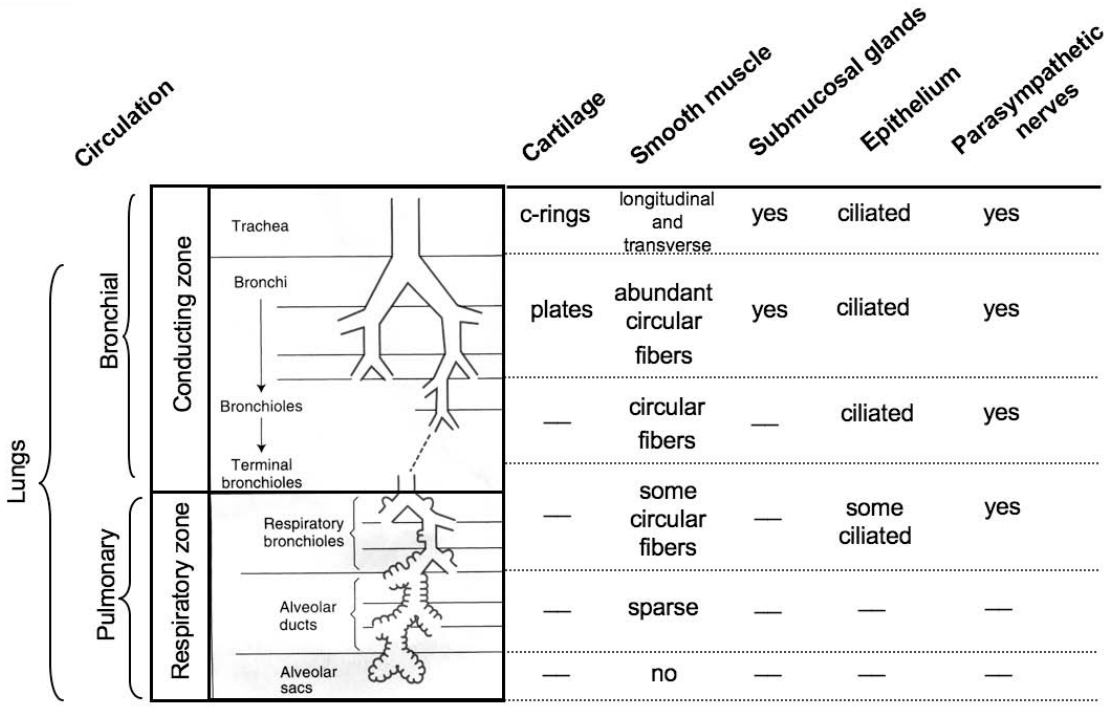


Figure 1.1. The conducting zone begins at the trachea and divides into the bronchi and bronchioles, ending at the terminal bronchioles (approximately 16 divisions). The respiratory zone consists of the respiratory bronchioles, alveolar ducts and sacs, where gas exchange occurs (approximately 7 divisions). Although the diameter and length of the bronchi decrease with each successive division, the sum of the cross-sectional area becomes greater as each airway branches. Adapted from West (West, 2005).

B. NEUROTRANSMISSION

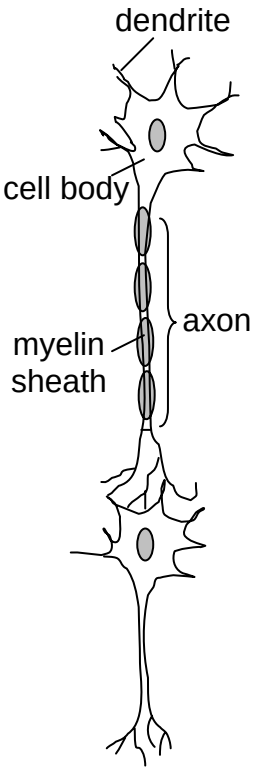
The nervous system is a communication network comprised of cells that transmit and integrate information about the external and internal environment. Nerve cells are comprised of a cell body, dendrites and axon. Neurons can receive signals from both axons and dendrites and transmit signals along the axon via propagation of action potentials. Some axons are covered with a myelin sheath, which increases the velocity of neurotransmission.

The present understanding of neurotransmission originated from work done by neuroanatomists in the nineteenth-century. In particular, Ramon y Cajal and Camillo Golgi, who shared the Nobel Prize in 1906, are recognized for their histological studies on the nervous system and neurotransmission theory. Based on these histological studies, Ramon y Cajal postulated that the nervous system is comprised of billions of separate neurons that are “dynamically polarized”, and receive and transmit signals unidirectionally. Cajal proposed that neurons communicate via specialized junctions called synapses, a term that was later defined by Charles Sherrington in 1897.

In 1897, Charles Sherrington introduced specific terminology to describe the organization of neuronal circuits (Sherrington, 1897). Sherrington defined the site of contact between two neurons as a *synapse*, which he discriminated from a neuronal *junction*, or the site of contact between a neuron and its target cell. A compact group of neuronal cell bodies is a ganglion; groups of ganglion are termed *ganglia*. *Preganglionic* nerves have axons that travel toward ganglia, whereas *postganglionic* nerves have cell bodies within ganglia and

axons that extend away from ganglia toward target cells or organs. Signaling between neurons is typically mediated by chemical neurotransmitters, which transmit the signal by binding to receptors on target cells. The terminology used in this thesis is based on Sherrington's definitions (Figure 1.2).

FIGURE 1.2. Neuroanatomy based on Sherrington's terminology.



PREGANGLIONIC NERVE	Cell body always in the CNS. Axon is myelinated. Releases acetylcholine.
GANGLION	Contains the axon terminus of preganglionic nerves and the cell bodies of postganglionic nerves.
SYNAPSE	Space between two nerves.
POSTGANGLIONIC NERVE	Cell body is outside of the CNS. Axon is not myelinated. Supplies target organ/cell.
JUNCTION	Space between the nerve and target organ/cell.

Figure 1.2. This is a schematic drawing based on Sherrington's terminology (Sherrington, 1897).

C. SENSORY AND AUTONOMIC NERVES IN THE AIRWAYS

1. Sources of airway nerves

The airways are supplied by afferent sensory and autonomic nerves, which regulate glandular secretion, baseline airway smooth muscle tone and bronchoconstriction. The majority of the afferent and autonomic nerves supplying the airways are within the vagus. The vagus nerves arise from the dorsal aspect of the medulla oblongata in the brainstem and descend through the neck into the thoracic cavity. From there, nerve fibers within the vagus branch into either the superior laryngeal or the recurrent laryngeal nerves, which then supply the trachea and main bronchi (Figure 1.3). The density of innervation in the lungs is greatest in the trachea and bronchi, decreasing peripherally toward the terminal bronchioles (Figure 1.1).

2. Afferent sensory nerves

Sensory nerves comprise approximately 80% of the nerve fibers within each vagus nerve (Agostoni *et al.*, 1957). These nerves relay information about environmental stimuli to the brain to elicit efferent output. In the lungs, nerve endings sense changes in pH, temperature, physical or chemical irritants and mechanical stretch (Carr *et al.*, 2003; Ho *et al.*, 2001). Sensory nerves are distributed in the airway epithelium, submucosal layer (Springall, 1995) and smooth muscle (Brouns *et al.*, 2006). Sensory nerve activation stimulates action potentials that are transmitted to the brain via the nodose and jugular ganglia (Kummer *et al.*, 1992) (Figure 1.4). Afferent nerves project to distinct regions within the nucleus tractus solitarius (Kalia *et al.*, 1980; Kalia, 1987), which then elicit

reflex responses via the efferent parasympathetic nerves, including cough, airway narrowing and mucus secretion (Figure 1.4) (Coleridge *et al.*, 1994).

FIGURE 1.3. Innervation of the trachea.

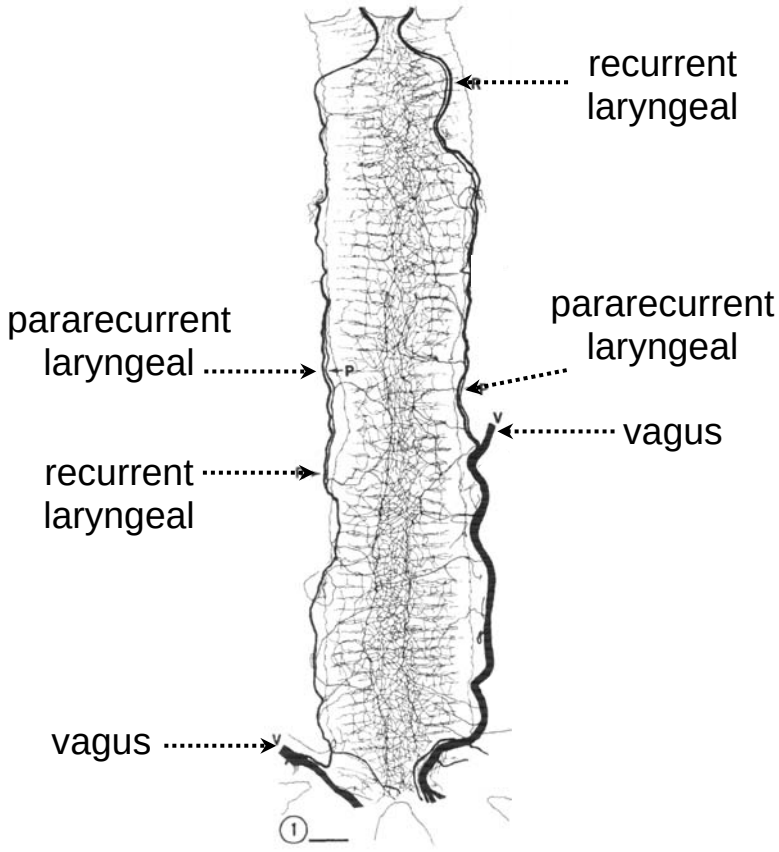


Figure 1.3. This tracing is a reconstruction of nerves and ganglia that were visible in a montage of photomicrographs depicting a ferret trachea tested histochemically for acetylcholinesterase activity (Baker *et al.*, 1986). The trachea was cut along the longitudinal axis and mounted with the trachealis muscle running longitudinally down the center. Sensory and parasympathetic nerves of the dorsal aspect were stained for acetylcholinesterase activity and traced from photomicrographs revealing the architecture and distribution of tracheal nerves. The vagus and the pararecurrent nerves supply the longitudinal nerve fibers, airway smooth muscle and glands. Adapted from Baker (Baker *et al.*, 1986).

Afferent sensory fibers innervating the airways fall into two categories based upon their physiology. A-fibers are mechanosensitive, whereas C-fibers are chemosensitive (Carr *et al.*, 2003). The A-fibers are further subclassified based upon their conduction velocity and action potential waveform in response to deep prolonged inhalation: slowly adapting stretch receptors (SARs) and rapidly adapting stretch receptors (RARs) (Ho *et al.*, 2001). In contrast, chemosensitive C-fibers are activated by tissue damaging stimuli and inflammatory mediators (Belvisi, 2003; Lee *et al.*, 2001). Within the airways, A-fibers are generally thinly myelinated and C-fibers are nonmyelinated.

The primary neurotransmitters released by sensory nerves in the lungs are neuropeptides. In particular, the tachykinin family of neuropeptides, which includes substance P, neurokinin A (NKA) and neurokinin B (NKB) are expressed by the majority of sensory nerves. The biological activity of tachykinins depends on their interaction with three specific receptors, the tachykinin NK_1, NK_2 and NK_3 receptors (Almeida *et al.*, 2004; Maggi, 1993). NK_1 receptors have the highest affinity for substance P, NK_2 receptors have the highest affinity for NKA and NK_3 have the highest affinity for NKB (Maggi, 2000). However, all tachykinins are full agonists at the three receptor subtypes, but with lower affinities than at the preferred receptor (Maggi, 1993). In the lungs, tachykinins increase vasodilation, mucosal secretion, neurotransmitter release, smooth muscle contraction and increase inflammation (De Swert *et al.*, 2006).

FIGURE 1.4. Innervation of the airways.

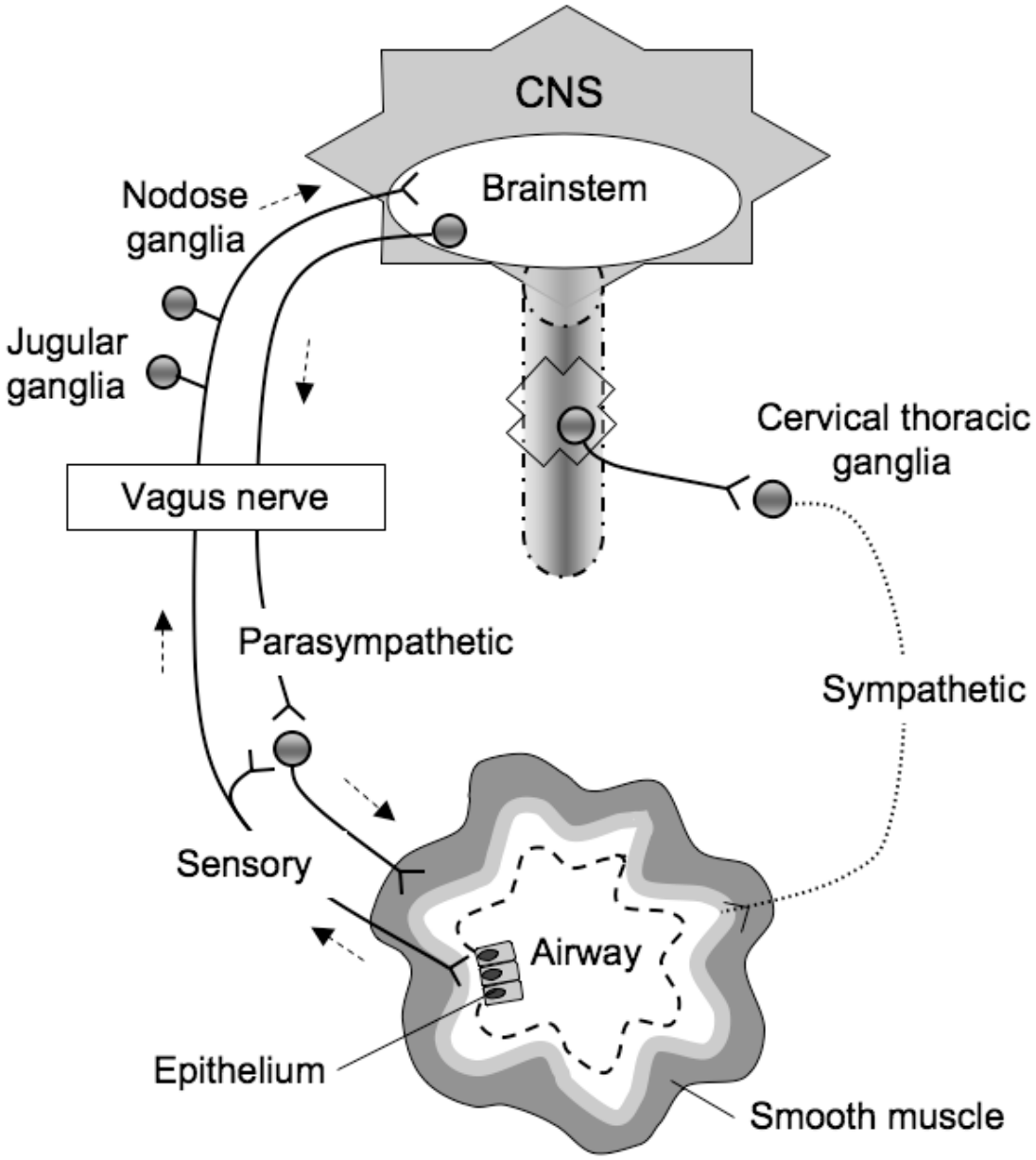


Figure 1.4. The vagus nerves supply airways with *sensory* and *parasympathetic* nerves. Sensory nerves can initiate a reflex via the CNS and can also mediate a peripheral reflex via stimulation of parasympathetic ganglia. In guinea pigs, but not humans, *sympathetic* nerves innervate airway smooth muscle. See text for references.

3. Autonomic efferent nerves

a. Sympathetic nerves

The distribution of sympathetic nerves supplying airway smooth muscle varies considerably among species. For example, guinea pigs (O'Donnell *et al.*, 1978), dogs (Knight *et al.*, 1981), cats (Dahlstrom *et al.*, 1966), goats, pigs, sheep and cows (Mann, 1971) have sympathetic innervation of the airways and airway smooth muscle (Canning, 2003). In contrast, rabbits (Mann, 1971) and humans (Pack *et al.*, 1984) have significantly less sympathetic innervation and the airway smooth muscle is not functionally regulated by sympathetic nerves (Barnes, 1986; Richardson, 1979).

Sympathetic preganglionic neurons supplying the lungs have cell bodies located in the ventral roots of the upper six thoracic segments of the spinal cord. These preganglionic nerves synapse at sympathetic ganglia that are located in the middle and inferior cervical ganglia and in the paravertebral ganglia. From the ganglia, postganglionic sympathetic nerves enter the lungs at the hilum (Belvisi, 2002).

In the lung, postganglionic sympathetic nerves release norepinephrine onto adrenergic receptors. Adrenergic receptors are seven transmembrane G-protein coupled receptors, initially divided into two subclasses, α and β, based upon rank order potency of activation by agonists (Ahlquist, 1948). Pharmacological, cloning and protein expression studies extended this classification into six α subtypes (Civantos Calzada *et al.*, 2001) and three β subtypes (Alexander *et al.*, 2008; Goodman *et al.*, 2001). In the lungs, α, β_1 and β_2 receptors are present (Goldie *et al.*, 1986; Spina *et al.*, 1989a; Spina *et al.*, 1989b)

and have variable effects on airway smooth muscle. Stimulation of α receptors in the lungs of dogs and humans causes smooth muscle contraction in vitro (Kneussl *et al.*, 1978; Leff *et al.*, 1986). In contrast, stimulation of β_1 receptors, present on airway parasympathetic nerves, relaxes smooth muscle by inhibiting parasympathetic activation (Danser *et al.*, 1987). However, the majority of adrenergic regulation of airway contractility is mediated by β_2 receptors, which relax airway smooth muscle (Kahn, 1907).

b. Parasympathetic nerves

i. Origins

The cell bodies of the preganglionic parasympathetic nerves originate in the nucleus ambiguous and extend to the trachea and bronchi via the vagus nerves where they supply parasympathetic ganglia located within the trachea and bronchi (Kalia, 1987; Richardson, 1979) (Figure 1.4).

ii. Ganglia

In contrast to sympathetic ganglia, which are located at a distance from the lungs, tracheal and bronchial parasympathetic ganglia are arranged in small clusters near the airways (Baker *et al.*, 1986; Baluk *et al.*, 1989; Fisher, 1964). Parasympathetic ganglia are distributed along the extrapulmonary trachea and bronchi and along intrapulmonary airways (Myers, 2001). At the ganglia, preganglionic parasympathetic nerves form defined synapses on postganglionic nerves. Stimulation of preganglionic nerves releases acetylcholine onto nicotinic receptors present on postganglionic nerves, mediating fast

excitatory postsynaptic action potentials. From these ganglia, short postganglionic fibers extend to the airway smooth muscle and mucosal glands (Richardson, 1979) and release acetylcholine at neuronal junctions.

iii. Parasympathetic control of airway tone

Unlike the preganglionic nerve endings, postganglionic nerve endings form a terminal meshwork with numerous sites of neurotransmitter release, called varicosities (Fisher, 1964). In the airways, these synapses are variable in location and close contacts between varicosities and muscle are rarely observed (Gabella, 1987). Acetylcholine release from parasympathetic nerves is triggered by a depolarizing stimulus: either physiologically via action potentials or mimicked in vitro by electrical field stimulation. Release is also controlled by complex prejunctional regulatory mechanisms, which are discussed in greater detail in section D of this chapter. It is important to note that postganglionic parasympathetic nerves are tonically active, and receive ongoing preganglionic input coming from the CNS, (Holtzman *et al.*, 1980; Kesler *et al.*, 1999). Thus, airway smooth muscle has a baseline continuous and passive partial contraction, called tone.

The parasympathetic nerves provide the dominant autonomic control of airway smooth muscle tone in animals and man (Boushey *et al.*, 1980; Nadel, 1977; Nadel *et al.*, 1984). This airway smooth muscle tone is regulated by tonic release of acetylcholine from the parasympathetic nerves (Widdicombe, 1966; Widdicombe, 1963b). In addition to maintaining airway smooth muscle tone, parasympathetic nerves mediate bronchoconstriction. Stimulation of parasympathetic nerves releases acetylcholine onto

muscarinic receptors on the airway smooth muscle, causing bronchoconstriction (Dixon, 1903; Nadel, 1977; Woolcock *et al.*, 1969a; Woolcock *et al.*, 1969b, Cabezas, 1971 #464), submucosal gland secretion (Baker *et al.*, 1983; Spencer *et al.*, 1964) and dilation of bronchial circulation (Widdicombe, 1963a), all of which are prevented by vagal blockade (Karczewski *et al.*, 1969; Widdicombe, 1966) or by pharmacological blockade with atropine (Nadel *et al.*, 1963; Severinghaus *et al.*, 1955). Parasympathetic regulation of airway tone and bronchoconstriction is present in the trachea, bronchi, bronchioles and terminal bronchioles. Parasympathetic nerves do not supply or control respiratory bronchioles or alveoli (Nadel *et al.*, 1971).

iv. Regulation of parasympathetic nerve function

Postganglionic parasympathetic nerve activity is regulated by several neural and non-neural mechanisms. Non-neuronal mediators that increase parasympathetic nerve activity include histamine (Kikuchi *et al.*, 1984), leukotrienes (Abela *et al.*, 1994), serotonin (Sheller *et al.*, 1982)) and prostaglandins (Inoue *et al.*, 1984; Tamaoki *et al.*, 1987). These inflammatory mediators are produced by immune cells within the lung, which have a pathological role in diseases characterized by inflammation and abnormal parasympathetic nerve function, such as asthma. Other nerve types, including sympathetic nerves (Baker *et al.*, 1983; Knight, 1980; Rhoden *et al.*, 1988) and inhibitory non-adrenergic non-cholinergic nerves (Hakoda *et al.*, 1990; Ito *et al.*, 1990), also modulate parasympathetic nerve function. There is also significant tachykinergic innervation of parasympathetic ganglia, which increases parasympathetic activity and thus smooth muscle contraction (Hall *et al.*, 1989; Watson *et al.*, 1993) (Figure 1.4).

However, perhaps the most significant control over parasympathetic nerve activity is provided by the prejunctional M_2 muscarinic receptors, which are discussed in further detail in section D of this chapter.

v. Parasympathetic nerves in other animals

The distribution and function of airway parasympathetic nerves is well conserved across species (Canning, 2006). In both guinea pigs and humans, parasympathetic nerves regulate airway smooth muscle tone, contraction and mucous secretion (Canning *et al.*, 2008; Kesler *et al.*, 1999; Nadel, 1977; Nadel *et al.*, 1984; Widdicombe, 1963a). For these reasons and those listed in the Methods chapter (section A), the guinea pig has been the most commonly used small animal species used for studies of airway function (Canning *et al.*, 2008).

4. Non-neuronal sources of acetylcholine in the lung

In addition to its role as a neurotransmitter, acetylcholine is also produced by a number of non-neuronal cells in the lung. These non-neuronal sources include structural cells (airway epithelium, endothelium and smooth muscle cells) and immune cells (mast cells, lymphocytes, macrophages, eosinophils and neutrophils) (Gwilt *et al.*, 2007; Kirkpatrick *et al.*, 2001; Klapproth *et al.*, 1997; Wessler *et al.*, 1998; Wessler *et al.*, 1999; Wessler *et al.*, 2001c). The mechanism of release from non-neuronal cells is completely different from that observed in nerves (section D in this chapter). Unlike neuronal cells, where acetylcholine is stored in vesicles, cells of the non-neuronal cholinergic system appear to

release acetylcholine via constituitive secretion (Wessler *et al.*, 2001a; Wessler *et al.*, 2001b).

D. MUSCARINIC RECPTORS IN THE LUNG

1. Neurotransmitter acetylcholine

Acetylcholine is synthesized in parasympathetic nerves endings by the enzyme choline acetyltransferase from two precursor molecules, acetyl-Coenzyme A and choline. Within nerves, acetylcholine is packaged into synaptic vesicles via the vesicular acetylcholine transporter. Depolarization-induced calcium influx triggers acetylcholine release, which then interacts with receptors on neurons or other target cells. Acetylcholine is rapidly hydrolyzed at synapses and junctions by the enzyme acetylcholinesterase, which is highly expressed in cholinergic synapses. Choline is taken back up by a choline transporter and recycled within the neuron.

2. Acetylcholine receptors

Acetylcholine interacts with two distinct classes of receptors, nicotinic and muscarinic. In 1914, Henry Dale distinguished between these two classes by demonstrating that each receptor was selectively activated by either nicotine or muscarine (Dale, 1914). In the lungs, both nicotinic and muscarinic receptors contribute to parasympathetic nerve function. Although these two receptors share the same ligand, they have major differences in their structure, effector function and kinetics.

a. Nicotinic receptors

Nicotinic receptors are expressed on postganglionic parasympathetic neurons in the ganglia (Racke *et al.*, 2004). They are ligand gated ion channels composed of five subunits arranged within the membrane to create a channel. There are multiple nicotinic

receptor subunit isotypes, and their combinations vary according to the cell type and developmental stage of the organism. In neurons, nicotinic activation causes a net influx of positively charged ions, which depolarizes the membrane and propagates an action potential.

b. Muscarinic receptors in the airways

i. Distribution in the lung

Muscarinic receptors (see section E of this chapter) are expressed in almost every cell type in the lungs and airways, including smooth muscle, glands, epithelial cells, endothelial cells, nerves and inflammatory cells. Three muscarinic receptor subtypes (M_1, M_2 and M_3) have been detected in the lungs of most mammals by subtype selective binding studies, immunocytochemistry and PCR (Gies *et al.*, 1989; Haddad *et al.*, 1996; Mak *et al.*, 1992; Mak *et al.*, 1990). Thus far, the only species reported to express M_4 in the lung are rabbits (Lazareno *et al.*, 1990; Mak *et al.*, 1993) and possibly pigs (Chelala *et al.*, 1998).

M_1 receptors are present in the parasympathetic ganglia, where they modulate neurotransmission in many species (Beck *et al.*, 1987; Bloom *et al.*, 1987; Kanemoto *et al.*, 2002; Lammers *et al.*, 1989; Yang *et al.*, 1991). Muscarinic facilitation at ganglia can be either excitatory or inhibitory (Ashe *et al.*, 1984). Additionally, M_1 receptors are expressed in the lung periphery and epithelium, but do not appear to be supplied by the parasympathetic nerves (Casale *et al.*, 1988; Gies *et al.*, 1989; Mak *et al.*, 1992; Mak *et al.*, 1990).

In the lung, 50-80% of muscarinic receptors in airway smooth muscle are M_2 (Haddad *et al.*, 1991; Roffel *et al.*, 1988). These M_2 receptors inhibit β receptor agonist induced adenylate cyclase activation and bronchodilation (Fernandes *et al.*, 1992; Sankary *et al.*, 1988). In addition, M_2 muscarinic receptors are present on postganglionic parasympathetic nerves. These autoinhibitory neuronal receptors were first demonstrated in guinea pig lung (Fryer *et al.*, 1984), but have been subsequently identified in cats (Blaber *et al.*, 1985), rats (Belmonte *et al.*, 1997), dogs (Brichant *et al.*, 1990), horses (Wang *et al.*, 1995), mice (Larsen *et al.*, 1994) and humans (Minette *et al.*, 1988; Minette *et al.*, 1989).

M_3 muscarinic receptors are present on airway smooth muscle where they make up 20-50% of the total muscarinic receptor population in the trachea. The exact percentage is species dependent (Haddad *et al.*, 1991; Roffel *et al.*, 1988). Stimulating M_3 muscarinic receptors causes smooth muscle contraction and bronchoconstriction (Roffel *et al.*, 1990a), which is mediated by $G_{q/11}$ phosphoinositide metabolism (Roffel *et al.*, 1990b) (see Section E in this chapter). Airway smooth muscle tone and bronchoconstriction are mediated by M_3 muscarinic receptors (Roffel *et al.*, 1990a). In addition to contracting smooth muscle, M_3 receptor stimulation increases vascular dilation (McMahon *et al.*, 1992) and mucus secretion (Baker *et al.*, 1985; Yang *et al.*, 1988).

ii. Role of muscarinic receptors in mediating bronchoconstriction

Perhaps the most significant role of M_2 muscarinic receptors in the lungs is to inhibit acetylcholine release from postganglionic parasympathetic nerves (Fryer, 1995; Fryer *et al.*, 1984) (Figure 1.5). The greatest density of muscarinic receptors in the airways is in parasympathetic ganglia (van Koppen *et al.*, 1985), but muscarinic receptors are also expressed along parasympathetic neurites (Fryer *et al.*, 1996). Neuronal M_2 receptors limit acetylcholine release thus, limiting airway smooth muscle contraction and bronchoconstriction (Fryer *et al.*, 1984; Minette *et al.*, 1988). The importance of inhibitory neuronal M_2 receptors becomes apparent when they are blocked with atropine, which increases acetylcholine release 5-8 fold (Baker *et al.*, 1992), or the selective M_2 antagonist gallamine, which increases bronchoconstriction in response to electrical field stimulation of the vagus nerve 8-10 fold (Fryer *et al.*, 1984). Conversely, stimulating neuronal M_2 receptors with agonists, such as pilocarpine, inhibits vagally induced bronchoconstriction by as much as 80-90% (Fryer *et al.*, 1984). Thus, inhibitory M_2 muscarinic receptors provide an important mechanism for controlling local release of acetylcholine.

FIGURE 1.5. Parasympathetic innervation of the airways.

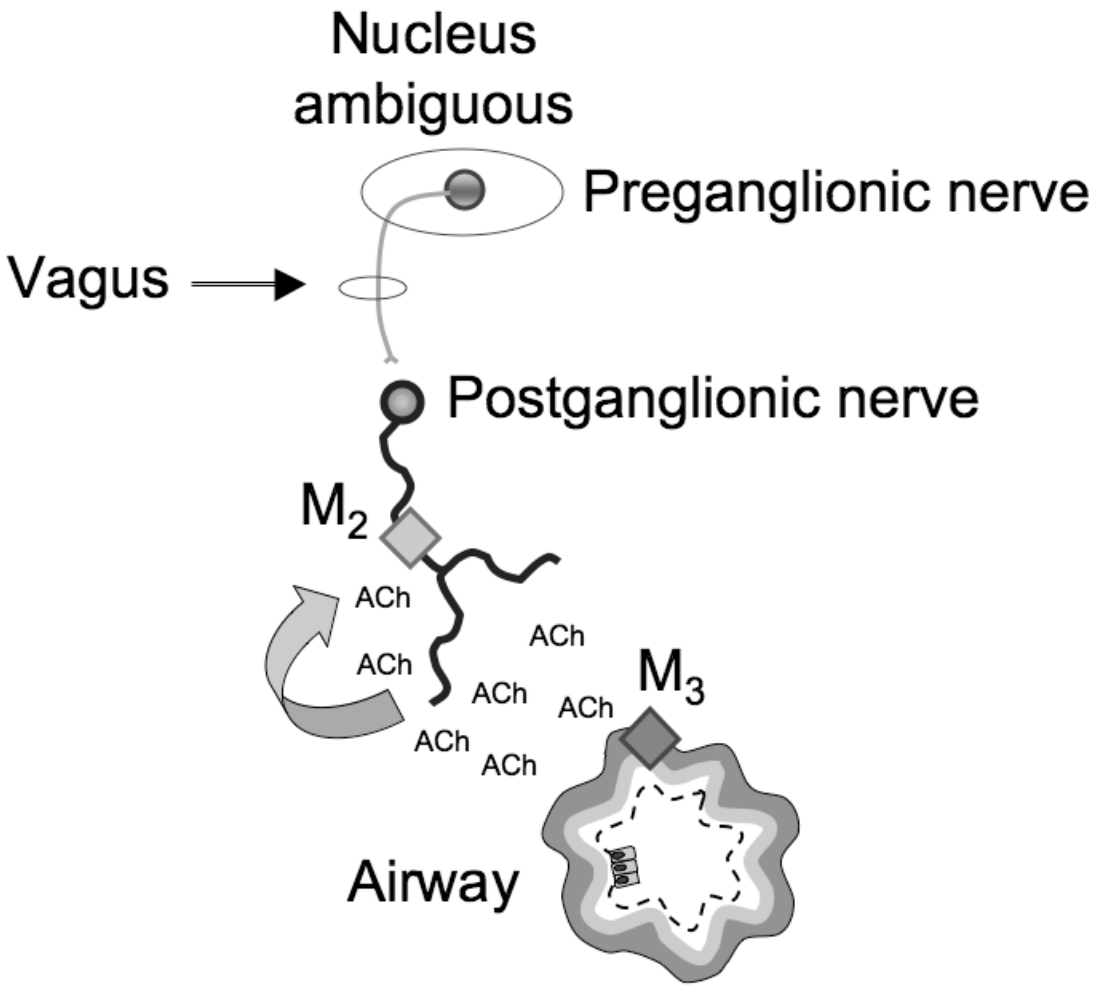


Figure 1.5. In the lungs, acetylcholine released by preganglionic parasympathetic nerves depolarizes postganglionic vagus nerves. Activation of postganglionic nerves releases acetylcholine onto M_3 muscarinic receptors on the airway smooth muscle, mediating contraction and bronchoconstriction. This pathway is regulated by the presence of prejunctional neuronal M_2 receptors that inhibit acetylcholine release and limit bronchoconstriction. See text for references.

E. MUSCARINIC RECEPTORS ON INFLAMMATORY CELLS

Inflammatory cells are known to express functional muscarinic receptors. Peripheral blood lymphocytes express M_3, M_4 and M_5 receptors, but not M_1 receptors (Tayebati *et al.*, 1999; Tayebati *et al.*, 2002). Acetylcholine promotes T lymphocyte cell survival and proliferation via muscarinic receptors by increasing T cell IL-2 cytokine production and IL-2 receptor expression (Nomura *et al.*, 2003). In mast cells, M_1 muscarinic receptors inhibit release of histamine, a potent inflammatory mediator implicated in the pathogenesis of asthma (Reinheimer *et al.*, 1997; Reinheimer *et al.*, 2000). However, inhibition of mast cell degranulation is species specific. For example, in humans it is inhibitory, whereas in rats and rabbits, muscarinic agonists stimulate mast cell degranulation (Masini *et al.*, 1985; Nemmar *et al.*, 1999). In human and bovine macrophages, acetylcholine stimulates release of leukotriene B_4 via M_3 muscarinic receptors (Profita *et al.*, 2005; Sato *et al.*, 1998). Neutrophils express M_3, M_4 and M_5 muscarinic receptor mRNA (Bany *et al.*, 1999), however the effects of muscarinic receptor stimulation on neutrophils are not known. Whether or not eosinophils express muscarinic receptors is not yet known and is the topic of this thesis. Thus, depending on the inflammatory cell, muscarinic receptors either promote or inhibit production of inflammatory mediators.

F. PROPERTIES OF MUSCARINIC RECEPTORS

1. General structure

Muscarinic receptors are G-protein coupled receptors that are divided into 5 distinct subtypes (M_1 – M_5) based upon their gene sequences. Each subtype couples preferentially to different classes of G-proteins, evoking different intracellular signaling pathways (Caulfield, 1993; Caulfield *et al.*, 1998; Hulme, 1990) (Table 1.1). They are comprised of seven alpha helical transmembrane domains linked together by three intracellular and three extracellular loops (Peralta *et al.*, 1987) and are encoded by five distinct, but intronless genes that are similar across mammalian species (Hall *et al.*, 1993). An important molecular distinction between the muscarinic receptor subtypes is the sequence divergence in the third intracellular loop, which is responsible for subtype specific coupling to diverse G-proteins (Bonner *et al.*, 1987; Peralta *et al.*, 1987; Wess, 1993).

2. Signaling via G-proteins

Agonist activation of muscarinic receptors induces conformational changes resulting in liberation of the three G-protein subunits, α, β and γ. The change in receptor conformation allows for GTP to be exchanged for GDP on the α subunit and promotes dissociation from the β and γ subunits. Signaling is initiated after the $\beta\gamma$ dimer separates from the α subunit, and each α subunit interacts with an effector protein or ion channel to stimulate or inhibit second messengers.

The G_α subunit is an important determinant of G protein effector specificity. In general, odd-numbered muscarinic receptor subtypes (M_1, M_3 and M_5) preferentially couple to G_α

proteins of the $G_{q/11}$ family, which activate membrane bound phospholipid enzymes, called phospholipases. The phospholipid enzymes associated with muscarinic receptor signaling are phospholipases C, A_2 and D (PLC, PLA_2 and PLD). Of all these phopholipases, PLC is the best characterized (Lanzafame *et al.*, 2003). Activation of membrane bound PLC catalyzes formation of two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Exton, 1996) (Figure 1.6). IP_3 leaves the plasma membrane and diffuses rapidly through the cytosol, where it binds to IP_3-gated calcium channels on the endoplasmic reticulum. Calcium is then released into the cytosol, further propagating the signal. The other cleavage product, DAG, remains embedded in the membrane and activates protein kinase C (PKC), which then phosphorylates downstream signaling molecules. This pathway is believed to be important to smooth muscle contraction in the lung.

FIGURE 1.6. Muscarinic receptor signaling via G-proteins.

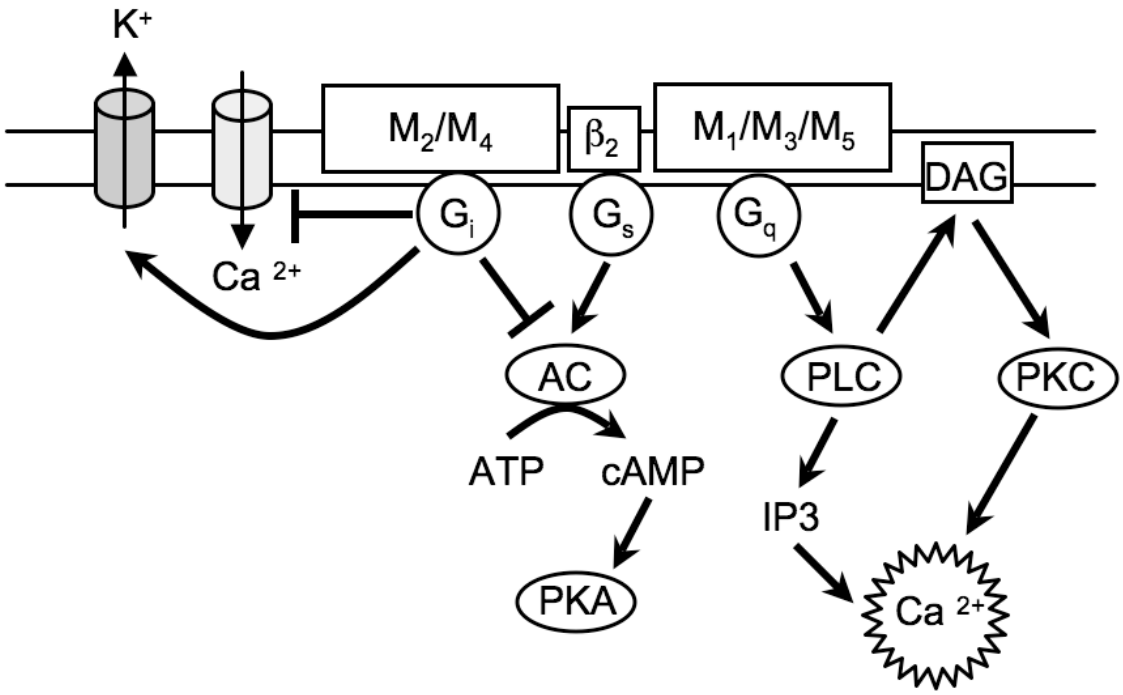


Figure 1.6. This is a simplified diagram that illustrates the classical signaling pathways regulated by the G_α subtypes: G_i, G_s and G_q. M_2 and M_4 muscarinic receptors directly couple to G_i, which inhibit adenylyl cyclase (AC), activate potassium channels and inhibit voltage-gated calcium channels. None of the muscarinic receptor subtypes are known to couple to G_s proteins. However, since activation of G_i opposes G_s signaling (shown here linked to a β_2 receptor), this pathway is also presented in this figure. M_1, M_3 and M_5 muscarinic receptors stimulate phospholipase C (PLC), which releases the second messengers inositol phosphate 3 (IP_3) and diacylglycerol (DAG). The signal is further amplified by DAG, which activates protein kinase C (PKC) and by IP_3, which releases calcium from intracellular stores.

The even numbered muscarinic receptors (M_2 and M_4) couple preferentially to G_α proteins belonging to the $G_{i/o}$ family, which inhibit adenylyl cyclase, an enzyme that converts adenosine triphosphate (ATP) to the second messenger cyclic adenosine monophosphate (cAMP) (Nathanson, 1987). In addition, the $\beta\gamma$ subunits directly couple to inwardly rectifying potassium channels (described below). Activation of these channels causes a decrease in cellular excitability. Thus, G_α signaling mediated by activation of M_2 and M_4 muscarinic receptors is considered inhibitory. In addition, due to their $G_{i/o}$ coupling, M_2 and M_4 receptors are sensitive to pertussis toxin (PTX). Pertussis toxin catalyzes ADP ribosylation of $G_{i/o,}$ thereby preventing intracellular signaling, even when an agonist is bound to the receptor. This characteristic is often used to discriminate between the other muscarinic receptor subtypes (M_1, M_3 and M_5), which are not susceptible to PTX mediated ribosylation due to their G_q coupling.

Another feature of muscarinic receptors is G-protein signaling and second messenger activation in the absence of agonists. This basal receptor signaling can be demonstrated in vitro, since activity is blocked by receptor antagonists (Jakubik *et al.*, 1995). However it is not known whether constituitive activity occurs under physiological conditions in vivo.

3. Ion channels

Depending on their G protein coupling and tissue expression, muscarinic receptors have been demonstrated to regulate several types of ion channels, including potassium, chloride, nonspecific cation and calcium channels (Beech, 1997; Lanzafame *et al.*, 2003). Of the many types of potassium channels, G Protein Inward Rectifying Potassium

(GIRK) channels, exhibit a basal level of potassium conductance that increases upon activation by G_i coupled receptors. In general, muscarinic receptors increase potassium channel activity, thereby decreasing cellular excitability, which is mediated by M_2 and M_4 muscarinic receptors (Figure 1.6) and has been well characterized in neurons and atrial myocytes (Caulfield, 1993; Caulfield *et al.*, 1998; Lanzafame *et al.*, 2003).

4. Calcium channels

Calcium is an important and ubiquitous second messenger. In many cells, elevation of calcium via muscarinic receptors is required for stimulation of signaling molecules PLA_2, PLD and PLC. Additionally, calcium is involved in regulation of the cytoskeleton, neurotransmitter release and enzyme activity (Berridge, 2007). Eukaryotic cells increase intracellular calcium by two mechanisms: either releasing compartmentalized calcium from intracellular stores, or by evoking calcium influx into the cell from the extracellular solution.

In *excitable* cells, which have the ability to propagate and spread action potentials to surrounding cells, such as neurons and muscle, calcium influx is generally mediated by voltage-operated calcium channels. Based upon on studies examining molecular biology, pharmacological profiles, single-channel ion conductance and voltage dependence, several different classes of calcium channels have been described, which are termed L-, T-, N-, P- and Q-type channels (Tsien *et al.*, 1995). These channels are activated during action potentials and generate inward calcium currents. It is well known that $G_{i/o}$ coupled muscarinic receptors (M_2 and M_4) inhibit voltage operated calcium channels in excitable

cells (Figure 1.6) (Caulfield, 1993; Caulfield *et al.*, 1998; Lanzafame *et al.*, 2003). In particular, muscarinic regulation of cell function via calcium channels has been described in neurons, cardiomyocytes, pacemaker cells in the heart and smooth muscle (Fernandez-Fernandez *et al.*, 2001; Hille *et al.*, 1995).

In non-*excitable* cells, such as immune cells, endothelium, epithelium and hepatocytes, voltage-operated channels are not expressed. Calcium influx in non-excitable cells regulates diverse processes as including exocytosis, contraction, enzyme control, gene regulation, cell proliferation, and apoptosis (Parekh *et al.*, 1997). In these cells, calcium influx is predominantly mediated by an alternative process, termed store-operated calcium influx (Clapham, 1995), which is initiated by depletion of intracellular calcium stores. Direct evidence in support of store-operated calcium influx was originally demonstrated using electrophysiological methods to measure calcium currents in mast cells, and was termed calcium release activated calcium current (I_{CRAC}) (Hoth *et al.*, 1992). Evidence for the presence of store-operated calcium currents has been demonstrated in numerous immune cells, including neutrophils (Demaurex *et al.*, 1994), lymphocytes (Dolmetsch *et al.*, 1994), macrophages (Malayev *et al.*, 1995) and eosinophils (Li *et al.*, 2002; Oshiro *et al.*, 2000).

The signaling mechanisms by which depletion of intracellular calcium stores activate extracellular calcium influx are not well understood, but there are two current models: direct coupling and indirect coupling. A direct coupling mechanism proposes a physical interaction between proteins in the plasma membrane and endoplasmic reticulum (Irvine,

1990). In a modified version of this hypothesis, depletion of intracellular calcium stores changes the conformation of the IP3 receptor on the endoplasmic reticulum, which leads to opening of store-operated calcium channels on the plasma membrane (Berridge, 1995).

Alternatively, indirect coupling proposes an intermediate signal transduction pathway that is mediated by second messenger molecule acting as a ligand to open calcium channels, affect calcium channel gating or stimulate insertion of calcium channels into the plasma membrane. One line of evidence supporting this particular model are studies demonstrating that a small low-molecular weight compound, termed calcium influx factor (CIF), is released from depleted calcium stores and subsequently activates calcium influx in several different non-excitable cells, including lymphocytes (Randriamampita *et al.*, 1995; Randriamampita *et al.*, 1993).

In granulocytic immune cells, there is also evidence that store-operated calcium channels are regulated by heterotrimeric G proteins (Jaconi *et al.*, 1993). In this particular study, the authors found that activation of calcium influx by fMet-Leu-Phe or by calcium ionophore was inhibited in HL-60 cells treated with a non-hydrolyzable guanosine phosphate analogue (GTPγS), suggesting that a GTP-sensitive protein is involved in signaling between intracellular calcium stores and plasma membrane calcium channels. Since GTPγS inhibits both heterotrimeric and small G proteins, the authors used fluoride to demonstrate that it is heterotrimeric G proteins and not small G proteins that inhibit store-dependent calcium influx. Fluoride mimics the effects of GTPγS on large G

proteins (Rodbell, 1992), but does not affect small G proteins (Kahn, 1991). Thus, it appears that in granulocytes, G proteins may regulate store-operated calcium channels.

5. Muscarinic receptor selectivity

The site of agonist binding to muscarinic receptors is within the outer half of the membrane embedded portion of the protein (Caulfield *et al.*, 1998). All muscarinic receptors have an aspartic acid residue on the N-terminal part of the third transmembrane domain, which is thought to interact with the polar headgroup of amine ligands, including acetylcholine. Agonist affinity for each receptor subtype is controlled by distinct amino acid sequences, which convey structural differences on the third intracellular loop. These structural differences influence the conformation of the agonist binding pocket, conferring selectivity for each receptor subtype (Wess, 1993).

Another characteristic of muscarinic receptors is the presence of separate binding sites, that when occupied, can alter agonist binding at the primary site (Caulfield, 1993). This *allosteric* site has been characterized in both binding studies and functional studies (Lazareno *et al.*, 1995). In particular, neuronal M_2 muscarinic receptor function is regulated by an allosteric interaction with eosinophil major basic protein (Jacoby *et al.*, 1993), which will be discussed in greater detail in section H.

Selectivity of antagonists for muscarinic receptors is dependent on their primary agonist binding site and their allosteric site (Clark *et al.*, 1976). Chimeric receptor studies have demonstrated that antagonist selectivity is due to both transmembrane and extracellular

domains (Wess, 1993). However, pharmacological characterization of muscarinic receptor subtypes has been plagued by the lack of antagonists with high selectivity for any single receptor subtype. Table 1.1 provides the pharmacological properties of the five muscarinic receptor subtypes. At present, there are no selective muscarinic receptor antagonists approved for treatment of asthma.

TABLE 1.1. Pharmacological properties of muscarinic receptor subtypes in humans.

	Muscarinic receptor subtypes					
	M_1	**M_2**	**M_3**	**M_4**	**M_5**	**source**
Antagonists **						
4-DAMP	8.6-9.2	7.8-8.4	8.9-9.3	8.4-9.4	8.9-9.0	*
AF-DX 116	6.4-6.9	7.1-7.2	5.9-6.6	6.6-7.0	6.6	*
AF-DX 384	7.3-7.5	8.2-9.0	7.2-7.8	8.0-8.7	6.3	*
Atropine	9.0-9.7	9.0-9.3	8.9-9.8	9.1-9.6	8.9-9.7	*
Darifenacin	7.5-7.8	7.0-7.4	8.4-8.9	7.7-8.0	8.0-8.1	*
Gallamine		6.5				#
Himbacine	6.9-7.4	8.0-8.3	6.9-7.4	8.0-8.8	6.1-6.3	*
Ipratropium		9.7	9.7			#
Major Basic Protein		4.8				ϕ
Methoctramine	7.1-7.8	7.8-8.3	6.3-6.9	7.4-8.1	6.9-7.2	*
MT3	7.1	<6	<6	8.7	6.3	*
MT7	9.8	<6	<6	<6	<6	*
Pirenzipine	7.8-8.5	6.3-6.7	6.7-7.1	7.1-8.1	8.9-9.7	*
Tiotropium		10.7	11			#
Agonists						
Acetylcholine	no discrimination of subtypes					
Carbachol	no discrimination of subtypes					
Methacholine	no discrimination of subtypes					
G-proteins	$G_{q/11}$	$G_{i/o}$	$G_{q/11}$	$G_{i/o}$	$G_{q/11}$	
Signaling	PLC	AC	PLC	AC	PLC	
	IP3, DAG Ca^{2+}, PKC	inhibition	IP3, DAG Ca^{2+}, PKC	inhibition	IP3, DAG Ca^{2+}, PKC	

Sources:

ϕ *J Clin Invest, Vol. 91, p1314-1318, April 1993.*

* *Pharmacological Research, Vol. 44, No. 3, 2001.*

 # Respiratory Research, 7:73, 2006.

** Antagonist affinity constants (log affinity constants; (-log K_i) for mammalian muscarinic receptors

G. ASTHMA AND INFLAMMATION

1. Asthma

a. Definition of asthma

Asthma is a heterogenous disease with several clinical subtypes and a wide spectrum of severity. Thus, the definition of asthma has been the subject of controversy and is often described as a syndrome, rather than a disease. The National Asthma Education Program Expert Panel Report, has defined asthma as: “Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular events play a role: in particular, mast cells, eosinophils, T lymphocytes, macrophages, neutrophils and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. The inflammation also causes an associated increase in the existing bronchial hyperresponsiveness to a variety of stimuli.” (National Heart, 2007).

For the purposes of this thesis, this discussion of asthma will focus on inflammation and airflow obstruction, or bronchoconstriction and the inflammatory mechanisms that underlie bronchoconstriction and airway hyperresponsiveness.

b. Asthma phenotypes

Traditionally, asthma has been divided into two general categories, *extrinsic* (allergic) asthma and *intrinsic* (non-allergic) asthma, depending upon the types of stimuli that

trigger exacerbations. In allergic asthma, the immune system produces antibodies in response to harmless environmental factors such as pollen, animal dander or dust mite particles. Inhalation of these particle triggers an asthmatic episode. By contrast, intrinsic asthma is not allergy-related, and is caused by anything except an allergy. Most patients with asthma have non specific hyperresponsiveness to inhaled stimuli, such as cigarette smoke or cleaning agents, taking aspirin, viral infections, stress, exercise and cold air (Smith *et al.*, 1995).

The National Asthma Education Program, which produces the US-based asthma treatment guidelines also classifies asthma by severity, a more commonly used scheme by most health professionals (National Heart, 2007). *Intermittent* asthma occurs periodically with few symptoms in between exacerbations. This type of asthma is usually associated with seasonal allergens or viral infections. Individuals with *persistent* asthma have continuous symptoms with frequent exacerbations. There is a spectrum of persistent asthma classifications, ranging from mild to moderate to severe. Persistent asthma is generally characterized by chronic abnormalities in lung function that may or may not be reversed pharmacologically. Thus, disease severity is linked to control of symptoms with medications.

2. Characteristics of asthma

There are three major features of asthma: recurrent episodes of reversible bronchoconstriction, localized inflammation and airway hyperreactivity.

a. Bronchoconstriction and airway remodeling

During an acute asthmatic exacerbation, the airway smooth muscle contracts in response to a variety of stimuli such as allergens or irritants. Allergen-induced acute bronchoconstriction results from IgE-dependent release of mediators from mast cells including histamine, leukotrienes and prostaglandins that directly contract airway smooth muscle (Busse *et al.*, 2001). Other stimuli, including cold air, dust, exercise and chemical irritants also cause acute bronchoconstriction that is mediated by a vagal reflex (see section C and D of this chapter). Responses to these types of stimuli can be attenuated by atropine in humans (Nadel *et al.*, 1965; Sheppard *et al.*, 1982; Simonsson *et al.*, 1972; Tinkelman *et al.*, 1976) and are abolished by vagotomy (severing vagus nerves) in experimental animals (Widdicombe *et al.*, 1962).

As asthma severity progresses and inflammation becomes persistent, other features limiting airflow develop, collectively called airway remodeling. Airway remodeling is a pathological feature in fatal asthma and is characterized by permanent structural changes in the tissues of the airways. It includes thickening of the sub-basement membrane, epithelial fibrosis, blood vessel proliferation and dilation, smooth muscle hypertrophy and mucous gland hyperplasia and mucous plugs due to hypersecretion (Holloway *et al.*, 1995). An important distinction between these two types of airflow obstruction is degree of reversibility. Drugs that relax smooth muscle reverse acute airway obstruction due to bronchoconstriction; in contrast, airway obstruction resulting from permanent structural and remodeling processes is less likely to respond to therapy (National Heart, 2007).

b. Airway hyperresponsiveness

i. Description

Airway hyperresponsiveness is the exaggerated bronchoconstrictor response to a wide variety of exogenous stimuli (Hargreave *et al.*, 1986). These stimuli include inhaled pharmacological agents (histamine, methacholine, etc.), as well as natural stimuli (exercise, cold air, etc.). The degree of hyperresponsiveness is commonly determined by assessing lung function before and after inhaling increasing concentration of an agent like methacholine or histamine. Thus, the more sensitive or responsive the airways are, the lower the amount of agent required to decrease lung function. Normal subjects will develop airway bronchoconstriction in response to inhaled agents, but asthmatic individuals have a much lower threshold to all agonists (Hopp *et al.*, 1985; Tepper, 1987), thus they are *hyperresponsive*. Multiple factors influence airway hyperresponsiveness, especially inflammation of the lungs and neuronal muscarinic receptor dysfunction (see sections D and E of this chapter).

ii. Role of parasympathetic nerves in airway hyperresponsiveness

Parasympathetic control of airway smooth muscle is clearly abnormal in patients with asthma (Cropp, 1975). There is no evidence suggesting that direct contraction of the smooth muscle to muscarinic receptor agonists is altered (Armour *et al.*, 1984; Roberts *et al.*, 1985; Taylor *et al.*, 1985). Since stimulation of parasympathetic nerves results in several physiological responses similar to asthma (see section D), and the site of bronchoconstriction in asthma corresponds to the area most densely innervated by parasympathetic nerves (Gross *et al.*, 1984; Nadel *et al.*, 1971), it is likely that these nerves play a prominent role in the pathogenesis of asthma.

In animal models of asthma, hyperreactivity is mediated by increased release of acetylcholine from the parasympathetic nerves onto M_3 muscarinic receptors on airway smooth muscle, increasing bronchoconstriction (Drazen *et al.*, 1975; Fryer *et al.*, 1991; Gold *et al.*, 1972; McCaig, 1987). Release of acetylcholine from postganglionic nerves is tightly controlled by inhibitory M_2 receptors on the nerves (Fryer, 1995; Fryer *et al.*, 1984; Minette *et al.*, 1988) (Figure 1.5, section D of this chapter). Neuronal M_2 receptors are dysfunctional in animal models of asthma and in some people with asthma. Loss of M_2 receptor function increases acetylcholine release and potentiates vagally induced bronchoconstriction in animals (Evans *et al.*, 2001; Fryer *et al.*, 1992; Fryer *et al.*, 2004; Fryer *et al.*, 1991; Lein *et al.*, 2005; Yost *et al.*, 1999) and in humans (Ayala *et al.*, 1989; Minette *et al.*, 1989). The best understood pathway for loss of neuronal M_2 receptor function involves an interaction between eosinophils, which are characteristic of asthma (discussed in section H of this chapter). In particular, a major mechanism of airway hyperreactivity is the blockade, by eosinophil proteins, of M_2 muscarinic receptors on the parasympathetic nerves (Elbon *et al.*, 1995; Evans *et al.*, 1997; Fryer *et al.*, 1992; Fryer *et al.*, 1984; Fryer *et al.*, 1991) (see section F of this chapter).

c. Airway inflammation

Inflammation has a central role in the pathophysiology of asthma. A clinical association between allergy, bronchial inflammation and asthma has been long known (Cockcroft *et al.*, 1977; Cooke, 1918). The association is so strong that it encompasses all phenotypes of asthma. Bronchoalveolar lavage fluid, tissue biopsies and autopsy specimens of deep

airways and muscle all demonstrate that inflammatory cells and inflammatory cell products are significantly increased in the airways of asthmatics. The major inflammatory cells found in asthmatic airways are mast cells, lymphocytes, macrophages, neutrophils and especially eosinophils.

3. Inflammatory cells in asthma

a. Mast cells

In asthma, mast cells can be activated by both allergen dependent and independent mechanisms. Mast cells are activated by binding of antigen specific immunoglobulin (Ig) E to its receptor (FCεRI) on the mast cell surface (Stanworth, 1971; Stanworth, 1972). Activation of mucosal mast cells releases mediators that contract the smooth muscle, including histamine, leukotrienes and prostaglandin D_2 (Boyce, 2003; Robinson, 2004).

Although the majority of mast cell initiated asthma exacerbations involve allergens, mast cells can also be activated by other stimuli, for example, exercise (O'Sullivan *et al.*, 1998) and aspirin (Mita *et al.*, 2001). Mast cells are increased in the airway smooth muscle of asthmatics (Brightling *et al.*, 2002) and mast cell mediators are increased in bronchoalveolar lavage of allergen challenged subjects (Wenzel *et al.*, 1988). Histamine contracts smooth muscle either directly (Drazen *et al.*, 1978) or via neurally mediated reflex bronchoconstriction (Benson *et al.*, 1977; Ellis *et al.*, 1992; Shore *et al.*, 1983).

b. Lymphocytes

Lymphocytes are a prominent cell type present in asthmatic airways. B lymphocytes produce antigen specific IgG, IgA and IgE, which promote allergic asthma response. The discovery of distinct subpopulations of T lymphocytes, T helper 1 and T helper 2 (Th1 and Th2) in allergic animal models may expand our understanding of the etiology of human asthma. Recent evidence in humans suggests that a shift in cytokine profile to Th2 subtype promotes eosinophilic inflammation, characteristic of asthma (Cohn *et al.*, 2004). This observation is supported by evidence that production of Th2 cytokines (IL-4, IL-5, IL-13) promotes eosinophilia and development of hyperresponsiveness (Cohn *et al.*, 1998; Mattes *et al.*, 2002). However, the majority of studies examining the role of Th1 and Th2 switching in asthma have been conducted in mouse models, and their role in human asthma is not completely understood.

c. Macrophages

Macrophages are the most numerous cells in the airways and are increased in asthmatics compared to normal subjects. Although macrophages do not play a significant role in antigen presentation, they are an abundant source of cytokines (IL-1β, tumor necrosis factor-α) that may amplify the inflammatory response (Peters-Golden, 2004) in asthma. Macrophage chemotactic proteins are increased in atopic asthmatics and correlate with increased airway hyperresponsiveness (Rozyk *et al.*, 1997). In guinea pigs, macrophage depletion prevents virus-induced airway hyperresponsiveness and neuronal M_2 receptor dysfunction (Lee *et al.*, 2004a). Thus, macrophages are likely to play a role in virus-induced asthma, which is clinically important because viruses are associated with the

majority of asthma exacerbations in children and adults (Atmar *et al.*, 1998; Johnston *et al.*, 1995).

d. Neutrophils

Although neutrophils are increased in airways and sputum during acute asthma exacerbations and in severe asthmatics (Fahy *et al.*, 1995), little is known about their pathological role in asthma. Neutrophil elastase proteins are implicated in the formation of mucous plugs in fatal asthma (Nadel *et al.*, 1999a) and increase goblet cell secretion in antigen challenged guinea pigs (Agusti *et al.*, 1998) and in humans with asthma (Nadel *et al.*, 1999b).

e. Eosinophils

Eosinophils have been associated with asthma since the early 1900s (Ellis, 1908). This association has become stronger over time. In 1922, it was observed that eosinophils were the predominant white blood cell found in the sputum of patients dying of fatal asthma (Huber *et al.*, 1922). In years following, the striking similarities between the eosinophilic lungs of humans with fatal asthma and sensitized guinea pigs established the guinea pig as a potential experimental model for studying asthma in man (Reviewed in (Kallos *et al.*, 1984)). In 1975, it was demonstrated that airway function (measured as forced expiratory volume in one second) was inversely related to the number of eosinophils in the blood (Horn *et al.*, 1975), followed by the finding that airway hyperreactivity was positively correlated with eosinophil number (Bousquet *et al.*, 1990).

Eosinophils are present in the airways of most asthmatics as measured in sputum, bronchoalveolar lavage, bronchial biopsy and post-mortem histology (Bousquet *et al.*, 1990; Ellis, 1908; Laitinen *et al.*, 1985; Ohashi *et al.*, 1992). In fatal asthma, many studies have found that eosinophils are the predominant infiltrating cell in the airways (Costello *et al.*, 1997; Dunnill, 1960; Gleich *et al.*, 1987; Huber *et al.*, 1922). There is ample evidence that eosinophils are in a highly activated state and direct evidence that eosinophil-derived proteins are localized in airway tissues obtained postmortem (Azzawi *et al.*, 1990; Bousquet *et al.*, 1990; Costello *et al.*, 1997; Filley *et al.*, 1982; Ohashi *et al.*, 1992). The ability of eosinophils to damage surrounding tissue with cytotoxic proteins is supplemented by the numerous inflammatory mediators they produce (Table 1.2). Additionally, it is well established that mediators originating from eosinophils in addition to other cells play a significant role in development of airway hyperresponsiveness in several different animal models of asthma, including antigen-challenge, ozone and virus-induced (Evans *et al.*, 1997; Evans *et al.*, 2001; Yost *et al.*, 1999) (Adamko *et al.*, 1999; Fryer *et al.*, 2006). The presence of eosinophils in allergen-induced asthma has been well established in humans and animal models. Their role in asthma is not well understood and may be considered controversial. See section H for discussion on anti-eosinophil treatments and eosinophil function asthma.

4. Animal models of allergic asthma

a. Modeling allergic asthma

In allergic asthma, inhalation of antigen causes immediate and reversible bronchoconstriction (Itkin *et al.*, 1963) that peaks after 10-20 minutes and recovers

spontaneously by 60-90 minutes (Robertson *et al.*, 1974). The immediate bronchoconstrictor response to inhaled antigen is initiated by resident mast cells that release histamine, leukotrienes and prostaglandins, which directly contract smooth muscle and mediate reflex bronchoconstriction via sensory nerve activation (Benson *et al.*, 1977; Ellis *et al.*, 1992; Shore *et al.*, 1983) (Figure 1.4).

The later phase is characterized by increased inflammation (Chung *et al.*, 1985) and increased airway responsiveness to inhaled histamine and methacholine (Cockcroft *et al.*, 1977). It is well known that susceptible individuals become sensitized to allergens and develop airway hyperreactivity upon subsequent exposures to these allergens. The development of animals models of airway hyperreactivity provide the means to study the mechanisms involved in the pathogenesis of allergic asthma and develop and test novel therapeutic agents. Few, if any established animal models exhibit all of the characteristics of allergic asthma (Abraham, 1995). The criteria set forth by the NIH indicate that an "ideal model of human asthma" should exhibit nonspecific airway hyperresponsiveness in vivo, development of a late bronchial response after allergen challenge, presence of airway eosinophilia and responsiveness to therapeutic drugs that have identical actions in humans (Wanner *et al.*, 1990).

b. Antigen challenge animal model

i. Antigen sensitization

The most commonly used small animal models of allergic asthma are not spontaneous, but rather are models of antigen provocation (Abraham, 1995). Since these animals are

not naturally allergic, it is necessary to introduce an allergy by controlled exposure of antigens through a process termed sensitization. Guinea pigs are likely the most frequently used species to study airway responsiveness (Abraham, 1995) due to their sensitivity to pharmacological mediators (Canning, 2003; Canning *et al.*, 2008), immunological responses (Canning, 2003; Canning *et al.*, 2008), small size and ease of handling. In guinea pigs, the process of sensitization to antigen stimulates production of specific IgG and IgE (Canning, 2003), a process that requires several weeks. In this species, the most commonly used antigens are ovalbumin and *Ascaris suum*. Different sensitization protocols (intraperitoneal versus inhalation; single versus multiple sensitizations) produce slightly different antibody responses and different pulmonary responses (Abraham, 1995).

ii. Description of antigen challenge

Controlled exposure to inhaled antigen, termed antigen challenge, occurs in a laboratory setting (Chapter II, Figure 2.1). Antigen challenge is characterized by two phases, an immediate, acute response followed by a latent, delayed response. The immediate response to antigen challenge is mediated by mast cells. Inhaled antigen activates mast cells, releasing numerous preformed mediators, in particular histamine, which immediately increases local blood flow, vessel permeability and bronchoconstriction (described in section G; 3a).

In addition to releasing histamine, activated mast cells also release lipid mediators that directly contract the airways, increase vascular permeability and mucus secretion. These

lipid mediators are synthesized from arachidonic acid. Enzymatic oxidation of arachidonic acid produces prostacyclin, prostaglandins, thromboxanes and leukotrienes. The metabolites are potent bronchoconstrictors and sustain inflammatory responses in the tissues (Kleeberger, 1995; Piper, 1984; Weichman *et al.*, 1982). Mast cells are also known to release TNFα, which activates endothelial cells, causing increased expression of adhesion molecules that promotes influx of inflammatory cells into the lung (Klein *et al.*, 1989). Additionally, mast cells are sources of chemokines and cytokines that collectively promote influx and activation of leukocytes and contribute to both the acute and late-phase response.

iii. Characteristics of events post antigen challenge

The late phase response to inhaled antigen is characterized by increased inflammation and airway hyperreactivity. In guinea pigs sensitized to ovalbumin, increased numbers of neutrophils and eosinophils are found in the bronchoalveolar lavage fluid during the late response (Tarayre *et al.*, 1990). Seventeen hours after antigen challenge, eosinophils are increased sixfold, and comprise approximately 49% of the inflammatory cells recovered 72 hours post antigen challenge (Hutson *et al.*, 1988). There are no significant differences in macrophages or lymphocytes in the bronchoalveolar lavage (Hutson *et al.*, 1988). Histologically, both neutrophils and eosinophils can be identified in the lung as early as 8 minutes after challenge (Dunn *et al.*, 1988) and by 6 hours, eosinophils are localized beneath the airway smooth muscle (Hutson *et al.*, 1988). Antigen challenge also activates eosinophils, releasing inflammatory mediators and granule proteins that damage airway tissue and mediate airway hyperreactivity (covered in more detail in section H).

Airway hyperresponsiveness following antigen challenge has been well characterized in guinea pigs (Lewis *et al.*, 1995a; Lewis *et al.*, 1995b; McCaig, 1987; Tarayre *et al.*, 1990). A single exposure to inhaled ovalbumin challenge increases vagally induced bronchoconstriction twenty-four hours later (Evans *et al.*, 1997; Fryer *et al.*, 2006; Lewis *et al.*, 1995a). Repeated antigen challenges produces hyperresponsiveness lasting up to two weeks, which is accompanied by increased tissue eosinophilia (Ishida *et al.*, 1989). In guinea pigs, antigen challenge increases vagally mediated airway hyperreactivity via eosinophils (Elbon *et al.*, 1995; Evans *et al.*, 1997; Evans *et al.*, 2001). The mechanism by which eosinophils mediate airway hyperreactivity is discussed in detail in the next section.

H. EOSINOPHILS AND THEIR INTERACTIONS WITH AIRWAY NERVES

1. Characteristics of eosinophils

a. Introduction

In 1879, Paul Ehrlich observed that a specific population of granulocytic leukocytes stained with a negatively charged, brominated fluoroscein compound, eosin. Thus, he named eosinophils for this eosin-staining property. Despite the discovery of eosinophils over a century ago, until the past few years, relatively little was known about their pharmacology and biochemistry compared to other leukocytes. The lack of knowledge is surprising given the critical role eosinophils are thought to play in host defense and allergic disease (Gleich *et al.*, 1984; Rothenberg *et al.*, 2006; Weller, 1994). It is likely that this lack of knowledge is attributed to the difficulty in acquiring pure populations of human eosinophils in substantial numbers to perform conclusive studies of effector functions (Giembycz *et al.*, 1999). In addition, the isolation process (Blom *et al.*, 1995; Ide *et al.*, 1994; Sedgwick *et al.*, 1996) and the source of eosinophils (Sedgwick *et al.*, 1992) can affect eosinophil behavior ex vivo, further confusing interpretation of any data derived from these cells. Indeed, the atopic status of the eosinophil donor is sufficient alone to affect in vitro responses (Sedgwick *et al.*, 2004). Despite this, the emergence of antibody-assisted negative selection techniques (Hansel *et al.*, 1991; Hansel *et al.*, 1989; Hansel *et al.*, 1990) has allowed for a substantial increase in the number of publications reporting detailed experiments examining human eosinophil function and pharmacology in the last two decades, (Giembycz *et al.*, 1999).

b. Morphology and granule proteins

Mature eosinophils are polymorphonuclear granulocytes filled with cytoplasmic membrane bound secretory granules. In general, mature eosinophils have a bilobed nucleus filled with partially condensed chromatin and are approximately 8 um in diameter (Dvorak, 1991; Sokol *et al.*, 1987). Eosinophils are well conserved in mammals and have also been identified in amphibians, birds, fish and reptiles (Spry, 1988). The cytoplasm of eosinophils is filled with spherical and ovoid granules (Figure 1.7). There are four distinct populations of secretory granules, which contain numerous proteins and enzymes. These distinct granule types are divided as follows: crystalloid granules, small granules, primary granules and secretory vesicles. The majority of granule proteins are stored in the *crystalloid granules*, which are named for the appearance of an electron-dense crystalline core surrounded by an electron-lucent matrix that is apparent when eosinophils are imaged by electron microscopy (Figure 1.7). The crystallized core is comprised of major basic protein (MBP) (Gleich *et al.*, 1973; Lewis *et al.*, 1978), surrounded by the non-crystallized proteins eosinophil peroxidase (EPO), eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN) (Egesten *et al.*, 1986; Peters *et al.*, 1986). Of these four highly basic proteins, MBP is the most abundant (Abu-Ghazaleh *et al.*, 1992). The primary granules contain Charcot-Leyden crystal (CLC) protein and the small secretory vesicles and granules contain lipid mediators and other enzymes (Table 1.2).

FIGURE 1.7. Photomicrograph of a human blood eosinophil.

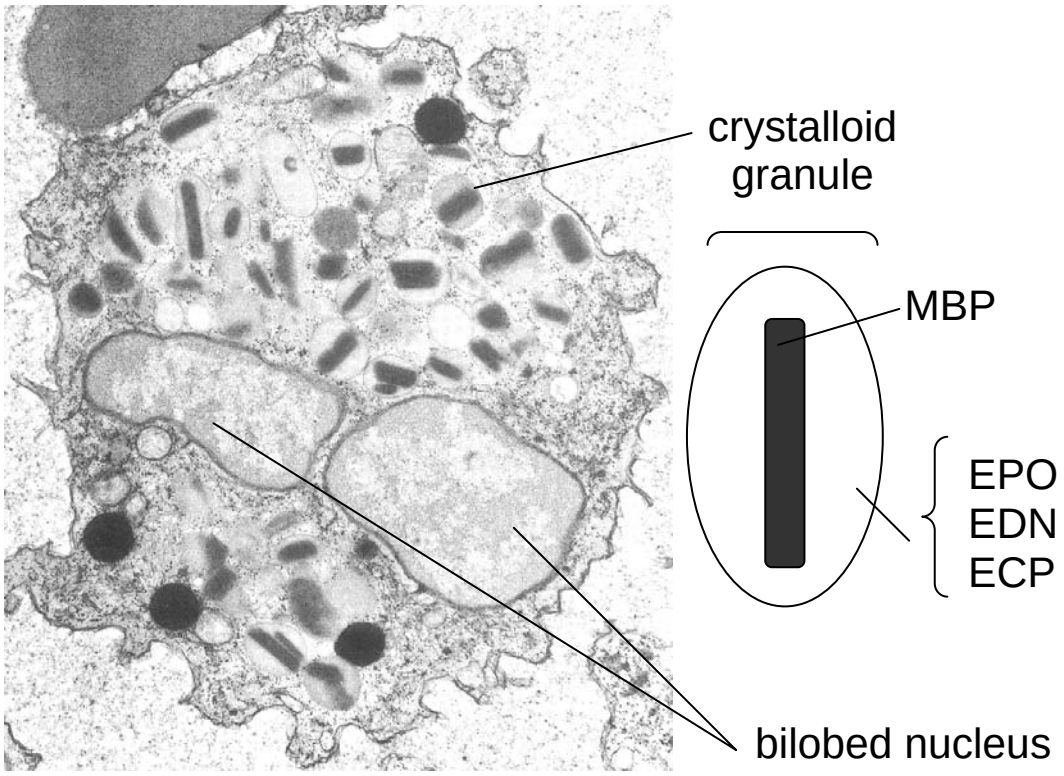


Figure 1.7. Mature eosinophils have a bilobed nucleus and numerous granules within their cytoplasm. The majority of eosinophil granule proteins are stored in the *crystalloid granules*, which are named for the appearance of an electron-dense crystalline core surrounded by an electron-lucent matrix that is apparent when eosinophils are imaged by electron microscopy (Dvorak, 1991). The most abundant granular protein, major basic protein (MBP), forms the dense core of the crystalloid granule and the other three proteins, eosinophil peroxidase (EPO), eosinophil derived neurotoxin (EDN) and eosinophil cationic protein (ECP) reside in the matrix. References in the text.

c. Life cycle and maturation

Eosinophils are terminally differentiated leukocytes produced in the bone marrow from pluripotent stem cells. Eosinopoeisis in the bone marrow occurs over 5 days and after release, mature eosinophils have a half-life of 13-18 hours in circulation (Kroegel *et al.*, 1994). However, eosinophil longevity is likely to be enhanced in the tissues based on in vitro evidence demonstrating that isolated human eosinophils co-cultured with either endothelial cells or conditioned media live up to two weeks (Rothenberg *et al.*, 1987). Eosinophils are predominantly tissue cells and do not reenter circulation, although there is some evidence that they act as antigen presenting cells, traveling from the airway lumen back into lymph nodes (Shi *et al.*, 2000; Wang *et al.*, 2007). The gastrointestinal tract, skin, lung and columnar epithelium are the principle sites of eosinophil accumulation (Rothenberg *et al.*, 2006). Three cytokines are particularly important for regulating eosinophil maturation, IL-3, IL-5 and GM-CSF (Lopez *et al.*, 1986; Lopez *et al.*, 1988; Rothenberg *et al.*, 1988; Takatsu *et al.*, 1994). Of these three, IL-5 is the most specific to the eosinophil lineage (Yamaguchi *et al.*, 1988) and stimulates eosinophil release from the bone marrow in humans (Collins *et al.*, 1995) and, mice (Dent *et al.*, 1990; Lee *et al.*, 1997; Mishra *et al.*, 2002; Tominaga *et al.*, 1991) guinea pigs (Palframan *et al.*, 1998a; Palframan *et al.*, 1998b).

d. Role of interleukin-5 in eosinophil expansion and function

Interleukin-5 has a critical role in controlling eosinophil production, which has been well established in transgenic mice (Dent *et al.*, 1990; Lee *et al.*, 1997; Mishra *et al.*, 2002; Tominaga *et al.*, 1991), guinea pigs (Palframan *et al.*, 1998a; Palframan *et al.*, 1998b)

and also in humans with hyperosinophilic syndrome (Owen *et al.*, 1989). Additionally, IL-5 primes eosinophils via ERK signaling (Bates *et al.*, 2000), which enhances eosinophil effector functions such as adhesion (Sedgwick *et al.*, 1995), chemotaxis (Bates *et al.*, 2000; Rothenberg *et al.*, 1996; Satoh *et al.*, 1997), superoxide anion generation (Sedgwick *et al.*, 1995) and degranulation (Carlson *et al.*, 1993; Takafuji *et al.*, 1996). Thus, the priming of circulating eosinophils by increased IL-5 levels in atopic individuals may explain the increased sensitivity and activity of eosinophils in vivo and in culture.

e. Eosinophil activation

In response to diverse stimuli (Table 1.2), eosinophil morphology becomes altered in a characteristic manner that is described as *activation.* These ultrastructural changes include decreased secondary granules, loss of crystalline cores of secondary granules and increased lipid bodies, which has been characterized in tissue and cultured human (Dvorak, 1991; Weller *et al.*, 1995) and guinea pig (Newman *et al.*, 1996) eosinophils. With regard to eosinophil function, the term activation refers to an agonist-specific cellular change in functional activity and is characterized by intracellular calcium transients, or fluctuations (Sedgwick, 1995). It is well established that this intracellular signaling is an important requirement for eosinophil functions such as adhesion, chemotaxis, secretion, degranulation and cytolysis (Giembycz *et al.*, 1999).

f. Mechanisms of eosinophil degranulation

At least three separate processes have been identified that result in the release of granule contents from eosinophils: *secretion, piecemeal degranulation* and *cytolysis*. Two

mechanisms have been described that regulate granule secretion in eosinophils. One of these processes, constituitive secretion, is characterized by the presence of small intracellular vesicles containing secretory mediators that are released in a stimulus-independent manner. The other process is regulated exocytosis, in which proteins are released in response to external stimuli (Giembycz *et al.*, 1999).

An alternative process commonly seen in human eosinophils is *piecemeal degranulation*. This process differs from secretion in that small protein containing vesicles bud off from the membrane and proteins are then released from these vesicles, and has been well characterized on an ultrastructural level (Dvorak, 1991; Dvorak *et al.*, 1993a; Tai *et al.*, 1981; Torpier *et al.*, 1988).

Another mechanism by which eosinophil proteins are released is *cytolysis*, which is characterized by membrane rupture, chromatolysis (chromatin lysing) and mitochondrial swelling. Eosinophil cytolysis is frequently observed in diseases characterized by eosinophilic inflammation, such as asthma (Costello *et al.*, 1997; Filley *et al.*, 1982; Laitinen *et al.*, 1985; Ohashi *et al.*, 1992), atopic dermatitis (Leiferman *et al.*, 1985) and inflammatory bowel disease (Dvorak *et al.*, 1980; Dvorak *et al.*, 1993a; Dvorak *et al.*, 1993b). In these regions of inflammation, “clusters of free eosinophil granules” (cfegs) within the surrounding tissue have been observed (Erjefalt *et al.*, 1998; Persson *et al.*, 1997a). Eosinophil cytolysis is reproducible in vitro by stimulation with IgA and IgG (Weiler *et al.*, 1996) or calcium ionophore (Fukuda *et al.*, 1985). Thus, in addition to

secretion and piecemeal degranulation, eosinophils undergo nonapoptotic lysis, which may be the final stage of eosinophil activation in vivo (Persson *et al.*, 1997a).

g. Eosinophil mediators

In addition to granule proteins, eosinophils secrete numerous other factors, including cytokines, chemokines, lipid mediators, oxygen radicals, cytotoxic proteins and neurotrophins (Giembycz *et al.*, 1999; Hogan *et al.*, 2008; Nassenstein *et al.*, 2003; Rothenberg *et al.*, 2006) (Table 1.2). Eosinophil mediator release is regulated by cell surface receptors (Figure 1.3) which ultimately produce a host of functional responses resulting in stimulation and suppression of eosinophil activity (Giembycz *et al.*, 1999; Hogan *et al.*, 2008; Nassenstein *et al.*, 2003; Rothenberg *et al.*, 2006). To date, there are more than twenty G protein-coupled receptors identified on eosinophils (Table 1.3).

FIGURE 1.8. Mechanism of eosinophil cytolysis.

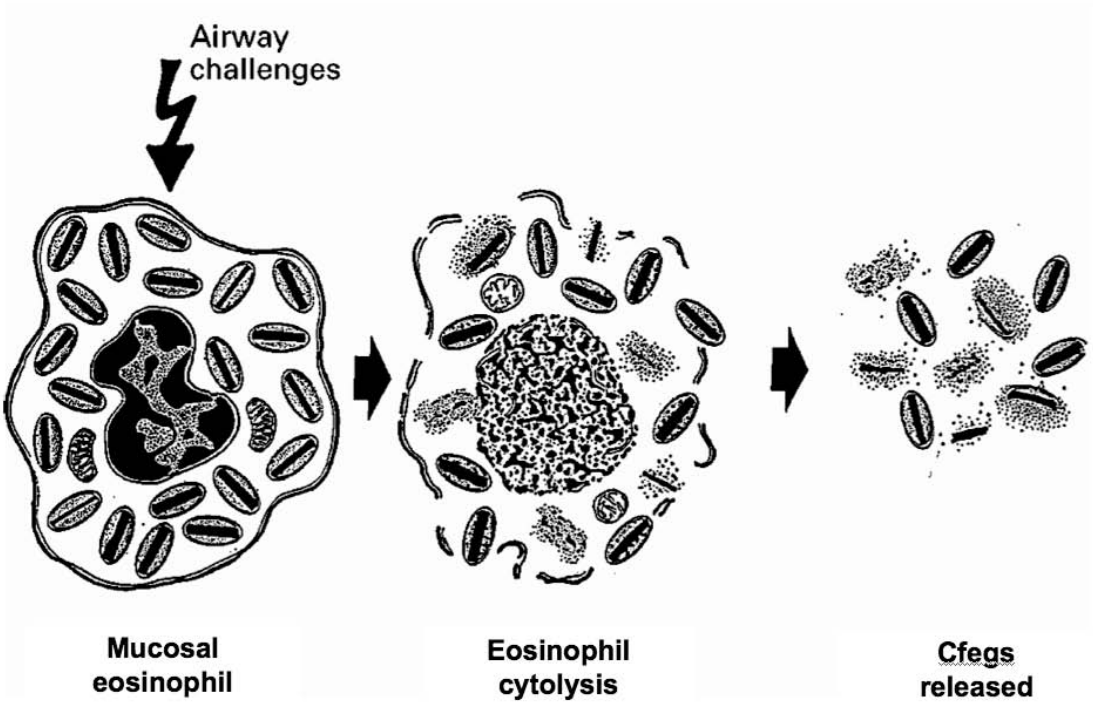


Figure 1.8. In response to allergens or other stimuli, mucosal **e**osinophils undergo cytolysis, which is characterized by membrane rupture, chromatolysis (chromatin lysing) and mitochondrial swelling. Following cytolysis, clusters of free eosinophil granules (Cfegs) are deposited in mucosal surfaces, such as the lamina propria, epithelium and lumen. Adapted from (Persson *et al.*, 1997a).

TABLE 1.2. Common eosinophil-derived mediators.

EOSINOPHIL-DERIVED MEDIATORS					
Granules	Lipids	Cytokines	Chemokines	Growth factors and neuro-mediators	Interferons
ECP	LTB4	IL-1α	GM-CSF	BDNF	INFγ
EDN	LTC4	IL-2	Eotaxin	NGF	TNFα
EPO	PAF	IL-3	MCP-1	NT3	
MBP	PGE2	IL-4	MCP-3	PDGF	
		IL-5	MCP-4	Substance P	
		IL-6	MIP-1α	TNFα	
		IL-8	RANTES	TNFβ	
		IL-10		VEGF	
				Leukemia inhibitory factor	
		IL-11			
		IL-12			
		IL-13			
		IL-16			
		IL-17			

Table 1.2. Abbreviations: BDNF, brain derived neurotrophic factor; ECP, eosinophil cationic protein; EDN, eosinophil derived neurotoxin; EPO, eosinophil peroxidase; GM-CSF, granulocyte /macrophage colony stimulating factor; IL, interleukin; INF, interferon; LTB, leukotriene B; MBP, major basic protein; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; NGF, nerve growth factor; NT3, neurotrophin 3; PAF, platelet activating factor; PDGF, platelet derived growth factor; PGE, prostaglandin E; RANTES, regulated on activation, normal T-cell expressed and secreted; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor. Data compiled from (Giembycz *et al.*, 1999; Hogan *et al.*, 2008; Nassenstein *et al.*, 2003; Rothenberg *et al.*, 2006).

TABLE 1.3. Receptors expressed by eosinophils.

EOSINOPHIL RECEPTORS			
Adhesion	G protein coupled	Cytokine and Chemokine	Neurotrophins
CD11a,b,c	Adenosine-1,2,3	GM-CSFR	p75NTR
CD18	β2-adrenergic	IFNγR	TrkA,B,C
αd integrin	BLT	IL-3R	
β7 integrin	C3aR	IL-5R	
	C5aR	TNFαRI and II	
	CCR1 and 3		
	CCR3		
	CXCR1/2		
	Cys-LT		
	fMLP		
	Histamine-1,2,3		
	NK1,2,3		
	PAF		
	VIP		

Table 1.3. Abbreviations: BLT, leukotriene B4; C3a complement, 3a; C5 complement, C5; CCR, CC-motif chemokine receptor; CXCR, CXC-motif chemokine receptor; Cys-LT, cysteinyl leukotriene; CD, cell determinant; fMLP, formyl-methionyl leucyl phenylalanine; GM-CSF, granulocyte /macrophage colony stimulating factor; IL, interleukin; INF, interferon; NK, neurokinin; PAF, platelet activating factor; p75NTR, p75 neurotrophin receptor; TNF, tumor necrosis factor; TRK, tropomyosin related kinase; VIP, vasoactive intestinal peptide. Data compiled from (Giembycz *et al.*, 1999; Hogan *et al.*, 2008; Nassenstein *et al.*, 2003; Rothenberg *et al.*, 2006).

2. Eosinophil interactions with parasympathetic nerves

a. Eosinophil recruitment to lungs

In asthma, there is increased production of cytokines and chemokines in the lungs, which collectively promote eosinophil trafficking from the blood. Of all the mediators implicated in recruiting leukocytes, only IL-5 and the eotaxins selectively regulate eosinophil trafficking (Rankin *et al.*, 2000; Zimmermann *et al.*, 2003). Eosinophils in the blood travel to the lung through the vasculature via L-selectin mediated "rolling" on the surface of the endothelium (Knol *et al.*, 1996; Teixeira *et al.*, 1995). In response to cytokine and chemokine gradients, eosinophils adhere more firmly to endothelium through binding of the eosinophil cell surface integrins very late antigen-4 (VLA-4) and CD11b/18 to members of the immunoglobulin superfamily of adhesion proteins: vascular-adhesion molecule (VCAM-1) and intercellular molecule 1 (ICAM-1), respectively. Eosinophils then transmigrate across the endothelial barrier and enter the interstitial matrix of the lung through a process called *diapedesis*. Allergen challenge increases expression of VCAM-1 and ICAM-1, and inhibiting either of these adhesion molecules attenuates eosinophil localization to lungs of antigen challenged mice (Broide *et al.*, 1998a; Broide *et al.*, 1998b; Nakajima *et al.*, 1994), underlying the importance of these adhesion molecules.

b. Adhesion to nerves

Eosinophils are clustered along the airway nerves in antigen challenged animals (Evans *et al.*, 1997; Evans *et al.*, 2001; Fryer *et al.*, 2006) and in patients with asthma (Costello *et al.*, 2000; Costello *et al.*, 1997). In antigen challenged guinea pigs, eosinophil recruitment

to airway nerves is mediated by eotaxin. Blocking CCR3 in antigen challenged guinea pigs prevents the antigen-induced localization of eosinophils around nerves without decreasing total tissue eosinophilia, thus eotaxin selectively mediates eosinophil recruitment to nerves (Fryer *et al.*, 2006).

Eosinophils adhere to parasympathetic nerves via ICAM-1 and VCAM-1, which are expressed by parasympathetic nerves. This interaction is mediated by the eosinophil counterligands CD11b/18 and VLA-4, respectively (Sawatzky *et al.*, 2002). It is also recognized that the process of eosinophil adhesion to cholinergic nerves via eosinophil CD11/18b and neuronal ICAM-1 activates eosinophils, measured by the release of eosinophil proteins EPO and LTC_4 (Kingham *et al.*, 2003; Kingham *et al.*, 2002; Sawatzky *et al.*, 2002). The physiological relevance of this interaction is also supported in vivo: inhibiting ICAM-1 expression with dexamethasone prevents eosinophil accumulation around airway nerves and protects neuronal M_2 receptor function, preventing airway hyperreactivity (Evans *et al.*, 2001). Thus, expression of adhesion molecules by the nerves and by eosinophils is integral to their dynamic interactions, including activation, adhesion and degranulation.

c. Major basic protein and M_2 receptor dysfunction

Following antigen inhalation, activated eosinophils degranulate near airway nerves, releasing major basic protein (MBP), an endogenous and selective antagonist for neuronal M_2 muscarinic receptors (Jacoby *et al.*, 1993), resulting in increased acetylcholine release and airway hyperreactivity (Fryer *et al.*, 1992; Fryer *et al.*, 1991)

(Figure 1.9). In antigen challenged guinea pigs, airway hyperreactivity is prevented by depleting eosinophils with antibody to IL-5 (Ab IL-5) (Elbon *et al.*, 1995), by blocking the CCR3 receptors (Fryer *et al.*, 2006), or by blocking eosinophil major basic protein (Evans *et al.*, 1997). Thus, airway hyperreactivity in antigen challenged guinea pigs is mediated by eosinophils and neuronal M_2 receptor dysfunction.

FIGURE 1.9. Eosinophils mediate airway hyperreactivity via major basic protein.

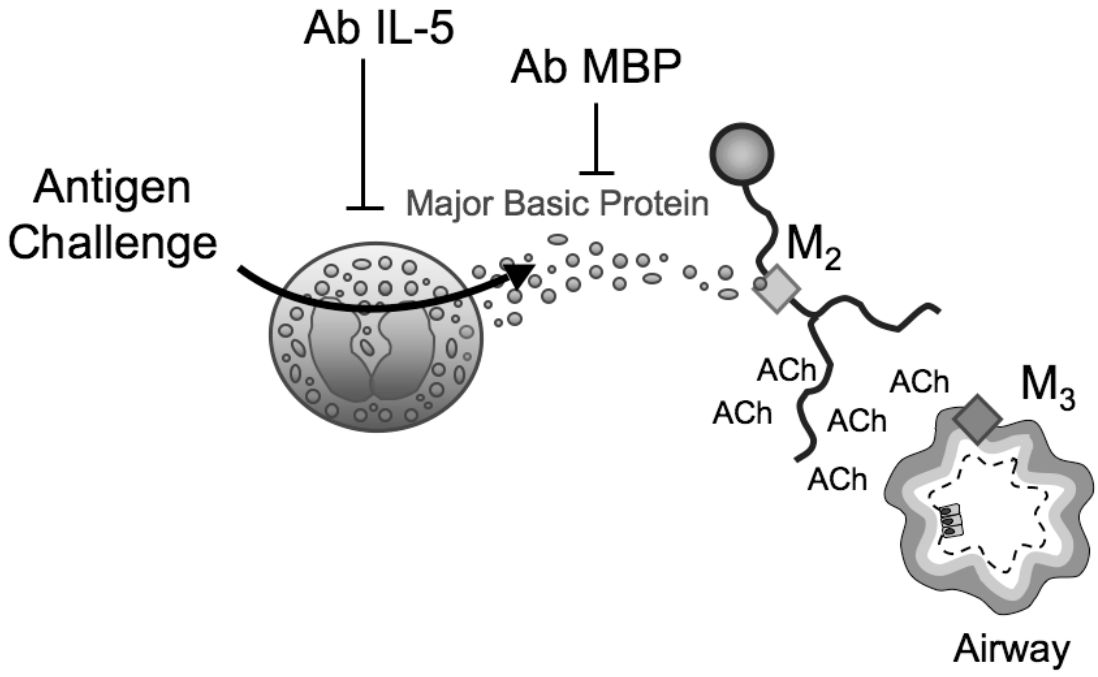


Figure 1.9. Antigen challenge releases eosinophil major basic protein (MBP) onto neuronal M_2 receptors on airway parasympathetic nerves, causing M_2 muscarinic receptor dysfunction. Loss of neuronal M_2 receptor function increases acetylcholine (ACh) release onto airway smooth muscle and increases bronchoconstriction. Both airway hyperreactivity and neuronal M_2 receptor dysfunction are protected by depleting eosinophils with antibody to IL-5 (Ab IL-5) or by neutralizing MBP (Ab MBP). References within the text.

3. Eosinophils and neuromediators

Eosinophils are a source of neuromediators, which may be important in asthma because eosinophils are found in close proximity to nerves. Eosinophils release several neuromediators, including the neurotrophins nerve growth factor (Solomon *et al.*, 1998), , brain-derived neurotrophic factor and neurotrophin-3 (Noga *et al.*, 2003) and the hematopoietic cytokine, leukemia inhibitory factor (Zheng *et al.*, 1999). In addition, there is an association between eosinophilic inflammation and increased neurotrophins in the lungs and bronchoalveolar lavage of humans and experimental animals (Namura *et al.*, 2007; Nassenstein *et al.*, 2003; Nassenstein *et al.*, 2005; Noga *et al.*, 2003; Noga *et al.*, 2005).

One effect of neuromediators is that they can alter neuronal neurotransmitter phenotype; this effect is termed neural plasticity. Neural plasticity is widely described in sensory nerves (de Vries *et al.*, 2006; Freund-Michel *et al.*, 2008; Frossard *et al.*, 2005; Undem *et al.*, 1999; Wu *et al.*, 2001; Wu *et al.*, 2003), but may also occur in parasympathetic nerves (Durcan *et al.*, 2006; Hazari *et al.*, 2007; Wu *et al.*, 2001; Wu *et al.*, 2003; Wu *et al.*, 2002). In particular, the neurotrophin nerve growth factor (NGF) is implicated in airway hyperresponsiveness (Freund-Michel *et al.*, 2008; Wu *et al.*, 2006). There are multiple sources of NGF in the lung, including bronchial epithelium, pulmonary fibroblasts, bronchial smooth muscle and several inflammatory cells, including eosinophils (Solomon *et al.*, 1998). In culture, eosinophils promote a cholinergic phenotype and increase expression of acetylcholine making enzymes in neurons (Durcan *et al.*, 2006; Sawatzky *et al.*, 2003). In addition, NGF enhances acetylcholine release in

forebrain cholinergic neurons (Auld *et al.*, 2001a) and increases cholinergic innervation and contractile response to electric field stimulation in murine trachea (Bachar *et al.*, 2004). Thus, one way in which eosinophils may induce neuronal plasticity and alter neurotransmitter content and release is via NGF.

4. Anti-eosinophil therapeutics and airway hyperreactivity

For many decades, eosinophils have been thought of as the key effector cells in asthma pathogenesis. This assumption is supported by the positive correlation between eosinophils and airway hyperreactivity (Bousquet *et al.*, 1990) and by the finding that abundant eosinophil MBP degranulation is present in bronchial biopsies from asthmatics (Filley *et al.*, 1982). Thus, it seems logical that specifically targeting eosinophils would prevent airway hyperresponsiveness in human asthma. This assumption is also supported by data from multiple studies employing experimental models of asthma (monkeys, mice, guinea pigs) that have demonstrated that reducing eosinophils by transgenic methods (Foster *et al.*, 1996; Kopf *et al.*, 1996; Lee *et al.*, 2004b), steroids (Evans *et al.*, 2001) or with IL-5 neutralizing antibodies (Elbon *et al.*, 1995; Hogan *et al.*, 1997; Mauser *et al.*, 1995) blocks antigen induced airway hyperresponsiveness.

Glucocorticoids are the most effective agents for reducing eosinophilia (Rothenberg, 1998) and are the most effective anti-inflammatory drug used for airway hyperresponsiveness in asthma (National Heart, 2007). However, therapies that are more targeted to eosinophils specifically, such as inhibiting IL-5 with anti-IL-5 antibodies, have had variable effects on airway hyperresponsiveness. Clinical trials in humans have

found that anti-IL-5 antibodies significantly decrease eosinophils levels in the blood (Kips *et al.*, 2003; Leckie *et al.*, 2000), but do not decrease airway hyperreactivity, leading the authors to conclude that eosinophils are not responsible for airway hyperresponsiveness in asthma. However, results of these studies are inconclusive because they were underpowered and had poor experimental design (Kay *et al.*, 2003; O'Byrne *et al.*, 2001).

One of these anti-IL-5 studies was a safety study (Kips *et al.*, 2003), thus a submaximal dose was used, making it difficult to evaluate the effectiveness on airway hyperresponsiveness (Kay *et al.*, 2003). In another anti-IL-5 study (Leckie *et al.*, 2000), the investigators measured airway function in mild asthmatics before and after allergen inhalation to determine baseline airway hyperresponsiveness. However, in two of the three occasions in which the authors measured baseline airway hyperresponsiveness prior to treatment with anti IL-5 antibodies, the authors failed to demonstrate any affect of allergen challenge on airway function. In other words, the positive control was ambiguous. Thus, the inability to demonstrate a significant effect in allergen induced airway hyperresponsiveness during the baseline period makes it impossible to interpret the effects of anti IL-5 treatment at later time points. Additionally, the individuals in this study were not used as their own controls, thus the number of subjects used to calculate statistical power was too few, leading some to conclude the study was underpowered (O'Byrne *et al.*, 2001). Furthermore, additional studies demonstrated that anti IL-5 antibodies induced only a modest reduction of eosinophils within the lung (Flood-Page *et al.*, 2003). This may have been because the dose of anti- IL-5 antibodies was insufficient,

or that survival of tissue eosinophils is regulated by IL-5 independent mechanisms. The exact reason for a different effect in one compartment compared to the other is unclear, but it may be due to changing sensitivities of eosinophils to IL-5 at different times over the course of the disease. This is supported by data suggesting that antigen-induced tissue eosinophilia can occur independently of IL-5, as seen in IL-5 deficient mice (Foster *et al.*, 1996; Hogan *et al.*, 1997). It is therefore reasonable to conclude that the studies using anti-IL-5 antibodies were flawed because the experimental designs were poor and there was no reduction in tissue eosinophils or MBP deposition.

Furthermore, a more recent study has demonstrated that anti-IL-5 antibodies are effective at reducing concurrent steroid therapy in asthma patient populations that are classified as moderate to severe with persistent symptoms (O'Byrne, unpublished data presented at 2007 International Eosinophil Society meeting), suggesting that patient criteria (mild versus severe) and the measures used to evaluate effectiveness are important factors in determining the efficacy of anti-IL-5 therapies. Thus, more recent data are supporting a role for eosinophils in mediating airway hyperreactivity in human asthma.

I. ANTICHOLINERGICS AND ASTHMA

1. Introduction to asthma therapeutics

There are five classes of medication that are generally used for treating asthma symptoms: glucocorticoids, cromolyn drugs, theophylline, β_2-adrenergic agonists and anticholinergics. Of these medications, glucocorticoids are the most potent at inhibiting inflammation and airway hyperresponsiveness (Bhagat *et al.*, 1985; Kerrebijn *et al.*, 1987; Szefler, 1991) and are the first-line anti-inflammatory agents used in asthma management (Asthma, 2006). The other most commonly used asthma medications are β_2-adrenergic agonists and anticholinergics. Inhaled β_2 agonists and anticholinergics and are used routinely to reverse acute bronchoconstriction during an asthmatic exacerbation, however, these drugs are less effective for treating chronic airway hyperresponsiveness and are not recommended for chronic treatment of stable asthma (National Heart, 2007).

2. Pharmacology of anticholinergic drugs used in asthma treatment

a. Atropine

Atropine is among the earliest of anticholinergic drugs to be purified and used in Western medicine (Goodman *et al.*, 2001). In clinical settings, atropine is administered via multiple routes, including oral, topical, intravenous and inhalation. Atropine is well absorbed into the systemic circulation by the gastrointestinal system, where it readily penetrates the blood brain barrier. The half-life of atropine is approximately 4 hours in humans (Goodman *et al.*, 2001).

To avoid central nervous system toxicities, several synthetic chemical derivatives were developed that contain a quaternary ammonium moiety (Figure 1.10), which limits

penetration across the blood brain barrier and decreases systemic side effects because of their poor absorption by the gastrointestinal system and the respiratory tract. These atropine analogues have a longer duration of action (Rominger, 1979) and retain their anticholinergic properties when aerosolized, thus allowing for these drugs to be targeted specifically to the lung. The most widely used synthetic derivative to be used in asthma treatment is ipratropium bromide.

b. Ipratropium

Ipratropium bromide has been used extensively in treatment of asthma and is administered via inhalation. Studies in asthmatics demonstrate that ipratropium provides effective bronchodilation during an acute asthma exacerbation compared to beta agonists alone (Baigelman *et al.*, 1977; Gross *et al.*, 1984) or when given in combination with beta agonists (Boushey, 1987). The effectiveness of ipratropium in treatment of asthma may be limited by the fact that it binds with similar affinities to both prejunctional neuronal M_2 muscarinic receptors and M_3 muscarinic receptors on smooth muscle (Table 1.1) (Maclagan *et al.*, 1989). Thus, even though ipratropium decreases bronchoconstriction by blocking M_3 receptors on airway smooth muscle, it increases acetylcholine release from the parasympathetic nerves by blocking neuronal M_2 receptors as well. In vivo studies have shown that ipratropium potentiates vagally induced bronchoconstriction in experimental animals via this mechanism (Fryer *et al.*, 1987; Groeben *et al.*, 1996) and possibly in humans as well (O'Callaghan *et al.*, 1989). Thus, drugs with improved selectivity for M_3 receptors would be clinically beneficial.

c. Tiotropium and other selective muscarinic antagonists

Several compounds with M_3 selectivity have surfaced in the last decade. Pfizer has developed two compounds, darifenacin and revatropate, which have a 5-fold and 50-fold selectivity for M_3 receptors over M_2, respectively (Alabaster, 1997). Similarly, another M_3 selective compound, tiotropium bromide, is currently used to treat chronic obstructive airway disease, COPD (Maesen *et al.*, 1993). Although tiotropium bromide has similar binding affinity for M_2 and M_3 muscarinic receptors (Haddad *et al.*, 1994), it dissociates more rapidly from M_2 receptors, which provides kinetic receptor selectivity for M_3 (Takahashi *et al.*, 1994). Functional studies on isolated airways indicate that tiotropium inhibits methacholine-induced contraction with a higher potency than either atropine or ipratropium (Takahashi *et al.*, 1994), which would suggest that tiotropium may be beneficial in asthma. However, the efficacy of tiotropium in long-term management of asthma has not been studied (Gross, 2004) and its use in asthma has not been approved by the Federal Drug Administration.

3. Rationale for using anticholinergics in asthma

Given that asthma and airway hyperreactivity are mediated by increased acetylcholine and bronchoconstriction, it is logical to assume that anticholinergics would prevent airway hyperreactivity. This is supported by studies in man demonstrating that antigen inhalation causes acute bronchoconstriction which is reversed by muscarinic receptor antagonists (Itkin *et al.*, 1970; Schultze-Werninghaus, 1981; Vastag *et al.*, 1976; Yu *et al.*, 1972). Furthermore, atropine or vagal blockade completely blocks antigen-induced bronchoconstriction in dogs, guinea pigs and primates (Gold *et al.*, 1972; Miller *et al.*,

1976; Mills *et al.*, 1970; Schutz *et al.*, 2004; Zimmermann *et al.*, 1976). Thus, studies in man and experimental animal models have all shown that anticholinergics prevent antigen-induced bronchoconstriction. Therefore it is surprising that anticholinergics are not used for chronic treatment of asthma.

4. The Expert Panel on Asthma's and anticholinergics

Asthma medications are categorized into two general classes: relief medications that rapidly reverse acute bronchoconstriction and long-term control medications that are taken on a daily basis that reduce airway tone and decrease the rate of asthmatic exacerbations. To reverse acute bronchoconstriction during an asthma exacerbation, the Expert Panel recommends β_2 agonists as the first line therapy for managing acute asthma. However, the Panel recognizes that combining anticholinergics with β_2 agonists provides an additive benefit for moderate or severe exacerbations. Additionally, anticholinergics are recommended as an alternative bronchodilator for patients that cannot tolerate beta agonists. However, anticholinergics have not demonstrated effectiveness in long-term management of asthma evaluated in terms of reducing the rate of exacerbations (airway hyperresponsiveness) and morbidity (Kerstjens *et al.*, 1992), and are not recommended for management of chronic asthma by the Expert Panel (National Heart, 2007).

5. Anticholinergics in chronic asthma

Use of anticholinergics in asthma has been relegated to acute situations; reversing bronchoconstriction during an asthmatic exacerbation. This class of drugs is not used for long-term management of asthma because there is a lack of clinical data demonstrating that they provide any added benefit in reducing asthma exacerbations and morbidity (National Heart, 2007). Thus, anticholinergics are not used as a preventative therapy for decreasing airway hyperresponsiveness.

The conclusion that anticholinergics are not entirely effective for treatment of chronic asthma is surprising given that cholinergic control of the airways is clearly abnormal in asthma (section G). Furthermore, it is unexpected given that animal studies have demonstrated that airway hyperresponsiveness is mediated by increased acetylcholine and bronchoconstriction (Section H). The reasons for this discrepancy between animal studies and clinical data from humans are not clear. It may be because asthma is more complicated than experimental animal models. Additionally, the lack of efficacy in preventing human airway hyperresponsiveness may be because anticholinergic drugs used for managing asthma thus far have been non-selective (Table 1.1). Lastly, the underlying reason for this discrepancy may be that anticholinergics have effects in human asthma that have not yet been recognized from animal studies.

FIGURE 1.10. Chemical structures of muscarinic receptor antagonists and agonists.

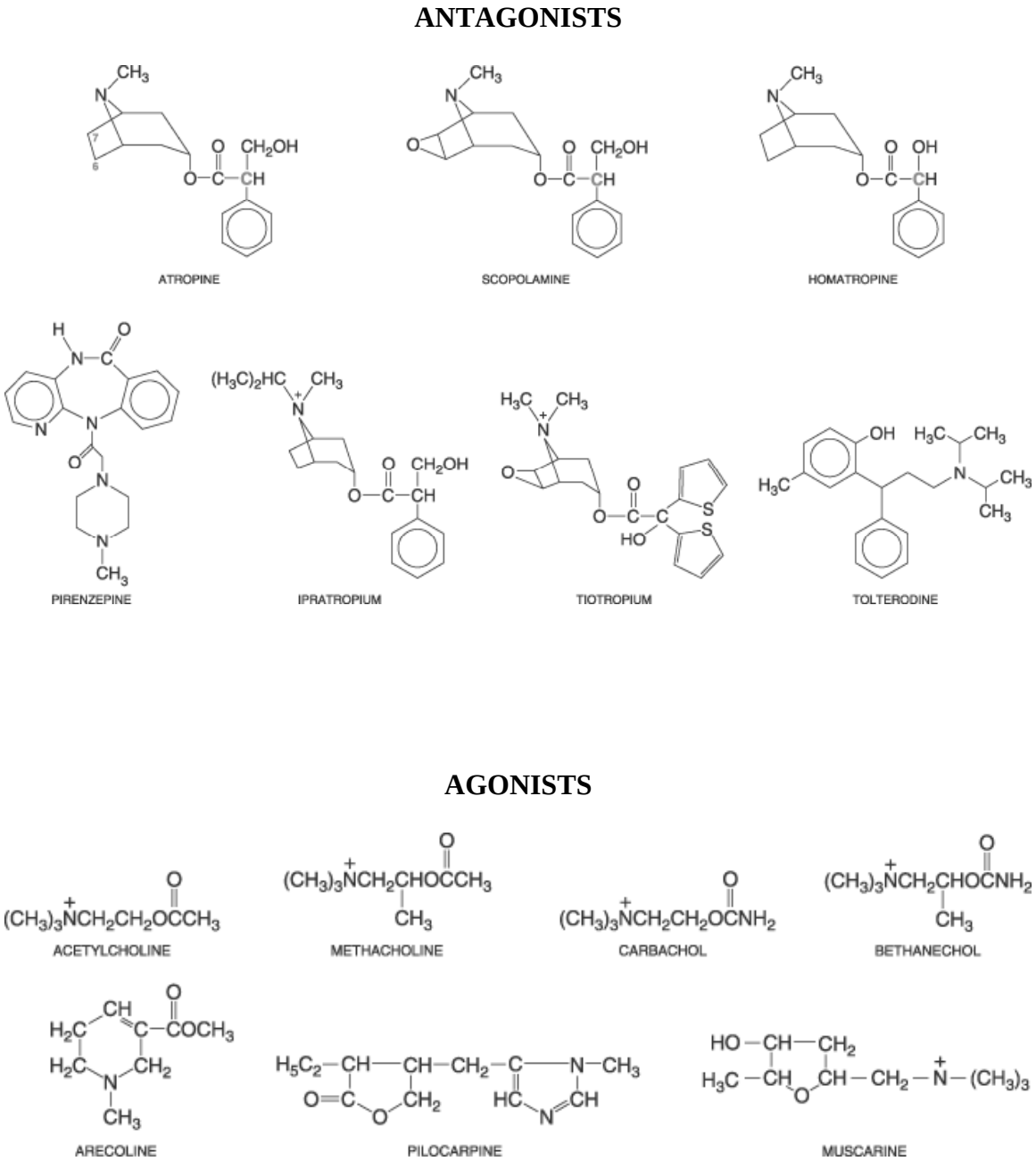


Figure 1.10. From (Goodman *et al.*, 2001).

J. HYPOTHESIS AND RESEARCH AIMS

Given that airway hyperreactivity is mediated by increased acetylcholine resulting in increased bronchoconstriction, anticholinergic medications would be expected to be highly effective treatments for asthma. However, use of anticholinergics in management of chronic asthma is not entirely effective and is not recommended by the NIH Expert Panel on the Management of Asthma. The reasons for the lack of efficacy of anticholinergic drugs in long-term asthma management are not understood.

Acetylcholine modulates function of some inflammatory cells, including mast cells, lymphocytes and macrophages. However, it is not known whether acetylcholine affects eosinophil function. Eosinophils are localized around airway nerves where they mediate airway hyperreactivity in antigen challenged guinea pigs. Antigen challenge releases eosinophil major basic protein onto neuronal M_2 receptors on airway parasympathetic nerves, causing M_2 receptor dysfunction, increased acetylcholine release onto M_3 receptors on airway smooth muscle and increased bronchoconstriction. Thus, airway hyperreactivity in antigen challenged guinea pigs is mediated by eosinophils, suggesting that recruitment and activation of eosinophils to airway nerves is relevant to human disease.

If acetylcholine inhibits eosinophil activation via muscarinic receptors, it might explain the lack of effect of anticholinergic drugs in human disease. Under normal conditions, acetylcholine would inhibit eosinophil activation, such that parasympathetic nerves and eosinophils exist in equilibrium. This equilibrium may be disrupted by antigen challenge,

and potentiated by the presence of anticholinergic drugs, which may explain why these drugs are not efficacious in asthma. *The overall hypothesis tested in this thesis is that eosinophils respond to acetylcholine via muscarinic receptors, and that activation of these receptors inhibits eosinophil function, changing interactions with airway parasympathetic nerves.* Specifically, these studies will determine which muscarinic receptor subtypes are present on guinea pig and human eosinophils and whether blockade of muscarinic receptors increases airway hyperreactivity, eosinophil recruitment to airway nerves and degranulation in airways of challenged guinea pigs.

FIGURE 1.11. Overall model of hypothesis tested within this thesis.

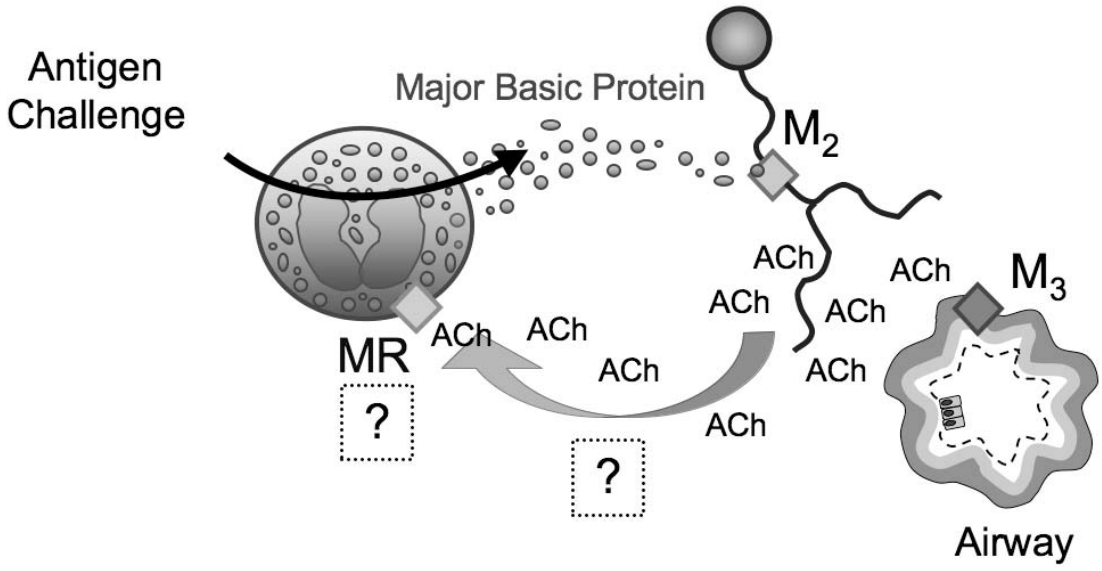


Figure 1.11. Eosinophils are localized around airway nerves in asthma and in antigen challenged guinea pigs. Antigen challenge releases the antagonist eosinophil major basic protein (MBP) onto neuronal M_2 receptors on airway parasympathetic nerves, causing M_2 muscarinic receptor dysfunction, increased acetylcholine (ACh) release onto M_3 receptors on airway smooth muscle and increased bronchoconstriction. This thesis will examine whether acetylcholine inhibits eosinophil function via muscarinic receptors (MR), thus providing an additional pathway in the complex interaction of inflammatory cells and nerves. The arrows shown in this diagram suggest the order of events, but do not necessarily exclude the steps in between.

CHAPTER II.

GENERAL METHODS

A. CHOICE OF ANIMALS VERSUS HUMANS

1. Rationale for guinea pig as an animal model of allergic asthma

The guinea pig has been used as an animal model for respiratory allergy for many decades. Guinea pigs exhibit bronchoconstriction upon exposure to histamine and experience both immediate onset and late onset reactions following allergen exposure. Allergen sensitization in guinea pigs can be either IgG and/or IgE dependent (Canning, 2003) and is associated with respiratory symptoms and eosinophilic inflammatory responses after allergen exposure. The docile nature and small size also make the guinea pig a good species for studying allergic asthma. In this thesis, guinea pigs were used as a model of airway hyperreactivity because their airways closely resemble the physiology and pharmacology of humans (Canning, 2003; Canning *et al.*, 2008; Richardson *et al.*, 1979).

The role of smooth muscle contracting mediators acetylcholine, substance P, tachykinins, leukotrienes, thromboxanes, prostaglandins and histamine in development of bronchoconstriction and airway hyperreactivity has been well studied in guinea pigs. The autonomic nerves innervating airway smooth muscle are similar to humans, with the exception that in guinea pigs, airway smooth muscle receives some sympathetic input, while humans do not. Therefore guinea pigs were chemically sympathectomized for studies performed in vivo. Although rodents such as mice and rats are also used as a small animal model for asthma, several key anatomical differences in airway innervation limit their usefulness. For example, mice and rats lack the protective cough reflex

(Widdicombe, 1998) and their airway smooth muscle does not have relaxant innervation (Canning, 2003).

Eosinophil influx, a marker of asthma in humans, occurs in the guinea pig. Agents that block eosinophilic influx also block allergen-stimulated airway hyperreactivity in the guinea pig. Extracellular eosinophil granules are present in airways of asthmatic humans and antigen challenged guinea pigs (Costello *et al.*, 2000; Costello *et al.*, 1997; Verbout *et al.*, 2007). These features of eosinophil degranulation in the airways are not present in mice, confirming that both mouse eosinophils and airway hyperresponsiveness are inherently different between mice and humans (Persson *et al.*, 1999). Lastly, in vitro, guinea pig eosinophils respond to the same pharmacological stimuli as human eosinophils (Giembycz *et al.*, 1999), making them a suitable model for human eosinophils.

2. Rationale for including tissue and eosinophils from human subjects

Animal models provide the means to indirectly study human disease and address mechanistic questions that would not be feasible or ethical in human subjects. However, it is imperative to demonstrate to a reasonable degree that conclusions based upon animal studies bear relevance to human disease. Therefore, whenever possible, I included human tissues. Since viable human eosinophils can be isolated from human blood, I used these cells for in vitro activation studies. All studies with human subjects were conducted in accordance with the Institutional Review Board committee at Oregon Health & Science University.

B. MEASUREMENT OF AIRWAY PHYSIOLOGY

1. Guinea pigs

Specific pathogen-free female Dunkin-Hartley guinea pigs (150-200 g) were shipped in filtered crates and kept in high-efficiency particulate-filtered air. Guinea pigs were fed a normal diet and handled in accordance with the standards established by the US Animal Welfare Acts set forth in NIH guidelines and approved by the Institutional Animal Care and Use Committees at Oregon Health & Science University.

2. Sensitization and challenge with antigen

Guinea pigs were sensitized to Grade V ovalbumin dissolved in sterile phosphate buffered saline (PBS; 6 mg/ml) every other day for a total of three intraperitoneal injections in approximately 0.5 ml volume, adapted from Fryer et al. (Fryer *et al.*, 2006). After the last ovalbumin injection, animals were housed in the care facility for at least three weeks to allow development of ovalbumin specific antibodies. On the day of antigen challenge, ovalbumin was dissolved in 80 ml PBS for one hour and clumps removed by filtering through gauze into a plastic cup containing 80 ul of Antifoam A, a foam suppressor. Guinea pigs were exposed to aerosolized ovalbumin (1.0 - 2.5%) in a constant flow exposure chamber (Figure 2.1) for five minutes or until signs of respiratory distress appeared, in which case antigen challenge was halted. Respiratory distress was defined as diaphragmatic breathing with exaggerated abdominal excursions and a fall in respiratory rate to below 25 per minute (Green, 1982). In the case of severe respiratory failure, light intermittent digital pressure was applied to the manubrium at a rate of 70-

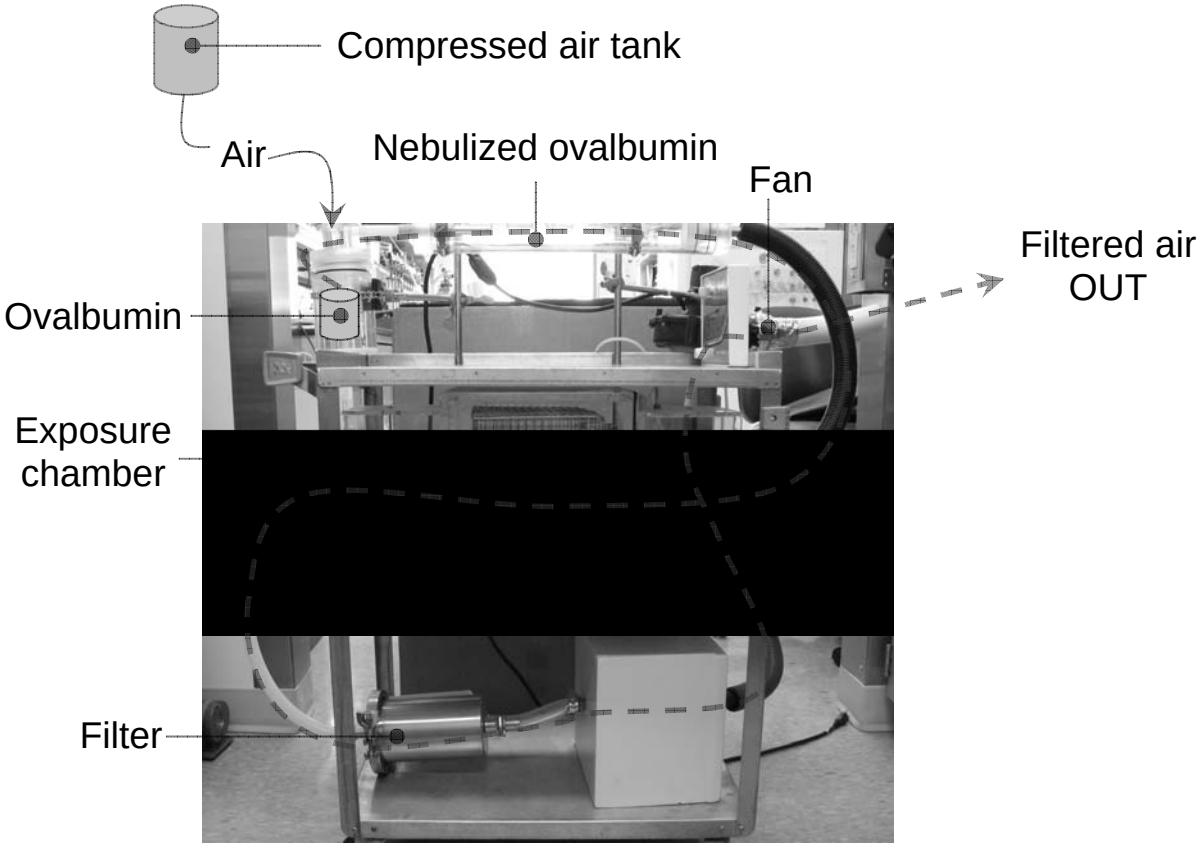
100 per minute to promote cardiac output and respiration to artificially ventilate guinea pigs (Green, 1982).

Guinea pigs are exquisitely sensitive to inhaled allergens, which makes them highly susceptible to anaphylactic shock. To partially protect against severe respiratory failure and subsequent death, guinea pigs were pretreated with the antihistamine pyrilamine (0.5 mg/kg, ip) one hour prior to antigen challenge. Pyrilamine does not prevent histamine release from mast cells, but antagonizes H_2 histamine receptors that contribute to respiratory distress and the acute anaphylactic response. Following antigen challenge, all guinea pigs were treated with the beta-2 agonist isoproternol (1 mg/kg, ip) to dilate airway smooth muscle and protect against severe respiratory failure due to anaphylaxis.

3. Treatments

Guinea pigs were pretreated with atropine (1 mg/kg, ip), a non-selective muscarinic antagonist, or saline one hour prior and again 6 hours after antigen challenge. In some animals, a rat monoclonal antibody to IL-5 (240 ug/kg, i.p.) was administered 4 days before the first injection of ovalbumin. Additionally, some animals received either a goat IgG antibody specific to recombinant human ß-nerve growth factor (10 ug/kg, ip; R & D Systems) or negative control Normal Goat IgG (10 ug/kg, ip; R & D Systems) one hour before antigen challenge. Blocking antibodies administered via intraperitoneal injection are absorbed into the bloodstream and distributed into the peripheral tissues, including the lungs and bone marrow. All specific antibodies used within this study demonstrated effects on physiology and histology, thus they were not tested in vitro.

FIGURE 2.1. Antigen exposure chamber.



Schematic representation:

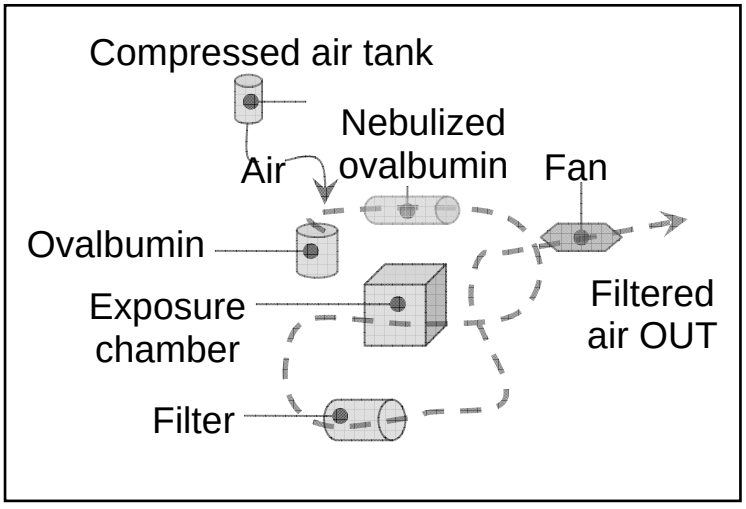


Figure 2.1. Guinea pigs are placed into an exposure chamber and exposed to 2.5% aerosolized ovalbumin for 5 minutes or until signs of respiratory distress occur. Air passes through a high efficiency particulate air filter and the exhaust fan releases clean air.

4. Anesthesia and surgical preparation

The resting physiological values for guinea pigs are provided in Table 2.1 (Green, 1982). Experiments were conducted 18-24 hours after antigen challenge or treatment. Guinea pigs were anesthetized with urethane (1.9 g/kg, i.p), a non-depolarizing drug that produces central nervous system depression and has minimal effect on respiratory and cardiovascular systems (Green, 1982). The dose used in these studies produces a deep anesthesia lasting 8-10 hours (Green, 1982), although none of these experiments lasted longer than 4 hours. Assessment of anesthetic depth was based on two well-established criteria: loss of blink reflex after touching corner of the eye and lack of kick reflex to firm hind paw pinch. One carotid artery and both jugular veins were cannulated to measure heart rate, blood pressure and administer drugs intravenously (Figure 2.2 and 2.3).

To eliminate spontaneous breathing patterns, respiratory muscles were paralyzed with continuous succinylcholine infusion (10 ug/kg/min, i.v.) (Green, 1982). During the entire procedure, body heat was maintained at 37˚C with a thermometer connected to a heating blanket. Since paralyzing agents were used, the depth and degree of anesthesia was monitored by observing heart rate and blood pressure fluctuations.

TABLE 2.1. Resting physiological parameters in guinea pigs.

Measurement	**Values**
Respiratory frequency (breaths/minute)	90 - 150
Tidal volume (ml)	1.0 – 4.0
Mean heart rate (beats/minute)	155 (130 – 190)
Arterial blood pressure (mmHg)	90/56
Blood volume; total (ml/kg bodyweight)	75
Blood volume; terminal exsanguination (ml/kg bodyweight)	35
Blood volume; safe maximum single sample (ml/kg bodyweight)	7

Table 2.1. Values obtained from (Green, 1982).

FIGURE 2.2. Anesthetized and ventilated guinea pig.

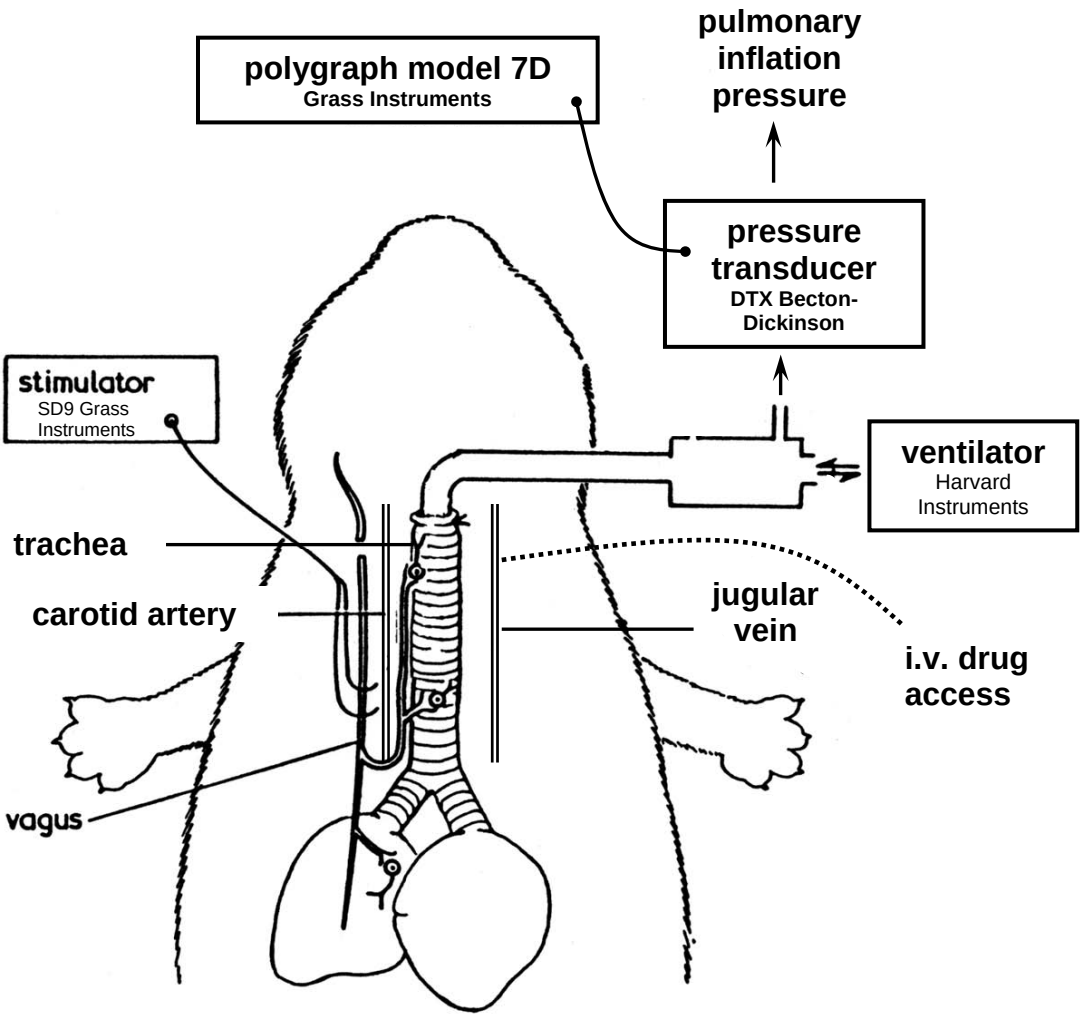
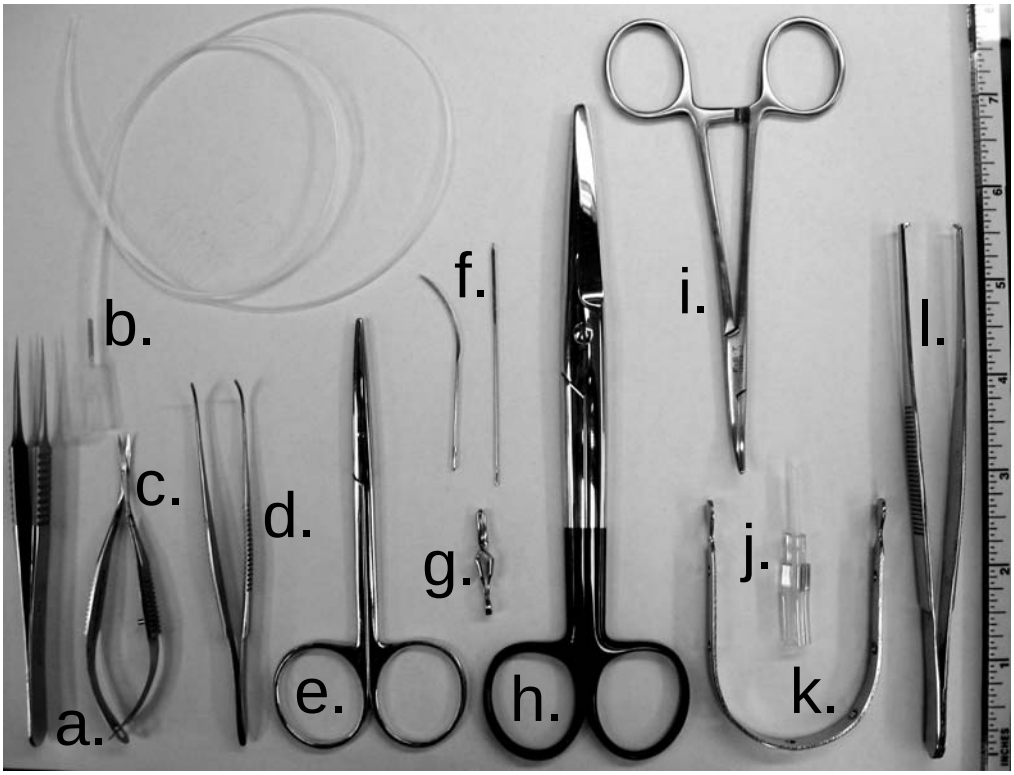


Figure 2.2. The trachea is flanked on each side by a carotid artery, jugular vein and vagus nerves. Although for the simplicity of the drawing, only one artery, jugular vein, and vagus nerve are shown. Both veins were cannulated for intravenous (i.v.) drug administration and both vagus nerves are attached to electrodes and stimulated. Figure adapted from (Fryer, 1986).

FIGURE 2.3. Surgical instruments used for in vivo experiments.



a. Dumont #5 forceps

b. vein/artery cannula

c. micro dissection scissors

d. hooked forceps

e. medium scissors

f. abdominal needles

g. bulldog clamp

h. large scissors

i. clamp scissors

j. trachea cannula (made from tubing)

k. neck collar

l. gripped claw forceps

5. Measurements of pulmonary inflation pressure

Bronchoconstriction was measured as the peak increase in pulmonary inflation pressure above the baseline inflation pressure produced by the ventilator (Dixon, 1903). Airway smooth muscle contraction results in changes in both resistance and compliance (Nadel *et al.*, 1965). However, changes in lung compliance are strongly correlated with changes in lung resistance and pulmonary inflation pressure (Ewart *et al.*, 1995; Fryer, 1986). Since changes in resistance and compliance provide the same information as changes in pulmonary inflation pressure, only pulmonary inflation pressure was measured in these experiments.

The trachea was cannulated and animals mechanically ventilated using a positive-pressure constant volume animal ventilator with tidal volume set at 1 ml per 100 g body weight and a respiratory rate of 100 breaths per minute (Green, 1982) (Figure 2.2 and 2.3). The pressure required to inflate the lungs, or pulmonary inflation pressure, was measured via a sidearm of the tracheal cannula using a pressure transducer. A positive pressure of 85 - 150 mmH_2O was needed for adequate ventilation of the animals, in accordance with average values for guinea pigs (Green, 1982).

The pulmonary inflation pressure signal from the driver was fed into the input of the preamplifier of a second channel on the polygraph, and the baseline pulmonary inflation pressure was subtracted electrically. Thus increased pulmonary inflation pressure was recorded on a separate channel at a higher sensitivity. This method allows increases in

pulmonary inflation pressure above baseline as small as 2 mm H_2O to be accurately measured. All signals were recorded on a polygraph.

6. Vagal nerve stimulation

Anesthetized, ventilated and paralyzed guinea pigs were vagotomized and the distal portions of vagi attached to platinum electrodes immersed in a pool of mineral oil (Figure 2.2). Electrical stimulation of the vagus nerves (1-25 Hz, 10 V, 0.2-ms pulse duration, for 5 seconds at 45-second intervals) produced frequency dependent bronchoconstriction and bradycardia that recovered on cessation of electrical stimulation (Figure 2.4). Atropine (1 mg/kg, i.v.) was given at the end of each experiment to confirm that vagally induced bronchoconstriction was cholinergically mediated.

All animals were chemically sympathectomized using guanethidine (2 mg/kg, i.v.) in order to deplete norepinephrine stores (Blaber *et al.*, 1985). Guanethidine releases norepinephrine resulting in temporary tachycardia and hypertension that returns to baseline within 20 minutes. Experiments testing vagally mediated bronchoconstriction and function of neuronal M_2 muscarinic receptors were conducted after heart rate and blood pressure had returned to baseline.

FIGURE 2.4. Effects of vagal nerve stimulation in pathogen free guinea pigs.

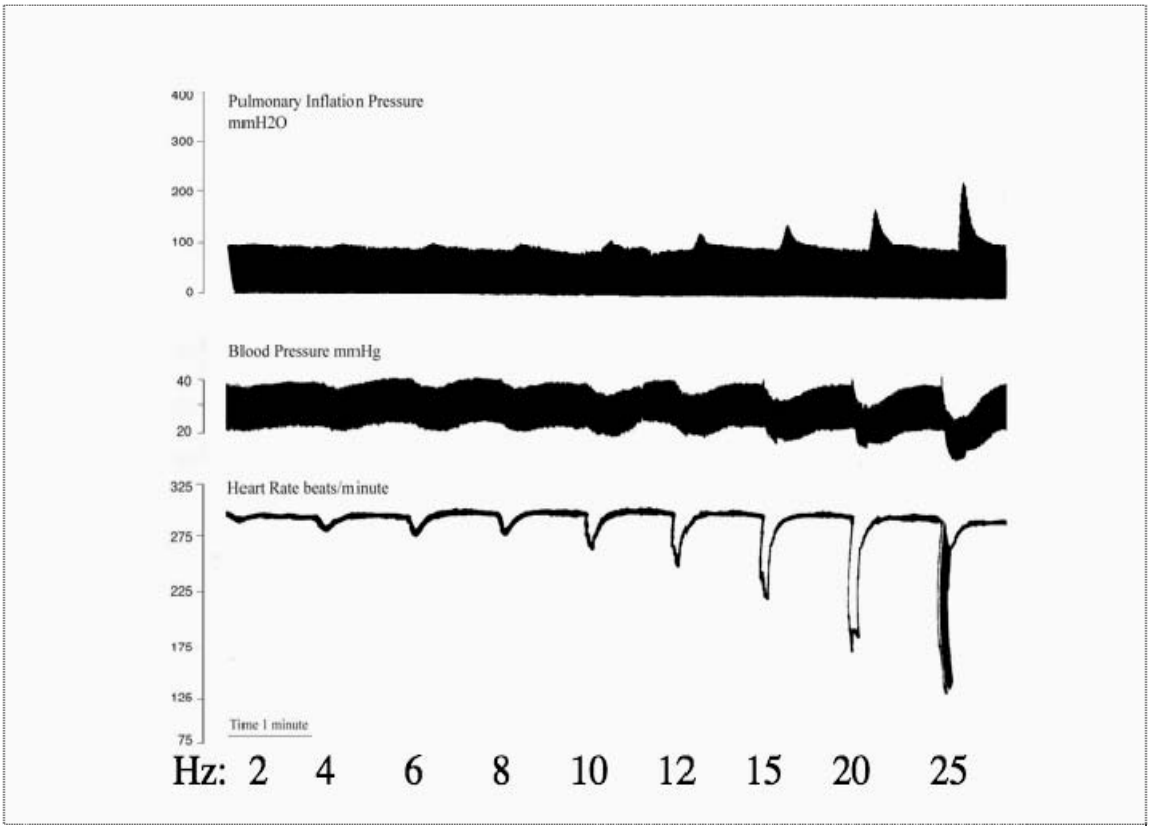


Figure 2.4. Depicted is an example of a recording made on a Grass polygraph showing pulmonary inflation pressure, blood pressure and heart rate in an anesthetized guinea pig. Vagal stimulation (0.2ms, 10 Volts, 5 seconds) caused frequency dependent bronchoconstriction (measured as an increase in pulmonary inflation pressure) and bradycardia (measured as a fall in heart rate) (2-25 Hz; bottom).

7. Muscarinic function on airway smooth muscle

Responsiveness of airway smooth muscle muscarinic receptors was measured by administering increasing doses of acetylcholine (1-10 ug/kg, i.v.) to vagotomized animals. This bypassed the vagus nerves that were cut to eliminate acetylcholine induced reflex responses (Fryer *et al.*, 1998; Wagner *et al.*, 1999). Exogenous administration of acetylcholine caused a dose dependent increase in bronchoconstriction.

8. Testing neuronal M_2 receptor function

a. Relationship between nerve stimulation intensity and neuronal receptor function

In the airways, acetylcholine release is under the local control of inhibitory M_2 muscarinic receptors present on postganglionic nerves that inhibit acetylcholine release (Fryer *et al.*, 1984). The function of neuronal muscarinic receptors is dependent on the stimulus intensity or the frequency at which the vagus nerve are stimulated (Duckles *et al.*, 1990; Fryer *et al.*, 1984). Neuronal M_2 receptors function best at higher frequencies (Blaber *et al.*, 1985), and the effects of antagonists are most apparent when the nerves are stimulated at higher frequencies (5 - 15 Hz). In contrast, it is easier to demonstrate the effect of exogenous agonists when the nerves are stimulated at lower frequencies since fewer receptors are occupied by the endogenous agonist acetylcholine (Fryer *et al.*, 1984; Fryer *et al.*, 1991; Starke, 1977). In guinea pigs, gallamine is an effective antagonist at 15 Hz and pilocarpine is an effective agonist at 2 Hz (Fryer *et al.*, 1984). Fifteen Hz was chosen as the maximum frequency because the vagus nerve normally fires at 12 - 15 Hz (Mitchell *et al.*, 1987).

b. M_2 muscarinic receptor agonist pilocarpine

M_2 receptor function was tested in anesthetized, ventilated, and paralyzed guinea pigs using the muscarinic agonist pilocarpine. Although all M_2 muscarinic receptor agonists have some affinity for other muscarinic receptor subtypes, ligand binding studies demonstrate that pilocarpine has a tenfold greater affinity for M_2 over M_3 (Caulfield, 1993). This is further supported by evidence that the physiological efficacy of pilocarpine is one hundredfold greater at M_2 than M_3 (Dixon, 1903).

Both vagus nerves were cut and the distal ends placed on platinum stimulating electrodes. Before pilocarpine administration, baseline responses to electrical stimulation (2 Hz, 0.2 ms, for 22 seconds at 40 second intervals) with voltage adjusted to elicit reproducible bronchoconstrictions between 20 - 40 mm H_2O were obtained. Once set, the voltage was not altered within each experiment.

Cumulative doses of pilocarpine (1 - 100 ug/kg, i.v.) were administered, and the effects on vagally induced bronchoconstriction were measured. Pilocarpine stimulates M_2 receptors, thus increasing doses of pilocarpine inhibit vagally induced bronchoconstriction in a dose dependent manner (Figure 2.5). In antigen challenged guinea pigs, M_2 receptors are not functional, and the ability of pilocarpine to inhibit vagally induced bronchoconstriction is lost (Fryer *et al.*, 1991).

FIGURE 2.5. Effects of pilocarpine on bronchoconstriction induced by vagal stimulation in guinea pigs.

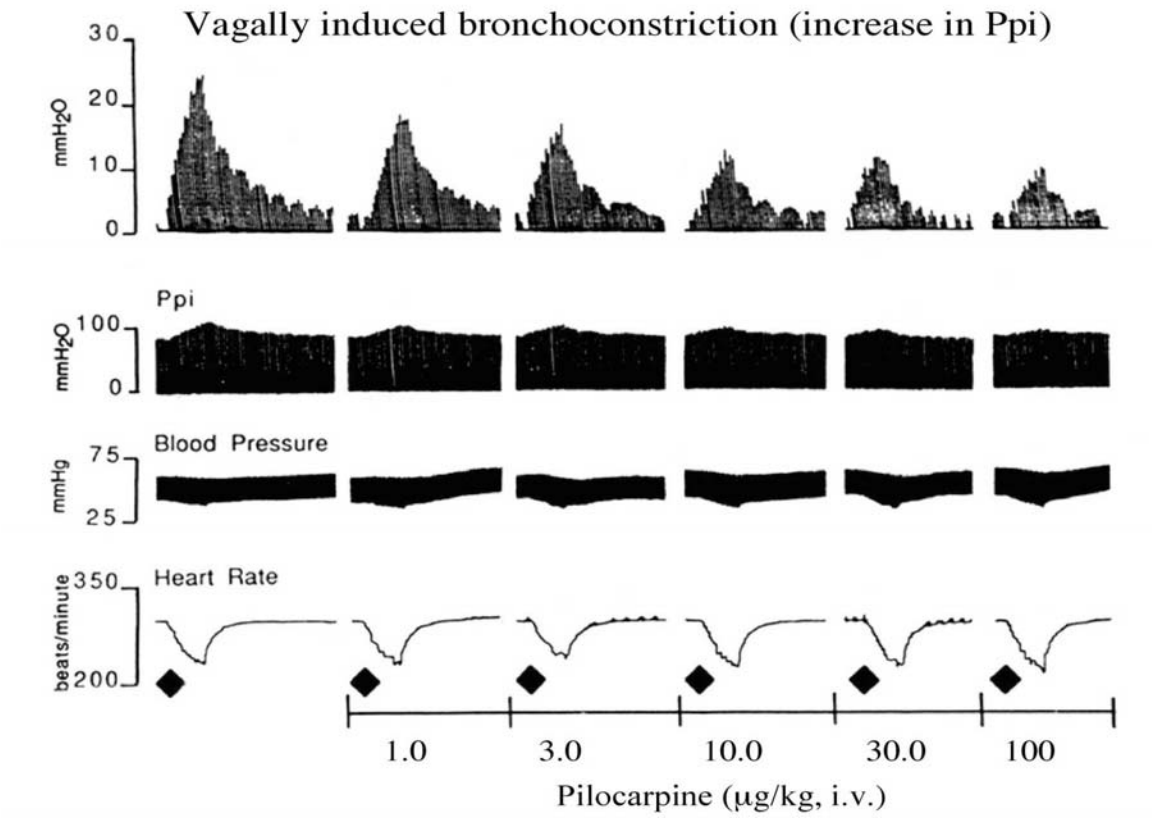


Figure 2.5. In pathogen free guinea pigs, electrical stimulation of the vagus nerves (2 Hz, 0.2 ms, 22 seconds; filled ◊) causes bronchoconstriction and bradycardia. The muscarinic agonist pilocarpine (1.0 – 100 ug/kg iv) stimulates M_2 muscarinic receptors, leading to dose dependent decreases in vagally induced bronchoconstriction, indicating that neuronal M_2 muscarinic receptors are functional. Figure adapted from (Fryer, 1986).

c. M_2 muscarinic receptor antagonist gallamine

The M_2 selective antagonist gallamine was used to test the ability of endogenous acetylcholine to stimulate muscarinic receptors. Vagi were stimulated electrically (15 Hz, 0.2-ms pulse duration, for 3 seconds at 40 second intervals) with voltage adjusted to elicit reproducible bronchoconstrictions between 8 - 18 mm H_2O prior to administration of gallamine.

In these gallamine experiments, vagi were stimulated at 15 Hz, thereby providing maximal activation of pre-junctional M_2 muscarinic receptors (Fryer *et al.*, 1987; Fryer *et al.*, 1984). The voltage of electrical stimulation in each experiment ranged from 1 - 20 V and was adjusted to produce repeated bronchoconstrictions of similar magnitude. Once set, the voltage did not vary within an experiment

Gallamine blocks M_2 receptors, thus cumulative doses of gallamine (0.1 - 10 mg/kg, i.v.) potentiate vagally induced bronchoconstriction in a dose dependent manner. When neuronal M_2 receptors are functional, gallamine blocks endogenous activation of M_2 muscarinic receptors by acetylcholine, thereby potentiating vagally induced bronchoconstriction by removing their inhibitory function. In antigen challenged guinea pigs, M_2 receptors are not functional, and the ability of gallamine to potentiate vagally induced bronchoconstriction is impaired (Fryer *et al.*, 1991).

d. Expression and interpretation of data

At least five consistent bronchoconstrictions in response to vagal stimulation were obtained during the control period. The average value of these five responses was used as a baseline measure of bronchoconstriction, termed B1. Similarly, averages of at least three consistent subsequent bronchoconstriction peaks were calculated after each dose of either agonist or antagonist, termed B2. All data were presented as the ratio of bronchoconstriction in the presence of increasing doses of pilocarpine or gallamine over bronchoconstriction in the absence of either drug respectively (B1 / B2). Since this is a ratio, a value equal to one represents no change in vagally induced bronchoconstriction. Similarly, a ratio less than one or greater than one represents inhibition or potentiation of vagally induced bronchoconstriction, respectively. Thus, the degree to which pilocarpine inhibits or gallamine potentiates vagally induced bronchoconstriction is a measure of neuronal M_2 receptor function.

9. Statistical analysis

All data are expressed as mean +/-SEM. In vivo responses to nerve stimulation, acetylcholine, pilocarpine and gallamine were analyzed using 2-way ANOVA for repeated measures. Baseline heart rates, blood pressures, pulmonary inflation pressures were analyzed using 1-way ANOVA. For ANOVA comparisons of multiple data sets (> 3 groups), a Bonferroni correction was applied. A p-value of less than 0.05 was considered significant. Statistical analyses were made with Kaleidagraph or Statview.

C. MEASUREMENT OF INFLAMMATION

1. Bronchoalveolar lavage leukocytes

After physiological measurements were made, lungs of guinea pigs were lavaged with five aliquots of 10 ml warm PBS containing 100 μM isoproternol via the tracheal cannula. Recovered lavage fluid was centrifuged, cells resuspended in PBS, and total cells counted using a hemocytometer. Aliquots of the cell suspension were spun down onto glass slides and stained for differential analysis.

2. Blood leukocytes

Blood cell differential counts were made from blood taken from the carotid artery via a heparinized syringe. Total blood leukocyte counts were made by lysing whole blood in 0.1 N hydrochloric acid and counting blood leukocytes with a hemocytometer.

3. Statistical analysis

Bronchoalveolar lavage and blood leukocytes were analyzed for statistical significance 1-way ANOVA with Bonferroni's correction using Kaleidagraph.

D. MEASUREMENT OF EOSINOPHIL PRESENCE AND ACTIVATION AT THE NERVES

1. Immunohistochemical detection of airway nerves and quantification of eosinophil accumulation

At the end of in vivo experiments, animals were given 2,500 units intravenous heparin, exsanguinated by perfusing the jugular vein with warm PBS while draining blood from the cannulated carotid artery. Once the lungs were cleared of blood, they were removed and inflated with zinc-buffered formalin and fixed overnight at 4°C. Transverse sections from two lobes of the lungs were embedded in paraffin for histology.

Nerves in the lungs were detected immunohistochemically in adjacent tissue sections using a mouse monoclonal antibody against PGP 9.5, adapted from Costello et al. (Costello *et al.*, 1997). Tissue sections were dewaxed, treated with antigen unmasking solution and blocked in 10% normal goat serum for one hour at 37°C. Slides were incubated at 4°C for twenty-four hours with primary antibody against PGP 9.5, diluted 1:1000 in 10% normal goat serum in PBS. Tissue sections were quenched with 3% H_2O_2 in methanol, blocked with CAS-Block and incubated for 90 minutes at 37°C with a secondary antibody, biotinylated goat anti-mouse IgG at 1:400 diluted in 5% normal goat serum. Slides were washed with PBS and incubated with an avidin-linked horseradish peroxidase (HRP) substrate. PGP 9.5 staining was visualized by incubating tissues with the chromagen diaminobenzidine and nickel, which stained airway nerves black. Eosinophils were visualized by counterstaining with a 1% solution of Chromotrope 2R for 45 minutes, which stained eosinophils red. Slides were washed in tap water,

dehydrated in graded ethanol solutions, cleared in xylene, and permanently mounted with Cytoseal-60.

The number of eosinophils within the walls of five different cartilaginous airways per animal was counted, using 3 - 5 animals per treatment group. Airways were photographed with a digital camera attached to an upright Nikon microscope, and airway area measured using Metamorph imaging software. Total area of smooth muscle within airway walls and below the lamina propria was measured and the total number of eosinophils within that area was counted in consecutive high-power fields. In addition, eosinophils within 8 um of an airway nerve (approximately the diameter of one eosinophil) were also counted. Thus, the number of eosinophils per millimeter^2 could be calculated for each treatment group, and the proportion of eosinophils associated or not associated with airway nerves could be determined.

2. Immunohistochemical detection of major basic protein in airways

a. Total eosinophil major basic protein deposition in the lung

Eosinophil major basic protein (MBP) in lung sections was detected using a rabbit monoclonal antibody against guinea pig MBP (Lewis *et al.*, 1976). Lungs were fixed, embedded, sectioned, dewaxed, treated with antigen unmasking solution and blocked as above. Slides were incubated at 4°C for twenty-four hours with Ab MBP, diluted 1:1000 in goat serum and then incubated for 90 minutes at 37°C with a fluorophore-conjugated goat anti-rabbit IgG. Slides were washed, mounted under aqueous media with nuclei

visualized by the fluorescent stain 4', 6-diamidino-2-phenylindole (DAPI) and stored at 4°C in the dark. Negative control slides were treated as above without primary antibody.

Slides were coded and all remaining steps were carried out under blinded conditions. Airways were photographed under identical conditions and images analyzed for MBP deposition using Metamorph imaging software. The airway lamina propria, smooth muscle, and any outer connective tissue attached or continuous with the smooth muscle were included in the analysis. Airway epithelium, cartilage, blood vessels, and alveoli were excluded.

Eosinophil MBP deposition was quantified using a technique adapted from Tuder *et.al.* (Tuder *et al.*, 2003). Fluorescence intensity in a 10-micron^2, non-cellular region of the lumen was measured in every airway. These values were averaged to obtain the mean background fluorescence, which was then subtracted from each image. MBP signal intensity was thresholded by measuring non-specific staining in a negative control (absence of primary antibody) using the tissue with the strongest MBP signal. A serial section from this MBP containing airway was stained without the primary antibody, and the average fluorescence measured. This value was used to set the lower limit of the calibration scale for all subsequent measurements. Intensity values above this negative control were included in the analysis; values below this threshold were excluded as non-specific. Thus, only fluorescence above background and separate from non-specific MBP staining was measured.

The total area of each airway was measured in microns^2. The area of positive signal above threshold within this area was measured, as was mean fluorescence intensity. Total intensity was calculated by multiplying the mean fluorescence intensity by the area of positive signal. Average fluorescence per micron^2 of each airway was determined by dividing total intensity by total area of the airway. Data are means of 4 - 5 animals per group, with replicates of five airways per animal.

b. Distribution of major basic protein in airways

Airways were stained for MBP and photographed in their entirety as described above. Mean fluorescence intensity across the epithelium, lamina propria, smooth muscle and outer connective/fibrous tissue of each airway was measured using the linescan tool in Metamorph. Each airway was sampled with four linescans, each consisting of three parallel lines within a 10-micron wide region (Figure 2.6). The four linescans were separated by 90 degrees (relative to the center of the airway lumen). Each linescan was drawn from the lumen to the fibrous tissues, and the intensity of each pixel along that line recorded. Data were sorted by anatomic region: epithelium, lamina propria, smooth muscle and fibrous tissue, and the average fluorescence intensity per pixel within each anatomic region calculated. Empty spaces were excluded. Background fluorescence and thresholding were calculated and subtracted as described above. Fluorescence intensity per pixel was converted to fluorescence intensity per micron. Data are mean of four animals per group, with replicates of four airways per animal, and 12 linescan measurements per airway.

3. Statistical analysis

Histological examination for tissue eosinophils and major basic protein content in the airways were analyzed by 1-way ANOVA with Fisher's LSD post-hoc correction using Kaleidagraph.

FIGURE 2.6. Method for measuring distribution of eosinophil major basic protein in the airways.

Figure 2.6. Airways were stained for MBP and mean fluorescence intensity across the epithelium, lamina propria, smooth muscle and outer connective/fibrous tissue of each airway was measured using the linescan tool in Metamorph. Each airway was sampled with four linescans, each consisting of three parallel lines within a 10-micron wide region. The four linescans were separated by 90 degrees (relative to the center of the airway lumen). Each linescan was drawn from the lumen to the fibrous tissues, and the intensity of each pixel along that line recorded. Data were sorted by anatomic region: epithelium, lamina propria, smooth muscle and fibrous tissue, and the average fluorescence intensity per pixel within each anatomic region calculated.

E. MUSCARINIC RECEPTOR EXPRESSION AND FUNCTION IN EOSINOPHILS

1. Eosinophil sources

Eosinophils from both guinea pigs and human subjects were used to investigate muscarinic receptor subtype expression and function. Guinea pig eosinophils are considered to be a good model for studies of eosinophil function since they respond to the same pharmacological stimuli as human eosinophils (Giembycz *et al.*, 1999) and they are readily obtained from multiple tissue locations (peritoneum, blood, lung).

a. Guinea pig eosinophils

i. Peritoneal eosinophil isolation

Female guinea pigs were anesthetized with ketamine (30 mg/kg, i.m.) combined with an analgesic xylazine (5 mg/kg, i.m.), which induces light surgical anesthesia that lasts for half an hour (Brown JN, 1989). The peritoneum was lavaged weekly with 50 ml warm sterile endotoxin-free PBS, administered via an 18 gauge catheter (Lindor *et al.*, 1981). Stable production of eosinophils occurred after 3 weeks. Peritoneal lavage fluid was collected, centrifuged and resuspended in 20 ml cold PBS. Resuspended cells were carefully layered over Percoll (density measured and adjusted to 1.090 g/ml with PIPES buffer) in a 50 ml conical vial and centrifuged (1400 rpm, 20 minutes, no acceleration or brake). Following centrifugation, lymphocytes and macrophages (density < 1.090) were localized at the interface between the PBS and Percoll, while eosinophils and red blood cells (density > 1.090) were localized in the cell pellet beneath the Percoll (Figure 2.7). Lymphocytes and macrophages were subsequently removed by vacuum aspiration.

Eosinophils and red blood cells in the cell pellet at the bottom of the conical tube were resuspended in 1 ml cold PBS. Red blood cells were removed by hypotonic lysis as follows: cells were treated for 30 seconds with 9 ml cold distilled water and then 1 ml 10X PBS was added to restore isotonicity. Cells were washed with cold PBS, centrifuged and resuspended in cold PBS. Cell viability (>95%) was verified by trypan blue exclusion and purity assessed by differential staining analysis.

ii. Blood eosinophil isolation

Blood was collected in a heparinized syringe by cardiac puncture of anesthetized pathogen-free female guinea pigs. Red blood cells were lysed with cold sterile ammonium chloride erythrocyte lysis solution (0.8% NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA). Approximately 0.5 ml of blood was diluted in 3 ml cold lysis buffer and placed on ice for 10 minutes. The cell suspension was centrifuged (300 x g, 10 minutes), resuspended in PBS and remaining leukocytes were spun down onto glass slides at 10,000 - 25,000 cells in 50 – 80 ul volume.

iii. Bronchoalveolar eosinophil isolation

The lungs of anesthetized pathogen-free guinea pigs were lavaged with 5 aliquots of 10 ml warm PBS containing 100 μM isoproternol via the tracheal cannula. Recovered lavage fluid was centrifuged (300 x g, 10 minutes) and resuspended in cold PBS. The cell suspension was spun down onto glass slides at 10,000 - 25,000 cells in 50 – 80 ul volume.

FIGURE 2.7. Guinea pig eosinophil isolation by density centrifugation.

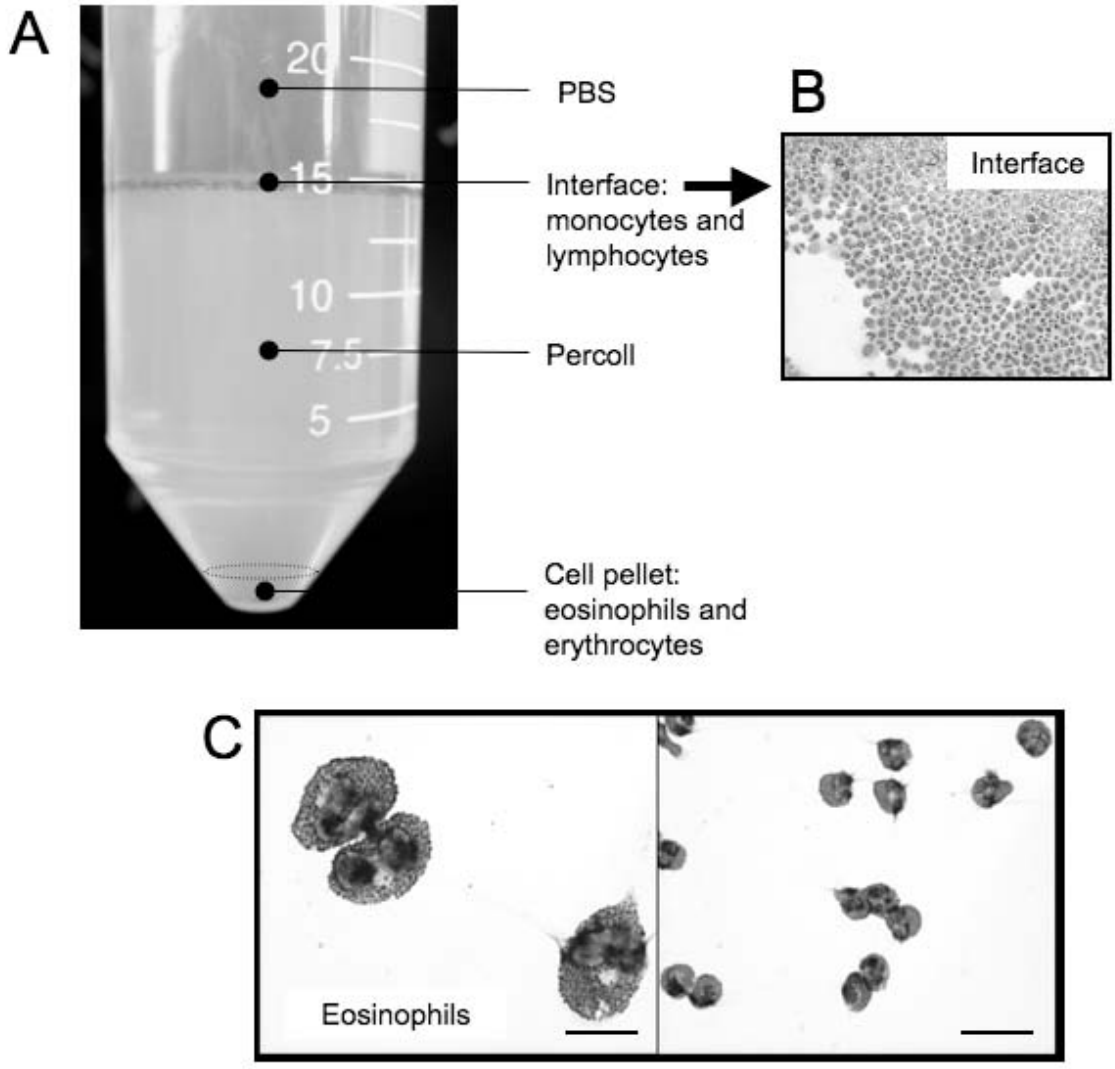


Figure 2.7. Eosinophils were separated from other peritoneal inflammatory cells based on density. Lavage fluid was layered over Percoll with density adjusted to 1.090 and centrifuged (A). Inflammatory cells with densities < 1.090 (monocytes, lymphocytes) at the PBS:Percoll interface were removed (B). Eosinophils and erythrocytes with densities > 1.090 were in the cell pellet. Erythrocytes were then removed by hypotonic lysis, yielding pure guinea pig peritoneal eosinophils (C). Calibration bars are 15 um (left) and 30 um (right).

b. Human eosinophils

Human eosinophils were isolated from blood taken from healthy adult volunteers. Donors were initially screened for percent circulating blood eosinophils from a blood smear sample (Table 2.1). Percent blood eosinophils was determined by counting the number of eosinophils relative to other leukocytes on a differentially stained glass slide. To obtain an accurate count, at least 250 total leukocytes were counted for each sample. All donors had eosinophil values within the normal range for humans (Giembycz *et al.*, 1999).

Human eosinophils were isolated using a protocol adapted from a commercially available eosinophil isolation kit. Approximately 120-180 ml blood was drawn from an arm vein of each donor. Blood was collected in sterile 60 ml syringes containing 1 ml of 0.5 M EDTA and then stored on ice for no longer than 10 minutes. All remaining steps were conducted in a sterilized laminar flow hood with all prepared solutions sterile-filtered. Blood was diluted 1:1 with cold buffer (PBS pH 7.2, 2.5% BSA and 2 mM EDTA). Approximately 20 ml diluted blood was carefully layered over 20 ml cold sterile Ficoll (density = 1.077) in a 50 ml conical vial. Depending on the volume of blood obtained from the donor, 12-18 conical vials were used.

TABLE 2.2. Percent blood eosinophils in human donors.

Donor #	% Eosinophils in blood smear
1	6.5
2	4.3
3	6.3
4	1.6
5	9.6
6	4.8
7	2.0
8	2.6
9	4.4
10	3.0
11	4.3

Table 2.1. Human eosinophils were isolated from blood taken from healthy adult volunteers. Donors were initially screened for percent circulating blood eosinophils from a blood smear sample. Percent blood eosinophils was determined by counting the number of eosinophils relative to other leukocytes on a differentially stained glass slide. To obtain an accurate count, at least 250 total leukocytes were counted for each sample. All donors had eosinophil values within the normal range for humans.

Granulocytes and red blood cells were separated from mononuclear cells by density centrifugation (600 x g, 30 minutes) in a tabletop centrifuge at room temperature. The plasma layer, the mononuclear cells that form a hazy layer at the plasma:Ficoll interface, and the Ficoll were carefully removed by aspiration, leaving the red cell pellet undisturbed (Figure 2.8). The cell pellet was resuspended with cold ammonium chloride erythrocyte lysis solution (0.8% NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA) and the 50 ml conical vial completely filled with lysis solution and placed on ice for 10 minutes. The centrifuge was held at 4˚C for all remaining steps. Following this initial lysis step, cells were centrifuged (300 x g, 10 minutes) and the supernatant was removed by aspiration.

Since not all red blood cells were lysed at this time point, I developed an additional lysis step to remove remaining red blood cells. Cell pellets were resuspended in 1 ml cold lysis solution on ice and all cells were combined into one conical vial and centrifuged again. The cell pellet was resuspended in cold buffer and cell number determined with a hemocytometer.

FIGURE 2.8. Human granulocyte isolation by density centrifugation.

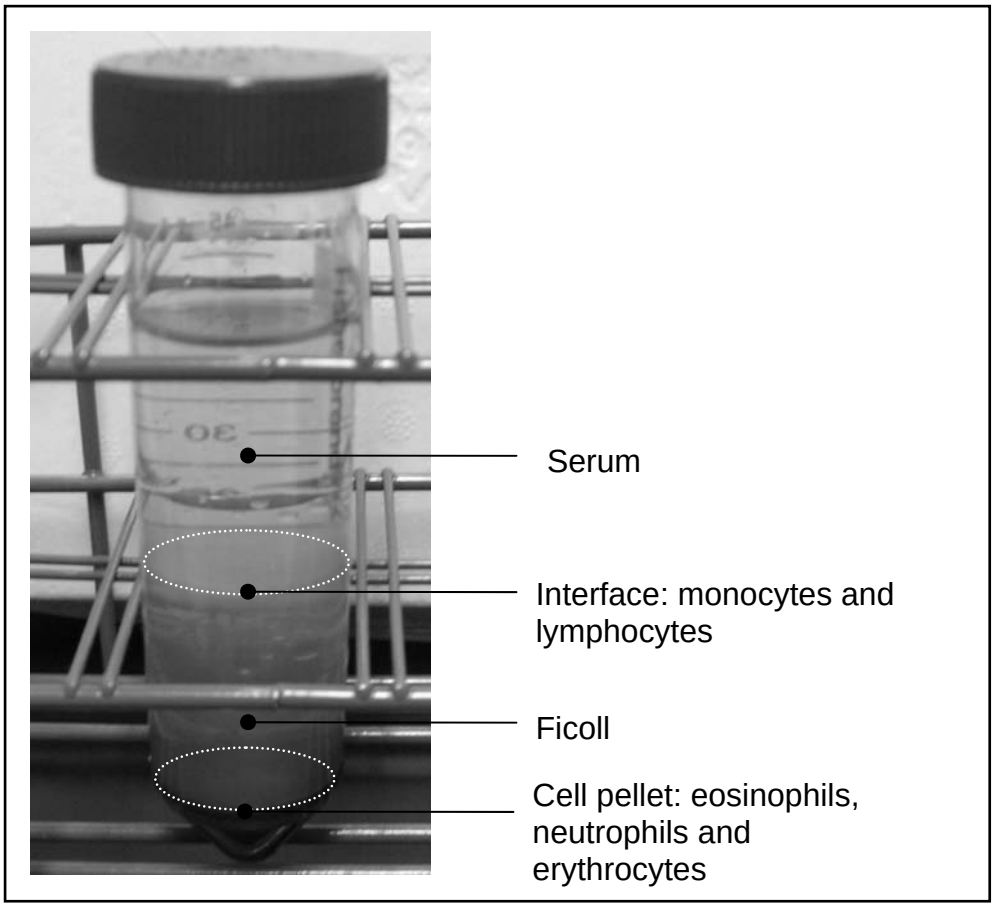


Figure 2.8. Granulocytes are separated from other blood leukocytes on the basis of their density. Diluted blood is layered over Ficoll with density adjusted to 1.077 and centrifuged. Inflammatory cells with densities less than 1.077 (monocytes, lymphocytes) localize at the PBS:Ficoll interface and are removed by vacuum aspiration. Eosinophils, neutrophils and erythrocytes with densities greater than 1.077 are localized in the cell pellet. Erythrocytes are removed by hypotonic lysis and eosinophils are separated from neutrophils by negative selection.

Cells were resuspended in dilution buffer at 40 ul per 10^7 total cells and incubated on ice for 10 minutes with an antibody cocktail at 10 ul per 10^7 total cells. This antibody cocktail contained biotin-conjugated antibodies to cell markers CD2, CD14, CD16, CD19, CD56, CD123 and CD235a and labels non-eosinophil cells including: T cells, B cells, NK cells, neutrophils, dendritic cells, monocytes, and erythroid cells. Following antibody labeling, the cell suspension was incubated on ice with 30 ul buffer and 20 ul Anti-Biotin Microbeads per 10^7 total cells for 15 minutes. The cell suspension was washed with 10 Volumes of dilution buffer, centrifuged (300 x g, 10 minutes) and resuspended in buffer at 500 ul per 10^8 cells.

The cell suspension was applied to a plastic column filled with magnetic beads secured within a magnetic block. The total number of cells applied to one column ranged between 15 - 25 x 10^8 total cells; multiple columns were used depending on the total number of cells. Columns were rinsed three times with 3 ml dilution buffer while in the presence of the magnetic field. The unlabeled eosinophil enriched effluent was collected on ice. Purity (>99%) and eosinophil viability (>99%) were assessed by differential staining and trypan blue exclusion, respectively. Efficiency of eosinophil separation from other granulocytes was determined by collecting the labeled cell fraction by removing the column from the magnetic block. These labeled cells were subsequently spun down on glass slides and stained to verify selectivity of the antibodies (Figure 2.9).

FIGURE 2.9. Separation of human eosinophils by negative selection.

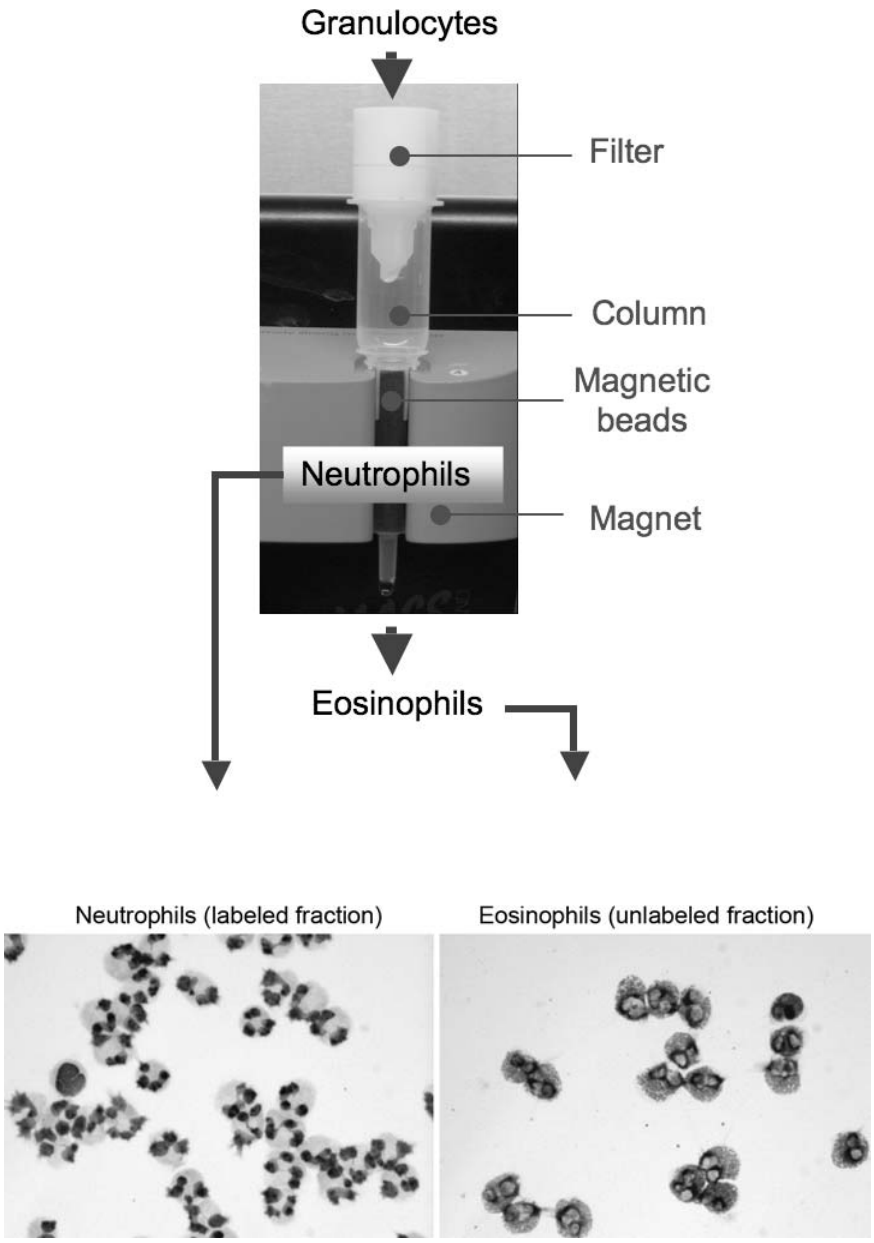


Figure 2.9. Granulocytes are incubated with antibodies conjugated to a magnetic bead and applied to a plastic column in the presence of a magnetic field. Labeled cells (mostly neutrophils) are retained on the column and unlabeled cells (eosinophils) are collected in the effluent.

2. Detection of muscarinic receptor mRNA in eosinophils

a. RNA isolation and cDNA synthesis

RNA isolation from guinea pig and human eosinophils was conducted using two different commercially available kits, RNeasy and RNAqueous -4PCR, respectively. Although the chemical reagents and RNA isolation methods provided by these kits are similar, they were not equally efficient in recovering eosinophil RNA. Despite using similar numbers of cells, the RNeasy kit yielded less RNA when used with human eosinophils compared to guinea pig eosinophils. The reason for this difference may be due to the tissue source of eosinophils used (peritoneal versus blood) or possibly species-specific differences in RNA content and endogenous RNases. Since RNA isolation from human eosinophils was more difficult compared to guinea pig eosinophils, I used the best performing kit, RNAqueous -4PCR.

i. Guinea pig peritoneal eosinophils

Pure samples (>99% purity by differential analysis) of 10^7 guinea pig eosinophils were flash frozen in liquid nitrogen and homogenized on ice in a rotor-stator. RNA was extracted from cell lysates using the RNeasy kit. During purification, RNA bound to the filter was treated with DNase I to enzymatically remove genomic DNA from the sample. Since no RNA isolation method can completely remove trace amounts of DNA below the limit of detection by RT-PCR, DNase I treatment is necessary. It is especially important that DNA is not present in RT-PCRs using primers that do not flank introns, because the RT-PCR products from RNA cannot be distinguished from contaminating DNA in this

case. Since muscarinic receptor genes do not have introns, a DNAse step was absolutely crucial.

One microgram of RNA was reverse transcribed into cDNA using Superscript III reverse transcriptase and oligonucleotide dT primers with a Px2 Thermal Cycler (Table 2.2). Negative control samples were generated by omission of reverse transcriptase.

ii. Human blood eosinophils

RNA was isolated from pure eosinophils using a commercially available kit, RNAqueous -4PCR. Preliminary experiments demonstrated that the quantity and quality of human eosinophil RNA was superior when RNA was harvested on the same day as eosinophils isolation. This may be due endogenous RNAses present in eosinophils (Hamalainen *et al.*, 1999; Rosenberg *et al.*, 1989). Thus, immediately following isolation from blood, 10^7 eosinophils were lysed with 500 ul lysis buffer and vortexed for 2 minutes. The lysate was transferred to a 2 ml microcentrifuge tube and thoroughly mixed with an equal volume of 64% ethanol. This lysate/ethanol mix was applied to a filter cartridge assembled in a collection tube, and centrifuged (13,000 x g; 30 seconds). The flow-through was discarded and the filter was washed two times with 700 ul Wash Solution#2/3. RNA was eluted from the filter in two sequential applications of preheated (70 – 80˚C) elution solution as follows: the filter was placed into a new 1.5 ml collection tube and 40 ul elution solution was pipetted directly onto the center of the filter, centrifuged (13,000 x g; 30 seconds) and RNA eluted one more time with 10 ul elution solution and centrifuged as before.

TABLE 2.3. Real time-polymerase chain reaction conditions.

Reverse transcription			
Stage	Temp (˚C)	Duration	Cycles
Denature	65	5:00	1
	4	1:00	1
Anneal	25	10:00	1
cDNA synthesis	50	50:00	1
Terminate	70	15:00	1
Stabilize	4	hold	1

Real time RT-PCR			
Stage	Temp (˚C)	Duration	Cycles
Hot Start Taq activation	95	15:00	1
Denature	94	:30	45
Anneal	58	1:00	
Extend	72	:30	
Dissociation	95	:15	1
Denaturation curve	55	1:00	1
	95	:15	1
	60	:15	1

Genomic DNA was removed using DNAse I treatment. Eosinophil RNA in elution buffer was mixed with 0.1 Volume of 10X DNase I Buffer and 1 ul DNase I and incubated for 30 minutes at 37˚C. DNase I must be removed from RNA used for RT-PCR because it has the potential to degrade DNA made in the process of PCR. To remove DNase I, 0.1 Volume DNase Inactivation Reagent was added and mixed by gently flicking the tube and incubated 2 minutes at room temperature. Following DNase I inactivation, the tube was centrifuged 10,000 x g for 1 minute and the RNA transferred to a new tube and kept on ice. On the same day, RNA yield was determined and 500 ng - 1 ug RNA was reverse transcribed into cDNA using Superscript III and oligonucleotide dT primers using a Px2 Thermal Cycler (Table 2.2). Negative control samples were generated by omission of reverse transcriptase.

b. Real-time PCR

PCR was carried out in triplicate samples using Quantitect SYBR green PCR. For guinea pig eosinophil RNA, the resulting PCR products were visualized using the Mx3000P real-time PCR system. For human eosinophil RNA, PCR was carried out on a 7500 Fast Real-Time PCR System. Threshold cycle number was measured in samples reverse transcribed in the presence and absence of reverse transcriptase to identify positive reactions. Primers specific to 18S rRNA and to C-C chemokine receptor 3 (CCR3; a chemokine receptor highly expressed by eosinophils) were used to validate the integrity and eosinophil origin of the mRNA, respectively. Although CCR3 is abundantly expressed by eosinophils, other leukocytes including lymphocytes and mast cells are reported to express this receptor (Pease *et al.*, 2006). Since eosinophil purity was always

99% or greater in RNA-based experiments, the relative contribution of RNA from these other cell types is negligible and it is justifiable to conclude that mRNA was eosinophil derived based upon CCR3 expression. In each experiment, standard curves were generated using CCR3 primers to verify the quality of the eosinophil RNA.

Primer pairs that yielded PCR products as indicated by real-time PCR were run on a 1.5% agarose gel to verify predicted product size. Resulting bands were excised, gel purified and identities confirmed by DNA sequencing and comparing to the published sequences.

mRNA isolated from sagittal sections of guinea pig brain was used as a positive control for guinea pig muscarinic receptors. Animals were sacrificed by a lethal overdose of pentobarbital (150 mg/kg i.p.) and brain tissue isolated and RNA stabilized in RNAlater solution. RNA was isolated from 450 mg brain tissue and purified using RNeasy Maxi Kit. Reverse transcription and PCR were conducted as described for guinea pig eosinophil RNA. Commercially available human brain cDNA was used to validate the human muscarinic receptor oligonucleotides. cDNA from human brain at 0.5 ng/ul was further diluted at 1:100 in 10 mM Tris pH 8 and run in tandem with reactions containing human eosinophil cDNA.

PCR primer pairs were designed using Primer3 and were based upon published (GenBank) guinea pig and human gene sequences. Primer pairs were designed to have similar melting temperatures and PCR products ranging from 150 - 250 base pairs (Table 2.3).

TABLE 2.4. Oligonucleotides for real-time polymerase chain reaction.

Species	Gene	5' sequence	3' sequence	Product size
Guinea pig	M1	TCATGAACCTGCTGCTCATC	GGTGATGATAGGCTGGGAGA	230
	M2	TTTTCCAATGCTGCTGTCAC	GGCATGTTGTTGTTGTTTGG	205
	M3	AATCTGGGGTACTGGCTGTG	GTCTGTGGGTTGATGTGTGC	246
	M4	GAGACCGTGGAGATGGTGTT	GAGAAGGCGCCTATGATGAG	173
	M5	TGTCATGAACCTCCTGGTGA	GTGGGCTCAGAGAGGAACTG	225
	CCR3	GCTCAACTTGGCCATTTCT	GAACACGGCATGAACAATG	202
Human	M1	GCTCTACTGGCGCATCTACC	GCCTTCGTCCTCTTCCTCTT	232
	M2	TTAAAGTCAACCGCCACCTC	CAAAGGTCACACACCACAGG	154
	M3	TCAACAAGCAGCTGAAGACG	ACAGAGGCATTGCTGGCTAC	184
	M4	GCCCACTAATGAAGCAGAGC	ACTGCCTGAGCTGGACTCAT	158
	M5	ACCAACAATGGCTGTCACAA	ACTCAGTGTCTGGGCTGCTT	159
	CCR3	TCTGCTGTGGATGGAGAGA	AGCATCTGGACCTGGTCAT	214
	18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	151

3. Detection of muscarinic receptor protein on eosinophils via immunocytochemistry

Eosinophils were spun down onto glass slides and fixed with fresh 3.7% paraformaldehyde diluted in PBS for 15 minutes. Slides were washed in 0.5% Triton-X in PBS followed by a PBS wash. Antigen retrieval was conducted by treating cells in 0.1% porcine trypsin diluted in 0.1% $CaCl_2$ at pH 7.8 for 7.5 minutes at 37˚C and rinsed with tap water for 30 seconds to remove trypsin. Non-specific binding was blocked by 10% normal goat serum for 45 minutes at 37°C, and cells were labeled for 24 hours at 4°C with primary antibodies, (polyclonal rabbit anti-M_1 receptor, anti-M_2 receptor, anti-M_3 receptor, anti-M_4 receptor or anti-M_5 muscarinic receptor) diluted in 10% normal goat serum. Primary antibody dilutions were based upon the manufacturer's recommendation and are listed in Table 2.5. The secondary antibody, Alexa Fluor 555 goat anti-rabbit IgG was incubated with the cells for 1 hour at 37°C diluted 1:1000 in 5% normal goat serum. Slides were rinsed in PBS and mounted under aqueous medium with DAPI to stain nuclei. Negative control slides consisted of 10% normal goat serum in the absence of primary antibody.

Cells were examined by fluorescence microscopy and photographed using identical microscope and camera settings. Several digital images per slide were taken to accurately reflect the overall staining. Eosinophils in heterogenous cell populations (guinea pig blood and bronchoalveolar lavage) were identified on the basis of their characteristic bilobed nucleus and autofluorescence (compared to other leukocytes) when visualized under 488nm excitation.

4. Eosinophil activation assay

a. Calcium indicator dye properties

Intracellular calcium transients in eosinophils were measured with Fluo-4, a synthetic non-charged lipophilic molecule that readily partitions across cell membranes. Nonspecific esterases within the cytosol cleave Fluo-4, exposing the calcium-binding site and rendering the molecule in a charged state that does not readily pass out of the cell membrane and remains within the cytosol. Fluo-4 responds to calcium binding by increasing fluorescence intensity without any spectral shift (Gee *et al.*, 2000). Thus, calcium released by intracellular stores or entering the cell from the extracellular medium via calcium channels, binds Fluo-4, increasing fluorescence within the cytosolic compartment. Fluorescence intensity is therefore proportional to the amount of calcium within the cytosol and bound to Fluo-4 (Gee *et al.*, 2000).

b. Loading eosinophils with calcium indicator dye, Fluo-4

Pure eosinophils from either human blood or guinea pig peritoneal lavage were resuspended at $1x10^6$/ml sterile Tyrode's solution (150 mM NaCl, 10 mM HEPES, 10 mM glucose, 4 mM KCl, 1.1 mM $CaCl_2$ and 1.1 mM $MgCl_2$) in the presence of the nicotinic receptor antagonist hexamethonium (0.1 μM). Eosinophils were incubated at 37˚C in 5 μM Fluo-4 for 30 minutes. Due to the photosensitivity of Fluo-4, tubes were covered in foil and experiments were carried out in a dark room. Stock concentrations of Fluo-4 (1 mM in DMSO) were stored at -20˚C and used within two weeks, according to manufacturer's recommendation.

Following incubation, eosinophils were pipetted onto 25 mm glass coverslips in 50 ul volume and incubated at room temperature for 30 minutes (Figure 2.10). Coverslips containing eosinophils were placed into a round imaging chamber, secured onto the stage of an inverted microscope and cells visualized under oil immersion with a 60X apoplanar objective lens. Serial photographs of eosinophils were taken with a digital camera mounted on the microscope. Cells were visualized by a mercury light source and fluorescence at 488 nm was recorded by the camera. An automated shutter connected to the microscope controlled exposure time. Imaging software was used to control the duration and frequency of the light exposure. Preliminary experiments were conducted to determine the optimal exposure time and frequency, and for all subsequent experiments exposure time was set to 500 ms every two seconds, with photographs acquired every two seconds.

FIGURE 2.10. Measurement of intracellular calcium in eosinophils.

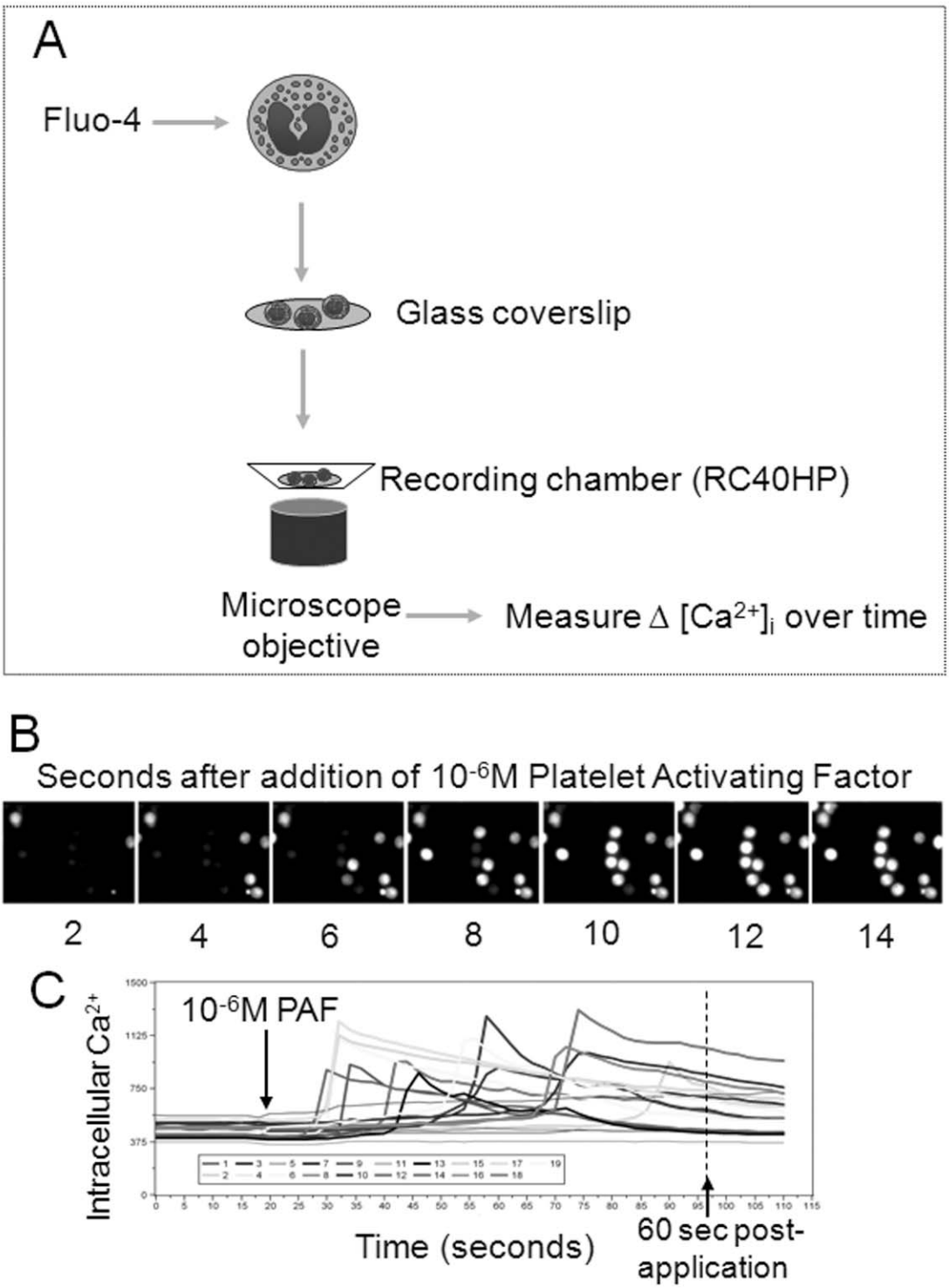


Figure 2.10. Eosinophils are incubated with a calcium indicator dye, Fluo-4, applied to glass coverslips mounted in a recording chamber and visualized by fluorescence microscopy (A). Shown are photographs of individual eosinophils (white circles) responding to platelet activating factor as a function of time (B). This is shown in the bottom trace, with each line representing individual cells (C) and amount of fluorescence intensity on the vertical axis and time on the horizontal axis. Data are expressed as the number of cells responding with increased fluorescence intensity within 60 seconds (dashed line). Prior to all experiments, eosinophils on glass coverslips were briefly illuminated to focus the objective lens and to identify a suitable cell density from which to record data. None of the cells within these experiments were adherent to each other. In each experiment, the average fluorescence intensity within entire individual eosinophils was measured over time. Baseline average fluorescence intensity of individual cells was recorded for twenty seconds and then compounds at 2X concentration in 50 ul volume were directly pipetted onto eosinophils.

c. Development of method to measure eosinophil activation

I used platelet-activating factor (PAF), a well-characterized mediator of intracellular calcium mobilization in human and guinea pig eosinophils (Giembycz *et al.*, 1999) to develop a method for quantifying eosinophil activation. PAF increases intracellular calcium through phosphatidyl inositol signaling initiated via activation of G-protein coupled receptors present on the cell membrane. The magnitude of the PAF-mediated intracellular calcium response is concentration dependent. Therefore, eosinophil activation by PAF can be quantified in multiple ways, including latency to response, absolute magnitude of response and number of responsive cells (Figure 2.10). There are artifactual variations in baseline intensity among cells, which have a number of possible origins, including: dye leakage, photo bleaching, non-uniform dye loading and cell-size differences. Based upon these limitations, I measured calcium transients in whole cells as a function of time and did not compare the absolute magnitude of the responses or quantify intracellular calcium content.

Preliminary experiments demonstrated that eosinophils respond to PAF by increasing intracellular calcium. These intracellular calcium responses were measured in individual cells by recording the average fluorescence intensity as a function of time (Figure 2.10). I validated this method for measuring eosinophil activation by demonstrating that the proportion of eosinophils activated by PAF within one minute of application is dose dependent.

d. Expression and interpretation of data

Eosinophil activation was calculated as the proportion of cells increasing fluorescence within one minute after compound application. All cells within the field of view were included in the analysis and data were expressed as a percent of total. For each compound tested, the total number of eosinophils analyzed ranged from 6 - 40. In experiments in which compounds were given in combination with PAF, data were normalized to treatment with PAF alone. These data were subsequently expressed as a ratio (percent activation with PAF alone) / (percent activation with PAF and experimental compound). Data collected from one experiment were averaged with replicates of n=3-7 for each experimental variable. Data acquired on separate days were averaged and are represented as the means +/- SEM.

e. Statistical analysis

Eosinophil activation data were analyzed for statistical significance by 1-way ANOVA with Dunnett's multiple comparison post-hoc test using Graphpad Prism v. 5.0a.

TABLE 2.5. Reagents and supplies.

Pharmacological Reagents		
Drugs	**Supplier**	**Formula wt**
acetylcholine	Acros Organics	181.66
atropine sulfate	Sigma-Aldrich	694.83
carbachol	Calbiochem	182.7
gallamine	Sigma-Aldrich	891.54
guanethidine	Sigma-Aldrich	296.4
hexamethonium bromide	Sigma-Aldrich	362.2
isoproternol HCl	Sigma-Aldrich	247.72
ketamine HCl	Bedford Laboratories	50 mg/ml
mamba toxin-3	Peptides International	7379.4
pentobarbital	Sigma-Aldrich	248.3
pilocarpine nitrate	Sigma-Aldrich	271.3
platelet-activating factor C-18	Cayman Chemical Company	
pyrilamine maleate	Sigma-Aldrich	401.5
succinylcholine	Sigma-Aldrich	397.3
urethane	Sigma-Aldrich	89.09
xylazine HCl	AmTech	100 mg/ml

Immunocytochemistry		
Antibodies	**Supplier**	**Dose/conc**
Rt x Ab IL-5 (TRFK-5)	BD Pharmingen	240 ug/kg
Gt x Ab betaNGF	R & D Systems	10 ug/kg
normal goat IgG	R & D Systems	10 ug/kg
Ms x Ab PGP9.5	Biogenesis	1/1000
Rb x Ab MBP	Gerald Gleich	1/1000
GtxRb IgG-Alexa Fluor 555/594	Molecular Probes	1/2000
Rb x Ab M1	Research & Diagnostics Antibodies	1/400
Rb x Ab M2	Research & Diagnostics Antibodies	1/2000
Rb x Ab M3	Research & Diagnostics Antibodies	1/800
Rb x Ab M4	Research & Diagnostics Antibodies	1/800
Rb x Ab M5	Research & Diagnostics Antibodies	1/800
Gt x Ms IgG biotinylated	Vector Laboratories	1/400
Normal goat serum	Vector Laboratories	5 - 10%

Other Reagents	
antigen unmasking solution	Vector Laboratories
CAS-Block	Zymed
chromotrope 2R	Sigma-Aldrich
Cytoseal-60	Richard-Allan Scientific
diaminobenzidine nickel substrate	Vector Laboratories
Hemacolor	EMD Chemicals, Inc.
phosphate buffered saline	Mediatech, Inc.
Triton-X 100	Roche
trypan blue	Gibco
Vectastain Elite ABC	Vector Laboratories

Commercially available reagents and kits		
Reagent	**Supplier**	**Formula wt (g/mol)**
agarose	Sigma-Aldrich	
Antifoam A	Sigma-Aldrich	
bovine serum albumin fraction V	Fisher Scientific	
$CaCl_2$	Sigma-Aldrich	147
chicken egg white ovalbumin grade II	Sigma-Aldrich	
Fluo-4	Molecular Probes	1096.95
glucose	Sigma-Aldrich	180.2
HCl (12N)	Fisher Scientific	
HEPES	Sigma-Aldrich	238.3
KCl	Sigma-Aldrich	74.55
$KHCO_3$	Fisher Scientific	100.12
$MgCl_2$ (1M)	Sigma-Aldrich	95.21
NH_4Cl	Fisher Scientific	53.49
PIPES	Sigma-Aldrich	302.37
Tris HCl	Fisher Scientific	121.14
trypsin porcine pancreas	Sigma-Aldrich	
DMSO	Fisher Scientific	
EDTA	Gibco	
Ficoll-Paque PLUS	GE Healthcare Bio-Sciences AB	
Percoll	Amersham	
RT-PCR Reagents		
DNase I	Qiagen	
Dnase Inactivation Reagent	Ambion	
FirstChoice PCR-Ready human brain cDNA	Ambion	
Gel Extraction kit	Qiagen	
Quantitect SYBR green PCR kit	Qiagen	
RNAlater	Qiagen	
RNAqeous -4PCR	Ambion	
Rneasy Maxi kit	Qiagen	
Rneasy Mini kit	Qiagen	
Superscript III reverse transcriptase	Invitrogen	
Turbo DNase I	Ambion	
Human Eos Isolation Supplies		
Eosinophil isolation kit	Miltenyi Biotec	
MACS separation columns LS	Miltenyi Biotec	
QuadroMACS column holder	Miltenyi Biotec	

CHAPTER III.

ATROPINE PRETREATMENT ENHANCES AIRWAY HYPERREACTIVITY THROUGH AN EOSINOPHIL DEPENDENT MECHANISM

ABSTRACT

Airway hyperreactivity in antigen challenged animals is mediated by eosinophil major basic protein (MBP) that blocks inhibitory M_2 muscarinic receptors on parasympathetic nerves, increasing acetylcholine release onto M_3 muscarinic receptors on airway smooth muscle. Acutely, anticholinergics block hyperreactivity in antigen challenged animals and reverse asthma exacerbations in man, but are less effective in chronic asthma. I tested whether atropine, given before antigen challenge affected hyperreactivity, M_2 receptor function, eosinophil accumulation and activation. Sensitized guinea pigs received atropine (1 mg/kg, i.p.) 1 hour before challenge and 6 hours later. Twenty-four hours after challenge, animals were anesthetized, vagotomized, paralyzed and ventilated. Airway reactivity to electrical stimulation of the vagi and to intravenous acetylcholine was not altered by atropine pretreatment in non-sensitized animals, indicating that atropine was no longer blocking post-junctional muscarinic receptors. Antigen challenge induced airway hyperreactivity to vagal stimulation that was significantly potentiated by atropine pretreatment. Bronchoconstriction induced by acetylcholine was not changed by antigen challenge or by atropine pretreatment. M_2 receptor function was lost in challenged animals, but protected by atropine pretreatment. Eosinophils in bronchoalveolar lavage and within airway tissues were significantly increased by challenge, but significantly reduced by atropine pretreatment. However, extracellular MBP in challenged airways was significantly increased by atropine pretreatment, which may account for reduced eosinophils. Depleting eosinophils with Ab IL-5 before challenge prevented hyperreactivity and significantly reduced MBP in airways of atropine pretreated animals. Thus, atropine pretreatment potentiated airway

hyperreactivity by increasing eosinophil activation and degranulation. These data suggest that anticholinergics enhance eosinophil interactions with airway nerves.

Data in this chapter have been published as:

Verbout et al., Atropine Pretreatment Enhances Airway Hyperreactivity in Antigen Challenged Guinea Pigs Through an Eosinophil Dependent Mechanism. *Am J Physiol Lung Cell Mol Biol* 292: 1126-1135, 2007.

Portions of the work presented within this chapter (Figures 3.1-3.8) were done in collaboration with another member of the Fryer lab, Jesse Lorton.

INTRODUCTION

Parasympathetic control of airway smooth muscle is clearly abnormal in patients with asthma (Cropp, 1975). Similarly, in animal models of asthma, hyperreactivity is mediated by increased release of acetylcholine from the parasympathetic nerves onto M_3 muscarinic receptors on airway smooth muscle, resulting in increased bronchoconstriction (Drazen *et al.*, 1975; Fryer *et al.*, 1991; Gold *et al.*, 1972; McCaig, 1987). A major mechanism of airway hyperreactivity is the blockade, by eosinophil proteins, of M_2 muscarinic receptors on the parasympathetic nerves (Elbon *et al.*, 1995; Evans *et al.*, 1997; Fryer *et al.*, 1992; Fryer *et al.*, 1984; Fryer *et al.*, 1991). These neuronal receptors normally limit release of acetylcholine. Loss of M_2 receptor function increases acetylcholine release and potentiates vagally induced bronchoconstriction in animals (Fryer *et al.*, 1992; Fryer *et al.*, 1991) and in humans (Ayala *et al.*, 1989; Minette *et al.*, 1989). Anticholinergic medications would therefore be expected to be highly effective treatments for asthma.

Anticholinergic drugs are used clinically to reverse acute bronchoconstriction (Sheppard *et al.*, 1982; Sheppard *et al.*, 1983; Yu *et al.*, 1972). During acute asthma, adding anticholinergic therapy to ß-agonists rapidly improves pulmonary function, decreases rates of hospitalization by 50% (Rodrigo *et al.*, 2000), and decreases the duration of hospital stay by more than one day (Brophy *et al.*, 1998). However, use of anticholinergics in management of chronic stable asthma has been disappointing (Cazzola *et al.*, 1998; Gross, 2006; Gross *et al.*, 1984; Jacoby *et al.*, 2001b; Westby *et al.*, 2004) and is not recommended by the NIH Expert Panel on the Management of Asthma

(National Heart, 2007). This may in part be due to substantial under-dosing, coupled with the non-selective nature of the muscarinic antagonists (Ward *et al.*, 1981). Inhalation of the muscarinic antagonist ipratropium bromide blocks post-junctional M_3 muscarinic receptors on airway smooth muscle, but also blocks neuronal M_2 receptors, thereby increasing acetylcholine release and potentially overcoming partial blockade of M_3 receptors. Indeed, ipratropium does potentiate vagally induced bronchoconstriction in experimental animals via this mechanism (Fryer *et al.*, 1987). Thus, addition of anticholinergic drugs has the potential to make airway reactivity worse or at the least cancel out the beneficial effects of post-junctional blockade. Alternatively, the poor performance of anticholinergics in chronic asthma may have to do with previously unrecognized effects of anticholinergics on the inflammatory response in the airways.

Eosinophilic inflammation of the lungs is a dominant feature of asthma (Gleich, 2000; Jacoby *et al.*, 2001a; Rothenberg *et al.*, 2006) and is associated with airway hyperreactivity in experimental animals (Elbon *et al.*, 1995; Lee *et al.*, 2004b; Mauser *et al.*, 1995). Eosinophils are recruited to airway nerves via CC-motif chemokine receptor 3 (CCR3) agonists (Fryer *et al.*, 2006), adhere to them via ICAM-1 and VCAM (Nie *et al.*, 2007; Sawatzky *et al.*, 2002), and release eosinophil major basic protein (MBP), an endogenous and selective antagonist for M_2 muscarinic receptors (Jacoby *et al.*, 1993). Thus, in antigen challenged guinea pigs, airway hyperreactivity is prevented by depleting eosinophils with antibody to IL-5 (Ab IL-5) (Elbon *et al.*, 1995), by blocking the CCR3 receptors (Fryer *et al.*, 2006), or by blocking eosinophil major basic protein (Evans *et al.*, 1997). Thus, I used a well-characterized model of airway hyperreactivity, antigen

challenged guinea pigs (Fryer *et al.*, 1998), to test whether the anticholinergic drug, atropine, would affect development of airway hyperreactivity and eosinophil activation if administered prophylactically.

EXPERIMENTAL DESIGN

Sensitization and challenge with antigen

Guinea pigs were sensitized to ovalbumin (10 mg/kg, i.p) every other day for a total of three injections. Three weeks after the last ovalbumin injection, some sensitized animals were challenged with an aerosol of 2.5% ovalbumin for 5 minutes or until signs of respiratory distress appeared, in which case antigen challenge was immediately halted.

Treatments

Guinea pigs were pretreated with atropine (1 mg/kg, i.p), a non-selective muscarinic antagonist or saline one hour prior to antigen challenge and again 6 hours after antigen challenge (18 hours prior to physiological measurements). In some animals, a rat monoclonal antibody to IL-5 (240 ug/kg, i.p.) was administered 4 days before the first injection of ovalbumin.

Measurements

Twenty-four hours after antigen challenge, pulmonary inflation pressure was measured as described in the methods. Vagal reactivity, airway smooth muscle responsiveness, neuronal M_2 receptor function and bronchoalveolar lavage was measured as described in the methods. Histological evaluation of eosinophil influx into the airway and major basic protein deposition and distribution were measured as described in the methods.

Statistics

All data are expressed as mean +/-SEM. In vivo responses to nerve stimulation, acetylcholine and pilocarpine were analyzed using 2-way ANOVA for repeated measures. Data sets were compared as follows: by group (control, sensitized, sensitized challenged), by drug (saline versus atropine) or antibody pretreatment (no Ab vs Ab IL-5). Baseline heart rates, blood pressures, pulmonary inflation pressures and bronchoalveolar lavage leukocytes were analyzed for statistical significance by 1-way ANOVA. Histological examination for tissue eosinophils and major basic protein content in the airways were analyzed by 1-way ANOVA with Fisher's LSD post-hoc correction. For comparisons of multiple data sets (> 3 groups), a Bonferroni correction was applied. A p-value of less than 0.05 was considered significant. In all figures, only comparisons that were statistically significant are indicated with symbols; all other comparisons did not have statistical significance and are not indicated with symbols.

RESULTS

Atropine pretreatment potentiated airway hyperreactivity in antigen challenged animals. Administration of atropine (1mg/kg, i.p.) one hour before and six hours after antigen challenge of guinea pigs did not alter resting pulmonary inflation pressure in any of the groups 24 hours later, by which time atropine had worn off (Table 3.1; see Table 2.1 in Chapter II for normal physiological values in conscious guinea pigs). In non-sensitized guinea pigs, electrical stimulation of both vagus nerves increased bronchoconstriction in a frequency dependent manner that was not changed by atropine pretreatment (Figure 3.1A). Vagally induced bronchoconstriction was not different in sensitized (not challenged) guinea pigs, regardless of whether they were pretreated with atropine or not (data not shown). However, vagally induced bronchoconstriction was significantly increased one day after antigen challenge of sensitized guinea pigs, compared to non-sensitized controls (see white circles versus white squares; Figure 3.1). In contrast to non-sensitized guinea pigs, atropine pretreatment further potentiated vagally induced bronchoconstriction in antigen sensitized and challenged animals (Figure 1B). Acetylcholine induced bronchoconstriction was not altered by antigen challenge or by atropine, demonstrating that atropine had worn off by this time (Figure 3.2A). Thus, atropine pretreatment exacerbates vagally mediated hyperreactivity in antigen challenged guinea pigs.

Atropine pretreatment did not change vagally mediated bradycardia. Atropine pretreatment did not change resting heart rate or blood pressure in any of the groups tested (Table 3.1). Electrical stimulation of both vagi resulted in frequency dependent

bradycardia in all groups. Vagally induced bradycardia was increased slightly, but not significantly, in the antigen challenged groups (Figure 3.3). This effect was not mediated by changes in the post-junctional muscarinic receptors on cardiac muscle since acetylcholine induced bradycardia was not different among groups (Figure 3.2B). Atropine pretreatment had no effect on vagally or acetylcholine-induced bradycardia in either non-sensitized or sensitized and challenged groups, demonstrating again that atropine had worn off by this time.

Antibody to IL-5 prevented airway hyperreactivity in atropine pretreated antigen challenged animals. To test the role of eosinophils in atropine-induced potentiation of airway hyperreactivity, guinea pigs were treated with Ab IL-5 prior to antigen sensitization. Antibody to IL-5 prevented antigen-induced hyperreactivity in atropine pretreated animals (Figure 3.4A) without altering acetylcholine-induced bronchoconstriction (data not shown). Ab IL-5 had no effect on vagally (Figure 3.4B) or acetylcholine-induced (data not shown) bradycardia. Thus, atropine pretreatment did not potentiate bronchoconstriction in Ab IL-5 treated animals.

Atropine pretreatment prevents M_2 muscarinic receptor dysfunction in antigen challenged animals. In non-sensitized guinea pigs, stimulating M_2 receptors with pilocarpine inhibited vagally induced bronchoconstriction in a dose dependent manner that was not affected by pretreatment with atropine (Figure 3.5A), demonstrating functional M_2 receptors. In contrast, the M_2 receptors did not respond to agonists in antigen challenged animals, since pilocarpine did not inhibit vagally induced

bronchoconstriction (Figure 3.5B). Pretreatment with atropine protected neuronal M_2 receptor function in antigen challenged animals (Figure 3.5B); this effect was not changed by reducing eosinophils with Ab IL-5 (Figure 3.5B). Thus it appears that the function of neuronal M_2 receptors does not contribute to atropine-induced potentiation of airway hyperreactivity in antigen challenged guinea pigs.

Effect of atropine pretreatment on pulmonary inflammation in antigen challenged guinea pigs. I assessed inflammation in bronchoalveolar lavage and in airway tissues. The total number of inflammatory cells in bronchoalveolar lavage was not different among groups or changed by atropine pretreatment (Table 3.1). However, antigen challenge significantly increased the number of eosinophils in the lavage fluid; an effect that was no longer present in animals pretreated with atropine (Figure 3.6; within box). Neither antigen challenge nor atropine pretreatment significantly altered any other inflammatory cell type in the bronchoalveolar lavage fluid (Figure 3.6). Thus, atropine pretreatment blocked antigen challenge induced eosinophilia in bronchoalveolar lavage fluid.

Quantitative analysis of eosinophils within the lungs also demonstrated that antigen challenge increased eosinophils in the airways and around nerves (Figures 3.7 - 3.8). As in the bronchoalveolar lavage, atropine pretreatment decreased eosinophils in airways and around nerves of sensitized and challenged animals. Thus, while atropine pretreatment increased airway hyperreactivity in antigen challenged guinea pigs, it

significantly decreased eosinophils in bronchoalveolar lavage, in the airway tissues and around airway nerves.

Analysis of eosinophil activation in the lungs. I measured eosinophil major basic protein deposition as an indicator of eosinophil activation in the lungs. Antigen challenge increased, though not significantly, major basic protein deposition throughout the airways (Figure 3.9A versus 3.9B; quantification in 3.9E). Atropine pretreatment before antigen challenge significantly increased major basic protein deposition within airways of antigen challenged guinea pigs (Figure 3.9B versus 3.9D; quantification in 3.9E), but had no effect in non-sensitized controls (Figure 3.9A versus 3.9C; quantification in 3.9E). Increased major basic protein deposition throughout airways of atropine pretreated and antigen challenged animals was prevented by Ab IL-5 (Figure 3.9F; quantification in 3.9E).

It appeared that the majority of major basic protein was located in the region of the lamina propria. Regional analysis of major basic protein content in the airways confirmed that regardless of treatment, most of the major basic protein was localized to the lamina propria (Figure 3.10). However, regardless of whether major basic protein was measured in the epithelium, lamina propria, smooth muscle or fibrous tissue, antigen challenge of sensitized animals with atropine pretreatment significantly increased major basic protein compared to all other groups (Figure 3.10F). There was also a significant increase in major basic protein in the fibrous tissue region of antigen challenged animals, and atropine pretreatment increased this further. It is worth noting that this region is where most of the nerve trunks are located (see Figure 3.7). Thus, eosinophils were more

activated in the presence of atropine in antigen challenged guinea pigs than in non treated animals and antigen challenge significantly increased major basic protein in regions of the lung that contain nerves.

TABLE 3.1. Baseline cardiovascular and pulmonary parameters.

Treatment	Group	Number	Heart Rate (beats/minute)	Blood Pressure (mmHg) Systolic	Diastolic	Pulmonary Inflation Pressure (mmH2O)	Total Inflammatory Cells (x 10^6)
Saline	Non-Sens	9	320 ± 9.6	47 ± 2.3	24 ± 1.8	100 ± 6.5	9.1 ± 1.9
	Sensitized	4	309 ± 5.2	45 ± 5.6	24 ± 3.2	98 ± 8.5	10.9 ± 1.6
	Sens/Chall	9	316 ± 7.8	48 ± 2.9	24 ± 2.1	86 ± 4.1	11.9 ± 1.8
Atropine	Non-Sens	6	304 ± 7.5	44 ± 2.7	19 ± 3.2	96 ± 6.1	13.0 ± 0.9
	Sensitized	8	293 ± 6.2	44 ± 2.2	22 ± 1.6	100 ± 7.1	12.0 ± 1.9
	Sens/Chall	5	314 ± 16.3	47 ± 2.9	24 ± 3.3	91 ± 6.4	13.5 ± 1.2
	Sens/Chall + AbIL-5	6	313 ± 4.4	45 ± 2.4	22 ± 2.3	104 ± 5.5	10.8 ± 0.7

Table 3.1. Pretreatment with atropine did not change resting heart rate, blood pressure, pulmonary inflation pressure or total bronchoalveolar lavage leukocytes among groups 24 hours after antigen challenge. Values are the mean +/-SEM.

FIGURE 3.1. Atropine pretreatment at the time of antigen challenge potentiated airway hyperreactivity in antigen challenged guinea pigs twenty-four hours later.

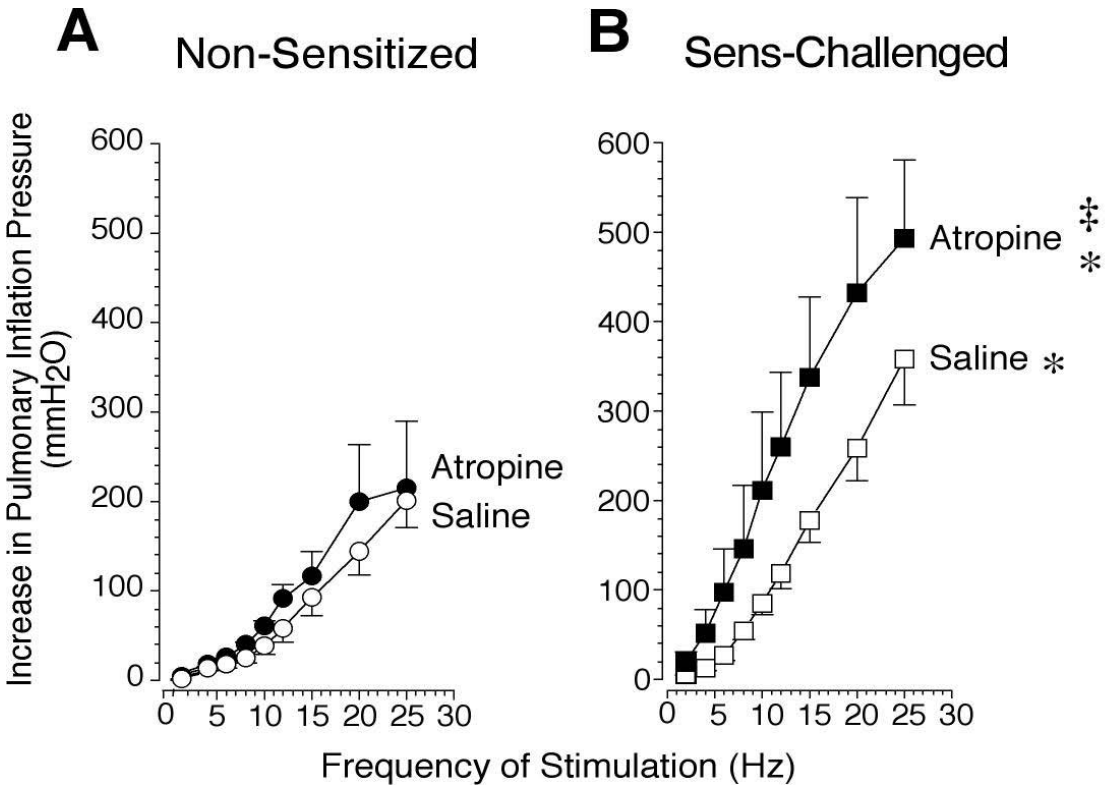


Figure 3.1. Electrical stimulation of both vagi caused frequency dependent bronchoconstriction (measured as an increase in pulmonary inflation pressure; mmH_2O) in non-sensitized guinea pigs (A, white circles) that was potentiated by antigen challenge (B, white squares). Atropine pretreatment one hour before antigen challenge potentiated vagally induced bronchoconstriction in sensitized challenged animals (B, black squares), but not in non-sensitized controls (A, black circles). *The entire frequency response is significantly different from respective frequency response in non-sensitized controls; ‡significantly different from challenged saline treated. Data are expressed as the mean +/- SEM, n=4-8.

FIGURE 3.2. Neither bronchoconstriction nor bradycardia are altered by atropine pretreatment in antigen challenged guinea pigs.

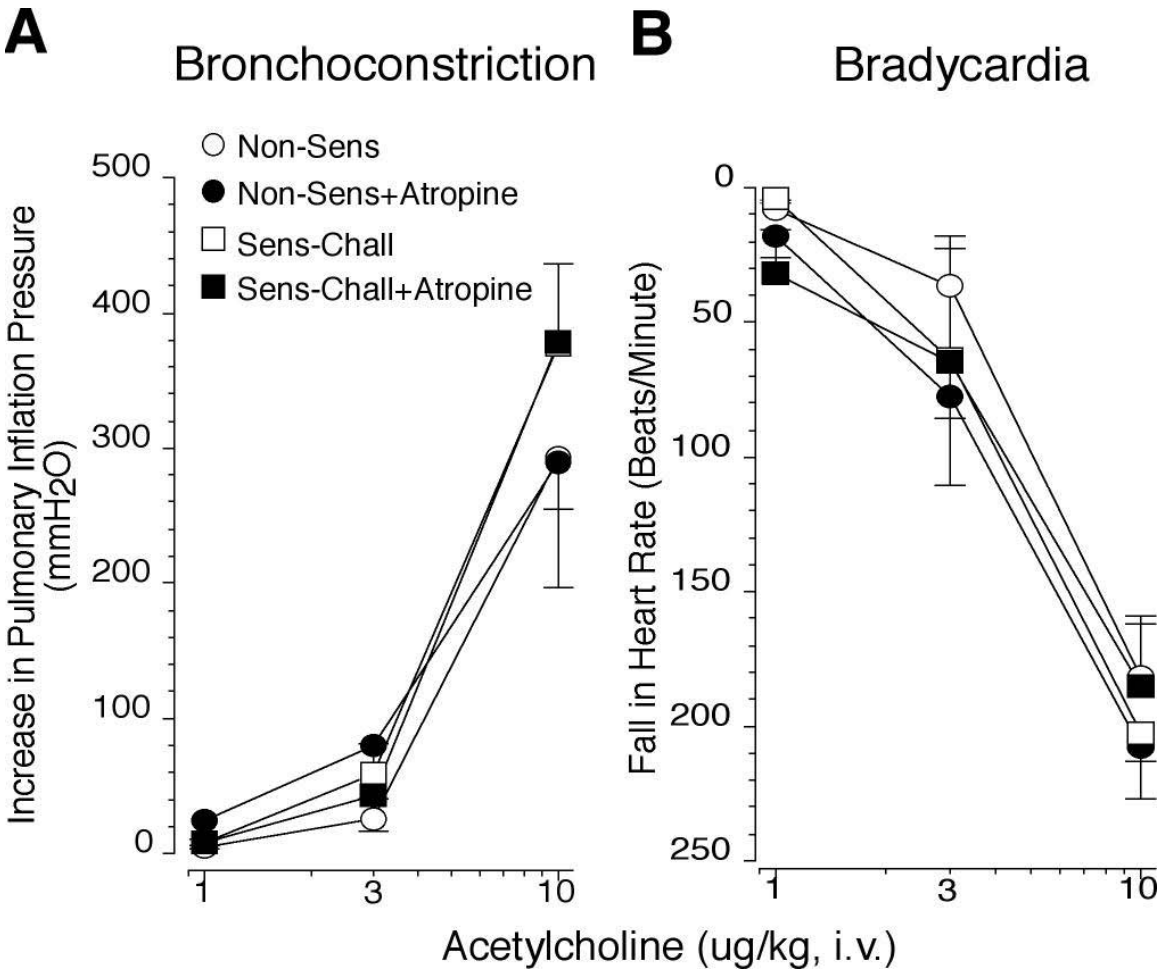


Figure 3.2. In vagotomized guinea pigs, acetylcholine induced bronchoconstriction, measured as an increase in pulmonary inflation pressure (A, white circles) and bradycardia, measured as a fall in heart rate (B, white circles) were not changed by atropine pretreatment (black symbols) or by antigen challenge (squares). Data are expressed as the mean +/- SEM, n=4-8.

FIGURE 3.3. Vagally induced bradycardia was not altered by atropine pretreatment.

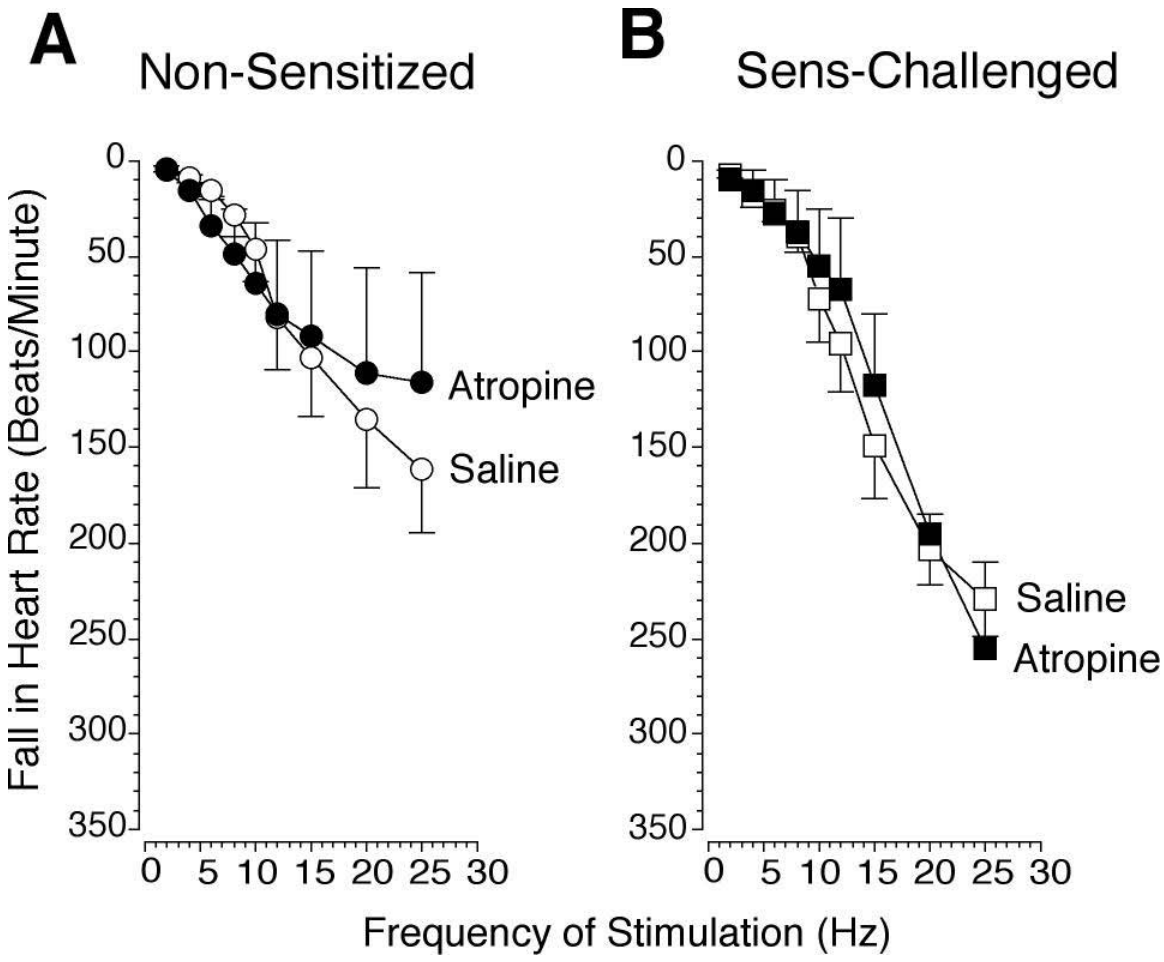


Figure 3.3. Electrical stimulation of both vagi caused a frequency dependent fall in heart rate, measured in beats per minute (A, white circles) that was not changed by atropine (black circles) given 24 hours previously. Vagally induced bradycardia was not significantly changed by antigen challenge (B, white squares) or by atropine pretreatment (black squares). Data are expressed as the mean +/- SEM, n=4-8.

FIGURE 3.4. Antibody to interleukin-5 prevented airway hyperreactivity in atropine pretreated antigen challenged guinea pigs.

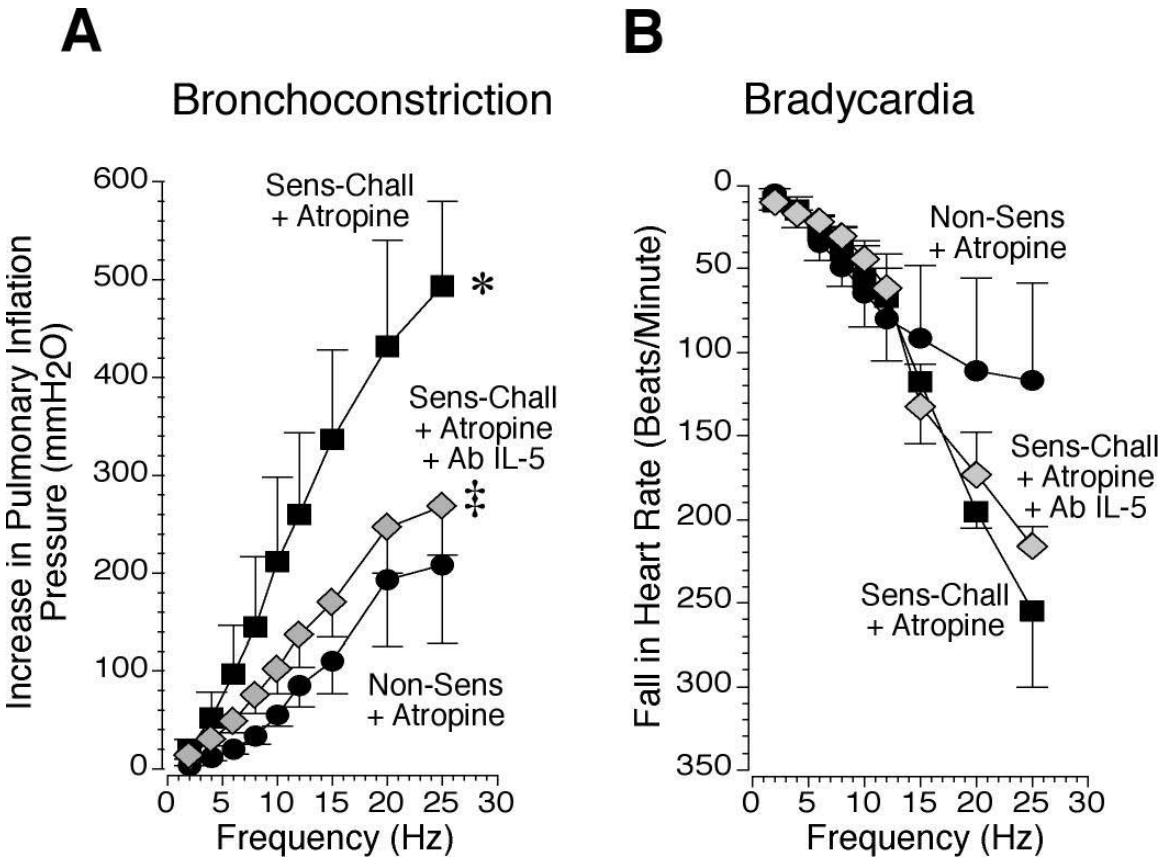


Figure 3.4. Electrical stimulation of the vagus caused bronchoconstriction (A) in animals treated with atropine 24 hours previously (black circles). Antigen challenge significantly potentiated vagally induced bronchoconstriction (black squares) that was blocked by treatment with antibody to IL-5 (gray diamonds). In the heart, vagally induced bradycardia (B, black circles) was not significantly altered by antigen challenge (black squares) or by antibody to IL-5 (gray diamonds). *The entire frequency response is significantly different from frequency response in non-sensitized controls. ‡Significantly different from sensitized challenged plus atropine. Data are the mean +/- SEM, n=4-8.

FIGURE 3.5. Neuronal M_2 receptor dysfunction was prevented by atropine pretreatment in antigen challenged guinea pigs.

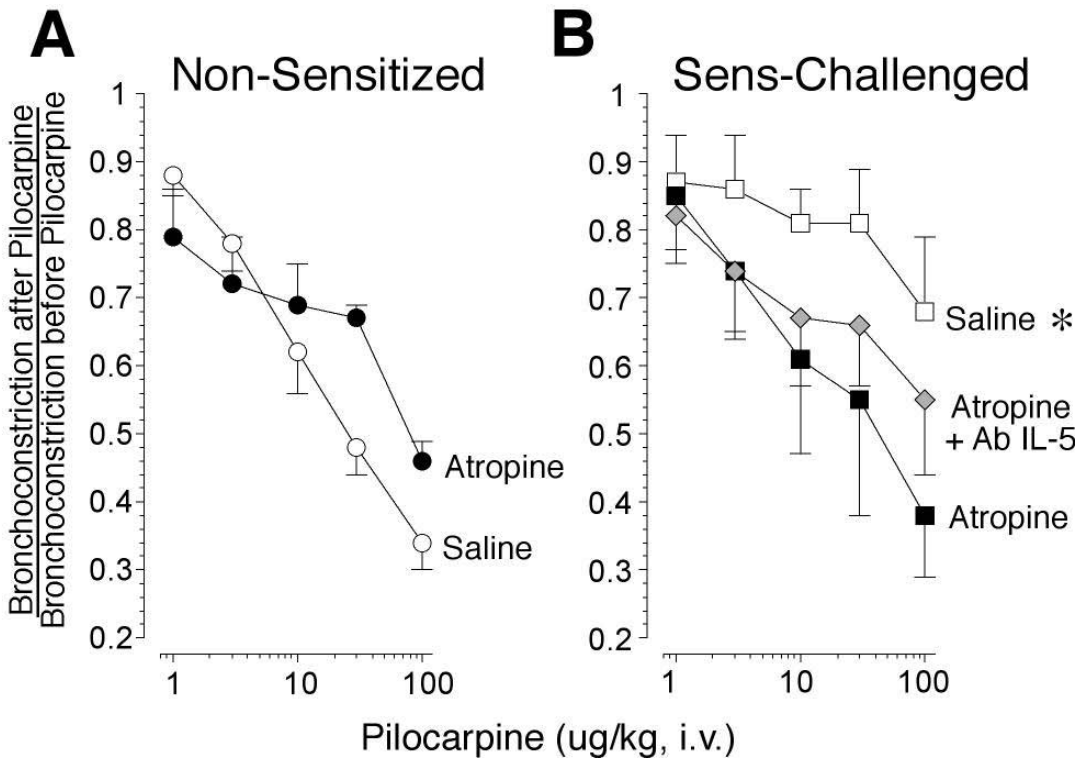


Figure 3.5. Pilocarpine inhibited vagally induced bronchoconstriction in non-sensitized guinea pigs (A) treated 24 hours previously with either saline (white circles) or atropine (black circles), demonstrating functional M_2 receptors. In contrast, pilocarpine did not inhibit vagally induced bronchoconstriction in antigen sensitized and challenged animals (B, white squares), demonstrating that M_2 receptors were no longer responding to agonists. In contrast, M_2 receptor dysfunction was prevented by pretreatment with atropine (B, black squares) and by antibody to IL-5 in addition to atropine pretreatment (B, gray diamonds) in antigen challenged guinea pigs. *The entire dose response is significantly different from dose response in non-sensitized controls. Data are expressed as the ratio of vagally induced bronchoconstriction in the presence of pilocarpine to

vagally induced bronchoconstriction in the absence of pilocarpine and are the mean +/- SEM, n=4-6.

FIGURE 3.6. Neither antigen challenge nor atropine pretreatment changed macrophages, neutrophils or lymphocytes in bronchoalveolar lavage fluid.

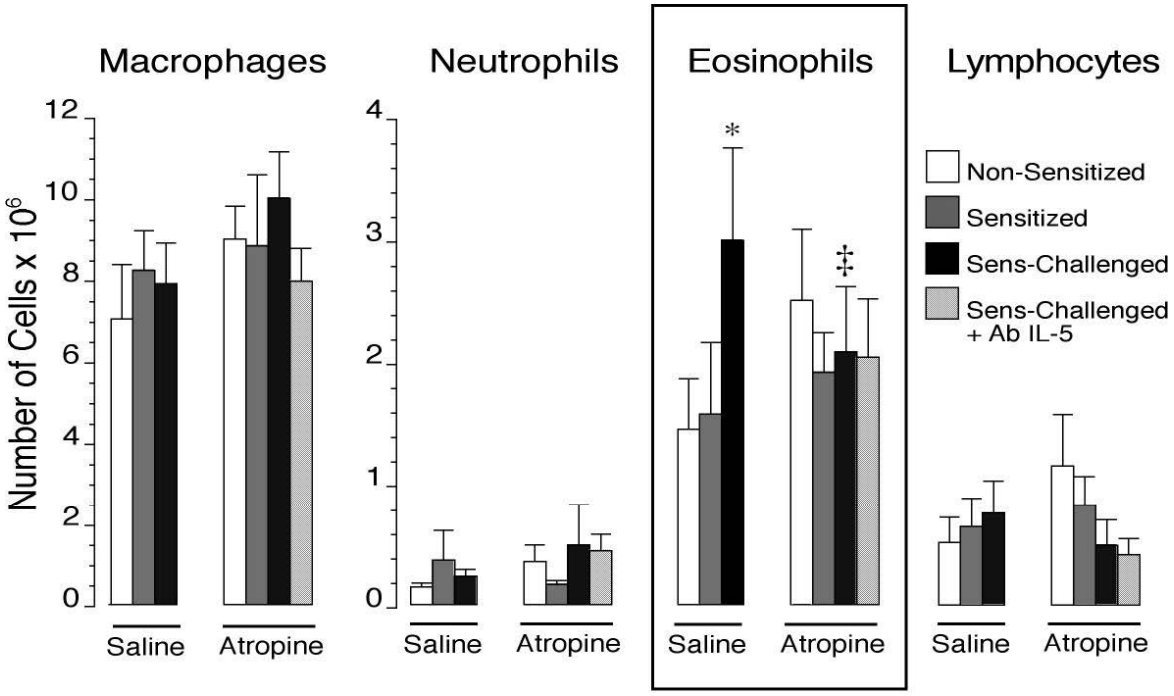


Figure 3.6. Antigen challenge increased eosinophils (black bar; saline group) compared to non-sensitized controls (white bars). Pretreatment with atropine significantly prevented the antigen induced increase in eosinophils (compare black to white bar in atropine group). *Significantly different from non-sensitized control; ‡significantly different from challenged in saline group. Data are expressed as the mean +/-SEM, n=4-8.

FIGURE 3.7. Atropine pretreatment decreased eosinophils in airways of antigen challenged guinea pigs.

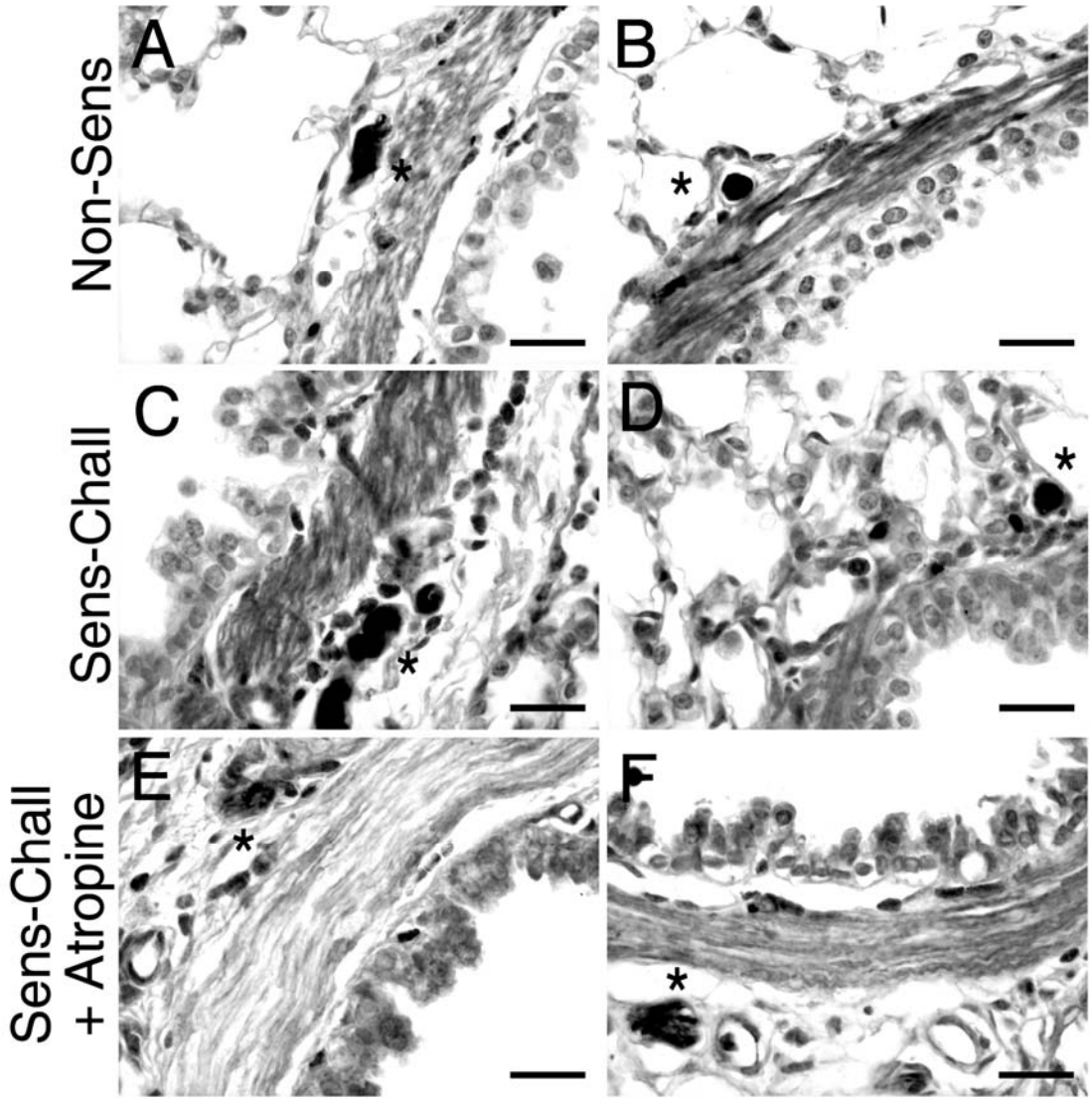


Figure 3.7. Shown are cross sections of guinea pig bronchi. Nerves were labeled with antibody to PGP 9.5 (black; nerves marked with asterisk) and eosinophils counterstained with Chromotrope 2R (red). There were few eosinophils in lungs or around nerves in non-sensitized guinea pigs (A-B). Following antigen challenge, eosinophils were present within smooth muscle and in close proximity to nerves (C-D). In contrast, animals treated

with atropine before antigen challenge had fewer eosinophils within the lungs or in close proximity to nerves (E-F). Photos are from 2 different animals per group and represent n=4-5. Scale bars are 50 um.

FIGURE 3.8. Atropine pretreatment decreased total eosinophils in the lungs and around nerves of antigen challenged guinea pigs.

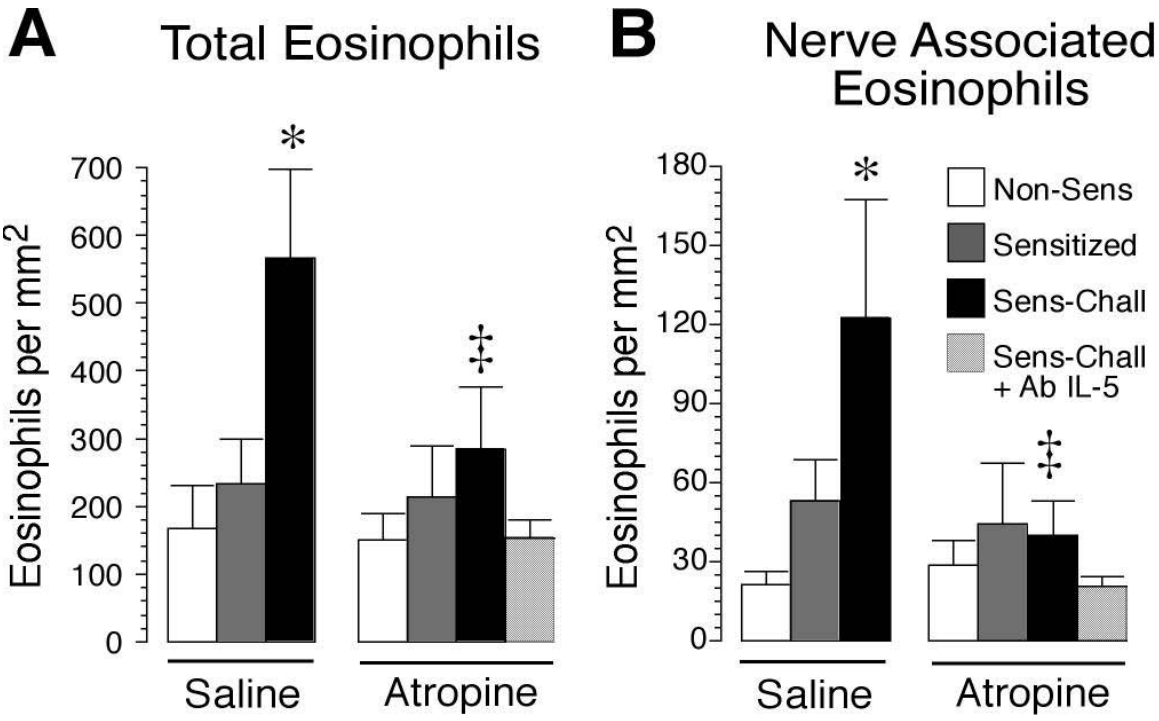


Figure 3.8. In saline pretreated animals (left panels of A and B), antigen challenge (black bars) significantly increased eosinophils in airways (A) and around nerves (B) compared to respective non-sensitized controls (white bars) and to respective sensitized animals (gray bars). Pretreatment with atropine prevented the antigen induced increase in eosinophils in airways (A) or around nerves (B). Animals treated with antibody to IL-5 prior to sensitization were not different from control. Data are expressed as the number of eosinophils per mm^2 of airway smooth muscle (A) and the number of eosinophils within 8 um of an airway nerve per mm^2 (B). *Significantly different from non-sensitized control; ‡significantly different from challenged saline treated. Data are expressed as the mean +/-SEM, n=3-5.

FIGURE 3.9. Atropine pretreatment increased eosinophil major basic protein deposition.

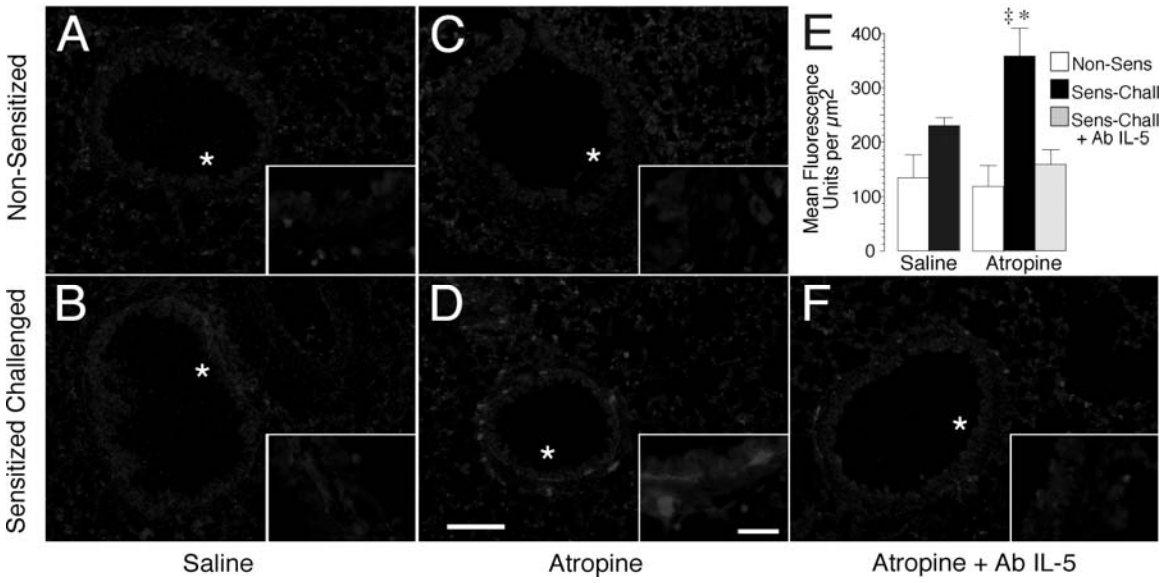


Figure 3.9. Airways were labeled with antibody to MBP (red) within airways (A-D, and F; increased magnification of area marked with * is shown in insets) of antigen challenged guinea pigs (nuclei are stained blue with DAPI). Airways of control guinea pigs contain some major basic protein (A), which was increased by antigen challenge (B). Atropine pretreatment did not increase major basic protein deposition in non-sensitized controls (C), but significantly increased major basic protein in challenged animals (D, quantification of data in E). Antibody to IL-5 prevented this increase in MBP deposition in challenged animals pretreated with atropine (F). Data (E) are expressed as mean fluorescence intensity in units/um^2, and are the mean +/-SEM, n=3-5. In E, *significantly different from non-sensitized atropine treated (white bar atropine group); ‡significantly different from challenged saline treated (black bar saline group). Scale bar 100 um; for insets scale bar 30 um.

FIGURE 3.10. Atropine pretreatment increased eosinophil major basic protein in airways of antigen challenged animals.

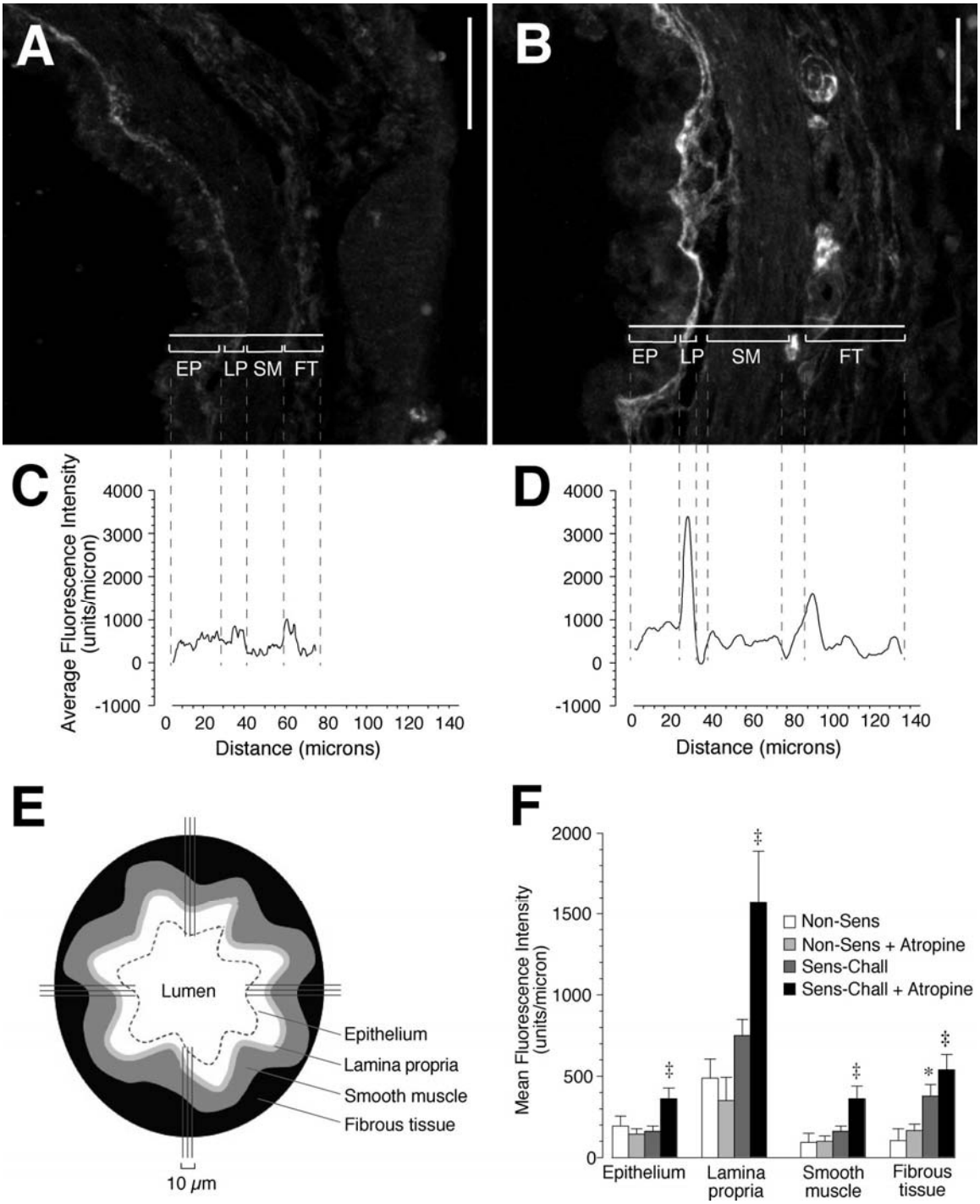


Figure 3.10. Eosinophil major basic protein was labeled with antibody to MBP and fluorescence was evaluated in gray scale. Shown are representative photographs of airways from control non-sensitized (A) and sensitized challenged atropine pretreated (B) animals. Mean fluorescence intensity was measured in a line extending across the epithelium (EP), lamina propria (LP), smooth muscle (SM) and fibrous tissue (FT) of each airway (C-D). Each airway was sampled with 4 sets of 3 lines taken 90 degrees relative to each other (E). Average fluorescence intensity (units/um) for each airway was calculated from these 12 measurements (F). *Significantly different from non-sensitized control (white bar); ‡significantly different from each of the other groups: non-sensitized control (white bars), non-sensitized atropine (light gray bars) and sensitized challenged (dark gray bars). Data are the mean +/-SEM; n=4 animals per group, with replicates of 4 airways per animal, and 12 measurements per airway.

DISCUSSION

The data presented here demonstrate that blocking muscarinic receptors with atropine at the time of antigen challenge potentiates challenge induced airway hyperreactivity twenty-four hours later, by which time atropine had worn off. The ability of atropine to potentiate airway hyperreactivity was specific to challenged animals, was not present in non-sensitized controls or in sensitized but not challenged animals, and was unrelated to neuronal M_2 receptor function. Atropine-induced potentiation was mediated at the level of the parasympathetic nerves supplying the airways, since acetylcholine induced bronchoconstriction was not potentiated by atropine pretreatment.

Pharmacologic blockade of muscarinic receptors by atropine was no longer apparent twenty-four hours later, since neither baseline pulmonary inflation pressure nor baseline heart rate was different between groups (Table 3.1). Furthermore, neither acetylcholine induced bronchoconstriction nor bradycardia in atropine-pretreated animals was different from controls at this time point (Figure 3.3). Thus, twenty-four hours after atropine administration, the post-junctional M_3 muscarinic receptors in the lung and the post-junctional M_2 muscarinic receptors in the heart appeared to be fully functional.

During these experiments, nine sensitized animals died during antigen challenge. This antigen challenge protocol has been used extensively and death is normally a very rare event. It may be significant that of the nine animals that died, eight were pretreated with atropine, supporting the observation that atropine pretreatment is not beneficial.

Histological examination of the lungs demonstrated acute bronchoconstriction. None of the animals that died during antigen challenge were included in final data analysis.

Airway hyperreactivity twenty-four hours after antigen challenge is vagally mediated (Fryer *et al.*, 1992; Fryer *et al.*, 1991). This is confirmed here since vagally induced bronchoconstriction was increased by challenge (Figure 3.1), while acetylcholine induced bronchoconstriction in vagotomized animals was not increased (Figure 3.2). The potentiating effect of atropine pretreatment on vagal hyperreactivity in antigen challenged animals was limited to the airways because vagally induced bradycardia was not altered by atropine pretreatment.

Dysfunction of neuronal M_2 muscarinic receptors causes vagally mediated hyperreactivity in antigen challenged animals since the negative feedback these receptors normally provide over acetylcholine release is absent (Elbon *et al.*, 1995; Evans *et al.*, 1997; Fryer *et al.*, 1991). Consistent with previous studies, neuronal M_2 receptors were dysfunctional in challenged guinea pigs because they did not respond to the muscarinic agonist pilocarpine (Figure 3.5). However, M_2 receptor function was protected by atropine pretreatment, since pilocarpine decreased vagally induced bronchoconstriction in challenged animals. These findings demonstrate that atropine induced potentiation of vagally mediated hyperreactivity is not linked to neuronal M_2 receptor dysfunction.

The mechanism by which atropine pretreatment prevented M_2 receptor dysfunction is not known. It may be that occupancy of M_2 receptors by atropine at the time of antigen

challenge prevented MBP from interacting with M_2 receptors, since previous studies have shown that eosinophil MBP binds to M_2 receptors (Jacoby *et al.*, 1993). Although atropine wears off by 24 hours, MBP may not be able to interact with M_2 receptors since MBP is highly cationic (Gleich *et al.*, 1974; Popken-Harris *et al.*, 1994), and therefore unlikely to travel far from the release by eosinophils. Histology demonstrated that MBP was not uniformly deposited in the lungs but was found in aggregates (Figures 3.9 - 3.10), probably at the sites of eosinophil activation.

Increased eosinophils are positively correlated with airway hyperreactivity in humans (Bousquet *et al.*, 1990). The role of eosinophils in airway hyperreactivity in antigen challenged guinea pigs and other experimental animals has been well established (Elbon *et al.*, 1995; Lee *et al.*, 2004b; Mauser *et al.*, 1995; Mita *et al.*, 2004; Obiefuna *et al.*, 2006). Eosinophils cluster along airway nerves in antigen challenged animals and in patients with fatal asthma (Costello *et al.*, 2000; Costello *et al.*, 1997; Evans *et al.*, 2001). When activated they release several preformed mediators including eosinophil MBP, eosinophil peroxidase, eosinophil derived neurotoxin and neurotrophins including nerve growth factor (Kita *et al.*, 1992; Solomon *et al.*, 1998). The dominant protein is MBP (Gleich *et al.*, 1973), which is an allosteric antagonist of M_2 muscarinic receptors (Jacoby *et al.*, 1993) in airways of challenged guinea pigs (Costello *et al.*, 1997; Lefort *et al.*, 1996; Pretolani *et al.*, 1994), and in patients with asthma (Costello *et al.*, 1997; Frigas *et al.*, 1981; Wardlaw *et al.*, 1988), causing airway hyperreactivity by blocking neuronal M_2 receptors (Evans *et al.*, 1997; Evans *et al.*, 2001). Depletion or inhibition of eosinophil migration to the lungs, or blockade of eosinophil major basic protein protects M_2 receptor

function and prevents airway hyperreactivity in antigen challenged animals (Elbon *et al.*, 1995; Evans *et al.*, 1997; Fryer *et al.*, 1997).

Consistent with previous studies (Costello *et al.*, 1997; Evans *et al.*, 2001; Fryer *et al.*, 2006), I found that antigen challenge increased eosinophils in bronchoalveolar fluid (Figure 3.6), in airway tissues and around airway nerves (Figures 3.7 – 3.8). Although atropine pretreatment potentiated airway hyperreactivity, it prevented the antigen induced eosinophil increase in both bronchoalveolar lavage and within the airways. Nonetheless, eosinophil activation, as assessed by MBP deposition, was increased by atropine in these airways. The mechanism of atropine-induced potentiation of airway hyperreactivity in challenged guinea pigs is linked to the presence of eosinophils, since this effect is abolished by pretreatment with Ab IL-5 (Figure 3.4). This illustrates that neither lavage nor histological analysis of eosinophil presence are sufficient to determine the role of eosinophils in the absence of a measure of eosinophil activation.

Interleukin-5 is chemotactic for eosinophils, activates eosinophils, prolongs eosinophil survival, and enhances degranulation (Giembycz *et al.*, 1999). Antibody to IL-5 decreases eosinophils in peripheral circulation, inhibits eosinophil migration to the lungs (Chand *et al.*, 1992; Gulbenkian *et al.*, 1992), and blocks airway hyperreactivity in antigen challenged animals (Elbon *et al.*, 1995; Mauser *et al.*, 1995). In these experiments, Ab IL-5 inhibited atropine-induced potentiation of airway hyperreactivity regardless of whether it was administered either before (Figure 3.4) or after sensitization (not shown). Although Ab IL-5 did not decrease eosinophils in bronchoalveolar lavage,

possibly because it was given before sensitization, it clearly prevented eosinophil activation (Figure 3.9).

Quantification of major basic protein revealed significantly greater deposition throughout airways of atropine pretreated challenged guinea pigs than in any other group (Figures 3.9 – 3.10). Regardless of treatment group, distribution of MBP was greatest in the airway lamina propria, which contains heparin as a structural component of the basement membrane (Yurchenco *et al.*, 1990). Since MBP is positively charged (Gleich *et al.*, 1976), it can interact with the negatively charged heparin (Jacoby *et al.*, 1993; Swaminathan *et al.*, 2005), which may explain the localization in the airway lamina propria. MBP was also significantly increased in the fibrous tissue both by antigen challenge alone and by atropine pretreatment. This may be important because eosinophils accumulate around airway nerves (Costello *et al.*, 1997; Evans *et al.*, 2001; Fryer *et al.*, 2006), which are found in this region. Thus, atropine pretreatment increased eosinophil activation during antigen challenge, which likely accounts for decreased eosinophil numbers in the lungs.

Eosinophil activation is a better determinant of airway hyperreactivity than eosinophil presence alone. In primates, although antigen induced hyperreactivity is associated with decreased eosinophils, it is positively correlated with extracellular eosinophil peroxidase in bronchoalveolar lavage, demonstrating eosinophil activation (Gundel *et al.*, 1992). In a clinical trial, an Ab IL-5 did not decrease airway hyperreactivity (Leckie *et al.*, 2000), consistent with it not decreasing either eosinophils or MBP deposition in the lungs

(Flood-Page *et al.*, 2003), despite depletion of peripheral eosinophils (Leckie *et al.*, 2000). Eosinophil peroxidase is increased in urine of animals and children during acute asthma exacerbations (Mita *et al.*, 2004; Obiefuna *et al.*, 2006) suggesting that eosinophil activation, rather than eosinophil number may better correlate with clinical status.

Thus, atropine pretreatment potentiated antigen induced vagal hyperreactivity and increased eosinophil major basic protein in the airways, which may be the result of increased eosinophil activation. The neuronal M_2 muscarinic receptor was not involved in atropine potentiation of antigen induced hyperreactivity, possibly because atropine blocked or protected neuronal M_2 receptors during antigen challenge. Without neuronal M_2 receptor dysfunction, the mechanism of eosinophil mediated atropine potentiation of antigen-induced hyperreactivity is unknown.

Eosinophils degranulate and release a variety of neuromediators, including nerve growth factor (Solomon *et al.*, 1998), leukemia inhibitory factor (Zheng *et al.*, 1999), and brain derived neurotrophic factor and neurotrophin-3 (Noga *et al.*, 2003), which may have either direct or indirect effects on parasympathetic nerves. It is possible that some of these neuromediator may alter parasympathetic nerve function. Alternatively, eosinophils near airway nerves are activated by chemotactic factors such as eotaxin (Fryer *et al.*, 2006; Jose *et al.*, 1994) and by ICAM-1 and VCAM expressed by these nerves (Sawatzky *et al.*, 2002). Neurotransmitters, such as substance P can also activate eosinophils (Kroegel *et al.*, 1990). There are no known endogenous controls to limit eosinophil activation at the parasympathetic nerves.

The data presented here provide support for the hypothesis that eosinophils express inhibitory muscarinic receptors that regulate their interaction with airway nerves. However, I cannot exclude that atropine may have effects on other cells, thus accounting for the results presented here. Muscarinic receptors are present on inflammatory cells including lymphocytes, macrophages and eosinophils (Fujii *et al.*, 2001; Ricci *et al.*, 2002; Sato *et al.*, 1998; Tayebati *et al.*, 2002; Verbout *et al.*, 2006). Should these muscarinic receptors be inhibitory, blockade by atropine would remove this inhibitory mechanism, thereby increasing inflammatory cell activity. At this point, this "inhibitory" muscarinic receptor could be on any cell that affects eosinophil function, including eosinophils themselves. Should these inhibitory muscarinic receptors be on eosinophils, blockade by atropine may directly affect eosinophil function, thereby increasing eosinophil activation and increasing eosinophil interactions with airway nerves (Figure 3.11). To support this model, it would be necessary to determine whether or not eosinophil function is regulated by acetylcholine.

In summary, pretreatment with atropine, a non-selective anticholinergic drug, potentiated vagally mediated hyperreactivity, an effect that was only observed in antigen challenged animals. The ability of atropine to potentiate airway hyperreactivity was dependent on the presence of eosinophils, since Ab IL-5 prevented both vagally mediated hyperreactivity and the concomitant increase in eosinophil activation. However, atropine pretreatment caused airway hyperreactivity through a mechanism independent from eosinophil MBP mediated M_2 receptor dysfunction. Whether this is mediated by muscarinic receptors on eosinophils or on a separate cell will be examined in Chapter V.

FIGURE 3.11. Eosinophils mediate airway hyperreactivity via multiple mechanisms.

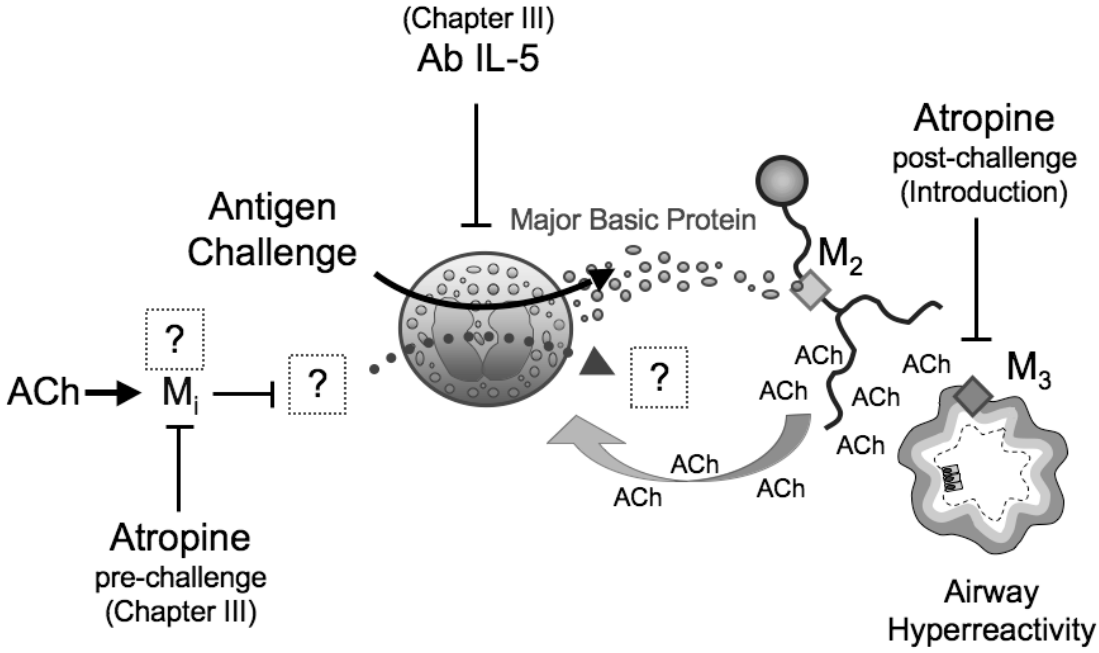


Figure 3.11. Antigen challenge releases eosinophil major basic protein (MBP) onto neuronal M_2 receptors on airway parasympathetic nerves, causing M_2 muscarinic receptor dysfunction, increasing acetylcholine (ACh) release onto airway smooth muscle, and increasing bronchoconstriction. It is known that airway hyperreactivity and neuronal M_2 receptor dysfunction are prevented by depleting eosinophils with antibody to IL-5 (Ab IL-5) or by neutralizing MBP (Ab MBP). Muscarinic blockade with atropine after antigen challenge is known to block airway hyperreactivity (for references see Chapter I). In contrast, muscarinic blockade at the time of antigen challenge increases eosinophil activation and protein release, leading to airway hyperreactivity, an effect that is not mediated by M_2 receptor dysfunction. Depleting eosinophils (Ab IL-5) decreases eosinophil activation and prevents atropine-enhanced antigen-induced airway hyperreactivity (Figure 3.4). This suggests the presence of a novel inhibitory pathway

that is controlled by acetylcholine: Inhibitory muscarinic receptors (MR_i) limit eosinophil activation either directly or indirectly via another cell. Since neuronal M_2 receptor function is not important in atropine-enhanced antigen challenge induced hyperreactivity, this suggests that there may be a separate eosinophil-derived factor (shown as (?)) that is increasing vagally induced bronchoconstriction. The arrows shown in this diagram suggest the order of events, but do not exclude the steps in between.

CHAPTER IV.

ATROPINE-ENHANCED, ANTIGEN CHALLENGE INDUCED HYPERREACTIVITY IN GUINEA PIGS IS MEDIATED BY NERVE GROWTH FACTOR

ABSTRACT

Eosinophils accumulate around airway nerves and cause airway hyperreactivity in antigen challenged guinea pigs. Acutely, anticholinergics inhibit bronchoconstriction in challenged animals and asthmatics, but are less effective in chronic asthma. Atropine, given to sensitized guinea pigs 1h before challenge significantly potentiates airway hyperreactivity and eosinophil activation measured 24h later. Neurotrophins such as nerve growth factor (NGF) are known to play a role in antigen-induced airway hyperreactivity. Since eosinophils produce NGF, I tested whether NGF mediates atropine-enhanced, antigen challenge-induced hyperreactivity. Antibody to NGF (Ab NGF) was administered to sensitized guinea pigs with and without atropine pretreatment (1 mg/kg; iv) 1h before challenge. Twenty-four hours after challenge, animals were anesthetized, vagotomized, paralyzed and ventilated. Electrical stimulation of both vagus nerves caused bronchoconstriction that was increased in challenged animals. Atropine pretreatment potentiated antigen challenge-induced hyperreactivity. Ab NGF did not alter eosinophils or inflammatory cells in any group, nor did it prevent hyperreactivity in challenged animals that did not receive atropine. However, Ab NGF did prevent atropine-enhanced, antigen challenge-induced hyperreactivity and eosinophil activation (assessed by immunohistochemistry). This effect was specific to NGF since animals given IgG remained hyperreactive. Thus, muscarinic blockade during antigen challenge exacerbates airway hyperreactivity via a mechanism involving both eosinophils and NGF. These data suggest that anticholinergics amplify eosinophil interactions with airway nerves via neurotrophins. Therefore, therapeutic strategies that target both eosinophil activation and

neurotrophin mediated inflammatory processes in allergic asthma are likely to be beneficial.

Data in this chapter have been submitted for publication as:

Verbout et al., Atropine-enhanced, antigen challenge-induced hyperreactivity in guinea pigs is mediated by nerve growth factor. [Submitted to: *Am J Physiol Lung Cell Mol Biol,* July 2008].

INTRODUCTION

In asthma, release of acetylcholine from parasympathetic nerves that control airway smooth muscle contraction is increased, leading to airway hyperreactivity (Cropp, 1975). The same is true in animal models of asthma (Drazen *et al.*, 1975; Fryer *et al.*, 1991; Gold *et al.*, 1972; McCaig, 1987). Acetylcholine release is inhibited by neuronal M_2 muscarinic receptors present on parasympathetic nerves and loss of M_2 function is characteristic of asthma in humans (Ayala *et al.*, 1989; Minette *et al.*, 1989) and of airway hyperreactivity in animals (Fryer *et al.*, 1992; Fryer *et al.*, 1991). Blockade of M_3 muscarinic receptors on airway smooth muscle should therefore be beneficial in asthma. However, it has been suggested that anticholinergic therapy may potentiate inflammatory mechanisms in airway disease (Gorski *et al.*, 1993) (Chapter III). Indeed, muscarinic blockade before and during antigen challenge exacerbates airway hyperreactivity measured in guinea pigs twenty-four hours later. This increased airway hyperreactivity is associated with increased eosinophil activation in the airways (Chapter III).

Eosinophilic inflammation of the lungs is a prominent feature of asthma and is associated with airway hyperreactivity in experimental animals. Antigen challenge increases eosinophils in the lungs and around airway nerves of guinea pigs (Costello *et al.*, 1997; Evans *et al.*, 2001)(Chapter III). Following antigen inhalation, activated eosinophils degranulate near airway nerves, releasing major basic protein (MBP), an endogenous and selective antagonist for neuronal M_2 muscarinic receptors (Jacoby *et al.*, 1993), resulting in increased acetylcholine release and airway hyperreactivity (Fryer *et al.*, 1992; Fryer *et al.*, 1991). Inhibition of eosinophil localization to airway nerves or neutralization of MBP

protects against airway hyperreactivity (Elbon *et al.*, 1995; Evans *et al.*, 1997; Evans *et al.*, 2001; Fryer *et al.*, 1997; Fryer *et al.*, 2006).

Atropine-enhanced, antigen challenge-induced hyperreactivity is vagally mediated, but it is not associated with M_2 receptor dysfunction (Chapter III). However, atropine-enhanced, antigen challenge-induced hyperreactivity is dependent on the presence of eosinophils, since eosinophil depletion with Ab IL-5 prevents hyperreactivity and eosinophil activation in the airways (Chapter III). Therefore atropine-enhanced, antigen challenge-induced hyperreactivity is mediated by other eosinophil-derived factors released during degranulation and cytolysis.

Allergic inflammation is associated with increased neurotrophins in the lungs and bronchoalveolar lavage of humans and experimental animals (Braun *et al.*, 1998; de Vries *et al.*, 1999; Undem *et al.*, 1999; Virchow *et al.*, 1998). In particular, nerve growth factor (NGF) is an inflammatory mediator that plays a role in allergic airway disease (Freund-Michel *et al.*, 2008). There are multiple sources of NGF in the lung, including bronchial epithelium, pulmonary fibroblasts, bronchial smooth muscle and several inflammatory cells, including eosinophils (Solomon *et al.*, 1998). NGF expression is increased in eosinophils isolated from allergic patients (Noga *et al.*, 2003; Noga *et al.*, 2005) and NGF concentration is positively correlated with allergic disease severity, airway hyperreactivity and eosinophil mediator release (Nassenstein *et al.*, 2005). NGF is known to mediate airway hyperreactivity by increasing substance P expression in sensory nerves (de Vries *et al.*, 2006; Undem *et al.*, 1999). This mechanism may also occur in

parasympathetic nerves; NGF has been demonstrated to induce substance P expression in parasympathetic airway nerves in guinea pigs (Hazari *et al.*, 2007) and ferrets (Wu *et al.*, 2006). Since eosinophils are a source of NGF and are located in close proximity to airway nerves, I tested whether NGF plays a role in eosinophil-mediated atropine-enhanced, antigen challenge-induced hyperreactivity.

EXPERIMENTAL DESIGN

Sensitization and challenge with antigen

Guinea pigs were sensitized to ovalbumin (10 mg/kg, ip) every other day for a total of three injections. Three weeks after the last ovalbumin injection, guinea pigs were pretreated with an antihistamine, pyrilamine (0.5 mg/kg, ip; Sigma-Aldrich) one hour before challenge to protect against acute anaphylaxis (Chapter II, pg 81) . Animals were then exposed to aerosolized ovalbumin (2.5%) for 5 minutes or until signs of respiratory distress appeared, in which case antigen challenge was immediately halted.

Treatments

Guinea pigs were pretreated with atropine (1 mg/kg, ip), a non-selective muscarinic antagonist, or saline one hour prior to antigen challenge and again 6 hours after antigen challenge. In some animals, either a goat IgG antibody specific to recombinant human ß-nerve growth factor (10 ug/kg, ip; R & D Systems) or negative control Normal Goat IgG (10 ug/kg, ip; R & D Systems) was administered one hour before antigen challenge.

Measurements

Twenty-four hours after antigen challenge, pulmonary inflation pressure was measured as described in the methods. Vagal reactivity, neuronal M_2 receptor function, peripheral leukocytes and bronchoalveolar lavage was measured as described in the methods. Histological evaluation of eosinophil influx into the airway and major basic protein deposition and distribution were measured as described in the methods. Data analysis and statistics were conducted as described in the methods.

Statistics

All data are expressed as mean +/-SEM. In vivo responses to nerve stimulation and gallamine were analyzed using 2-way ANOVA for repeated measures. Data sets were compared as follows: by group (control versus challenge), by drug (saline versus atropine) or antibody pretreatment (no Ab vs Ab NGF). Baseline heart rates, blood pressures, pulmonary inflation pressures, bronchoalveolar lavage and blood leukocytes were analyzed for statistical significance by 1-way ANOVA. Histological examination for tissue eosinophils and major basic protein content in the airways were analyzed by 1-way ANOVA with Fisher's LSD post-hoc correction. For comparisons of multiple data sets (> 3 groups), a Bonferroni correction was applied. A p-value of less than 0.05 was considered significant. In all figures, only comparisons that were statistically significant are indicated with symbols; all other comparisons did not have statistical significance and are not indicated with symbols.

RESULTS

Treatments. All sensitized guinea pigs exhibited symptoms of acute respiratory distress with acute antigen challenge. While all challenged animals were pretreated with antihistamine (pyrilamine: 0.5 mg/kg; ip) one hour before antigen challenge and given isoproterenol (1 mg/kg, ip) immediately following challenge, twenty-eight out of forty-nine animals died within one hour after antigen challenge. This is unusually high for the laboratory and mortality was significantly greater in the animals treated with atropine (69.6%*; significantly different from saline pretreatment) or Ab NGF (63.6%) compared to challenge with saline pretreatment (28.6%) and challenge with both atropine and Ab NGF (37.5%). Non-sensitized control guinea pigs that received pyrilamine or isoproterenol 24 hours previously were not different from untreated control animals in any parameter tested.

Pulmonary baselines. Twenty-four hours after antigen challenge, neither resting pulmonary inflation pressure, nor any other parameter measured was different among any group when physiological measurements were made (Table 4.1).

Airway hyperreactivity. In non-sensitized non-challenged control animals, electrical stimulation of both vagus nerves increased bronchoconstriction in a frequency-dependent manner (open circles, Figure 4.1A) that was not changed by Ab NGF given 24 hours previously (open squares, Figure 4.1B). Since atropine pretreatment does not alter vagally induced bronchoconstriction in control guinea pigs (Chapter III), this group was not included in this study. Antigen challenge of guinea pigs significantly increased vagally

induced bronchoconstriction, which was further potentiated by atropine pretreatment (Figure 4.1A).

Atropine-enhanced, antigen challenge-induced hyperreactivity was prevented in animals that received Ab NGF prior to challenge (gray squares, Figure 4.1B). Pretreatment with IgG did not prevent atropine-enhanced, antigen challenge-induced hyperreactivity (at 25 Hz maximum bronchoconstriction was 350±-30; n=3). In contrast, Ab NGF alone did not prevent airway hyperreactivity in challenged animals (black squares, Figure 4.1B). Thus, atropine-enhanced, antigen challenge-induced hyperreactivity in antigen challenged guinea pigs is mediated by NGF.

Bradycardia. Electrical stimulation of both vagus nerves caused frequency dependent bradycardia that was not altered by antigen challenge or by atropine pretreatment (Figure 4.2A). Antibody to NGF did not affect bradycardia among any groups (Figure 4.2B). Thus, vagal hyperreactivity following antigen challenge is limited to the airways and does not extend to the heart.

M_2 receptor function. Gallamine, an M_2 selective antagonist, potentiated vagally induced bronchoconstriction in control guinea pigs in a dose-dependent manner, demonstrating functional M_2 receptors (open circles, Figure 4.3A). In antigen challenged animals, vagally induced bronchoconstriction was not potentiated by gallamine, demonstrating that M_2 receptors were dysfunctional (black circles). By contrast, M_2 receptor dysfunction appeared to be partially protected in atropine pretreated animals,

suggesting that atropine-enhanced, antigen challenge-induced hyperreactivity is not mediated by neuronal M_2 receptor dysfunction. Pretreatment with Ab NGF did not significantly affect the ability of gallamine to potentiate vagally induced bronchoconstriction in challenged animals with or without atropine pretreatment. In contrast, the ability of gallamine to potentiate vagally induced bronchoconstriction in control animals given Ab NGF was modestly decreased; however, this effect was not statistically significant (Figure 4.3B).

Inflammation. There were no differences among leukocyte populations in the blood of control, challenged or challenged plus atropine-pretreated guinea pigs regardless of Ab NGF pretreatment (Figure 4.4). Neither were there were any differences in bronchoalveolar lavage cells among control, challenged or challenged plus atropine-pretreated guinea pigs regardless of Ab NGF pretreatment (Figure 4.5).

Histological staining revealed that eosinophils in airways and around nerves were increased by antigen challenge (Figure 4.6 and 4.7). Atropine pretreatment decreased total eosinophils in the airways (Figure 4.7A) and around nerves (Figure 4.7B) in antigen challenged guinea pigs. Antibody to NGF did not significantly alter antigen-induced increase in eosinophils (black bars) or the atropine mediated decrease in antigen-induced eosinophilia (gray bars).

Major basic protein deposition. Atropine pretreatment increases eosinophil MBP deposition in airways of challenged guinea pigs. In contrast, Ab NGF decreased MBP in atropine pretreated animals (Figure 4.8).

TABLE 4.1. Baseline cardiovascular and pulmonary parameters.

Treatment	Group	n	Heart Rate (beats/minute)	Blood Pressure (mm Hg) Systolic	Diastolic	Pulmonary Inflation Pressure (mm H_2O)
- Ab NGF	Control	6	299 ± 5	39 ± 3	21 ± 2	128 ± 9
	Challenge	5	295 ± 8	46 ± 2	25 ± 2	136 ± 10
	Challenge + Atropine	5	293 ± 9	44 ± 3	19 ± 1	140 ± 10
+Ab NGF	Control	5	284 ± 5	38 ± 2	22 ± 3	129 ± 14
	Challenge	4	291 ± 9	43 ± 2	24 ± 4	153 ± 33
	Challenge + Atropine	5	293 ± 4	45 ± 3	22 ± 1	135 ± 5

Table 4.1. Pretreatment with atropine, antibody to nerve growth factor (Ab NGF) or both in combination did not change resting heart rate or systolic blood pressure among groups 24 hours after antigen challenge. Values are the mean +/-SEM.

FIGURE 4.1. Atropine-enhanced, antigen challenge-induced hyperreactivity is reversed by pretreatment with antibody to nerve growth factor.

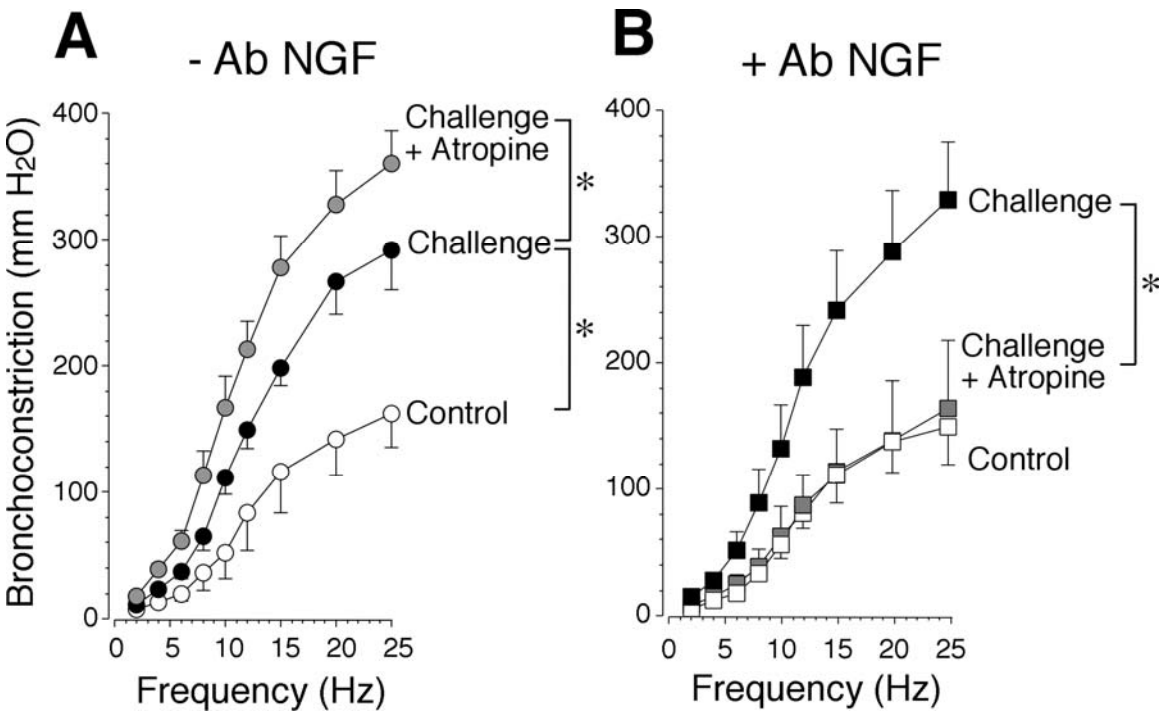


Figure 4.1. In non-sensitized non-challenged control animals, electrical stimulation of both vagus nerves increased bronchoconstriction (measured as an increase in pulmonary inflation pressure) in a frequency-dependent manner (white circles, A) that was not changed by Ab NGF given 24 hours previously (white squares, B). Antigen challenge of guinea pigs significantly increased vagally induced bronchoconstriction, which was further potentiated by atropine pretreatment (A). Atropine-enhanced, antigen challenge-induced hyperreactivity was prevented in animals that received Ab NGF prior to challenge (gray squares, B). Pretreatment with IgG did not prevent atropine-enhanced, antigen challenge-induced hyperreactivity (at 25 Hz maximum bronchoconstriction was 350±-30; n=3). In contrast, Ab NGF alone did not prevent airway hyperreactivity in challenged animals (black squares, B). *The entire dose response is significantly different

from comparison dose response. Data are expressed as the mean +/- SEM, n=4-6.

FIGURE 4.2. Neither atropine nor antibody to nerve growth factor altered vagally induced bradycardia.

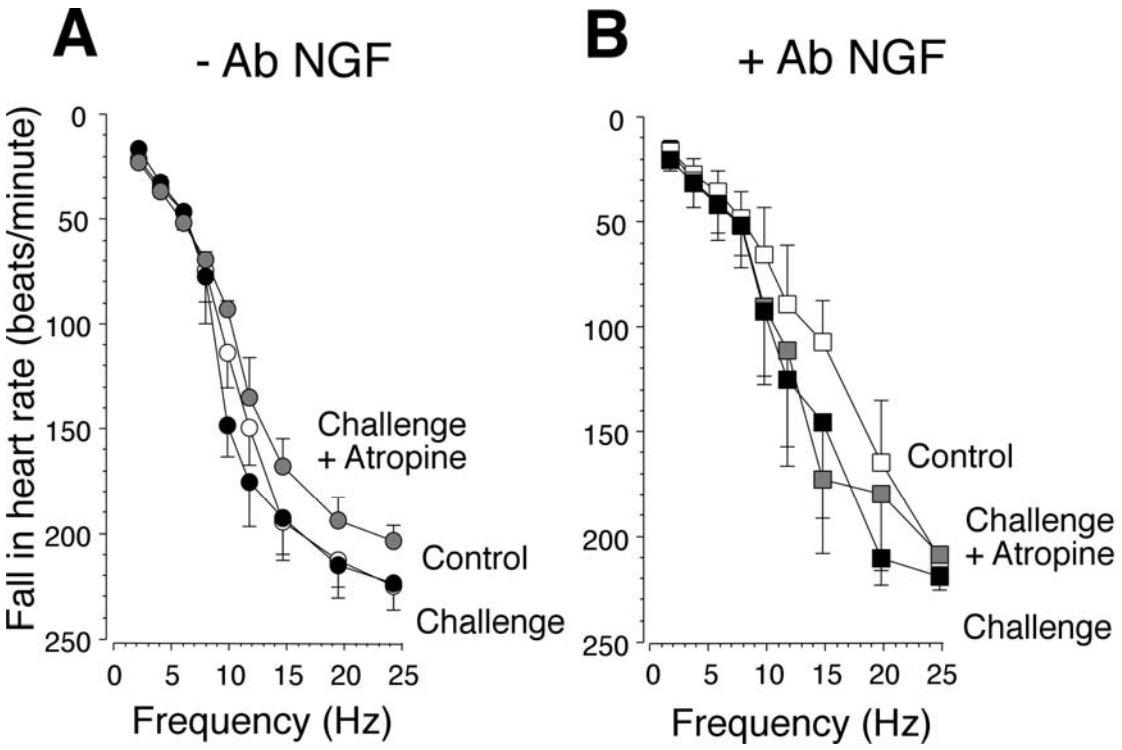


Figure 4.2. Electrical stimulation of both vagus nerves caused frequency dependent bradycardia (measured in beats per minute) (A, white circles) that was not changed by antigen challenge (black circles) or by atropine pretreatment (gray circles). Vagally induced bradycardia was not significantly altered by pretreatment with Ab NGF in control (B, white squares) challenged (black squares) or in challenged plus atropine treated guinea pigs (gray squares). Data are expressed as the mean +/- SEM, n=4-6.

FIGURE 4.3. Neuronal M_2 receptor dysfunction was prevented by atropine pretreatment in antigen challenged guinea pigs.

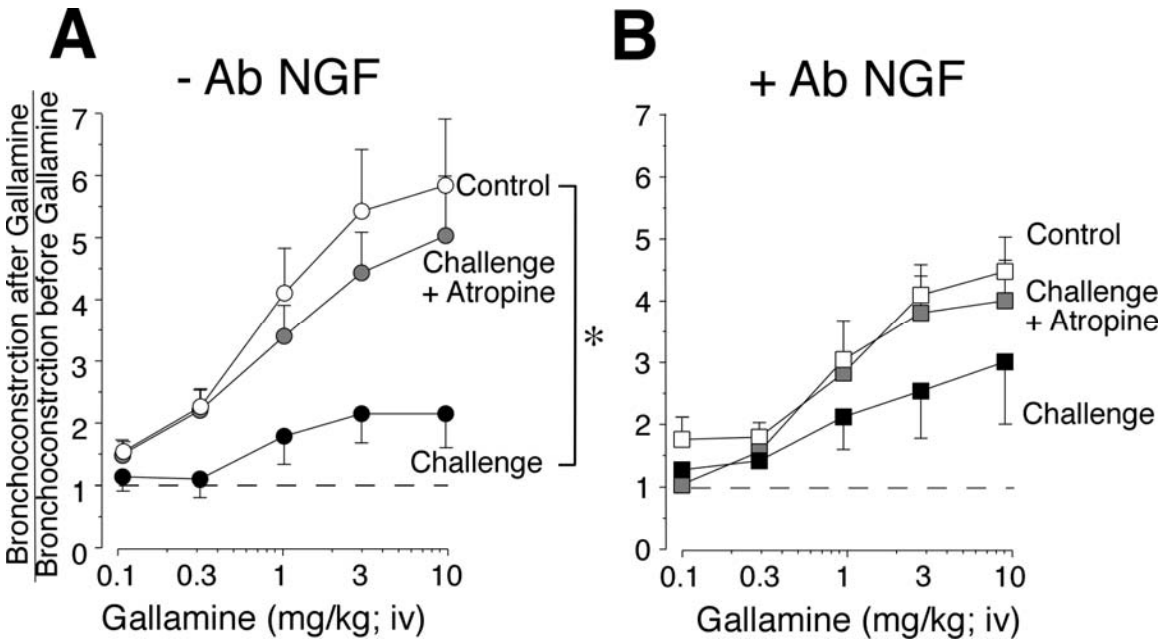


Figure 4.3. Gallamine, an M_2 selective antagonist, potentiated vagally induced bronchoconstriction in control guinea pigs in a dose-dependent manner, demonstrating functional M_2 receptors (white circles, A). In antigen challenged animals, vagally induced bronchoconstriction was not potentiated by gallamine, demonstrating that M_2 muscarinic receptors were dysfunctional (black circles). By contrast, neuronal M_2 receptor function appeared to be partially protected by atropine pretreatment in challenged animals (gray circles) and was not significantly different from control animals, suggesting that atropine-enhanced, antigen challenge-induced hyperreactivity is not mediated by neuronal M_2 receptor dysfunction. Pretreatment with Ab NGF did not significantly affect the ability of gallamine to potentiate vagally induced bronchoconstriction in challenged animals with or without atropine pretreatment. In contrast, the ability of gallamine to potentiate vagally induced bronchoconstriction in control animals given Ab NGF was modestly decreased;

however, this effect was not statistically significant (B). *The entire dose response is significantly different from dose response in controls. Data are expressed as the ratio of vagally induced bronchoconstriction in the presence of gallamine to vagally induced bronchoconstriction in the absence of gallamine and represent the mean +/-SEM, n=4-8.

FIGURE 4.4. Neither atropine pretreatment nor antibody to nerve growth factor changed circulating leukocytes.

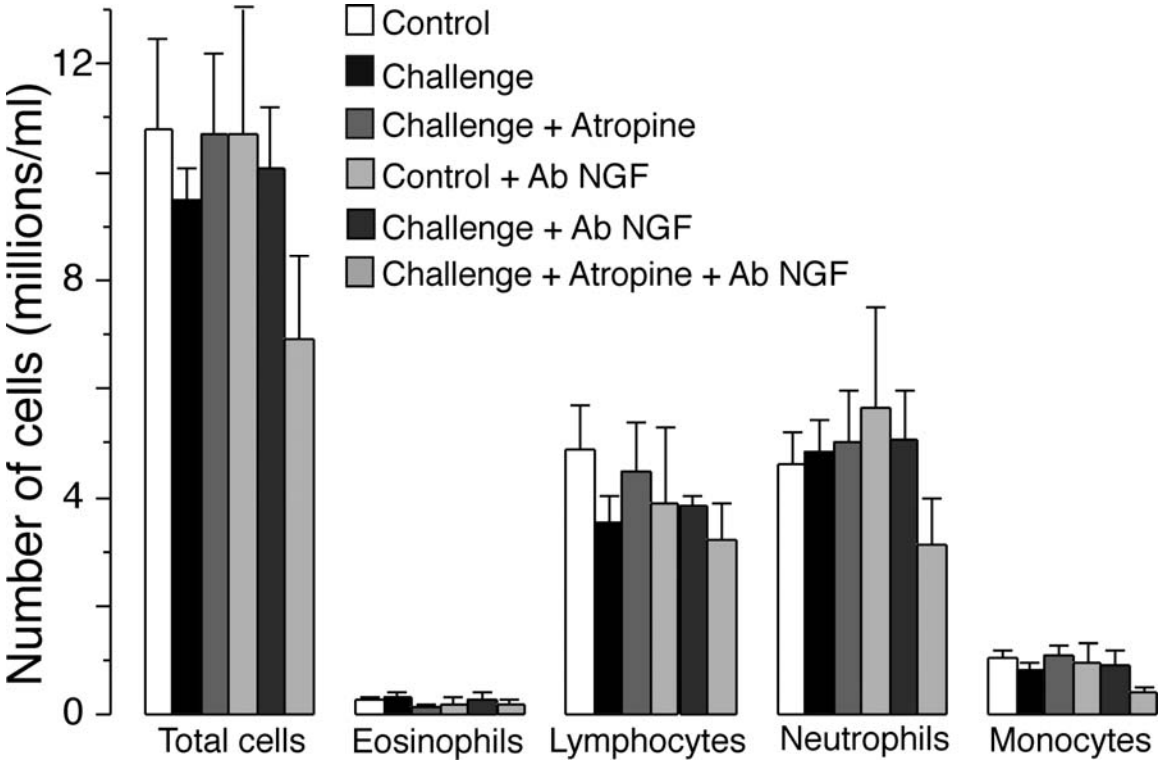


Figure 4.4. There were no significant differences in circulating leukocyte populations among control, challenged or challenged plus atropine-pretreated guinea pigs regardless of Ab NGF pretreatment. Data are expressed as the mean +/-SEM, n=4-6.

FIGURE 4.5. Neither atropine pretreatment nor antibody to nerve growth factor changed bronchoalveolar lavage leukocytes.

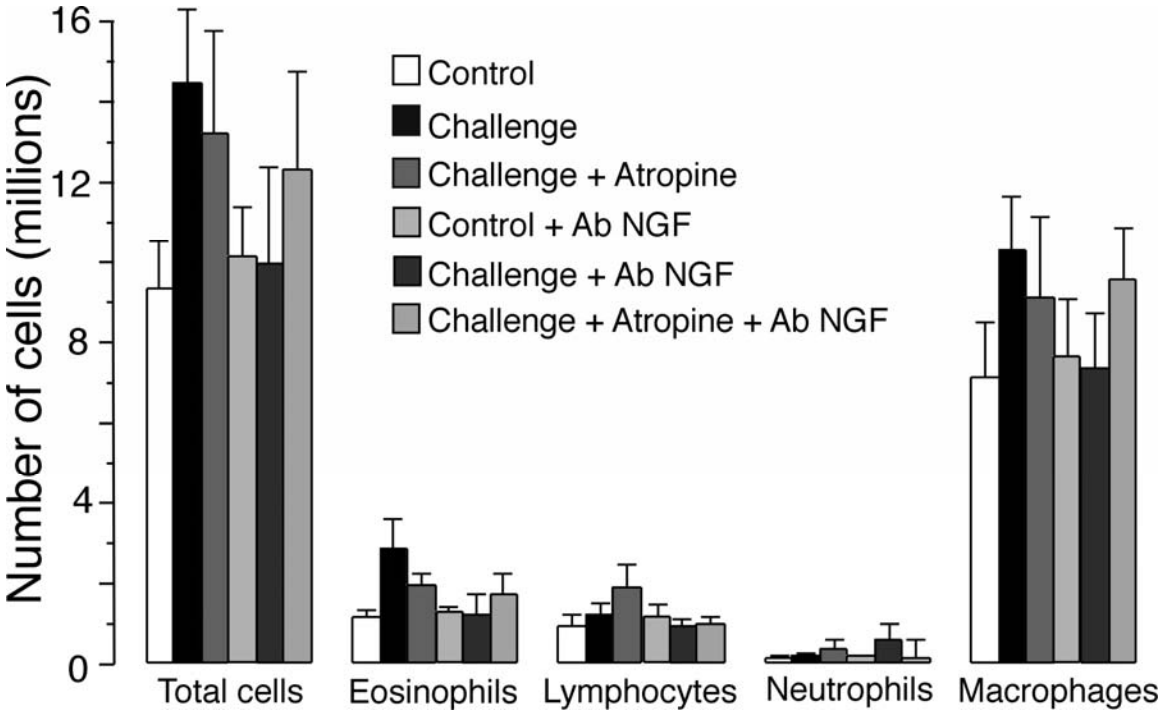


Figure 4.5. There were no significant differences in bronchoalveolar lavage leukocyte populations among control, challenged or challenged plus atropine-pretreated guinea pigs regardless of Ab NGF pretreatment. Data are expressed as the mean +/-SEM, n=4-6.

FIGURE 4.6. Atropine pretreatment decreased eosinophils in airways of antigen challenged animals.

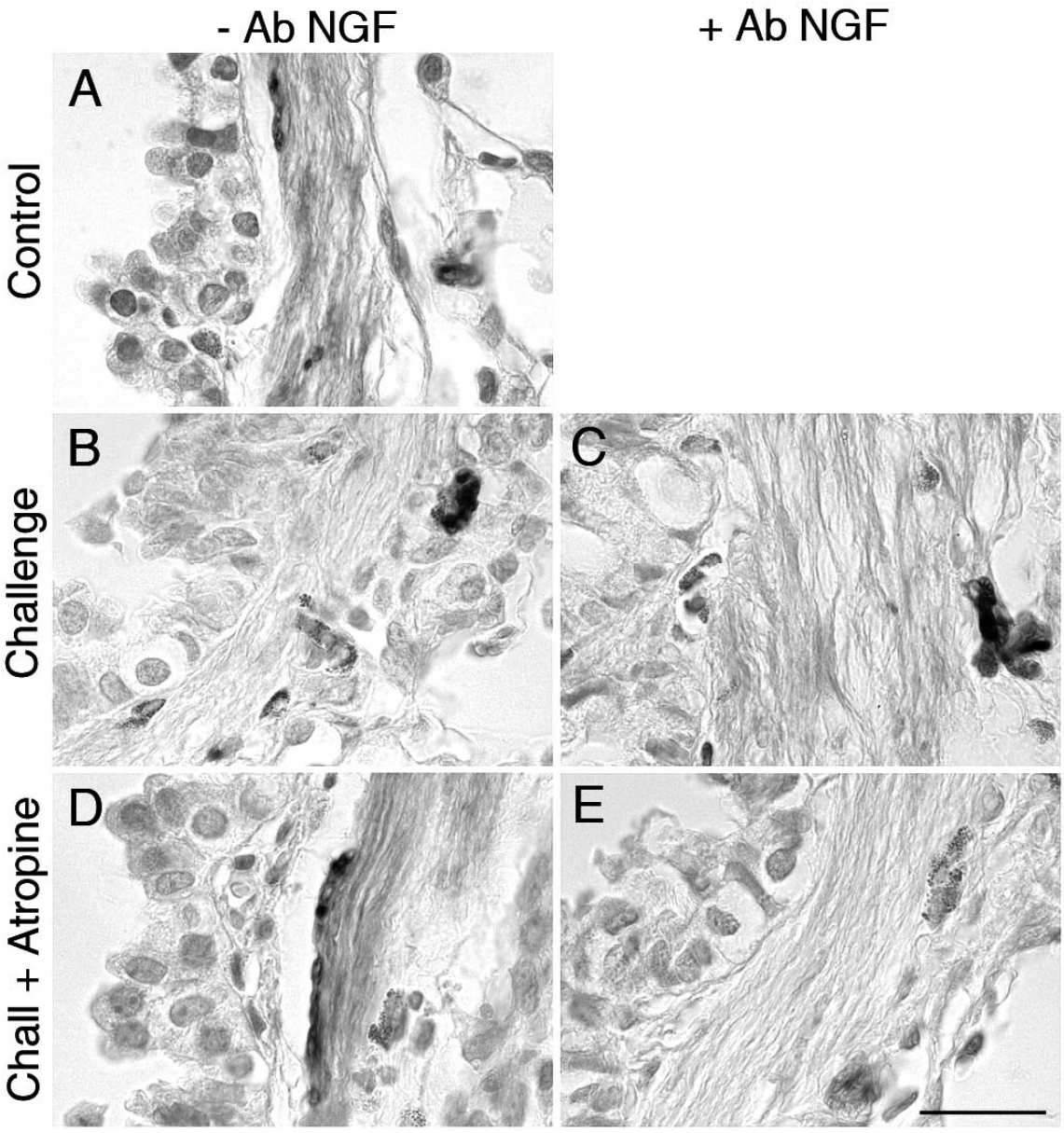


Figure 4.6. Shown are cross sections of guinea pig bronchi. Nerves were labeled with antibody to PGP 9.5 (black) and eosinophils counterstained with Chromotrope 2R (red). There were few eosinophils in lungs or around nerves in control guinea pigs (A). Following antigen challenge, eosinophils were present within smooth muscle and in close

proximity to nerves (B), which was not altered by pretreatment with antibody to nerve growth factor (C). In contrast, animals treated with atropine before antigen challenge had fewer eosinophils within the lungs or in close proximity to nerves (D), which was not changed by addition of antibody to nerve growth factor. Photos are representative of an n=4-5 per group; scale bar is 50 um.

FIGURE 4.7. Atropine pretreatment decreased total eosinophils in the lungs and around nerves of antigen challenged guinea pigs.

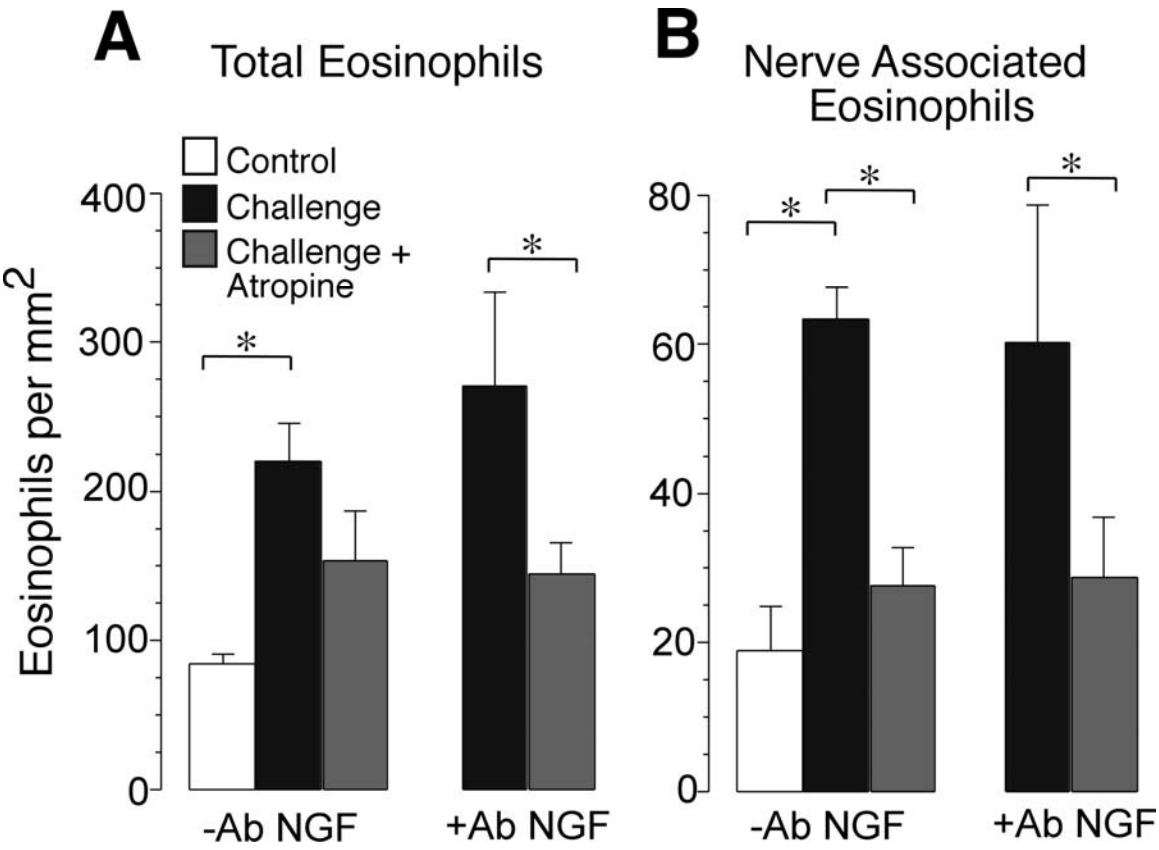


Figure 4.7. Antigen challenge (black bars) significantly increased eosinophils in lungs (A) and around nerves (B) compared to control (white bars). Pretreatment with atropine (gray bars) decreased the antigen induced increase in eosinophils in airways (A) and especially around nerves (B). Antibody to NGF did not significantly alter antigen-induced increase in eosinophils or the atropine mediated decrease in antigen-induced eosinophilia. Data are expressed as the number of eosinophils per mm^2 of airway smooth muscle (A) and the number of eosinophils within eight um of an airway nerve per mm^2 (B). *Significantly different. Data are expressed as the mean +/-SEM, n=4-5.

FIGURE 4.8. Antibody to nerve growth factor decreased eosinophil major basic protein deposition in challenged animals pretreated with atropine.

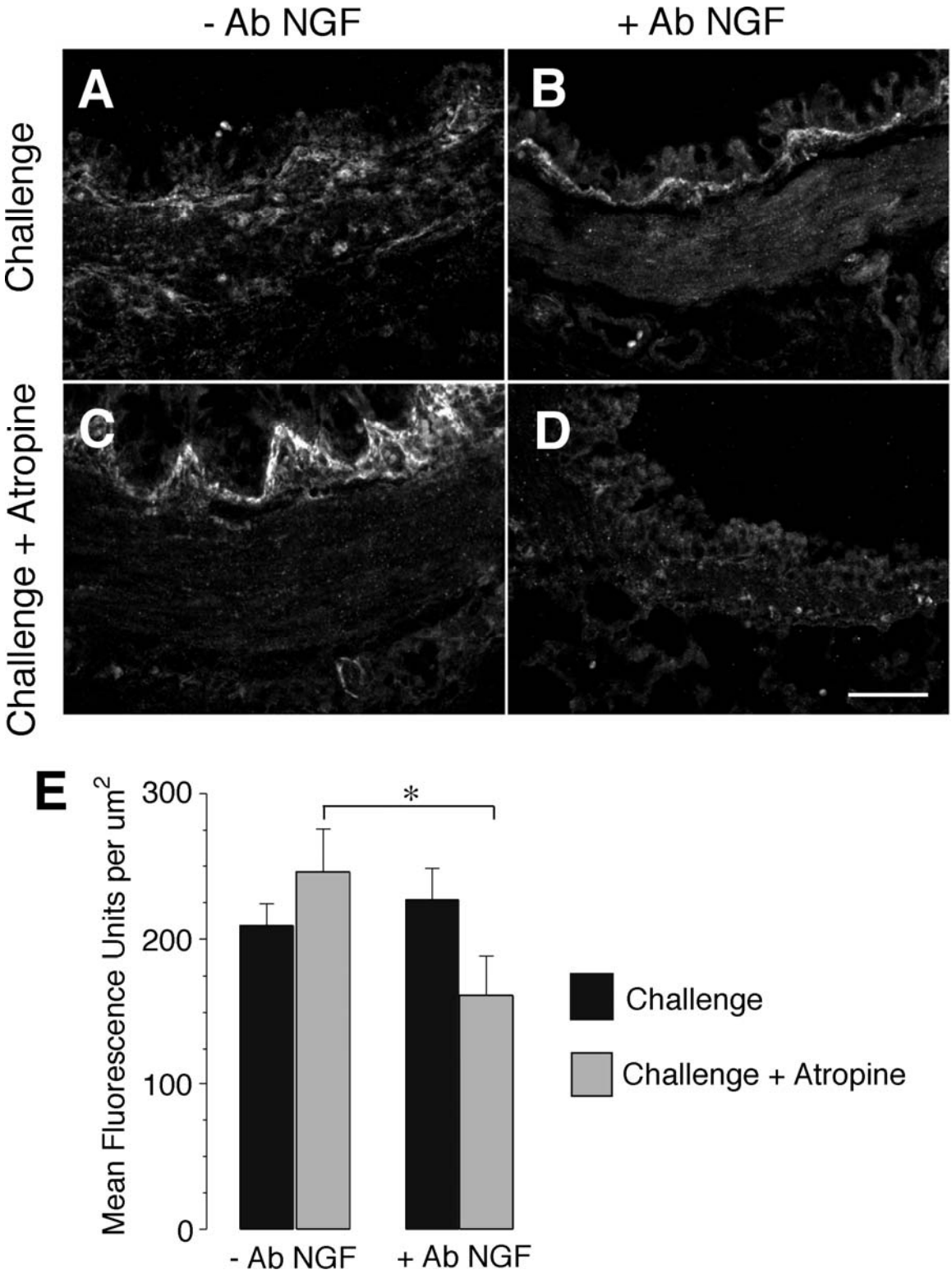


Figure 4.8. Eosinophil major basic protein (MBP) deposition within airways of challenged guinea pigs was labeled using antibody to MBP and fluorescence intensity was evaluated in gray scale. Shown are representative photographs of airways from challenged animals (A) that were pretreated with either Ab NGF (B), atropine (C) or both (D). Quantification (E) of MBP deposition in airways of challenged animals demonstrated that pretreatment with Ab NGF did not decrease MBP deposition in challenged guinea pigs (black bars). In contrast, Ab NGF decreased MBP deposition in atropine pretreated animals (gray bars). Data (E) are expressed as mean fluorescence intensity in Units/um^2, and are the mean +/-SEM, n=3-5, with replicates of five airways per animal. *Significantly different; scale bar 50 um.

DISCUSSION

Pretreatment with Ab NGF did not significantly alter any baseline parameter (Table 4.1; see Table 2.1 in Chapter II for normal physiological values in conscious guinea pigs). However, blocking NGF prior to antigen challenge completely prevented atropine-enhanced, antigen challenge-induced hyperreactivity (Figure 4.1). This protective effect was specific to blocking NGF, since guinea pigs that received non-specific IgG remained hyperreactive. In contrast, Ab NGF did not prevent hyperreactivity in antigen challenged animals that were not treated with atropine. Thus, atropine-enhanced, antigen challenge-induced hyperreactivity is mediated by NGF.

Antigen challenge caused acute bronchoconstriction and death in twenty-eight out of forty-nine animals. It may be important that atropine pretreatment doubled mortality in antigen challenged guinea pigs (28.6% to 69.6%), which coincided with potentiation in airway hyperreactivity and increased eosinophil activation 24 hours later. In animals given both Ab NGF and atropine prior to challenge, mortality was reduced (69.6% to 37.8%), which likewise coincided with protection from airway hyperreactivity and decreased eosinophil activation. Thus, mortality risk mirrors the physiologic response 24 hours later.

Antigen induced airway hyperreactivity is mediated by loss of neuronal M_2 receptor function (Elbon *et al.*, 1995; Evans *et al.*, 1997; Fryer *et al.*, 1992; Fryer *et al.*, 1984; Fryer *et al.*, 1991). However, as previously demonstrated (Chapter III), atropine-enhanced, antigen challenge-induced hyperreactivity was not associated with neuronal

M_2 receptor dysfunction, whereas non-atropine treated animals did have dysfunctional M_2 muscarinic receptors (Figure 4.3). Thus, atropine-enhanced, antigen challenge-induced hyperreactivity occurs independent of M_2 muscarinic receptor function and neuronal M_2 function/dysfunction does not appear to be significantly altered by Ab NGF. These data support the previous observation that atropine-enhanced, antigen challenge-induced hyperreactivity occurs independent of M_2 muscarinic receptor function, and reveal a potential alternate mechanism involving NGF.

Antigen challenge increases eosinophil activation in the lung (Evans *et al.*, 1997; Gundel *et al.*, 1992). As previously described (Chapter III), atropine pretreatment further increases eosinophil activation measured by increased major basic protein deposition in the lungs (Figure 4.8) coupled with loss of intact eosinophils in airway tissues (Figure 4.6 and 4.7) and in airway bronchoalveolar lavage (Figure 4.5). Blocking NGF did not significantly affect eosinophil numbers in the peripheral blood (Figure 4.4), airways (Figure 4.6 and 4.7), or in bronchoalveolar lavage (Figure 4.5) in any group tested. Neither did Ab NGF affect eosinophil activation in airways of challenged guinea pigs that did not receive atropine (Figure 4.8E), consistent with Ab NGF having no protective effect on airway hyperreactivity (Figure 4.1B) or neuronal M_2 receptor function (Figure 4.3B). In contrast, Ab NGF significantly reduced MBP deposition in atropine pretreated antigen challenged guinea pigs, coincident with protection from antigen-induced airway hyperreactivity. Ab NGF did not appear to affect any inflammatory cell population in blood or bronchoalveolar lavage. Thus, atropine-enhanced, antigen challenge-induced

hyperreactivity and concomitant increased eosinophil activation in airways are mediated by NGF.

The findings presented here differ from a study demonstrating that inhibiting NGF with a TrkA antagonist prevents airway hyperreactivity in antigen challenge guinea pigs (de Vries *et al.*, 2006). One possible explanation for this difference may be the method employed to inhibit NGF. There are two NGF receptors: the higher affinity tropomyosin-related kinase A (TrkA) receptor and the lower affinity receptor p75 (p75NTR). The relative contribution of these two receptor types to asthma is not completely clear, although airway hyperreactivity has been associated with either receptor, depending on the study (Freund-Michel *et al.*, 2008; Kerzel *et al.*, 2003; Tokuoka *et al.*, 2001). It may also be significant that TrkA and p75NTR are expressed by both eosinophils (Nassenstein *et al.*, 2003) and parasympathetic nerves (Hazari *et al.*, 2007); either of which are potential targets for NGF. In this study, I inhibited NGF with a neutralizing antibody, thereby preventing NGF interactions with both TrkA and p75NTR receptors. This difference may explain why my results differ from those of de Vries et al.; it may be that inhibiting TrkA, but not p75NTR (de Vries *et al.*, 2006) prevents antigen induced airway hyperreactivity, whereas blocking NGF interactions with both receptors has an entirely different effect.

Another important difference between this study and that of de Vries et al. is the method by which hyperreactivity was measured. In this study, bronchoconstriction in response to electrical stimulation of the vagus nerves was measured in vagotomized, paralyzed and

ventilated guinea pigs. Thus, the experiments described here examine airway smooth muscle contraction that is mediated by the parasympathetic nerves. By contrast, de Vries et al. measured smooth muscle contraction in response to intravenous histamine and methacholine in non-vagotomized, spontaneously breathing guinea pigs. It is known that histamine (Costello *et al.,* 1999) and methacholine (Wagner *et al.,* 1999) induce reflex bronchoconstriction via the vagus nerves. It is therefore reasonable to assume that de Vries et al. were measuring reflex bronchoconstriction mediated by sensory nerves and parasympathetic nerves, whereas the measurements described here reflect hyperreactivity mediated by parasympathetic nerves. By subtraction, de Vries et al. measured TrkA mediated increases in either sensory nerve sensitivity or neuropeptide release, whereas my data measure changes in parasympathetic nerves that are NGF independent (in the absence of atropine); neither of which are mutually exclusive.

The data presented here demonstrate that blockade of muscarinic receptors during antigen challenge increases eosinophil activation and potentiates airway hyperreactivity via a mechanism involving NGF. Although NGF is a key effector in this pathway, it is not clear which targets are affected by Ab NGF. The data presented here strongly suggest that eosinophils are a key candidate. However, I cannot rule out the possibility that NGF affects other cells, in particular parasympathetic nerves. Thus, NGF may be affecting eosinophils, parasympathetic nerves or possibly both.

The mechanism by which NGF influences airway hyperreactivity in this model is not known. One possible explanation is that NGF directly regulates eosinophil activation.

This is supported by in vitro studies demonstrating that NGF receptors on eosinophils promote survival (Nassenstein *et al.*, 2003), chemotaxis, activation and degranulation (Solomon *et al.*, 1998). In vivo, the number of eosinophils in bronchoalveolar lavage is higher after antigen challenge in transgenic mice overexpressing NGF in the lung compared to wild-type mice (Path *et al.*, 2002). The data presented here support a model in which NGF acts upstream of eosinophils, since blocking NGF reduced eosinophil activation in airways of atropine-pretreated animals (Figure 4.8). It is also possible that NGF acts via an autocrine mechanism since eosinophils themselves produce NGF (Solomon *et al.*, 1998). Thus, it may be that in the presence of atropine, NGF induces eosinophil release of a neuromediator that alters nerve function (Figure 4.9). However, I cannot exclude the possibility that NGF may indirectly affect eosinophil activation via another cell type not identified within this study. Numerous cells within the lungs express NGF receptors, including neuronal cells, inflammatory cells, fibroblasts and smooth muscle cells (Freund-Michel *et al.*, 2008), any of which may be part of this pathway.

It is not clear from these data how NGF and atropine are interacting in this model. One possibility is that in vivo, muscarinic agonists cooperate with NGF to stabilize eosinophils and limit eosinophil degranulation, since in PC12 cells, cell survival is increased when muscarinic agonists are combined with NGF (Wu *et al.*, 2006). If cell survival could be translated to decreased eosinophil degranulation, then a similar interaction may explain these data. For example, if muscarinic agonists inhibit NGF induced eosinophil activation, then in the absence of muscarinic antagonists, Ab NGF is ineffective, which is consistent with the data presented here (Figure 4.8). Thus, it may be

that in vivo, NGF dependent eosinophil activation is inhibited by muscarinic agonists. There are multiple sources of acetylcholine in the lung: parasympathetic nerves, bronchial epithelium, endothelium, smooth muscle, pulmonary fibroblasts and inflammatory cells (Gwilt *et al.*, 2007; Racke *et al.*, 2004). Whether eosinophil activation is inhibited by acetylcholine is not yet known.

A second possibility is that NGF directly affects parasympathetic nerve function. It is known that NGF plays a role in antigen-induced airway hyperreactivity (Freund-Michel *et al.*, 2008). NGF is increased in bronchoalveolar lavage fluid of allergic asthmatics (Undem *et al.*, 1999; Virchow *et al.*, 1998) and in eosinophils from allergic humans (Noga *et al.*, 2003; Noga *et al.*, 2005). Overexpression of NGF in transgenic mice causes sensory hyperinnervation (Hoyle *et al.*, 1998) and in vivo, NGF potentiates airway hyperreactivity in guinea pigs (de Vries *et al.*, 1999) and mice (Braun *et al.*, 1998), which has been associated with a process termed neural plasticity (de Vries *et al.*, 2006; Hazari *et al.*, 2007; Undem *et al.*, 1999).

Neural plasticity is a phenomenon characterized by changes in nerve structure and function in response to stimuli, such as inflammation. Neural plasticity has been studied in relation to pain (Woolf *et al.*, 2000) and airway hyperreactivity (Bonham *et al.*, 2006; Dey, 2003; Jacoby, 2003). Alterations in airway nerve function can be rapid and reversible, for example, increased excitability after exposure of neurons to NGF (Hazari *et al.*, 2007). Additionally, NGF has been demonstrated to have longer lasting effects on

nerve function, including increased expression of neuropeptides (Lindsay *et al.*, 1989; Skoff *et al.*, 2006) and nonspecific cation channels (Chuang *et al.*, 2001; Ji *et al.*, 2002).

The role of nerve plasticity in development of airway hyperreactivity has been well studied in sensory nerves. NGF is known to mediate airway hyperreactivity by increasing substance P expression in sensory nerves in guinea pigs and mice (de Vries *et al.*, 2006; de Vries *et al.*, 2001; Quarcoo *et al.*, 2004; Undem *et al.*, 1999), an effect that is blocked by TrkA receptor antagonists (de Vries *et al.*, 2006) and neurokinin (NK)-1 receptor antagonists (de Vries *et al.*, 1999; Quarcoo *et al.*, 2004) (the substance P receptor), strongly suggesting the involvement of tachykinins. This mechanism may also occur in parasympathetic nerves; NGF has been demonstrated to induce substance P expression in parasympathetic airway nerves in guinea pigs (Hazari *et al.*, 2007) and ferrets (Wu *et al.*, 2006). It may be that parasympathetic nerves also undergo phenotypic switching similar to sensory nerves and produce tachykinins. Thus, the mechanism of atropine-enhanced, antigen challenge-induced hyperreactivity that is mediated by NGF may involve induction of substance P expression and neural plasticity, as has been described in antigen challenged animals (Costello *et al.*, 1998; Undem *et al.*, 1999) and asthmatics (Nieber *et al.*, 1992).

Alternatively, it may be that eosinophil-derived NGF enhances the cholinergic phenotype. In culture, eosinophils promote acetylcholine synthesis in human cholinergic neuroblastoma cells by increasing expression of choline acetyltransferase and vesicular acetylcholine transferase within 24 hours (Durcan *et al.*, 2006). It is known that NGF has

both rapid and long-term effects on increasing acetylcholine production in forebrain cholinergic neurons. Acutely, NGF increases acetylcholine release in forebrain cholinergic neurons within one hour (Auld *et al.*, 2001b), but also increases choline acetyltransferase activity, acetylcholine content and both constituitive and stimulated acetylcholine release following 96 hours treatment (Auld *et al.*, 2001a). In addition, NGF increases cholinergic innervation and contractile response to electric field stimulation in murine trachea after four days treatment (Bachar *et al.*, 2004). Thus, atropine-enhanced, antigen challenge-induced hyperreactivity that is mediated by NGF may result from eosinophil-derived NGF increasing acetylcholine release and thus increasing vagally mediated airway hyperreactivity.

These data demonstrate that cholinergic blockade during antigen challenge significantly affects airway nerve function through a mechanism involving eosinophils and NGF. Thus, I have identified a second pathway by which eosinophils cause vagally mediated airway hyperreactivity in antigen challenged guinea pigs. It is well known that antigen challenge releases eosinophil MBP onto airway nerves, causing neuronal M_2 receptor dysfunction, resulting in airway hyperreactivity (Elbon *et al.*, 1995; Evans *et al.*, 1997; Evans *et al.*, 2001; Fryer *et al.*, 1991; Jacoby *et al.*, 1993) (Figure 4.9). Both airway hyperreactivity and neuronal M_2 receptor dysfunction are prevented by depleting eosinophils with antibody to IL-5 (Elbon *et al.*, 1995) or by neutralizing MBP (Evans *et al.*, 1997). NGF does not play a role in this pathway since Ab NGF does not prevent antigen-induced airway hyperreactivity in the absence of atropine. In contrast, NGF is important when muscarinic receptors are blocked.

Muscarinic blockade with atropine at the time of antigen challenge increases eosinophil activation and protein release, leading to airway hyperreactivity that is not mediated by neuronal M_2 receptor dysfunction (Chapter III). Depleting eosinophils (Chapter III), or inhibiting NGF decreases eosinophil activation and prevents atropine-enhanced antigen-induced airway hyperreactivity. One possibility for how this may occur involves the presence of a negative regulatory pathway that is controlled by acetylcholine. In this pathway, inhibitory muscarinic receptors (M_i) inhibit release of an eosinophil-derived neuromediator (Figure 4.9). This eosinophil-derived neuromediator, which may be NGF or another factor (see Table 1.2), induces a change in parasympathetic nerve activity leading to hyperreactivity through a mechanism that does not involve neuronal M_2 receptor dysfunction.

There is growing evidence that NGF and other neurotrophins have significant roles in development of allergic asthma and other chronic inflammatory diseases, such as inflammatory bowel disease, atopic dermatitis and allergic rhinitis (di Mola *et al.*, 2000; Dou *et al.*, 2006; Namura *et al.*, 2007; Sanico *et al.*, 2000). If neurotrophins are under the negative regulation of muscarinic receptors on eosinophils in the lungs, anticholinergic drugs may promote inflammation. However, it will be important to determine which muscarinic receptor subtype regulates eosinophil activity, given that the non-selective drug atropine increases vagally induced bronchoconstriction.

FIGURE 4.9. Role of nerve growth factor in atropine-enhanced antigen challenge induced hyperreactivity.

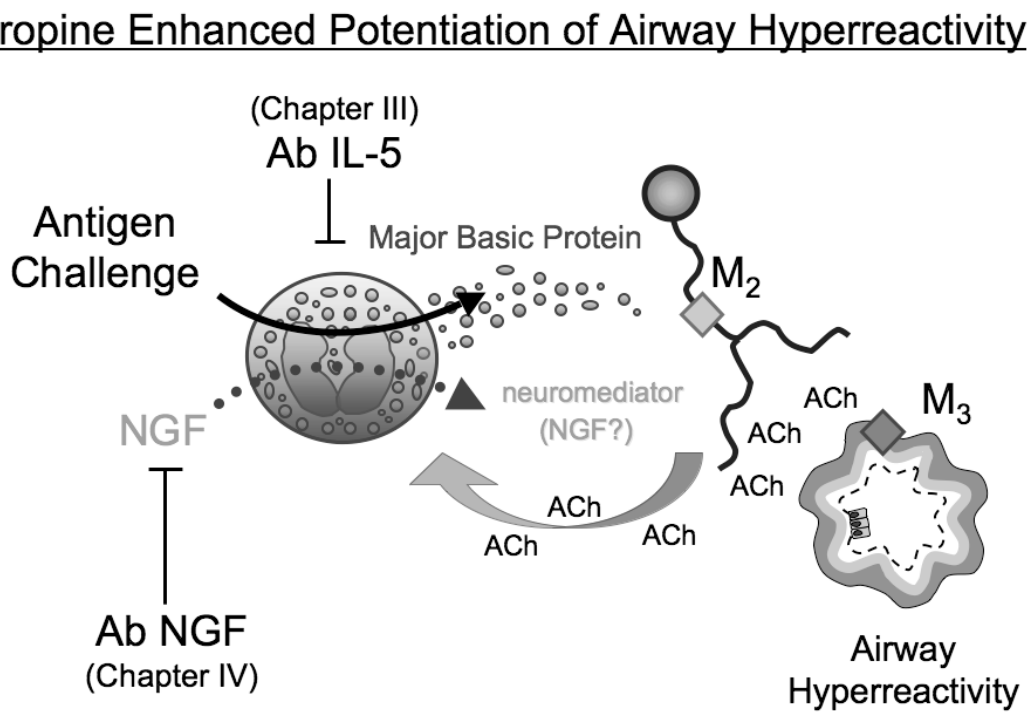


Figure 4.9. Role of eosinophils and nerve growth factor in airway hyperreactivity. Antigen challenge releases eosinophil major basic protein (MBP) onto neuronal M_2 receptors on airway parasympathetic nerves, causing M_2 muscarinic receptor dysfunction. Loss of neuronal M_2 receptor function increases acetylcholine (ACh) release onto airway smooth muscle and increases bronchoconstriction. Both airway hyperreactivity and neuronal M_2 receptor dysfunction are protected by depleting eosinophils with antibody to IL-5 (Ab IL-5) or by neutralizing MBP (Ab MBP). NGF does not play a role in this pathway since Ab NGF does not prevent antigen-induced airway hyperreactivity. In contrast, NGF is important when muscarinic receptors are blocked. Muscarinic blockade with atropine at the time of antigen challenge increases eosinophil activation and protein release, leading to airway hyperreactivity that is not mediated by M_2 receptor dysfunction

(dashed lines). Depleting eosinophils (Ab IL-5) or inhibiting NGF (Ab NGF) decreases eosinophil activation and prevents atropine-enhanced antigen-induced airway hyperreactivity. One possible mechanism involves a negative regulatory pathway that is controlled by acetylcholine: Inhibitory muscarinic receptors (M_i) limit NGF and subsequently inhibit release of an eosinophil-derived neuromediator. This factor (which may be NGF or another neurotrophin) induces a change in parasympathetic nerve activity leading to hyperreactivity through a mechanism not involving neuronal M_2 receptor dysfunction. The arrows shown in this diagram suggest the order of events, but do not exclude the steps in between.

CHAPTER V.

ROLE OF MUSCARINIC RECEPTORS ON EOSINOPHILS

ABSTRACT

Eosinophils are clustered around parasympathetic nerves in asthmatic humans and animal models of asthma. Antigen challenge releases eosinophil major basic protein, an antagonist for neuronal M_2 receptors. Eosinophil mediated blockade of inhibitory M_2 muscarinic receptors on parasympathetic nerves increases acetylcholine release, which increases bronchoconstriction. Given that eosinophils are associated with cholinergic parasympathetic nerves, I tested whether eosinophils could be regulated by acetylcholine. Eosinophils were isolated from guinea pig peritoneal lavage, guinea pig blood and human blood. RT-PCR and immunocytochemistry demonstrated that guinea pig eosinophils express M_3, M_4, but not M_1, M_2 or M_5 muscarinic receptors, whereas human eosinophils express M_3, M_4 and M_5, but not M_1 or M_2 muscarinic receptors. To test whether muscarinic receptors regulate eosinophil activation, eosinophils were loaded with a calcium indicator (fluo-4; 5 μM) in the presence of a nicotinic receptor antagonist hexamethonium (0.1 μM) and increases in intracellular calcium were measured via fluorescence microscopy. Eosinophil activation was measured as the number of cells responding to an agonist within 1 minute. Platelet-activating factor (PAF) (0.01 – 10 μM) increased the number of responding cells in a dose-dependent manner. Neither acetylcholine (1 nM – 10 μM) nor carbachol (1 nM – 10 μM) increased intracellular calcium in any eosinophils. However, the number of eosinophils responding to PAF (1 μM) was dose-dependently decreased by acetylcholine (0.01 – 3 μM; the max decrease was 49% of control at 3 μM), an effect that was reversed by muscarinic blockade with either atropine (1 μM) or the M_4 selective antagonist, MT-3 (0.002 – 20 nM). These data demonstrate that eosinophils express three muscarinic receptor subtypes and M_4

muscarinic receptors inhibit human eosinophil activation in vitro. Agonists that stimulate this inhibitory pathway may therefore be useful for treatment of asthma.

Data in this chapter have been published in abstract form:

Verbout et al., Muscarinic Receptors on Eosinophils Inhibit PAF-induced Eosinophil Activation. American Society for Pharmacology and Experimental Therapeutics Meeting; *Recent Advances in Muscarinic Receptor Pharmacology and Therapeutics*, 2008.

INTRODUCTION

Activated eosinophils play an important role in the pathogenesis of several inflammatory diseases, including asthma (Hogan *et al.*, 2008). In response to diverse stimuli, eosinophils are recruited to sites of inflammation, where they adhere and release numerous pro-inflammatory mediators. In these environments, eosinophils secrete cytokines, chemokines, lipid mediators, proteases, growth factors and cytotoxic proteins that initiate or exacerbate disease states (Rothenberg *et al.*, 2006). In vivo, eosinophils mediate airway hyperreactivity in antigen challenged guinea pigs via release of major basic protein that blocks neuronal M_2 receptors on parasympathetic nerves, increasing acetylcholine release and bronchoconstriction (Elbon *et al.*, 1995; Evans *et al.*, 1997). Thus, the mechanisms underlying eosinophil recruitment, localization and mediator release in disease states are important areas of research.

Eosinophils accumulate around cholinergic nerves in human and animal models of allergic diseases such as asthma, rhinitis and eosinophilic gastroenteritis (Costello *et al.*, 1997; Gleich, 2000; Hogan *et al.*, 2002; Rothenberg *et al.*, 2001; Sawatzky *et al.*, 2002). In the lung, parasympathetic cholinergic nerves release acetylcholine, mediating ganglionic transmission, smooth muscle contraction and mucus secretion (Racke *et al.*, 2004). Immune cells within the lung, including mast cells, lymphocytes and macrophages are likewise regulated by acetylcholine (Gwilt *et al.*, 2007).

Some inflammatory cells are known to express functional nicotinic and muscarinic receptors. Acetylcholine promotes T lymphocyte cell survival and proliferation via

muscarinic receptors by increasing T cell IL-2 cytokine production and IL-2 receptor expression (Nomura *et al.*, 2003). In mast cells, muscarinic receptors inhibit release of histamine, a potent inflammatory mediator implicated in the pathogenesis of asthma (Reinheimer *et al.*, 1997; Reinheimer *et al.*, 2000). In human macrophages, acetylcholine inhibits release of tumor necrosis factor (TNF)-α via nicotinic receptors (Borovikova *et al.*, 2000; Wang *et al.*, 2003), but stimulates bovine alveolar macrophages and human sputum macrophages to release leukotriene B_4 via muscarinic receptors (Profita *et al.*, 2005; Sato *et al.*, 1998). Thus, depending on the inflammatory cell, acetylcholine either promotes or inhibits production of inflammatory mediators. The effects of acetylcholine on neutrophils and eosinophils are not well characterized.

In antigen challenged guinea pigs, eosinophils accumulate around airway cholinergic nerves where they mediate airway hyperreactivity via release of major basic protein. In vivo, blockade of muscarinic receptors during antigen challenge increases eosinophil activation in airways (measured by eosinophil major basic protein deposition) and makes airway hyperreactivity substantially worse (Chapter III and IV). Collectively, these data suggest that eosinophil activation is regulated by muscarinic receptors. If muscarinic receptors on eosinophils inhibit eosinophil activation, these receptors would potentially be an important target in inflammatory diseases characterized by eosinophil activation, such as asthma. Thus, this study characterizes muscarinic receptor subtype expression and function in eosinophils.

EXPERIMENTAL DESIGN

Eosinophils

Eosinophils were obtained from guinea pigs and humans as described in the methods. Healthy adult volunteers were screened for percent circulating blood eosinophils (Chapter II, Table 2.2) and recruited as blood donors. All subjects underwent blood drawing between 8:00 and 9:00 a.m. and had blood eosinophils within normal range (2 – 10%)(Giembycz *et al.*, 1999).

Detection of muscarinic receptors in eosinophils

Muscarinic receptor subtypes were identified using RT-PCR and immunocytochemistry as described in the Chapter II. Oligonucleotide sequences (Table 2.4) and antibody concentrations (Table 2.5) are also provided in Chapter II.

Eosinophil activation assay

Increased intracellular calcium was measured to quantify guinea pig peritoneal and human blood eosinophil activation in response to acetylcholine (0.001 - 10 μM), carbachol (0.001 - 10 μM) and platelet-activating factor (0.001 - 10 μM), as described in the method chapter. In some experiments, eosinophils were pretreated for one hour with the non-selective muscarinic receptor antagonist, atropine (1 μM) or the M_4 selective antagonist, mamba toxin-3 (0.002 - 20 nM) prior to calcium measurements. Data analysis was conducted as described in Chapter II.

Statistics

Data are represented as the means +/-SEM, unless otherwise indicated. Eosinophil activation data were analyzed for statistical significance by 1-way ANOVA with Dunnett's multiple comparison post-hoc test. A p-value of less than 0.05 was considered significant. In all figures, only comparisons that were statistically significant are indicated with symbols, all other comparisons did not have statistical significance, and are not indicated with symbols.

RESULTS

In guinea pigs, muscarinic receptor subtype expression was evaluated in eosinophils derived from three sources: peritoneal lavage, blood and bronchoalveolar lavage, while expression in human eosinophils was only evaluated from the blood. RT-PCR was performed on guinea pig peritoneal eosinophils and human blood eosinophils because they were available in sufficient numbers. Evaluation of muscarinic receptor subtype protein expression by immunocytochemistry required fewer cells, and was therefore conducted on eosinophils from both species.

Guinea pig peritoneal and blood eosinophils express M_3 and M_4 muscarinic receptors. Guinea pig eosinophils (Figure 5.1A) obtained from peritoneal lavage (>99% purity) were screened for muscarinic receptor mRNA. RT-PCR demonstrated that guinea pig peritoneal eosinophils express mRNA for M_3 and M_4 receptors, but not M_1, M_2 or M_5 muscarinic receptors (Figures 5.1B and 5.2). M_3 and M_4 products were confirmed by the appearance of bands at 246 base pairs (M_3) and 173 base pairs (M_4) on agarose gels (Figure 5.1B) and by subsequent DNA sequencing. Specificity of all five guinea pig muscarinic receptor primers was verified with guinea pig brain cDNA (Figure 5.2). That RNA originated from eosinophils was confirmed by demonstrating the presence of mRNA for CCR3, an abundantly expressed eosinophil protein (Figure 5.2). Immunocytochemistry confirmed that only M_3 and M_4 muscarinic receptors are expressed on peritoneal guinea pig eosinophils (Figures 5.1C and 5.1D), since labeling with anti-M_3 and anti-M_4 antibodies demonstrated antigen specific labeling, whereas anti-M_1, anti-M_2 and anti-M_5 antibodies did not. Additionally, bronchoalveolar lavage

eosinophils from a single pathogen free guinea pig were evaluated for muscarinic receptor expression by immunochemistry in a preliminary experiment (n=1), which confirmed the expression of M_4 muscarinic receptors. However, in contrast to eosinophils from the blood and peritoneal lavage, bronchoalveolar lavage eosinophils did not appear to be labeled by anti-M_3 antibodies, nor did they demonstrate antigen-specific labeling for M_1, M_2 or M_5 muscarinic receptors. Thus, guinea pig eosinophils from peritoneal lavage and blood express M_3 and M_4 muscarinic receptors, and bronchoalveolar lavage eosinophils express only M_4 muscarinic receptors.

Human blood eosinophils express M_3, M_4 and M_5 muscarinic receptors. Expression of M_3, M_4 and M_5 muscarinic receptors in purified (>99%) human blood eosinophils (Figure 5.3A) was verified by RT-PCR and immunocytochemistry. RNA for M_3, M_4 and M_5 muscarinic receptors was confirmed by the appearance of bands at 184 base pairs (M_3), 158 base pairs (M_4) and 159 base pairs (M_5) on agarose gels (Figure 5.3B) and by subsequent DNA sequencing. Specificity of all five human muscarinic receptor primers was verified with human brain cDNA (Figure 5.3A). That RNA originated from eosinophils was confirmed by demonstrating the presence of CCR3 mRNA (Figure 5.3D). Fluorescence immunocytochemistry confirmed that M_3, M_4 and M_5 muscarinic receptors are expressed on human blood eosinophils since labeling with anti-M_3, anti-M_4, and anti-M_5 antibodies demonstrated antigen specific labeling (Figure 5.3C), whereas anti-M_1 and anti-M_2 antibodies did not. Thus, human blood eosinophils express M_3, M_4 and M_5 muscarinic receptors.

Muscarinic receptors on eosinophils do not increase intracellular calcium signaling. Preliminary data indicated that the percent of eosinophils mobilizing intracellular calcium after treatment with PAF was dose dependent in guinea pig (Figure 5.4A) and human (Figure 5.5A and B) eosinophils. The EC50 values for PAF induced calcium mobilization in guinea pig and human eosinophils were similar, 4.63 nM and 13.56 nM, respectively and close to reported values in the literature (Kroegel *et al.*, 1989). However, neither guinea pig (Figure 5.4B) nor human eosinophils (Figure 5.5C and D) mobilized intracellular calcium in response to stimulation with either carbachol or acetylcholine. The maximum dose of carbachol and acetylcholine used in these experiments was 10 μM. This is nearly 100 times the EC50 (Roux *et al.*, 1997) and thus should be a maximally effective dose.

Muscarinic stimulation inhibits PAF-induced eosinophil activation. In human eosinophils, the percent eosinophils activated by PAF at either 0.1 μM or 1 μM was decreased in the presence of acetylcholine (1 μM) (Figure 5.6). Furthermore, acetylcholine inhibited PAF-induced eosinophil activation in a dose-dependent manner (Figure 5.7A) that was reversed by atropine (Figure 5.6 and Figure 5.7A), confirming that muscarinic receptors mediate inhibition. Acetylcholine inhibition of eosinophil activation was also reversed by pretreatment with the highly selective M_4 muscarinic receptor antagonist mamba toxin-3 (MT3) (Figure 5.7B; Table 1.1; see Chapter I) in a dose dependent manner (Figure 5.8A), demonstrating that M_4 muscarinic receptors mediate inhibition. Pretreatment with MT3 did not alter eosinophil responses to PAF, vehicle

control, or acetylcholine (5.8B), demonstrating that MT3 does not change baseline responses to stimuli.

FIGURE 5.1. Guinea pig eosinophils express M_3 and M_4, but not M_1, M_2 or M_5 muscarinic receptors.

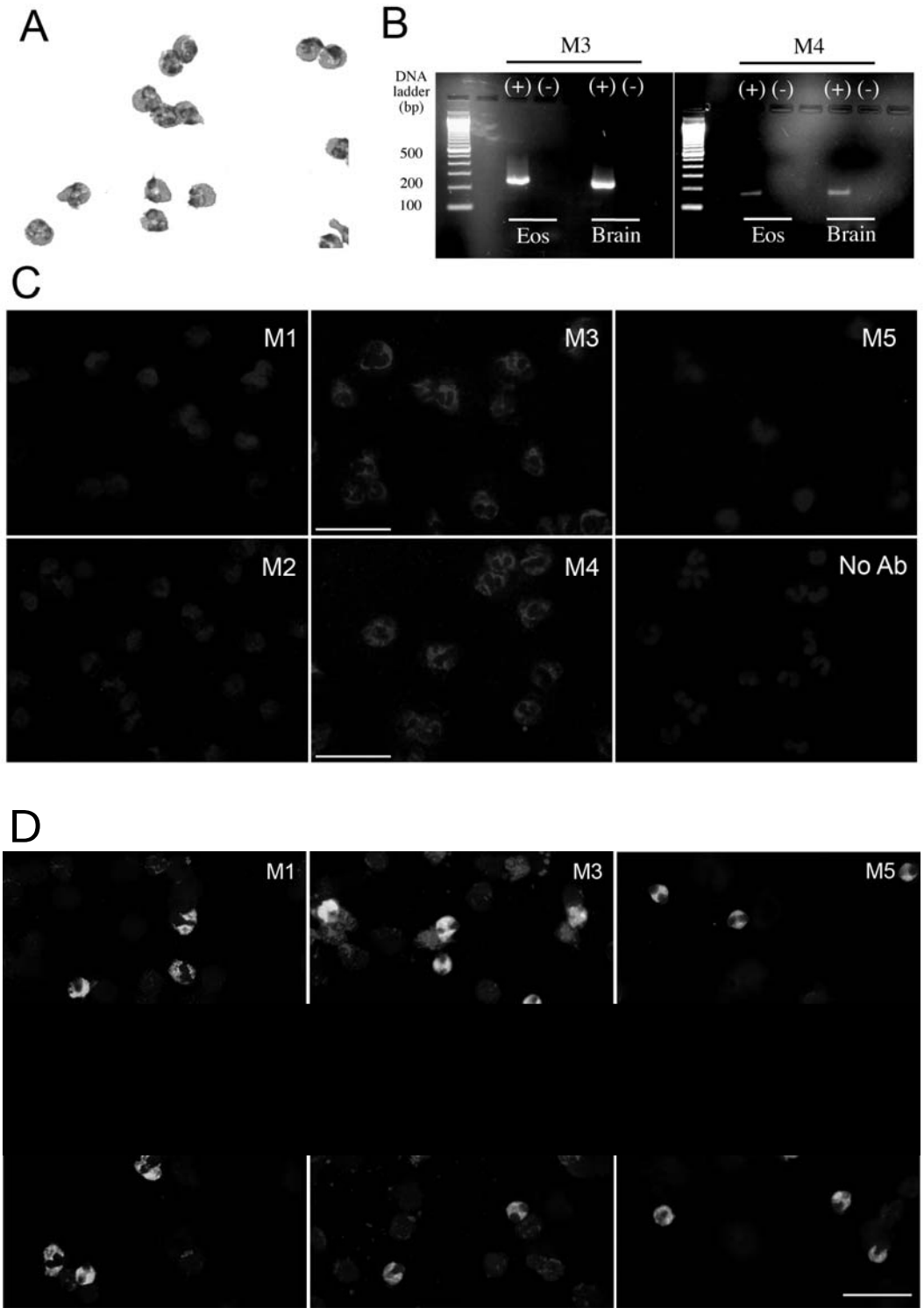


FIGURE 5.1. (continued)

Figure 5.1. RNA isolated from 10^7 pure (>99%) peritoneal guinea pig eosinophils (A) was reverse transcribed in the presence (+) and absence (-) of RT and cDNA was screened by RT-PCR using primers specific to M_1, M_2, M_3, M_4 and M_5 guinea pig muscarinic receptor sequences. Brain RNA was used as a positive control for all muscarinic receptor subtypes. PCR products were run on gels and sequenced,

demonstrating expression of M_3 (173 bp) and M_4 (246 bp), but not M_1, M_2 or M_5 (not shown) muscarinic receptor RNA (B). Immunocytochemistry confirmed protein expression of both M_3 and M_4 receptors in peritoneal (C) and blood (D) eosinophils. Neither M_1, M_2 nor M_5 muscarinic receptors were observed in eosinophils by immunocytochemistry (C and D). Preliminary data in bronchoalveolar lavage eosinophils (E; marked with white arrows) appeared to express M_4 receptors, but not M_1, M_2, M_3 or M_5 muscarinic receptors (n=1). Nuclei are stained blue with DAPI and positive antibody labeling is red. In (D and E), eosinophils were identified on the basis of their characteristic bilobed nucleus and autofluorescence (compared to other leukocytes) when visualized under 488 nm excitation (green), thus positive labeling appears as yellow (overlay of green and red). Scale bar is 50 μm.

FIGURE 5.2. Specificity of primers for guinea pig muscarinic receptor subtypes was verified with guinea pig brain cDNA.

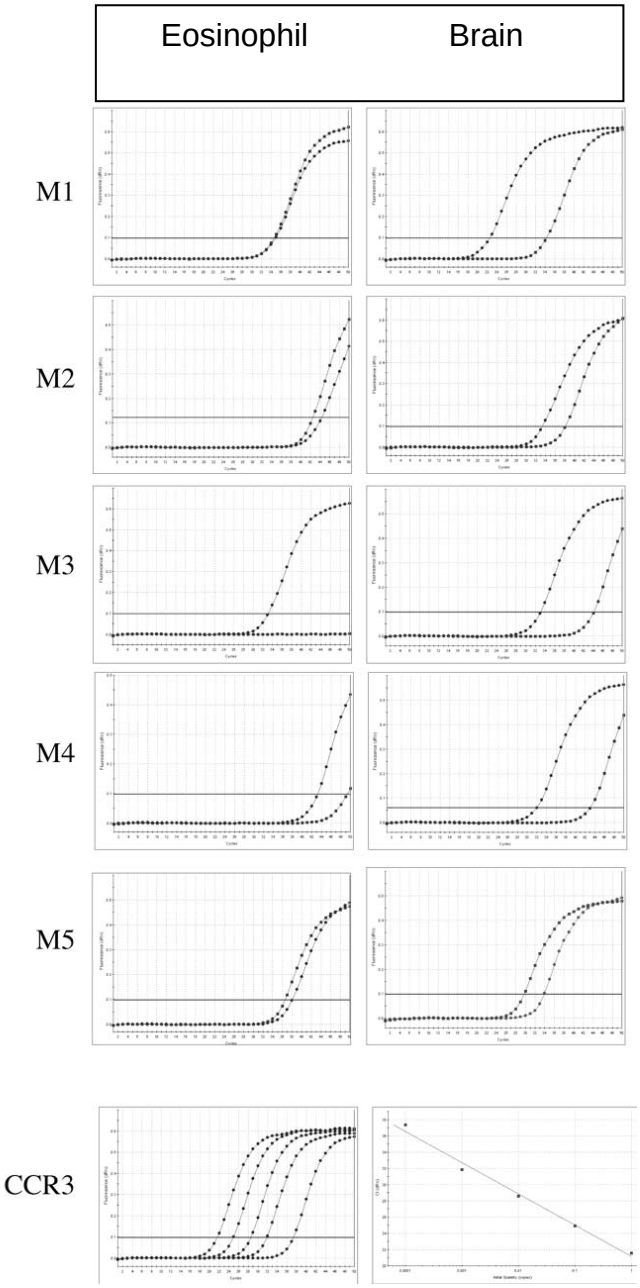


Figure 5.2. Shown are representative amplification plots for real-time PCR experiments in the presence (blue lines) and absence (red lines) of reverse transcriptase. CCR3 expression in guinea pig eosinophils was also evaluated; tenfold serial dilutions (1 to 1/10,000) are shown at the bottom left and plotted as a standard curve on bottom right.

FIGURE 5.3. Human eosinophils express M_3, M_4, and M_5, but not M_1 or M_2 muscarinic receptors.

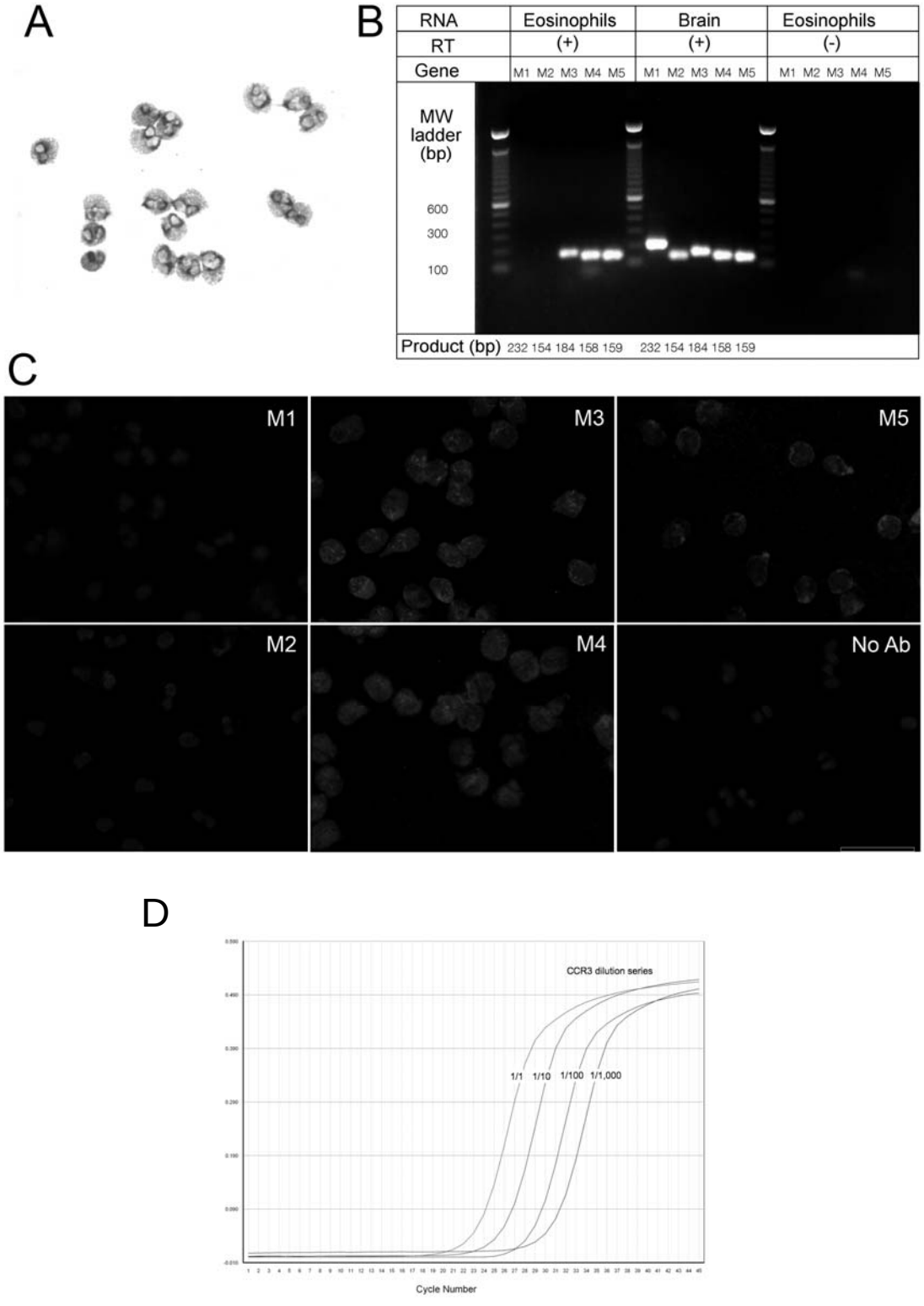


Figure 5.3. RNA isolated from 10^7 pure (>99%) eosinophils (A) was reverse transcribed in the presence (+) and absence (-) of RT and cDNA was screened by RT-PCR using primers specific to M_1, M_2, M_3, M_4 and M_5 human muscarinic receptor sequences. Brain cDNA was a positive control for all muscarinic receptor subtypes (B) and CCR3 was a positive control for eosinophil cDNA (D). In (D), shown is a representative amplification plot for CCR3 with tenfold serial dilutions (1 to 1/1,000). PCR products were run on gels and sequenced, demonstrating expression of M_3 (184 bp), M_4 (158 bp), and M_5 (159 bp) but not M_1 (232 bp) or M_2 (154 bp). Immunocytochemistry (C) confirmed protein expression of M_3, M_4, and M_5 (antibody labeling red; nuclei stained blue with DAPI), but not M_1, M_2 muscarinic receptors; scale bar is 50 μm.

FIGURE 5.4. Muscarinic receptor agonists do not activate guinea pig eosinophils.

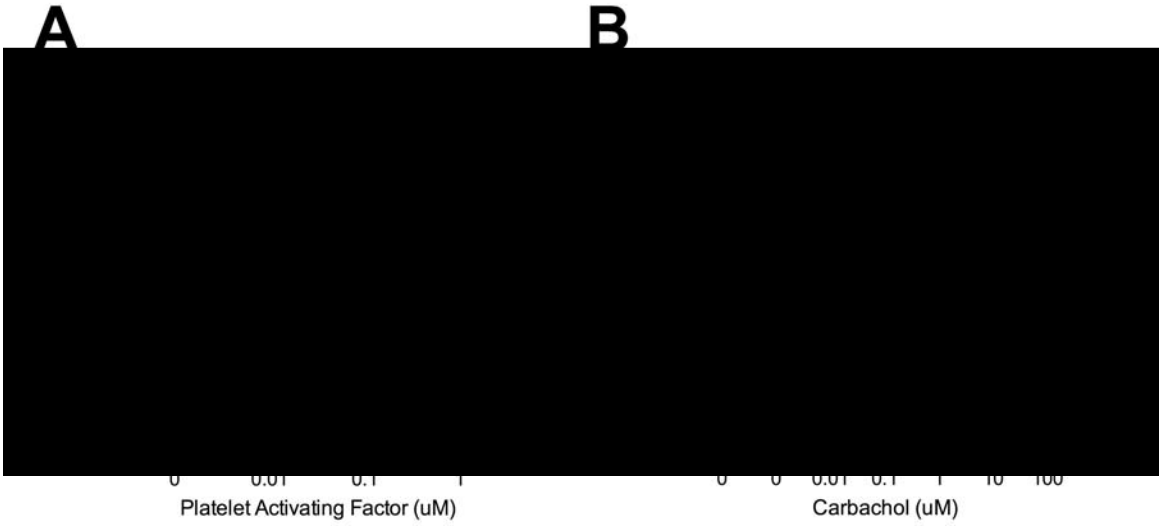


Figure 5.4. Guinea pig peritoneal eosinophils were activated by platelet activating factor (PAF) (0.01 – 1 μM) in a dose dependent manner (A) with an EC50 = 4.63 nM (log EC50 = 8.33). In contrast, neither carbachol (0.01 – 100 μM), nor acetylcholine (not shown) mobilized intracellular calcium above vehicle control (white bar; B). In B, eosinophils were treated with PAF (0.1 μM, black bar) to confirm eosinophils could respond to agonists. Eosinophil activation was calculated as percent eosinophils increasing intracellular calcium within one minute of agonist application. Data are expressed as the mean +/- SD.

FIGURE 5.5. Muscarinic receptor agonists do not activate human eosinophils.

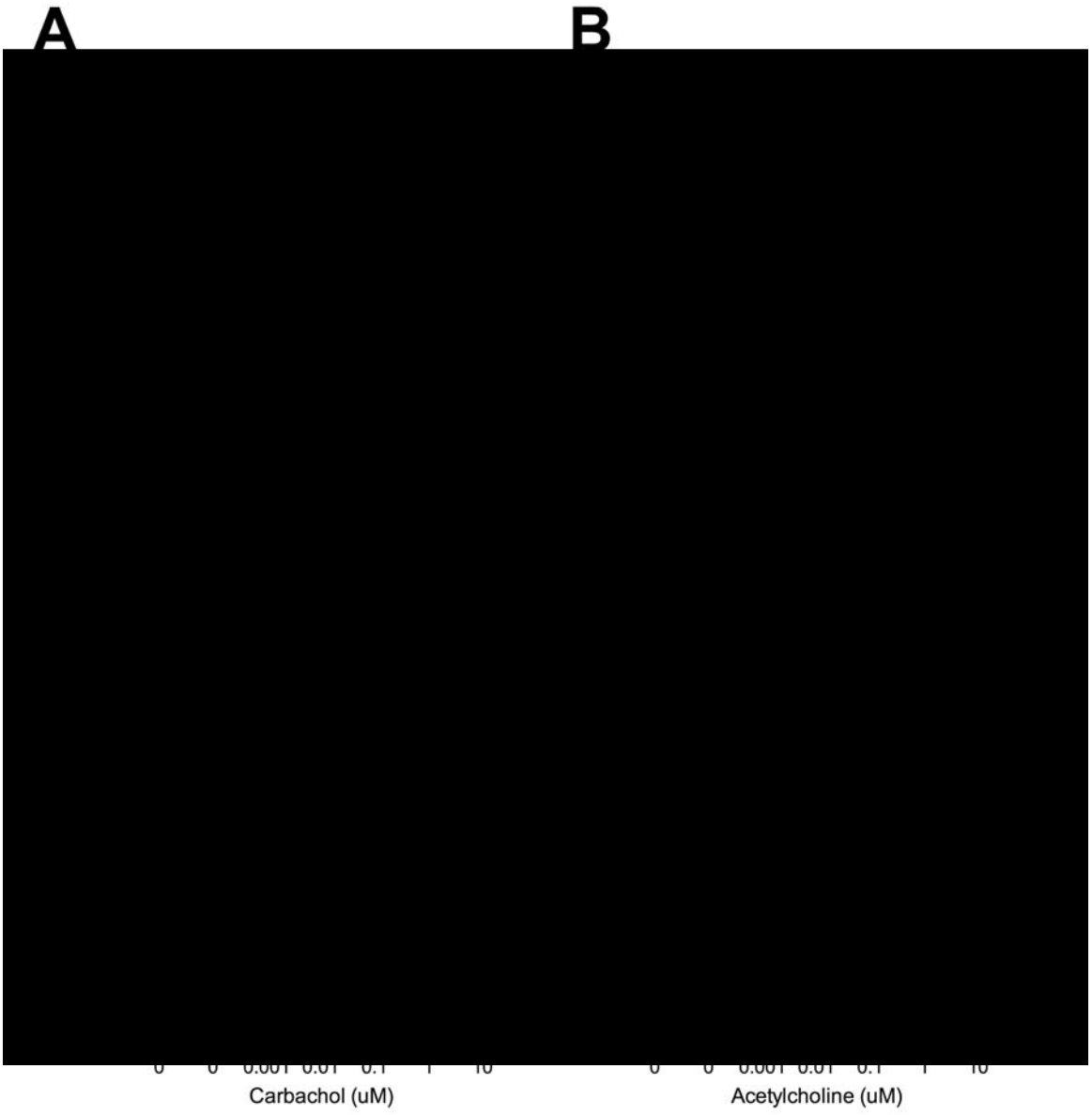


Figure 5.5. Human eosinophils were dose-dependently activated by PAF (A and B), with an EC50 = 13.56 nM (log EC50 = 7.86). In contrast, neither carbachol (C) nor acetylcholine (D) increased the number of eosinophils responding with increased Ca^{2+} above vehicle (white bar). In (C) and (D), eosinophils were treated with PAF (1 μM, black bar) to confirm eosinophils could respond to agonist. Eosinophil activation was

calculated as percent eosinophils increasing intracellular calcium within one minute of agonist application. Data are expressed as the mean +/- SD.

FIGURE 5.6. Muscarinic receptors inhibit platelet activating factor induced eosinophil activation.

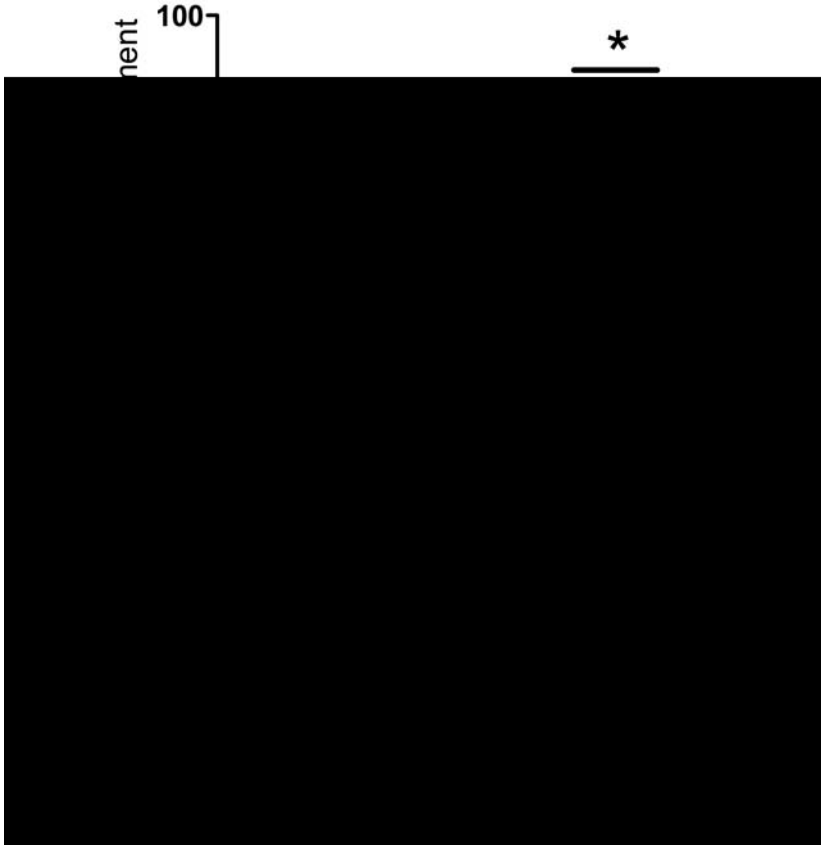


Figure 5.6. The number of human blood eosinophils activated by PAF at 0.1 μM and 1 μM (white bars) was decreased in the presence of acetylcholine (1 μM; black bars) and reversed by muscarinic blockade by atropine (1 μM; gray bars). Eosinophil activation was calculated as percent eosinophils increasing intracellular calcium within one minute of agonist application. Data are expressed as the mean +/- SEM, n=4. *Statistically significant ($p<0.05$) compared to PAF (1 uM) alone.

FIGURE 5.7. Acetylcholine inhibition of human eosinophil activation is mediated by M_4 muscarinic receptors.

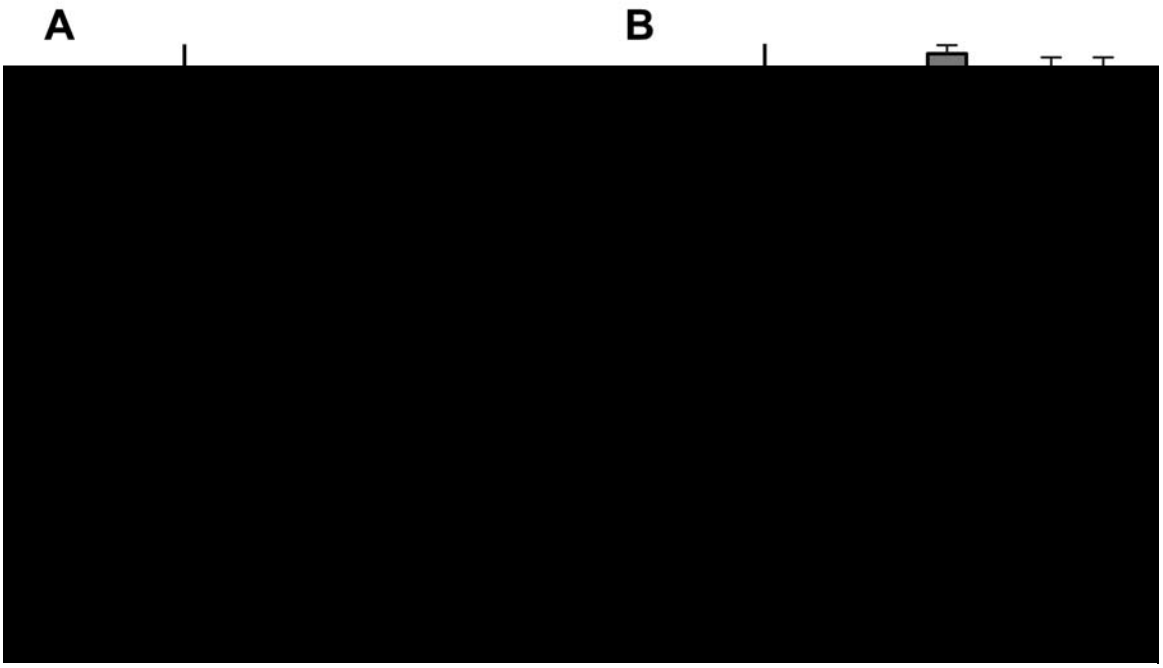


Figure 5.7. Acetylcholine inhibited human eosinophil activation induced by PAF (white bar) in a dose dependent manner, which was reversed by muscarinic blockade with atropine (black bar) (A). The ability of acetylcholine to inhibit eosinophil activation was completely prevented by blocking M_4 muscarinic receptors with mamba toxin-3 (MT3) (B). Addition of MT3 to atropine did not alter activation by PAF. Activation was calculated as percent eosinophils increasing intracellular calcium within one minute of agonist application and normalized to treatment with PAF alone. Data are expressed as a ratio (percent activation with PAF alone) / (percent activation with PAF plus agonist) and represent the mean +/- SEM, n=3. *Statistically significant ($p<0.05$) compared to PAF alone.

FIGURE 5.8. Mamba toxin-3 dose-dependently reverses the inhibitory effect of acetylcholine on platelet activating factor-induced human eosinophil activation.

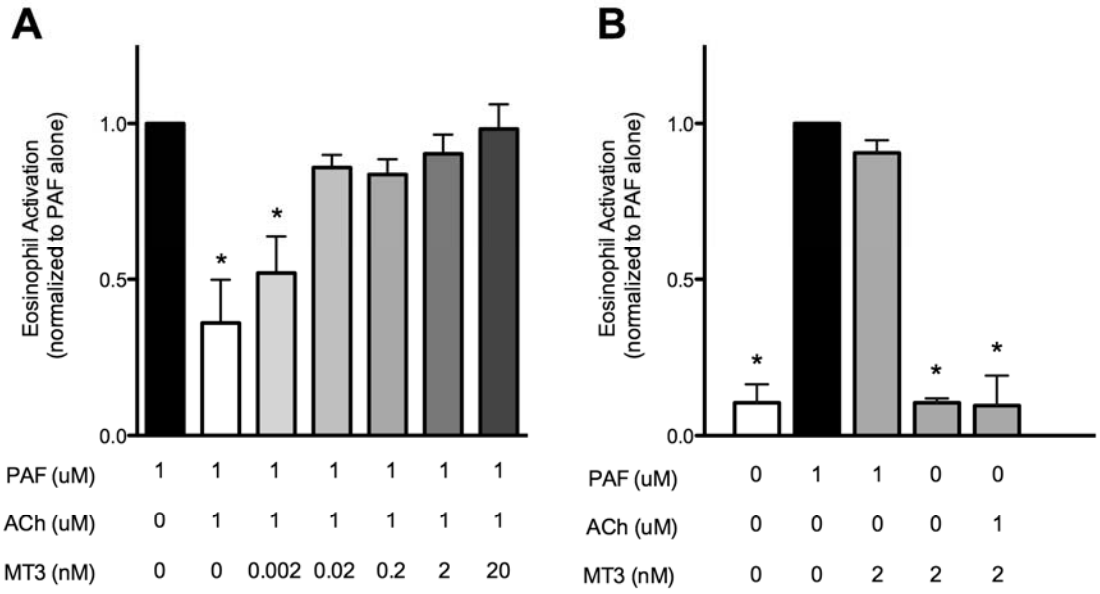


Figure 5.8. MT3 dose-dependently reverses the inhibitory effect of acetylcholine on PAF-induced human eosinophil activation (A). Pretreatment with MT3 did not alter responses to PAF, vehicle control, or acetylcholine (B). Activation was calculated as percent eosinophils increasing intracellular calcium within one minute of agonist application and normalized to treatment with PAF alone. Data are expressed as a ratio (percent activation with PAF alone) / (percent activation with PAF plus agonist) and represent the mean +/- SEM, n=4 (A), n=2 (B). *Statistically significant ($p<0.05$) compared to PAF alone.

DISCUSSION

The data presented in this paper demonstrate that eosinophils express functional muscarinic receptors. Guinea pig eosinophils obtained from peritoneal lavage and blood express M_3 and M_4 muscarinic receptor subtypes; preliminary data indicate that guinea pig bronchoalveolar lavage eosinophils may only express M_4 muscarinic receptors. Human blood eosinophils express three muscarinic subtypes: M_3, M_4 and M_5. Thus, differences in muscarinic receptor subtype expression are species specific and may possibly be tissue dependent.

In these studies, RT-PCR was used to test for expression of all five muscarinic receptor subtypes in guinea pig and human eosinophils. In all experiments, RNA was extracted from freshly isolated, viable, purified (>99%) eosinophils (Figure 5.1A and 5.3A). It is possible that preparations included RNA from other immune cells, some of which express muscarinic receptors (See Chapter I, section E), however the relative contribution of RNA from other cell types is less than 1%. Other potential sources of contamination include platelets, however there are no reports that platelets express muscarinic receptors or that platelet function is regulated by muscarinic receptors (Offermanns, 2006).

Eosinophil cDNA quality was evaluated during RT-PCR using CCR3 primers because CCR3 is abundantly expressed by human (Ponath *et al.*, 1996) and guinea pig eosinophils (Sabroe *et al.*, 1998). Although other immune cells are reported to express this receptor (Pease *et al.*, 2006), the relative contribution of RNA from these cell types is negligible since eosinophil purity was always 99% or greater. Therefore, in each experiment,

standard curves were generated using CCR3 primers to verify the quality of eosinophil RNA (Figure 5.2 and 5.3D).

Muscarinic receptor genes, like most other mammalian G-protein coupled receptors do not contain introns in the coding sequence (Gentles *et al.*, 1999). To eliminate genomic DNA contamination, RNA preparations were treated with DNase I, and all PCR reactions were carried out with cDNA samples synthesized in the presence and absence of reverse transcriptase as a control (Figures 5.1B, 5.2 and 5.3B). Since RT-PCR was carried out in real-time using SYBR green detection of PCR products, it was possible to obtain melting temperatures for each product, which were used to verify the primer specificity. Positive reactions were run on agarose gels (Figure 5.1B and 5.3B) and confirmed with sequencing. In the case of negative PCR reactions with eosinophil cDNA (M_1, M_2 and M_5 in guinea pig; M_1 and M_2 in human), primers were validated within experiments with positive controls, brain cDNA (Figures 5.2 and 5.3B). Muscarinic receptor RNA expression was confirmed with three independent experiments in guinea pig eosinophils and two in human eosinophils.

Immunocytochemistry confirmed protein expression of M_3 and M_4 muscarinic receptors in guinea pig peritoneal and blood eosinophils (Figure 5.1C and D) and muscarinic subtypes M_3, M_4 and M_5 in human eosinophils (Figure 5.3C). The antibodies used to detect muscarinic receptors in this study recognize the intracellular carboxyl terminal sequences. Thus, eosinophils were permeabilized during antibody detection, and antigen labeling reflects both cytosolic and cell surface muscarinic receptor localization.

Eosinophil effector functions including chemotaxis, adhesion and degranulation are mediated via G-protein coupled receptors. It is well established that intracellular calcium mobilization is an important requirement for eosinophil activation induced by agonists such as PAF, leukotrienes and eotaxin (Elsner *et al.*, 1995; Elsner *et al.*, 1996; Giembycz *et al.*, 1999). Therefore, I developed a functional assay to test whether or not muscarinic agonists activate eosinophils. Intracellular calcium transients were measured in eosinophils via fluorescence microscopy (see Methods chapter). PAF, a well-characterized eosinophil activator (Kroegel *et al.*, 1991; Kroegel *et al.*, 1989), was used to validate the assay. I have confirmed that PAF-induced eosinophil activation is dose-dependent in guinea pig (Figure 5.4A) and human (Figure 5.5A) eosinophils, with similar EC50 values (Figure 5.4A and 5.5B), close to reported values in the literature (Kroegel *et al.*, 1989).

In contrast, muscarinic receptor agonists did not increase eosinophil responses above vehicle control in either species (Figures 5.4B, 5.5C and D). It is not likely that the lack of calcium signaling in response to acetylcholine was due to degradation by acetylcholinesterases, because experiments conducted with carbachol, a non-hydrolyzable muscarinic agonist yielded the same result. In addition, eosinophils responded to PAF (black bars; Figures 5.4B, 5.5C and D), therefore eosinophils were not unresponsive because they were dead in these experiments. Thus, muscarinic receptors do not increase intracellular calcium signaling in guinea pig peritoneal or human blood eosinophils.

In contrast, PAF-induced human eosinophil activation was inhibited in the presence of acetylcholine, an effect reversed by muscarinic blockade with atropine (Figure 5.6). This inhibitory effect of acetylcholine was evident at submaximal concentrations of PAF (0.1 μM and 1 μM), and was not mediated by nicotinic receptors, since all experiments were conducted in the presence of hexamethonium. The ability of acetylcholine to inhibit human eosinophil activation was dose dependent and decreased eosinophil activation by 49% of maximal response at 3 μM acetylcholine (Figure 5.7A).

Pretreatment with the M_4 selective antagonist, mamba toxin-3 (MT-3; 20 nM) eliminated the inhibitory effect of acetylcholine at all doses tested (0.01 – 10 μM; Figure 5.7B). There was no additional effect when MT3 was combined with atropine, suggesting that other muscarinic receptor subtypes are not involved. MT3 did not increase baseline activation of eosinophils by vehicle control or PAF (Figure 5.7B), ruling out any nonspecific effects on eosinophil activation. Dose-response experiments (0.002 – 20 nM; MT3) performed with a submaximal acetylcholine concentration (1 μM) demonstrated that M_4 antagonism by MT3 is dose-dependent (Figure 5.7A).

MT3 is a competitive, reversible antagonist with a reported K_d of 8.7 (Table 1.1; see Introduction chapter), equating to an EC_{50} of approximately 2 nM. Thus, experiments were carried out using MT3 in the range of 0.2 – 20 nM. However, muscarinic blockade by MT3 remained maximally effective at 100-fold lower concentrations than the reported EC_{50}. The very high potency of MT3 could be explained by blockade of the other

receptor subtypes. However, MT3 is highly selective for M_4 receptors, therefore it is not likely that M_3 and M_5 muscarinic receptors contribute in a significant way.

An alternative explanation for the antagonistic effect of MT3 at lower than expected concentrations may be related to receptor number. It may be that the number of M_4 receptors expressed on eosinophils is very low. In other cell systems in which receptor numbers are few, such as airway smooth muscle, the agonist potency for β_2 receptors is greatly impacted by low receptor number (McGraw *et al.*, 1999; Whaley *et al.*, 1994). Thus, if this were the case in eosinophils, a low concentration of antagonist would be sufficient to completely abrogate signaling and a greater concentration of agonist may be necessary to overcome the effect of an antagonist.

The presumptive function of M_3 and M_5 receptors on eosinophils remains unknown. Blockade of M_4 receptors did not uncover the baseline response to acetylcholine, therefore it is unlikely M_4 receptors exert a dominant effect over the other two muscarinic receptor subtypes. All available M_3 selective antagonists discriminate poorly between M_3 and M_4 subtypes, and an antagonist with greater than tenfold selectivity for M_5 has yet to be identified (Caulfield *et al.*, 1998; Eglen, 2005)(Table 1.1; Chapter I).

Physical disturbance of eosinophils when pipetting solutions containing either vehicle or agonists on the cells caused a small proportion to increase intracellular calcium. It may be important that acetylcholine and carbachol alone did not decrease this baseline response. This finding suggests that acetylcholine inhibition acts on receptor-mediated stimuli.

There are at least two functionally distinct pathways by which PAF increases intracellular calcium. The first pathway is characterized by activation of GTP-binding proteins to stimulate formation of inositol 1,4,5-triphosphate (IP_3) (Dent *et al.,* 1993; Honda *et al.*, 1991; Honda *et al.*, 1994). This IP_3-mediated pathway causes a transient increase in intracellular calcium that is mediated by calcium release from intracellular calcium stores (Kroegel *et al.*, 1991). Following release from intracellular stores, PAF induces a prolonged and sustained intracellular calcium elevation in eosinophils, which is mediated by influx of extracellular calcium (Kroegel *et al.*, 1989; Oshiro *et al.*, 2000).

The mechanism by which M_4 receptors decrease PAF induced intracellular calcium in eosinophils is not known, but may involve inhibiting G protein receptor signaling pathways, extracellular calcium influx via channels or both. M_4 receptors couple preferentially to $G_{i/o}$ proteins, which classically inhibit adenylyl cyclase and couple directly to activate potassium channels and inhibit non-selective ion channels (Chapter I, section F). M_4 mediated inhibition in eosinophils may be similar to another $G_{i/o}$ protein coupled receptor, A_3 adenosine receptors. A_3 receptors inhibit eosinophil chemotaxis (Knight *et al.*, 1997; Walker *et al.*, 1997), superoxide anion generation and eosinophil peroxidase release (Ezeamuzie *et al.*, 1999), all of which are regulated by intracellular calcium signaling. There is also evidence that G proteins may switch between G_s and $G_{i/o}$ coupling under certain circumstances, as is the case of adrenergic receptors on macrophages (Magocsi *et al.*, 2007), thus it may be that M_4 receptors on eosinophils couple to other signaling pathways that limit eosinophil activation.

It is also known that M_4 receptors inhibit calcium currents in some cell types (Cuevas *et al.*, 1997; Hille *et al.*, 1995), supporting a role for M_4 receptor inhibition of intracellular calcium in eosinophils. In non-excitable cells such as eosinophils, extracellular calcium influx enters via channels present on the plasma membrane and is initiated by depletion of intracellular calcium stores (Clapham, 1995; Fukuda *et al.*, 1985; Li *et al.*, 2002; Oshiro *et al.*, 2000). The signaling mechanisms by which depletion of intracellular calcium stores activate extracellular calcium influx in eosinophils are not well understood, but currently there are two models: direct coupling and indirect coupling. A direct coupling mechanism proposes a physical interaction between proteins in the plasma membrane and endoplasmic reticulum (Irvine, 1990). In a modified version of this hypothesis, depletion of intracellular calcium stores changes the conformation of the IP_3 receptor on the endoplasmic reticulum, which leads to opening of store-operated calcium channels on the plasma membrane (Berridge, 1995). Thus there are multiple ways by which M_4 receptors may inhibit intracellular calcium.

In granulocytic immune cells, there is evidence that store-operated calcium channels are regulated by heterotrimeric G proteins (Jaconi *et al.*, 1993). In this particular study, the authors found that activation of calcium influx by fMet-Leu-Phe or by calcium ionophore was inhibited in HL-60 cells treated with a non-hydrolyzable guanosine phosphate analogue (GTPγS), suggesting that a GTP-sensitive protein is involved in signaling between intracellular calcium stores and plasma membrane calcium channels. Since GTPγS inhibits both heterotrimeric and small G proteins, the authors used fluoride to demonstrate that it is heterotrimeric G proteins and not small G proteins that inhibit store-

dependent calcium influx. Fluoride mimics the effects of GTPγS on large G proteins (Rodbell, 1992), but does not affect small G proteins (Kahn, 1991). Thus, it appears that in granulocytes, G proteins may regulate store-operated calcium channels, supporting a role for M_4 mediated inhibition of calcium channels in eosinophils. Thus, it may be that PAF activates calcium channels, which may be functionally opposed by M_4 receptor signaling.

Although the experiments described here do not specifically identify the mechanism by which M_4 receptors inhibit intracellular calcium in eosinophils, future experiments may clarify which signaling pathways are important. For example, these experiments could be repeated using other eosinophil activators, such as calcium ionophore and eotaxin, both of which increase intracellular calcium, but have very different mechanisms of action. These experiments could also be conducted in the presence of calcium channel blockers, which would determine whether or not M_4 receptors inhibit calcium channels. Lastly, future experiments should address whether acetylcholine modulates key eosinophil functions such as chemotaxis, adhesion or mediator release.

The preliminary observation that guinea pig bronchoalveolar lavage eosinophils may only express M_4 muscarinic receptors (Figure 5.1E) and do not express M_3 receptors, as seen in peritoneal and blood eosinophils (Figure 5.1C and D) suggests that there may be tissue dependent differences in muscarinic receptor subtype expression. Although these data are preliminary, it suggests that muscarinic receptor expression is labile and may be regulated by the local environment. Whether or not these differences in receptor subtype

expression convey any difference in eosinophil function, or whether or not they also occur in human eosinophils is not known. However, future experiments may clarify whether or not these tissue dependent differences are important.

Given that eosinophils are localized around cholinergic parasympathetic nerves in asthma, it may be that acetylcholine released from these nerves plays a role in regulating eosinophil activation. In addition to its role as a neurotransmitter, acetylcholine is also produced by a number of non-neuronal cells in the lung. These non-neuronal sources include structural cells (airway epithelium, endothelium and smooth muscle cells) and immune cells (mast cells, lymphocytes, macrophages, eosinophils and neutrophils) (Gwilt *et al.*, 2007; Kirkpatrick *et al.*, 2001; Klapproth *et al.*, 1997; Wessler *et al.*, 1998; Wessler *et al.*, 1999; Wessler *et al.*, 2001c). Thus, there are multiple sources of acetylcholine in the lung, any of which may play a role in limiting eosinophil activation in vivo.

Acetylcholine interacts with two separate classes of receptors, muscarinic and nicotinic. Nicotinic receptors are ligand gated ion channels that are structurally distinct from muscarinic receptors (Conti-Tronconi *et al.*, 1994; Racke *et al.*, 2004). Eosinophils also express nicotinic receptors and stimulation with a nicotinic agonist dimethylphenylpiperazinium (DMPP) decreases PAF-induced leukotriene and metalloprotease release from human blood eosinophils, suggesting that nicotinic receptors on eosinophils have an anti-inflammatory role.

In keratinocytes, both muscarinic and nicotinic pathways negatively regulate adhesion (Nguyen *et al.*, 2004). Pharmacological blockade of muscarinic receptors in keratinocytes increases phosphorylation of adhesion molecules E-cadherin, beta-catenin, and gamma-catenin, thereby decreasing adhesion. Similarly, nicotinic inhibition with antisense oligonucleotides decreases expression of these same adhesion molecules, thereby also decreasing cell adhesion. Simultaneous inhibition of these two pathways significantly potentiates abnormalities in keratinocyte adhesion in vitro and in vivo. Although the experimental protocol described here specifically excludes nicotinic effects since all cells were treated with hexamethonium, it appears that in non-eosinophils, nicotinic and muscarinic receptors can regulate cell function in a synergistic manner. However this is not a mechanism here, since hexamethonium was present in all experiments, but this mechanism may be important in vivo.

In summary, guinea pig peritoneal and blood eosinophils express M_3 and M_4 muscarinic receptors; human blood eosinophils also expressed these two subtypes and additionally express M_5 muscarinic receptors. Muscarinic stimulation did not increase intracellular calcium in eosinophils, but acetylcholine inhibited PAF-induced activation (5.9). These data are in agreement with in vivo data demonstrating that muscarinic blockade enhances eosinophil activation in antigen challenged guinea pigs (Chapter III and IV). Acetylcholine inhibition was mediated by M_4 muscarinic receptors, since MT3 blocked the effects of acetylcholine. The function of M_3 and M_5 muscarinic receptors remains unknown. Thus, muscarinic regulation of eosinophil function may be important in inflammatory diseases characterized by increased eosinophil activation, such as asthma, in which anticholinergic drugs are routinely used to treat acute asthmatic exacerbations.

FIGURE 5.9. Eosinophil activation is inhibited by M_4 muscarinic receptors.

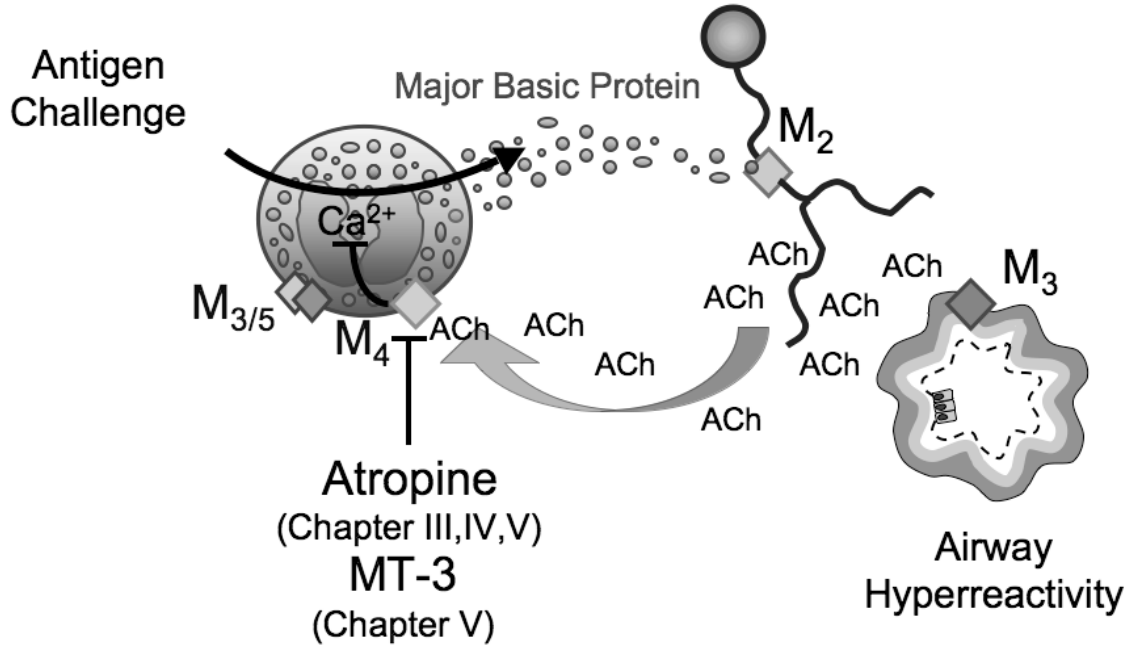


Figure 5.9. In vivo, eosinophils mediate airway hyperreactivity in antigen challenged guinea pigs via major basic protein that blocks neuronal M_2 receptors on parasympathetic nerves, increasing acetylcholine release and bronchoconstriction (Elbon *et al.*, 1995; Evans *et al.*, 1997). In vitro, acetylcholine inhibits PAF-induced eosinophil activation in guinea pig and human eosinophils. In human eosinophils, this effect is mediated by M_4 muscarinic receptors, since mamba toxin-3 reverses the inhibitory effect of acetylcholine on eosinophil activation. The role of M_3 and M_5 muscarinic receptors remains unknown. Thus, acetylcholine attenuates eosinophil activation, suggesting that airway nerves interact with eosinophils via muscarinic receptors. The arrows shown in this diagram suggest the order of events, but do not exclude the steps in between.

CHAPTER VI.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

The novel results presented in this thesis demonstrate that human and guinea pig eosinophils express muscarinic receptors, which function to inhibit eosinophil activation in vitro (Chapter V). Furthermore, I have demonstrated that muscarinic blockade during antigen challenge significantly increases eosinophil activation and subsequent vagally mediated hyperreactivity in guinea pigs (Chapter III and IV) via a mechanism involving eosinophils (Chapter III) and NGF (Chapter IV). These data suggest that the inhibitory muscarinic receptors I have identified in vitro are functional in vivo.

Previous work has demonstrated that antigen-induced airway hyperreactivity in guinea pigs is mediated by eosinophil MBP and neuronal M_2 receptor dysfunction (Chapter I). In this thesis, I have potentially identified an alternative pathway by which eosinophils contribute to airway hyperreactivity in atropine-enhanced antigen challenged guinea pigs that is not mediated by loss of neuronal M_2 function (Chapter III). In this newly identified pathway, NGF appears to play a role since anti-NGF antibodies prevent atropine-enhanced antigen-induced airway hyperreactivity (Chapter IV). Thus, eosinophils mediate airway hyperreactivity via at least two different mechanisms, one involving MBP blockade of neuronal M_2 receptors and the other involving NGF (Figure 6.1), which may produced by multiple sources in the lung, including immune cells, neurons, fibroblasts, epithelial cells, smooth muscle and also eosinophils (Groneberg *et al.*, 2004; Hahn *et al.*, 2006; Solomon *et al.*, 1998).

The data presented in Chapter IV suggest that NGF is likely to be acting upstream of eosinophils, since anti-NGF antibodies prevent increased eosinophil activation observed in the airways of challenged animals pretreated with atropine. Furthermore, the observation that Ab NGF only prevents increased eosinophil activation and airway hyperreactivity when muscarinic receptors are blocked (Chapter IV) reveals a novel and complex interaction.

The mechanism by which NGF influences airway hyperreactivity in this model is not known. One possibility is that NGF directly affects eosinophil activation in the airways. It is known that NGF promotes eosinophil survival, activation and degranulation via TrkA and p75NTR (Nassenstein *et al.*, 2003; Noga *et al.*, 2002). Thus, one possible explanation for the results presented here is that NGF regulates release of an eosinophil derived factor capable of altering nerve function, and that release of this neuromediator is inhibited by acetylcholine (Figure 6.1).

Eosinophils are known to produce numerous neuromediators that affect nerve function (Chapter I, Table 1.2), including leukemia inhibitory factor (Zheng *et al.*, 1999), brain-derived neurotrophic factor (Noga *et al.*, 2003), neurotrophin-3 (Noga *et al.*, 2003) and NGF (Solomon *et al.*, 1998). Since NGF activates eosinophils in vitro (Nassenstein *et al.*, 2003), it would be possible to test in vitro whether or not acetylcholine modulates NGF induced eosinophil mediator release. Eosinophil degranulation can be quantified in vitro using a variety of biochemical assays. The experiments described above could be performed in the absence and presence of blocking antibodies and antagonists for TrkA,

p75NTR and muscarinic receptors, which would confirm specificity for these particular receptors.

The mechanism by which eosinophils increase vagally mediated hyperreactivity independently of blocking M_2 function is not known, however it may involve neural plasticity. Airway hyperreactivity associated with neural plasticity is widely described in sensory nerves (de Vries *et al.*, 2006; Dinh *et al.*, 2004; Freund-Michel *et al.*, 2008; Frossard *et al.*, 2005; Undem *et al.*, 1999; Wu *et al.*, 2001; Wu *et al.*, 2003), but may also occur in parasympathetic nerves (Durcan *et al.*, 2006; Hazari *et al.*, 2007; Wu *et al.*, 2001; Wu *et al.*, 2003; Wu *et al.*, 2002). One potential alteration in parasympathetic nerves is induction of substance P expression, a potent bronchoconstrictor (Wu *et al.*, 2006; Wu *et al.*, 2003; Wu *et al.*, 2002). Thus, it may be that atropine-enhanced antigen-induced hyperreactivity is mediated by substance P.

Alternatively, it may be that the mechanism of atropine-enhanced antigen induced airway hyperreactivity involves enhancement of the cholinergic phenotype, rather than substance P induction. Eosinophils have been shown to increase expression of several cholinergic enzymes regulating the synthesis and storage of acetylcholine, including choline acetyltransferase and vesicular acetylcholine transferase (Durcan *et al.*, 2006), providing a potential mechanism by which eosinophils can enhance cholinergic activity of parasympathetic nerves in vivo. The mechanism by which eosinophils promote an increased cholinergic phenotype is not known, but may involve both eosinophil adhesion to nerves and release of soluble mediators (Durcan *et al.*, 2006). Thus, atropine-

enhanced, antigen challenge-induced hyperreactivity that is mediated by NGF may result from eosinophil-derived factors increasing acetylcholine release and thus increasing vagally mediated airway hyperreactivity.

To distinguish between these two possibilities the following experiments could be done. Neurokinin antagonists block antigen-induced hyperreactivity in the absence of atropine pretreatment by preventing eosinophil activation (Costello *et al.*, 1998; Evans *et al.*, 2000), thus it is not possible to test whether potentiated airway hyperreactivity is mediated by substance P in vivo. Alternatively, this possibility could be tested in vitro using cultured parasympathetic nerves (Fryer *et al.*, 1996). Specifically, these experiments could determine whether activated eosinophils induce substance P production in parasympathetic nerves via release of soluble mediators. Similarly, it would be possible to test for increased acetylcholine production in parasympathetic nerves cultured in the presence of eosinophils. Follow-up experiments could determine whether eosinophil adhesion to nerves is required for neurotransmitter production using a transwell chamber system.

The data presented within this thesis provide a potential mechanism by which muscarinic receptors on eosinophils may regulate their interactions with parasympathetic nerves in the context of asthma. However, there are alternative interpretations of the data presented here. Thus, even though the Ab IL-5 data suggest that atropine potentiation of airway hyperreactivity is mediated by eosinophils (Chapter III), it may be that increased eosinophil activation and subsequent airway hyperreactivity is not directly mediated by blockade of muscarinic receptors on eosinophils, and instead may involve an indirect,

intermediate step. This indirect pathway may involve some other structural or inflammatory cell not identified within these experiments, especially since epithelial cells (Andrew *et al.*, 2003), smooth muscle cells (Wen *et al.*, 2003) and neutrophils (Dewachi *et al.*, 2006; Suttmann *et al.*, 2003) express IL-5 receptors. Additionally, though NGF appears to be involved in atropine-enhanced antigen induced airway hyperreactivity (Chapter IV), there is no direct evidence demonstrating that NGF directly affects eosinophil activation in vivo. NGF regulates function of numerous other cell types within the lung, including lymphocytes, mast cells, neutrophils, macrophages, neurons and fibroblasts (Freund-Michel *et al.*, 2008; Groneberg *et al.*, 2004), any of which may potentially be involved in this pathway. Thus, there may be intermediate steps not identified within the scope of this work. Nonetheless, the data presented in this thesis demonstrate that atropine pretreatment induces a change in nerve function, resulting in increased vagally-induced bronchoconstriction independent of neuronal M_2 receptor dysfunction that depends upon the presence of eosinophils and NGF. A potential mechanism that integrates these observations is shown in Figure 6.1.

Muscarinic receptors on eosinophils may have other important roles in vivo. The data presented here demonstrate that acetylcholine inhibits PAF induced intracellular calcium. It is well known that intracellular calcium signaling is a key second messenger that regulates several eosinophil effector functions, such as chemotaxis, adhesion and superoxide anion generation (Chapter I). Thus, it may be that M_4 muscarinic receptors on eosinophils inhibit other eosinophil effector functions not tested in this thesis. In addition,

the putative function of M_3 and M_5 muscarinic receptors on eosinophils remains unknown, and these receptor subtypes may also have significant roles in the airways. Futhermore, the muscarinic receptor subtypes expressed in the airways may be different from those expressed in the blood. This is supported by preliminary data (Chapter V) that in guinea pigs, bronchoalveolar lavage eosinophils do not appear to express M_3 receptors. Similarly, it is possible that the role of muscarinic receptors on eosinophils in the lungs of asthmatics may be very different compared to normal individuals. Eosinophils in the airways are likely to be influenced by their microenvironment, in particular by inflammatory mediators. Indeed, bronchoalveolar lavage eosinophils from asthmatic humans are more highly activated state (Sedgwick *et al.*, 1992; Sedgwick *et al.*, 1995; Sedgwick *et al.*, 2004). Given that eosinophils in airways of asthmatic humans are more highly activated, it is also possible that muscarinic regulation of eosinophil function may be different in asthma as well.

It is becoming increasingly evident that eosinophils participate in neuroimmune interactions modulating function of peripheral nerves (Durcan *et al.*, 2006; Raap *et al.*, 2008). Not only are eosinophils a source of neuromediators (Chapter I, Table 1.2), but eosinophils also have the capacity to respond to neuropeptides and neuromediators released by peripheral nerves, including substance P (El-Shazly *et al.*, 1996; Foster *et al.*, 2003), calcitonin gene related peptide (Dunzendorfer *et al.*, 1998; Numao *et al.*, 1992), vasoactive intestinal peptide (Dunzendorfer *et al.*, 1998; Numao *et al.*, 1992), brain derived neurotrophic factor (Nassenstein *et al.*, 2003; Raap *et al.*, 2005), neurotrophin-3 (Nassenstein *et al.*, 2003; Noga *et al.*, 2002) and NGF (Nassenstein *et al.*, 2003; Noga *et*

al., 2002; Raap *et al.*, 2008). The data presented in this thesis provide evidence that acetylcholine, a parasympathetic neurotransmitter, may also affect eosinophil function, possibly as part of a negative feedback loop. Thus, it may be that under normal conditions, eosinophils and parasympathetic nerves exist in equilibrium, and that acetylcholine limits eosinophil activation in the presence of parasympathetic nerves. This relationship may be part of a complex interplay between the immune system and the nervous system, in which nerves selectively recruit eosinophils to participate in mechanisms relating to nerve development, repair or growth. This bi-directional communication may be important in the context of asthma, given the close proximity of eosinophils to parasympathetic nerves. Under inflammatory conditions, such as asthma, eosinophil and nerve interactions are clearly altered, causing eosinophils to release proteins that change nerve function. This equilibrium between eosinophils and parasympathetic nerves appears to be further disrupted by anticholinergics, which increases eosinophil activation and may influence the types of neuromediators they produce.

A majority of this discussion has focused on parasympathetic regulation of eosinophil function via acetylcholine because of their proximity to airway nerves in asthma. However, there are additional non-neuronal sources of acetylcholine with the lung. These include the airway epithelium, endothelium, smooth muscle and immune cells (Chapter I, section C). Therefore, it is possible that acetylcholine regulation of eosinophil function in the lung may also occur in non-innervated areas such as the epithelial layers, and may also be mediated by these non-neuronal acetylcholine sources.

The implications of these findings are clinically relevant, considering the poor performance of anticholinergics in management of chronic asthma. In asthmatics, anticholinergic drugs have proven to be effective bronchodilators when given during an asthma exacerbation. However, these drugs are not as effective as predicted from animal studies and are not recommended for chronic treatment of asthma (National Heart, 2007). The findings described in this thesis provide a tantalizing explanation for this paradox, and suggest that timing of anticholinergic administration is very important. Anticholinergics given after antigen challenge are effective bronchodilators, but if administered before challenge, as would be the case if given chronically, would make hyperreactivity worse.

The findings presented here also suggest new alternative approaches to developing more effective treatments for asthma. In asthma, the most commonly used anticholinergic drug, ipratropium, blocks all muscarinic receptors with equal affinity. Thus, non-selective anticholinergics will enhance eosinophil activation in asthma, which may explain the lower than expected clinical efficacy in chronic treatment of asthma. Development of anticholinergics that selectively target M_3 receptors on airway smooth muscle while simultaneously sparing beneficial M_4 receptors on the eosinophils and beneficial M_2 receptors on parasympathetic nerves would be important. The findings presented here demonstrate that muscarinic blockade at the time of antigen challenge increases airway hyperreactivity and eosinophil activation in vivo. The findings of this thesis may translate into strategies for identifying drugs that simultaneously decrease asthma exacerbations

and limit eosinophil activation in the airways. Future experiments could include using selective antagonists for each muscarinic receptor subtype (Chapter I, Table 1.1), which would clarify the individual contribution of each receptor subtype.

In summary, this work has identified a potentially novel interaction between the parasympathetic nerves and eosinophils in which parasympathetic nerves limit eosinophil activation. I have provided evidence supporting my hypothesis that eosinophils respond to acetylcholine via muscarinic receptors, and that activation of these receptors inhibits eosinophil function, changing interactions with airway parasympathetic nerves. While it has been known for many years that eosinophils affect parasympathetic nerve function, I have identified a reciprocal mechanism by which nerves may inhibit eosinophil function. This observation is important because it may explain why anticholinergic drug treatments that should be effective in managing asthma are not, despite the current body of knowledge in this field and may provide a rationale for new approaches to pharmacological control of the lungs. This newly identified interaction between the parasympathetic nerves and eosinophils may be also be important in other chronic inflammatory diseases characterized by eosinophil localization to cholinergic nerves, including, rhinitis and eosinophilic gastroenteritis (Costello *et al.*, 1997; Gleich, 2000; Hogan *et al.*, 2002; Rothenberg *et al.*, 2001; Sawatzky *et al.*, 2002). These findings are important because they contribute to our understanding of the complex interactions between the immune and nervous systems and because they will influence future therapeutics for treating diseases characterized by increased eosinophil activation, such as asthma.

FIGURE 6.1. Summary of major findings and their significance to asthma.

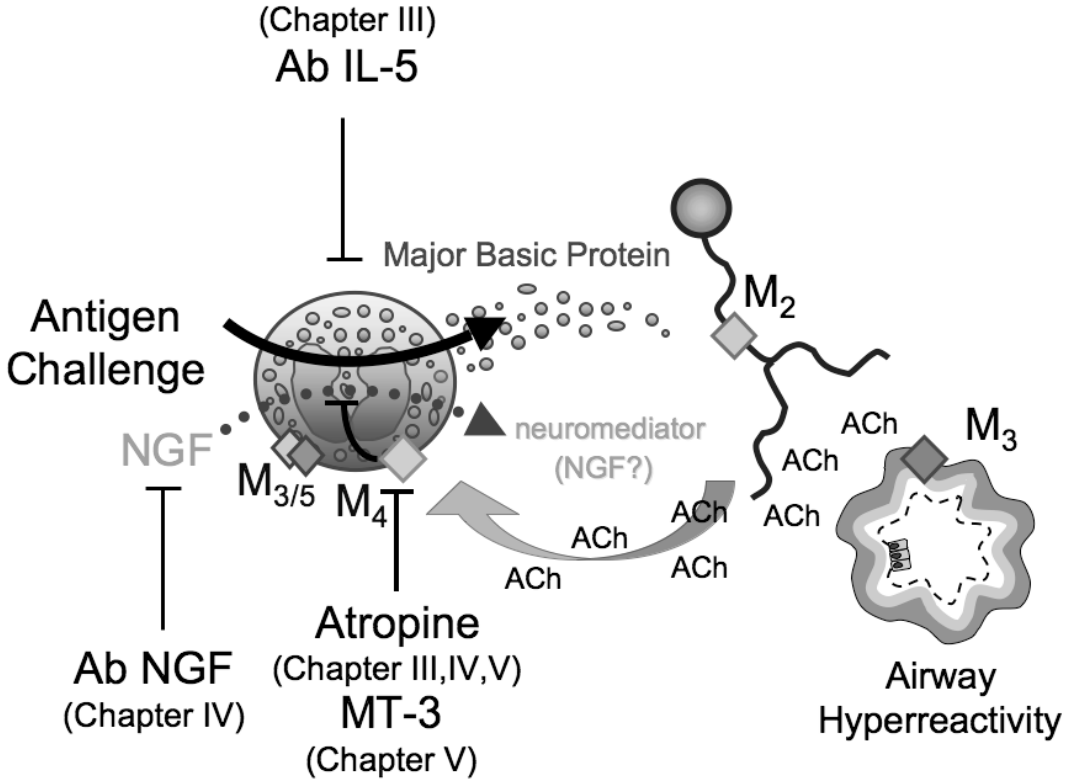


Figure 6.1. Using an animal model of asthma, I have demonstrated that when given prophylactically, anticholinergics increase eosinophil activation in the airways, thereby making airway hyperreactivity significantly worse (Chapter III and IV). This paradoxical effect is explained the presence of muscarinic receptors on eosinophils, which I have shown inhibit PAF-induced eosinophil activation in vitro (Chapter V). I have also identified that it is the M_4 muscarinic receptor subtype that is responsible for this inhibitory effect on eosinophil activation (Chapter V). Since anticholinergic drugs also block eosinophil M_4 muscarinic receptors, these drugs would hypothetically disrupt this inhibitory pathway. Disruption of this inhibitory pathway in asthma thereby increases eosinophil activation and release of eosinophil derived neuromediator. This eosinophil

derived factor (which may be NGF or another neuromediator) induces a change in parasympathetic nerve activity leading to hyperreactivity through a mechanism not involving neuronal M_2 receptor dysfunction. The arrows shown in this diagram suggest the order of events, but do not exclude the steps in between.

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