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CHOLINERGIC AND ADRENERGIC RESPONSES OF BRONCHIAL RINGS AND PERIPHERAL LUNG STRIPS FROM IMMUNOGLOBULIN E-PRODUCING AND CONTROL RABBITS

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CHOLINERGIC AND ADRENERGIC RESPONSES OF BRONCHIAL RINGS AND PERIPHERAL LUNG STRIPS FROM IgE-PRODUCING AND CONTROL RABBITS

by

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ABSTRACT

Asthmatic or allergic humans have enhanced cholinergic and decreased beta-adrenergic responsiveness, and these individuals generally possess elevated serum IgE levels. A rabbit model of IgE-mediated allergy, wherein in vivo studies demonstrated enhanced cholinergic pupil responsiveness, was used here to test whether cholinergic hyperresponsiveness or beta-adrenergic hyporesponsiveness was demonstrable in isolated airway smooth muscle from rabbits with elevated serum IgE levels. The contractile response to methacholine or the relaxant response to isoproterenol showed no difference between IgE-producing and control rabbits. To determine the muscarinic subtype(s) mediating cholinergic contraction in rabbit airway smooth muscle, inhibition studies compared the classical muscarinic antagonist atropine with the M_1 receptor subtype antagonist pirenzepine. These studies indicated the M_1 muscarinic receptor subtype did not mediate contraction of airway smooth muscle induced by either exogenous methacholine or endogenously released acetylcholine. The results revealed muscarinic contraction of airway smooth muscle to be mediated by an M_2 (pirenzepine low-affinity) receptor subtype.

CHAPTER 1

INTRODUCTION

Intent of Study

Allergic asthma is characterized by bronchial hyperreactivity and by increased levels of serum IgE antibodies. Whether these two characteristics are inter-related is not known. An animal model of IgE-mediated allergy is used in this investigation to determine whether the induction of elevated serum IgE levels promotes hyperreactive airways.

The underlying physiologic mechanism for the airway hyperreactivity has also not been established. The degree of airway reactivity may involve abnormalities in the airway smooth muscle itself or in the nerves which regulate its tone. The autonomic nerves which innervate the lungs terminate at the bronchial smooth muscle cells on which there are various types and subtypes of receptors. By examining these receptors and agents which may promote or inhibit their ability to produce certain responses, one may be able to draw conclusions about the nature of airway hyperreactivity or about agents which may control this reactivity.

Airway smooth muscle contractility and the receptors which mediate that contractility may be assessed through functional *in vitro* tissue bath studies. The first goal of this investigation is to analyze whether elevated serum IgE levels are associated with an enhanced bronchial contraction and/or decreased bronchial relaxation. The second major goal is to examine certain receptors of the airway smooth muscle, namely muscarinic receptors, and their ability to bind certain drugs. By examining three drugs - methacholine a muscarinic agonist , atropine (a classical antagonist) and pirenzepine a nonclassical antagonist with selective affinity for certain muscarinic receptors - we can distinguish the subtype of muscarinic receptors responsible for airway contraction.

IgE-Antibody - Mediated Hypersensitivity

IgE antibody is present in very low concentrations in the serum of normal individuals (less than 0.2µg/ml serum). The serum IgE concentration is elevated in some allergic or asthmatic individuals and in patients with some parasitic infections such as schistosomiasis. The only known biological importance of IgE antibody is its ability to mediate hypersensitivity allergic reactions (Nisonoff, 1982) and possibly its capacity to help protect against parasitic infestation.

Besides being in the serum, IgE antibodies are bound to the membranes of mast cells in tissues or to basophils in blood. These antibodies can bind to the cell membranes via a site near the

carboxyterminal end of the heavy chains, called the Fc portion. An allergen (or antigen) can crosslink two antibody molecules at a site near the aminoterminal ends of the heavy and light chains, called the Fab portion. This crosslinking causes the mast cells or basophils to release preformed pharmacological mediators, such as histamine, and newly synthesized mediators such as leukotrienes, prostaglandins and thromboxanes which can produce hypersensitivity or allergic reactions characterized by smooth muscle constriction, increased vascular permeability, cellular infiltration, and other changes. Since there are mast cells located in the airway epithelium, these adverse effects may be responsible for airway bronchoconstriction, airway edema, and other reactions of allergic asthmatics (Austen and Orange, 1975). Allergic reactions may actually be the body's first line of defense against injury because they cause inflammation which then stimulates tissue repair.

Pinckard, Halonen, and Meng (1972) have developed a rabbit model of IgE-mediated allergy. By immunizing neonatal rabbits and continuing on a specific schedule thereafter, the rabbits will produce preferentially IgE antibody to a given antigen. With this model the antigen exposure can be controlled and any allergic reactions to this antigen can be attributed to the IgE antibody.

Airway Hyperreactivity is Characteristic of Asthmatics

The most distinguishing feature of asthmatic individuals is their bronchial hyperreactivity. These individuals bronchoconstrict after inhalation of agents or drugs, such as smoke, cold air,

histamine, or acetylcholine, in doses which have no effect on normal individuals. This increased bronchial reactivity is sometimes called "nonspecific" bronchial hyperreactivity in contrast to the bronchospasm initiated by immunologically specific antigens (Boushey et al., 1980).

An asthmatic may have extrinsic or intrinsic asthma. Extrinsic is also called allergic, atopic, or immunologic asthma, and attacks usually occur during pollen seasons, in the presence of animals, on exposure to house dust, or in response to other allergens. Intrinsic is also called nonallergic or idiopathic asthma and these subjects have recurrent bronchial obstruction to nonspecific irritants, unrelated to any known allergen exposure (Terr, 1974).

It is not known how the condition of bronchial hyperreactivity arises. It may result from an abnormality in the airway smooth muscle or possibly in the nerves which regulate its tone. It is also interesting that subjects with allergic asthma, allergic rhinitis, urticaria, or atopic dermatitis generally have elevated serum IgE levels as compared to nonallergic individuals (Boushey, 1982). It has been found that both allergic individuals and asthmatics have cholinergic hyperreactivity (Nadel, 1976). The significance of the relationship between bronchial hyperreactivity and elevated serum IgE levels has not yet been established, though.

Smith, Shelhamer and Kaliner (1980) have shown that allergic humans also exhibit cholinergic hyperreactivity when pupillary constriction is assessed. Thus, asthma is associated with both pupillary and bronchial cholinergic hyperreactivity. Since, in the

IgE-mediated allergic rabbit model, cholinergic responses are also heightened in pupillary studies (Halonen and Kaliner, 1984), it seemed possible that this allergic rabbit model might also have heightened bronchial cholinergic responses. In vitro experiments assessing airway smooth muscle contraction are performed in this investigation to examine this possibility.

The beta-adrenergic theory of the abnormality in bronchial asthma has been postulated by Szentivanyi (1968). According to this theory, allergic individuals have a reduced responsiveness to beta-adrenergic stimulation and therefore possess a decreased ability to bronchodilate when their airways are constricted.

It has been shown by Rinard et al.(1979) in an asthmatic dog model that beta-adrenergic receptor function is impaired in airway smooth muscle tissues such that isoproterenol, a beta agonist, does not produce as much of a relaxing effect in these dogs as it does in normal "nonasthmatic" dogs. We herein examined whether this beta-adrenergic abnormality is present in the airways of the allergic rabbit model.

The Autonomic Nervous System

The baseline bronchial tone and at least part of the bronchial constriction or dilation which occurs in response to certain agents is thought to be regulated by the autonomic nervous system. The autonomic nervous system regulates the activities of structures which are under involuntary control. It provides innervation to the heart,

blood vessels, glands, viscera and smooth muscles, and regulates such functions as heart action, blood pressure, respiration, metabolism, digestion, sweating, and vision.

The autonomic nervous system is composed of two divisions: the sympathetic division and the parasympathetic division. The sympathetic nerves control general responses that result from various kinds of stress and is said to be the "fight or flight" response. The parasympathetic nerves are organized for local discharge and are concerned with restoring energy rather than expending energy; therefore, these nerves control the so-called "housekeeping" functions of the body. These two divisions then work in concert to keep the body functioning normally.

The sympathetic division has short preganglionic and long postganglionic fibers, with each preganglionic fiber able to fire many postganglionic fibers. The parasympathetic division has long preganglionic fibers which are connected 1:1 to short postganglionic fibers. In both cases the preganglionic fibers emerge from the brain or spinal cord and the postganglionic fibers provide innervation to the effector cells, such as the smooth muscle cells.

In the autonomic nervous system there are two major neurotransmitter substances, acetylcholine and norepinephrine. These substances distinguish the types of neurons: acetylcholine is the neurotransmitter for cholinergic neurons and norepinephrine is the neurotransmitter for adrenergic neurons.

When a neurotransmitter substance is released from a postganglionic nerve fiber, or when a substance is administered exogenously, the molecules combine at sites called receptors on the cells of the effector organ. A substance which activates these sites is called an agonist and these substances produce a detectable change, be it an increase or decrease in function. A substance which binds to the receptor and inhibits or blocks the response of the agonist is called an antagonist.

The cholinergic neurons are subdivided into two receptor types based on observations that two chemicals mimicked differently the actions of acetylcholine at different sites of nerve impulse transmission. The chemical nicotine was found to mimic acetylcholine at "nicotinic" sites and the chemical muscarine was found to mimic acetylcholine at "muscarinic" sites. The nicotinic sites occur at all autonomic ganglia, the adrenal medulla, and at the neuromuscular junction of somatic nerves and skeletal muscle. The muscarinic sites occur at effector cells in the parasympathetic division.

As mentioned, the neurotransmitter at adrenergic receptors is norepinephrine, but epinephrine, which can be released from the adrenal medulla and enter the blood stream, can also influence adrenergic receptors. Adrenergic receptors are subdivided into two types: alpha and beta. Alpha receptors generally produce an excitatory effect (e.g. arteriole constriction, contraction of iris radial muscle) except in the gut and beta receptors generally produce an inhibitory effect (e.g. relaxation of bronchial smooth muscle,

dilation of arterioles) except in the heart. These are then also divided further into subtypes: $alpha_1$, $alpha_2$, $beta_1$, and $beta_2$. These subtypes are classified as to whether certain agonists or antagonists can interact at these sites.

Muscarinic Receptors

Acetylcholine acts as an agonist at cholinergic muscarinic receptors of bronchial smooth muscle, as previously described. Methacholine, an analogue of acetylcholine, can also act at these receptors. In vitro studies using isolated bronchial rings and peripheral lung strips is a widely used method to examine the effects of drugs at these receptor sites (Hooker, Calkins and Fleisch, 1977, Lulich, Mitchell and Sparrow, 1976). This method of using isolated rings of smooth muscle was first developed for blood vessels by Bevan (1962), Nielsen and Owman (1971), and Bevan and Osher (1972). Spirally cut strips were used for larger tissues such as larger blood vessels and trachea (Furchgott, 1960, Patterson, 1958, Fleisch and Calkins, 1976, Furchgott and Bhadrakom, 1953), but the ring preparation can measure responses in smaller vessels and airways in a manner more closely approximating their in vivo response. Reactions of both cholinergic and adrenergic receptors have been studied in this fashion (Hooker et al., 1977, Lulich et al., 1976, Rinard et al., 1979).

Cholinergic muscarinic antagonists, such as atropine, have been shown to inhibit competitively the contractile response to muscarinic agonists (Fleisch and Calkins, 1976, Drazen and Schneider, 1977) in

bronchial smooth muscle. Recently it has been proposed that there are subtypes of muscarinic receptors (Hammer et al., 1980, Hammer and Giachetti, 1982, Watson, Roeske and Yamamura, 1982, Birdsall, Burgen and Hulme, 1978, Birdsall and Hulme, 1983, Hammer and Giachetti, 1984). These subtypes are based on binding studies with agonists and with antagonists such as pirenzepine, in which these agents exhibit high and low affinity binding in different tissues (Hammer et al., 1980, Birdsall et al., 1980, Hammer and Giachetti, 1982, Watson et al., 1982). Receptor subtypes which have a high affinity for pirenzepine are termed M_1 receptors and those with low affinity for pirenzepine are called M_2 receptors as described by Hammer et al. (1980), Birdsall et al. (1980), and Watson et al. (1982). At M_1 receptors, the potency of pirenzepine is within one order of magnitude to atropine, but at M_2 receptors pirenzepine is up to 200 times less potent than atropine (Hammer, 1980). M_1 receptors can be found in discrete areas of the mammalian brain and in peripheral ganglia, and M_2 receptors have been found in heart, smooth muscle of the upper gastrointestinal tract and bladder, and other peripheral organs (Hammer et al., 1980, Hammer and Giachetti, 1982, Watson et al., 1982, Birdsall and Hulme, 1983).

There are different types of techniques available to assess selective muscarinic action of different drugs in different organs. One approach involves <u>in vivo</u> studies in man or animals. A second

type is *in vitro* functional studies using isolated tissues, and a third technique is receptor ligand binding to cell membranes. In order to prove receptor selectivity, uniform concensus in the results of all of these techniques is desired (Hammer and Giachetti, 1984).

Subtypes of muscarinic receptors have not been fully characterized in mammalian lung tissue. Our group has also recently shown (Lawrence, Halonen and Yamamura, 1984) that radiolabeled quinuclidinyl benzilate (QNB) can identify cholinergic muscarinic receptors in rabbit lung homogenate by binding to all muscarinic cholinergic receptors with equal affinity. Tivol, Halonen and Yamamura (1985) and Lawrence et al., (1984) have shown that pirenzepine can displace ^{3}H-QNB binding from lung homogenate with high affinity. Tivol has also shown direct binding of ^{3}H-pirenzepine to peripheral lung and that atropine and pirenzepine can inhibit ^{3}H-pirenzepine binding. Also in our group, *in vivo* studies by Bloom et al., (unpublished) have used vagal stimulation to increase airway resistance and decrease heart rate in the anesthetized rabbit. Atropine or pirenzepine was infused to inhibit these responses and it was found that atropine and pirenzepine have similar potencies in inhibiting an increase in airway resistance (atropine 17-fold more potent) but atropine is much more potent in blocking the decreased heart rate response as compared to pirenzepine (200-fold more potent). These studies suggest that muscarinic receptors in rabbit lung may belong to the M_1 subtype. To determine whether these M_1 receptors mediate the smooth muscle contraction of airway tissues, *in vitro* functional studies using isolated tissues in tissue baths were performed in this investigation.

In these functional studies, one way to compare the potencies of certain drugs is by comparing their pA_2 values. The pA_2 value is the -log concentration of the antagonist needed to produce a two-fold increase in the ED50 (effective dose 50%) of the agonist (Furchgott, 1967). The pA_2 values can also compare the potency of a drug in two or more different types of tissues. Heathcote and Parry (1980) compared the effect of pirenzepine, atropine, and other drugs in reducing gastric acid secretion in rats and in increasing pupil diameter of mice. The concentration of atropine needed to do this was about the same in both situations, but an approximately three-fold higher dose of pirenzepine was needed to increase pupil diameter than was needed to reduce acid secretion. Hirschowitz, Fong and Molina (1983) showed the existence of high affinity M_1 receptors in the stomach and low affinity M_2 receptors in the heart rate of the dog. Another group has shown that pirenzepine and atropine differ in potency by about 10 in gastric secretion, but by 100 in gastric smooth muscle contraction by comparing pA_2 values (Del Tacca et al., 1984), atropine being more potent in each case.

In addition to functional studies using isolated tissues and applying agonists to produce smooth muscle contraction, tissues can be subjected to electrical field stimulation and contractile responses measured due to the excitatory cholinergic nerves present. Electrical field stimulation is an excellent method to produce stimulation in smooth muscle cells because all cells are activated simultaneously,

directly, and equally (Sperelakis, 1978). Also, this system can be effectively controlled. It can be shown that the nerve, and not the smooth muscle itself, is being stimulated by adding tetrodotoxin which is a nerve blocker. No response should be seen if only the nerves are being stimulated when tetrodotoxin is present.

Studies have shown that electrically stimulated contractile activity of airway smooth muscle is atropine- and tetrodotoxin-sensitive and is enhanced by anticholinesterase agents (Davis et al., 1982 in human tracheal and first-order bronchial smooth muscle, Jones, Hamilton and Lefcoe, 1980 in guinea pig trachea, Taylor, Pare and Schellenberg, 1984 in guinea pig tracheal trains). It has been shown that parasympathetic fibers innervate the lungs (Richardson, 1979) and that in vivo electrical stimulation of vagal nerves causes bronchoconstriction but is inhibited by atropine (reviewed by Nadel, 1980). We used this type of study in rabbit airways to further assess the participation of muscarinic receptor subtypes in bronchoconstriction induced by cholinergic (postganglionic) nerves.

CHAPTER 2

METHODS AND MATERIALS

Animals and Immunizations

California rabbits, ages 3-4 months, of either sex were used as the source of lung tissues for these experiments. Rabbits were either sensitized or controls. Sensitized rabbits were immunized with the antigen horseradish peroxidase (HRP) within 24 hours of birth, and periodically thereafter according to the schedule in Table 1, thereby producing preferentially IgE antibody to this antigen (Pinckard, Halonen, Meng, 1972).

Control Rabbits, either California or New Zealand White, were also used in homologous passive cutaneous anaphylaxis assays, which are described below.

Most rabbits were maintained for three months at Olson's Blue Ribbon Ranch, Tucson, Arizona, and thereafter were maintained in the Division of Animal Resources, College of Medicine, University of Arizona. Some rabbits were also maintained, immunized, and received from Penn Acres, Wimberley, Texas.

Table 1. Immunization schedule for rabbits.

Rabbits were immunized neonatally as follows:

Day	Antigen
0, 7, 14	1 mg of soluble HRP, i.p.
21	1 mg of alum HRP, i.p.
35, 49, 63	1 mg of soluble HRP, i.p.
77	1 mg of alum HRP, i.p.

Antigen

The antigen used for immunizations was horseradish peroxidase (HRP; Type II, Sigma Chemical Co., St. Louis, MO). A 10mg/ml stock solution of HRP was prepared in pyrogen-free 0.85% saline solution, sterilized by Millipore filtration, and stored at -20°C. Alum precipitated HRP was prepared by diluting the stock HRP solution to 4 mg/ml, adding an equal volume of Alhydrogel (1.3% aluminum oxide, Superfos Export Co., Copenhagen, Denmark) and mixing gently by inversion.

Antibody Determinations

To determine rabbit serum anti-HRP IgE titers, homologous passive cutaneous anaphylaxis (PCA) assays were performed (Halonen et al., 1976). Two-tenths millliliter volumes of sera or serum dilutions were injected intracutaneously onto the shaved backs of California or New Zealand White control, approximately 3-month-old, rabbits. Each sample was done in duplicate on 2 separate rabbits. After a sensitization period of 72 hours, 2 ml of 2% Evans Blue dye (diluted in saline) was injected into a marginal ear vein of the rabbit. A 30 minute period followed this to allow for any non-specific bluing to occur. If it did occur, those areas were marked. Five-tenths millliliter of 10mg/ml HRP was then injected into the rabbit's other marginal ear vein and after 30 minutes the animals were killed by pentobarbital (3g) intracardiac injection and the blue areas read from the external side of the skin. A serum or serum dilution was

considered positive if the diameter of the bluing was greater than 5mm. The largest dilution of serum which produced a positive response in both rabbits was the titer of that serum. Each rabbit received up to 40 different serum injections, three of which were dilutions of a standard serum which was known to be positive for anti-HRP IgE.

Isolated Airway Tissue Bath Studies

Animals were sacrificed either by cervical dislocation followed by exanguination or by decapitation. After removal of the heart and lungs, they were placed in a dish containing Krebs Solution and oxygenated with 95% O_2/ 5% CO_2 during the dissection of the tissues. The composition of the Krebs Solution is given in Table 2. For studies using peripheral lung strips, the lungs were perfused with 100 ml physiological saline solution before removal of the heart and lungs.

Three types of airway tissues were used: rings of mainstem bronchi (MSB), rings of intrapulmonary bronchi (IPB), and peripheral lung strips (PLS), whose sizes were approximately 3-5 mm diam., 3-5 mm long; 1-2 mm diam., 3-5 mm long; and 2x3x10 mm, respectively (Hooker, Calkins, Fleisch, 1977, Lulich, Mitchell, Sparrow, 1976). MSB rings were taken just beyond the corina and just before entering the lungs. Two rings could be obtained from each bronchus. IPB rings and PLS were obtained from either the right or left lung lower lobes. The IPB tissues were 1/2-1/3 distance from the bottom of the lower lobe, and PLS were cut from the peripheral margin of the lobe. The PLS which

Table 2. Composition of Krebs Solution.

Krebs Stock (g/liter)		Krebs Solution (1 liter)	
NaCl	28.55	250 ml	Krebs Stock
$NaHCO_3$	8.59	750 ml	d.d. H_2O
KCl	1.54	2 g	dextrose
$MgSO_4 \cdot 7H_2O$	1.20	1.6 ml	1M $CaCl_2$
KH_2PO_4	0.66		
Bring up to 1 liter with d.d. H_2O and pH to 7.4.			

were used were free of any obvious blood vessels. Tissues were from either freshly sacrificed rabbits or tissues which had been dissected and cut into the appropriate sizes and refrigerated overnight at $4^{o}C$ in 50ml air-tight tubes filled with Krebs Solution that had previously been bubbled with $95\%O_2/5\%CO_2$ (Ghelani, Holroyde, Sheard, 1980).

The tissues were then suspended in 10ml jacketed tissue baths containing Krebs Solution bubbled with $95\%O_2/5\%CO_2$ maintained at $37^{o}C$. Two stainless steel triangular wires which were each attached to 000 suture were placed through the MSB and IPB rings. PLS were tied on each end with 000 suture. One end was stationary at the bottom of the bath and the other suture attached to a strain gauge transducer (Statham UC3), which measured force isometrically. The force displacement was measured on a model DR-8 recorder from Electronics for Medicine, Inc., White Plains, N.Y.

Tissues were given an initial tension during a 45 min. stretching period, or until tissues stabilized: PLS=0.3g, IPB=0.75g, and MSB=2.0g (based on prior optimal tension studies).

Methacholine Responses

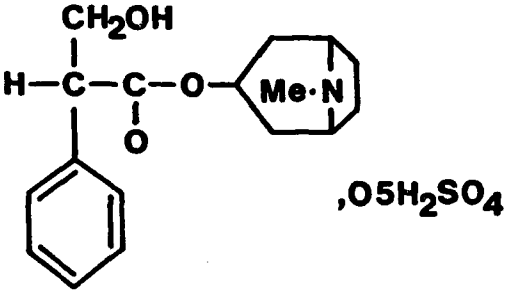
These studies were performed in collaboration with and in the laboratory of Dr. John Palmer. Cumulative concentrations of methacholine (Sigma Chemical Co., St. Louis, MO) were added to each bath at 5 min. intervals (100µl of the first concentration and 90µl of each following concentration). Methacholine was dissolved in Krebs Solution. A concentration-response curve was then constructed using the maximum g response to methacholine as 100% response for each tissue.

EC50 (Effective Concentration 50% i.e., the concentration required for a half-maximal response) values were calculated for each tissue. Tissues from control vs. IgE-producing rabbits were then compared.

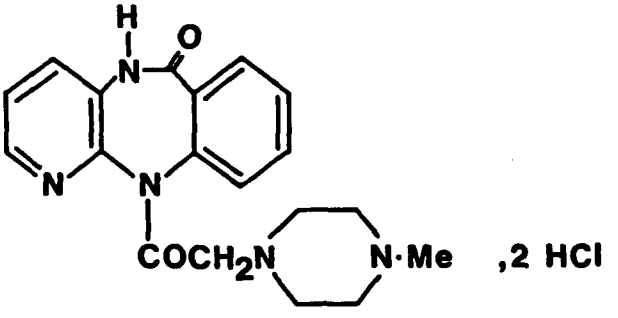
Responses to Atropine and Pirenzepine

Cholinergic antagonists (when used) were added to the baths during the stretching period. Atropine sulfate (Merck and Co., Inc., Rahway, N.J.) and pirenzepine (a gift from Boehringer Ingelheim, Ridgefield, CT) dilutions were made in Krebs Solution. Structures of these muscarinic antagonists are given in Figure 1. Atropine concentrations used were 10^{-9}, 10^{-8}, and 10^{-7}M. Pirenzepine concentrations used were 10^{-7},10^{-6},10^{-5}, and 10^{-4}M. EC50 values for methacholine were obtained at each antagonist concentration.

To determine antagonist potency and whether it was acting competitively, Schild plots were made by plotting log (dose ratio-1) of agonist vs. -log [antagonist], where the dose ratio is the EC50 for the agonist in the presence of an antagonist divided by the EC50 in the absence of the antagonist, a method from Furchgott(1967). Slopes were determined by regression analysis of all points from individual tissues treated with each agonist. The x-axis intercept was the pA_2 value. The pA_2 value is the negative log of the antagonist concentration needed to shift the dose-response curve 2-fold to the right. The pA_2 values of atropine and pirenzepine were then compared. The pA_2 values for airway tissues from control and IgE-producing rabbits were also compared.



Atropine Sulphate



Pirenzepine Dihydrochloride

Figure 1. Structures of Atropine and Pirenzepine.

Electrical Field Stimulation of Airway Tissues

These studies were performed in collaboration with and in the laboratory of Dr. Sue Duckles. MSB and IPB tissues were used for these experiments and were prepared and suspended in Krebs solution in much the same manner as previously described. The baths used here were 50 ml baths. In addition, 2 one-inch platinum electrodes were placed one on each side of the airway ring. The 2 electrodes were parallel and were connected to a stimulator (Grass S44) which produced the stimulation. The transducers were Statham UC2 and the recorders were Soltec 220 and Soltec 1242.

Tissues were stimulated until a peak response was seen, usually 1-2 min., and then the stimulation turned off when the response began to subside. The tissue usually relaxed below the baseline and was left to rest for 10 min. before being stretched back to the baseline and then stimulated again.

A tissue stretch study was performed prior to doing the experiments to determine the amount of initial baseline tension to be placed on the tissue in order to obtain optimal contractile responses to electrical field stimulation. Arbitrary stimulator settings were used (which others had used) first: 15V,16Hz, 0.3 msec. For MSB, tissues were stretched to 1g until stable (approximately 45 min.) then stimulated and the response recorded. Then the tissue was stretched to 2g until stable (approximately 15 min.), stimulated and the response recorded. This same procedure was repeated for 3 and 4g.

The 1g tension gave the optimal contractile g response, so it was used for the experiments. A similar type of study was done for IPB tissues, which were stretched at 0.5,0.75,1.0, and 1.5g. A tension of 0.5g gave the optimal contractile response; therefore, this tension was used for further experiments.

A study was also done to determine optimal stimulator settings for frequency, voltage, and duration. A frequency-response curve was first established. The voltage and duration remained constant (15V,0.3msec.) and the frequency was varied at 2,4,8,16,32, and 64 Hz. A frequency which gave approximately one-half the maximal response was chosen, that being 16 Hz. A setting which gave a half maximal response was used, rather than maximal response, because we wanted to be on the ascending portion of the curve for subsequent inhibition studies. A voltage-response curve was also constructed. The frequency and duration were kept constant (16Hz,0.3msec.) and the voltage was varied at 10,15,20,25,30, and 40V. The curve reached a plateau between 20-40V; therefore, 25V was chosen for future studies. At 16 Hz and 25V, maximal response was reached at 0.3 msec., so this duration setting was used.

Stimulations at 16Hz,0.3msec,25V were quite reproducible over the length of the experiment. To determine that only the nerves, and not the smooth muscle itself, were being stimulated by the electrodes, tetrodotoxin (Cal-Biochem-Behring Co., La Jolla, CA) at $3x10^{-7}$g/ml, a nerve blocker, was added to the baths prior to stimulation. Tetrodotoxin completely inhibited the contractile response (Taylor, Pare, Schellenberg, 1984).

In the electrical field stimulation studies, atropine or pirenzepine was added cumulatively to the baths. The lowest concentration of antagonist was added to the bath and after 10 min. the tissue was stimulated. This procedure was followed for each higher concentration. Concentration-response curves were generated by percent inhibition of the original responses before the addition of the antagonist. The IC50's (i.e., the concentration which produces 50% inhibition) of atropine and pirenzepine in inhibiting the response were then compared.

Isoproterenol Studies

For the isoproterenol hydrochloride (Sigma Chemical Co., St. Louis, MO) relaxation studies, a baseline tension was first induced by adding a concentration of methacholine which was known to produce a half-maximal contraction. Once this was established, cumulative concentrations of isoproterenol were added at 5 min. intervals to produce tissue relaxation. Isoproterenol was dissolved in 0.5% sodium metabisulfite as an antioxidant. The sodium metabisulfite was prepared in Krebs Solution. Results were expressed as percent relaxation from the methacholine-induced tension. Control and IgE-producing rabbits were compared in their relaxation responses.

Pirenzepine Relaxation Studies

In order to assess whether pirenzepine had any relaxing effect on tissues, histamine (Sigma Chemical Co., St. Louis, MO) at 10^{-4}M or phenylephrine, an $alpha_1$-adrenergic agonist, (Sterling-Winthrop

Research Institute, Rensselaer, NY) at 10^{-4}M were added to the tissue baths to get a preliminary tone prior to beginning the methacholine concentration-response curves in the presence of the various pirenzepine concentrations. Histamine was dissolved in Krebs Solution and brought to pH 7.4 with 10M NaOH. Phenylephrine was also prepared in Krebs Solution.

Statistical Methods

The results are expressed as means $\pm$S.E.M. Student's t-test was used to determine the statistical significance of the difference between two means. A P value of ≤ 0.05 was accepted as indicating a significant difference. Maximum g responses for the tissues treated with atropine or pirenzepine were analyzed for differences using 2-way analysis of variance (ANOVA). Two-way ANOVA was also used to determine whether the maximum g responses among individual baths were significantly different. Means and S.E.M.'s for all results are listed in Appendix A.

CHAPTER 3

RESULTS

Contractile Responses to Methacholine in Airway Tissues from IgE-Producing vs. Control Rabbits

Cumulative concentration-response curves were established for methacholine contractile activity in peripheral lung strips from 10 IgE-producing and 10 control rabbits (Fig. 2). Similar responses were assessed in intrapulmonary bronchi tissues from 9 IgE-producing and 15 control rabbits (Fig. 3).

As shown in Table 3, IgE-producing rabbits did not differ from controls in either the concentration of methacholine which induces a half-maximal response (EC50) or in the maximum force generated by methacholine. Interestingly, peripheral lung strips exhibited the same EC50 as did the bronchial rings despite the marked differences in the amount of force generated by these tissues. Methacholine maximum contraction of peripheral lung strips was about 31% of the maximum attainable with 0.03M $BaCl_2$. In contrast, intrapulmonary bronchi are apparently maximally contracted by this cholinergic agonist.

Homologous PCA assays on sera from each of the IgE-producing rabbits demonstrated that all were producing anti-HRP IgE antibodies with titers ranging from 1 to 40.

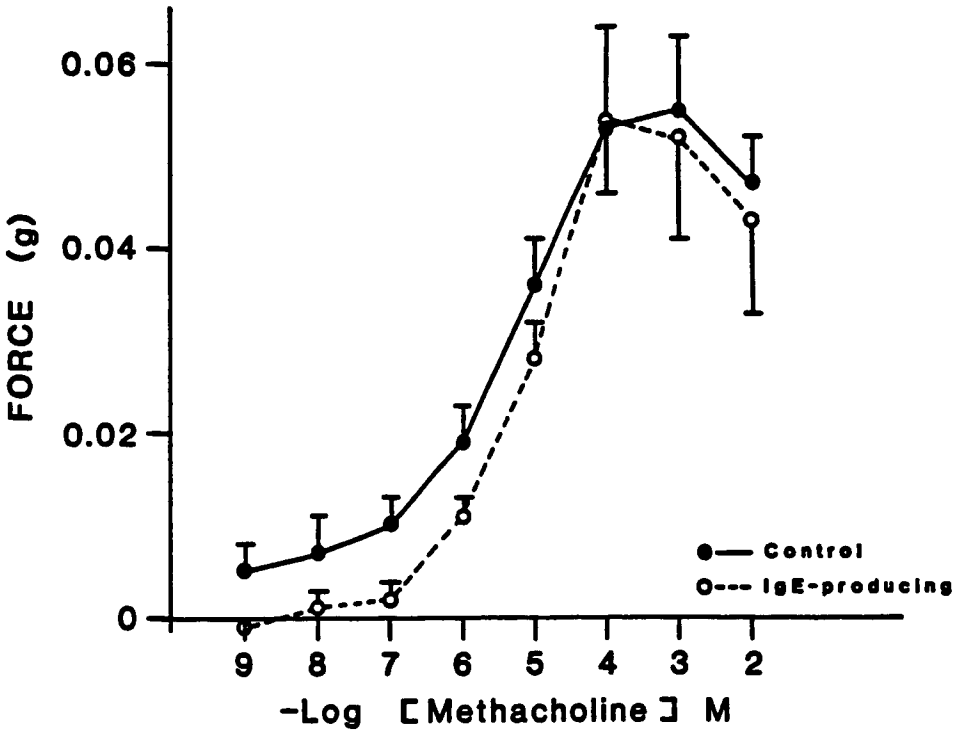


Figure 2. Methacholine concentration-response curves in rabbit peripheral lung strips from 10 IgE-producing and 10 control rabbits. (Means ± S.E.M.). Contractile force was measured isometrically in strips under 0.3g baseline tension. Methacholine was added cumulatively at 5 min. intervals.

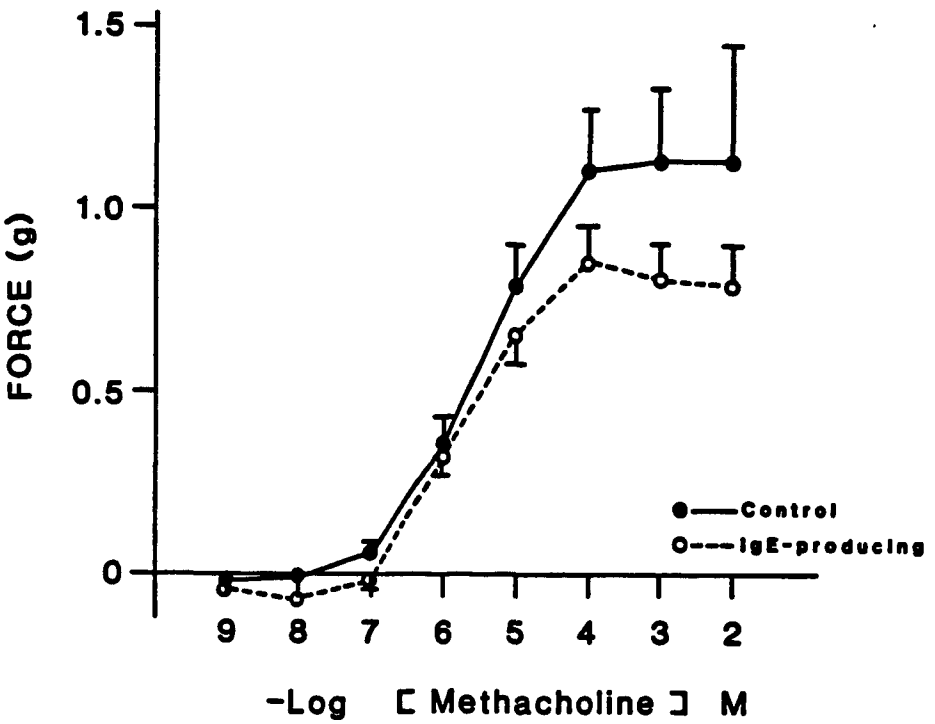


Figure 3. Methacholine concentration-response curves in rabbit intrapulmonary bronchial rings from 9 IgE-producing and 15 control rabbits. (Means ± S.E.M.). Contractile force was measured isometrically in tissues under 0.75g baseline tension. Methacholine was added cumulatively at 5 min. intervals.

Table 3. Comparison of Methacholine Contractile Activity in Peripheral Lung Strips and Bronchial Rings of IgE-producing vs. Control Rabbits.

	-log EC50	Maximum Response(g)	% of $BaCl_2$ maximum
Peripheral lung strips			
IgE Rabbits (10)	5.46±0.31	0.057±.011	29.22±2.84
Control Rabbits (10)	5.61±0.19	0.057±.008	33.22±2.29
	NS	NS	NS
Intrapulmonary bronchial rings			
IgE Rabbits (9)	5.54±0.07	0.94±0.12	
Control Rabbits (15)	5.54±0.07	1.19±0.21	
	NS	NS	

Antagonism of Methacholine Contractile Activity by Atropine and Pirenzepine

Atropine shifted the methacholine concentration-response curve to the right in peripheral lung strips for both IgE-producing (n=8) and control (n=6) rabbits (Figs. 4 and 5). To determine if atropine antagonism was competitive, two analyses were performed. First, the maximum g response of the tissues treated with atropine were found not to differ from the maximum g responses of the untreated tissues. Second, a Schild plot was constructed and the slope was determined for each rabbit. There was no significant difference between the IgE-producing and control rabbits in their Schild slopes (Table 4). The slopes of unity indicate that atropine is acting as a competitive antagonist at a single site. Composite Schild plots for the IgE-producing and control rabbits are shown in Figures 6 and 7.

The pA_2 value (the -log concentration of the antagonist required for a 2-fold shift to the right of the agonist concentration-response curve) is also derived from the Schild plot as a measure of antagonist potency. Values in Table 4 demonstrate that atropine is a very potent antagonist and acts similarly on tissues from IgE-producing and control rabbits.

Pirenzepine, a muscarinic antagonist with differential affinities for muscarinic receptors in various tissues, also shifted the methacholine concentration-response curve to the right in peripheral lung strips for both IgE-producing (n=10) and control (n=10) rabbits (Figs. 8 and 9). The competitive nature and the potency of

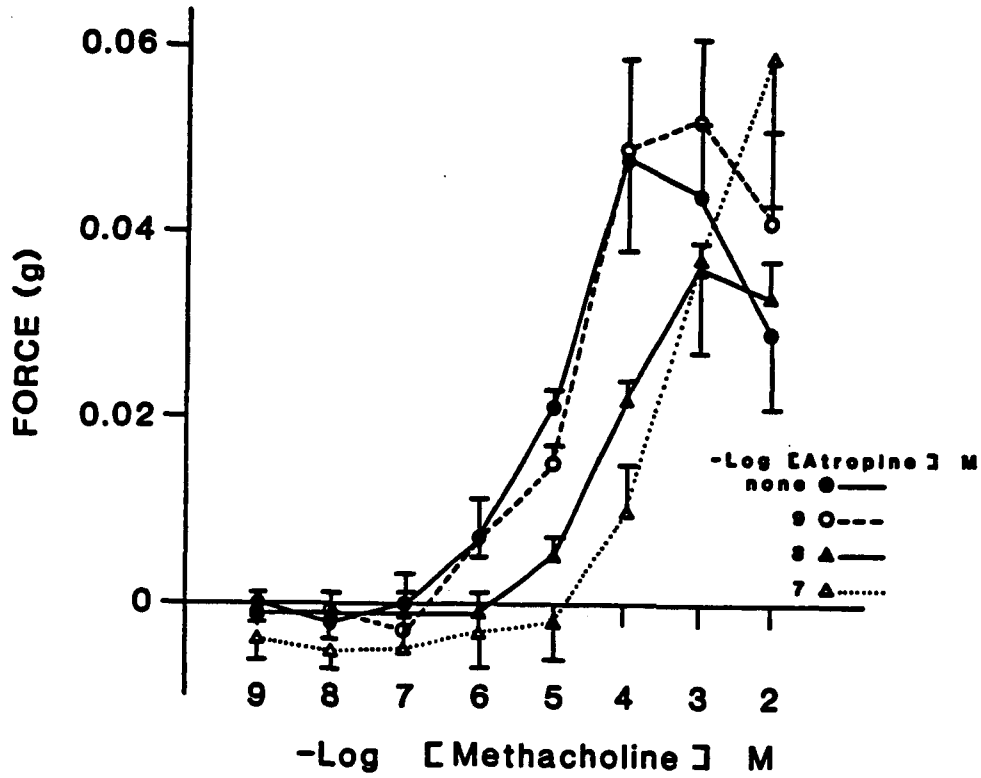


Figure 4. Atropine antagonism of methacholine contractility in peripheral lung strips of IgE-producing rabbits. Values are expressed as means ± S.E.M. (n=8). Experimental conditions are as in Figure 2.

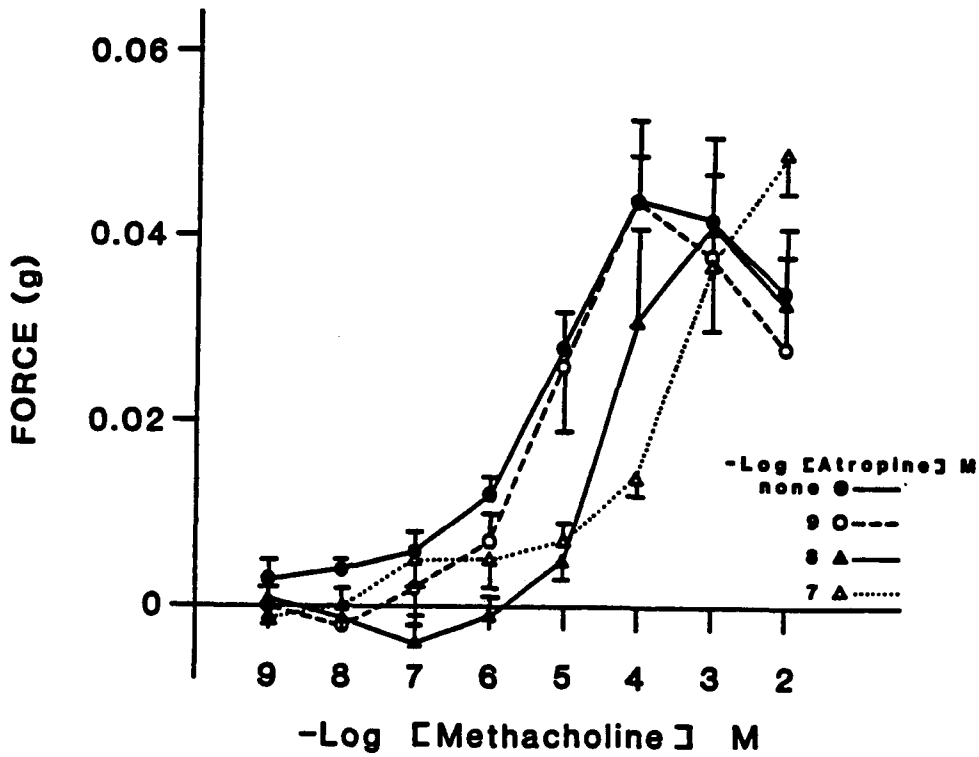


Figure 5. Atropine antagonism of methacholine contractility in peripheral lung strips of control rabbits. Values are expressed as means ± S.E.M. (n=6). Experimental conditions are as in Figure 2.

Table 4. Means ± S.E.M. for Schild Slopes and pA_2 Values for Atropine and Pirenzepine Antagonism of Methacholine Contractile Activity in Peripheral Lung Strips.

	n	Schild Slope	pA_2
Atropine			
IgE Rabbits	8	0.86±0.03	9.16±0.37
Control Rabbits	6	1.07±0.08	8.82±0.13
		NS	NS
All Rabbits	14	0.95±0.05	9.02±0.22
		NS	$p < .001$
Pirenzepine			
IgE Rabbits	10	0.72±0.09	7.68±0.28
Control Rabbits	10	1.03±0.26	7.29±0.52
		NS	NS
All Rabbits	20	0.87±0.14	7.49±0.29

NS = not statistically significant

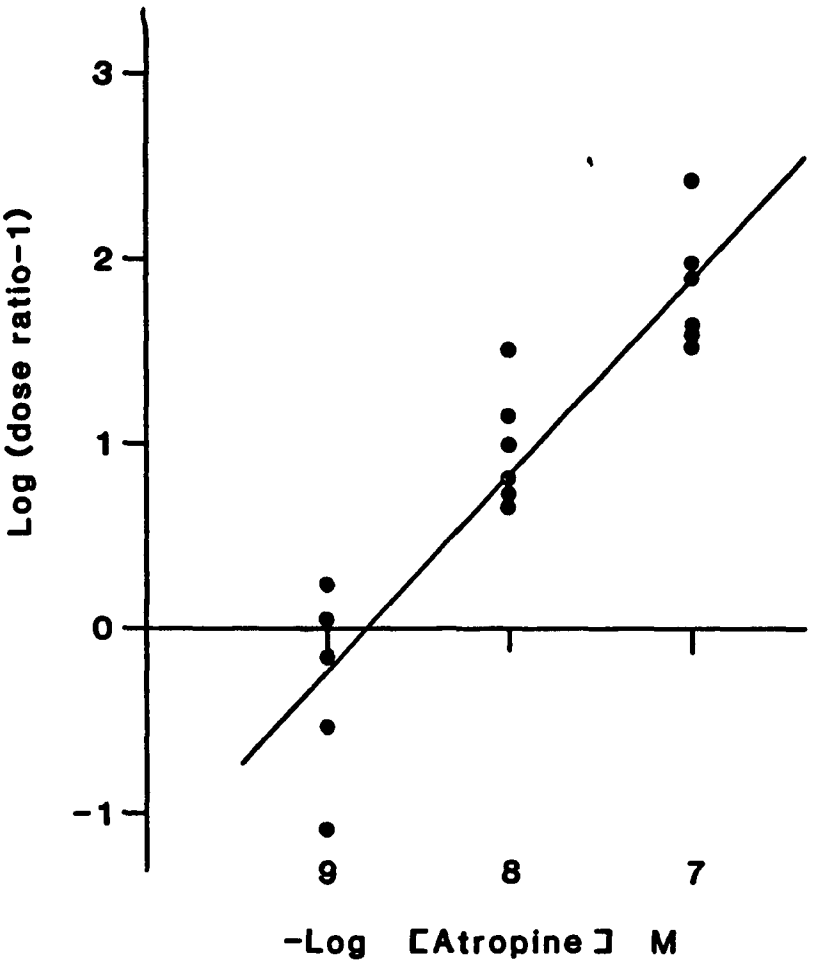


Figure 6. Schild plot for atropine antagonism of methacholine contraction in peripheral lung strips for 6 control rabbits. Each point represents one tissue. In some cases, values could not be obtained in each of the 6 rabbits for all concentrations of atropine. The slope is 1.07 and pA_2 value is 8.79.

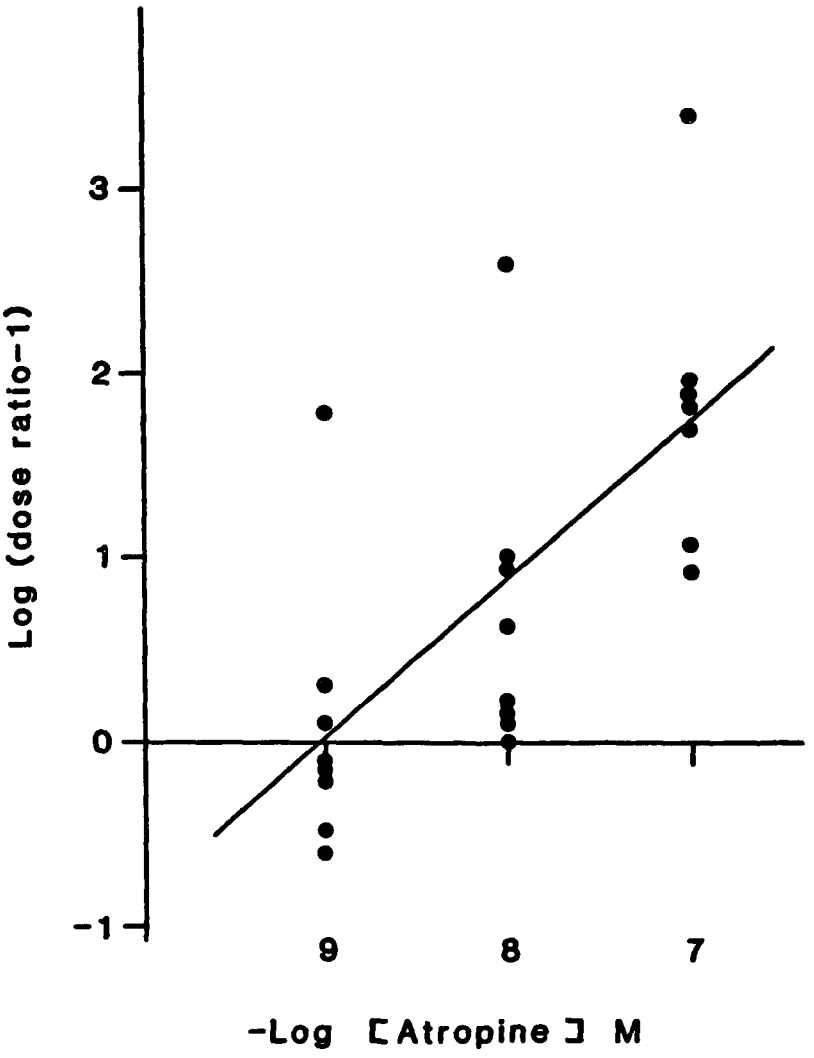


Figure 7. Schild plot for atropine antagonism of methacholine contraction in peripheral lung strips for 8 IgE-producing rabbits. Each point represents one tissue. In some cases, values could not be obtained in each of the 8 rabbits for all concentrations of atropine. The slope is 0.86 and the pA_2 value is 9.02.

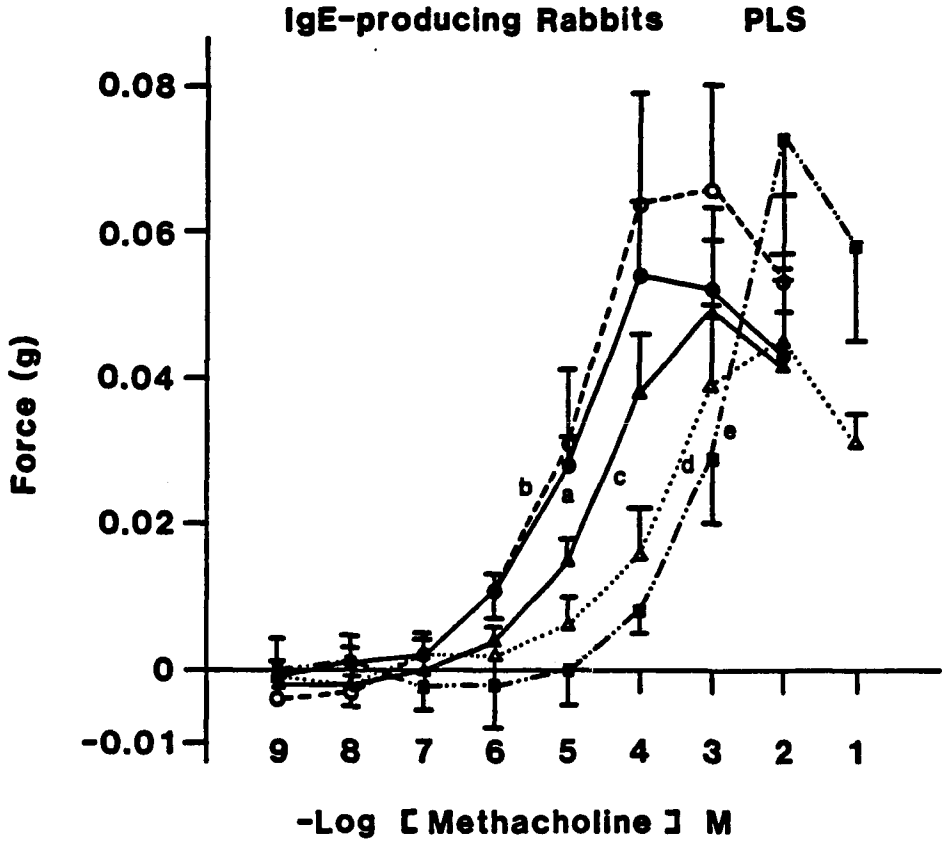


Figure 8. Pirenzepine antagonism of methacholine contractility in peripheral lung strips of IgE-producing rabbits. a) No pirenzepine. b) 10^{-7}M pirenzepine. c) 10^{-6}M pirenzepine. d) 10^{-5}M pirenzepine. e) 10^{-4}M pirenzepine. Values are expressed as means ± S.E.M. (n=10). Experimental conditions are as in Figure 2.

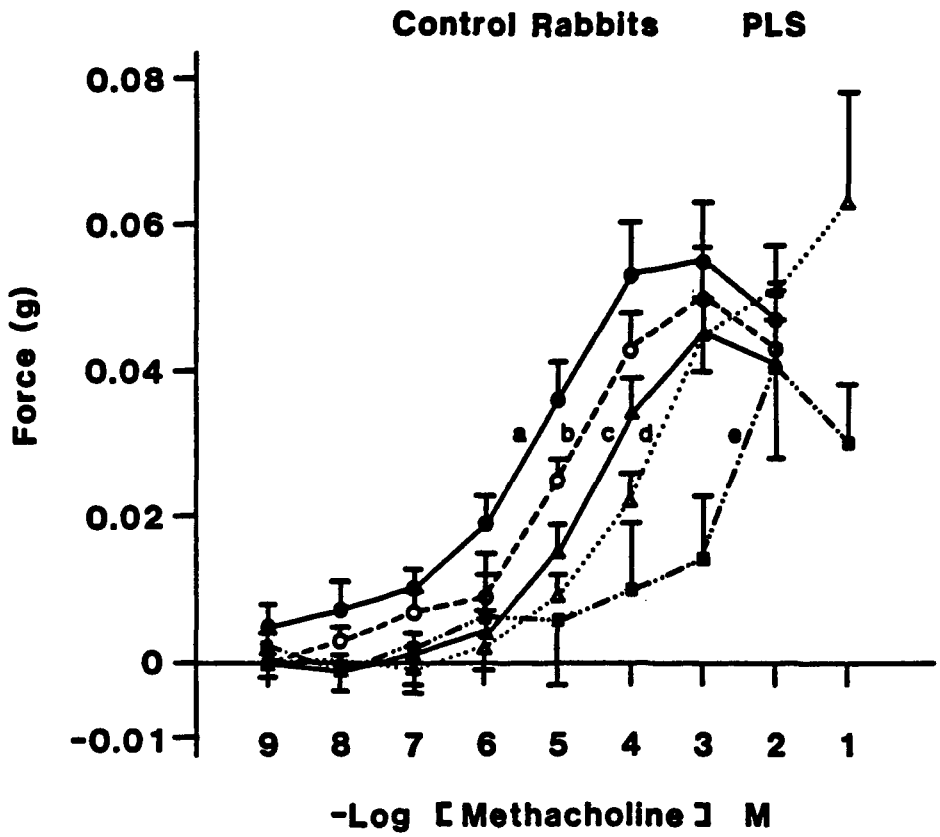


Figure 9. Pirenzepine antagonism of methacholine contractility in peripheral lung strips of control rabbits. a) No pirenzepine. b) 10^{-7}M pirenzepine. c) 10^{-6}M pirenzepine. d) 10^{-5}M pirenzepine. e) 10^{-4}M pirenzepine. Values are expressed as means $\pm$ S.E.M. (n=10). Experimental conditions are as in Figure 2.

pirenzepine antagonism for methacholine contractile activity were assessed by the same methods used with atropine. First it was determined that the maximum g responses in the tissues treated with pirenzepine were not significantly different from those of untreated tissues. Then, Schild plots were constructed and yielded the slopes and pA_2 values given in Table 4. There was no significant difference between the IgE-producing and control rabbits in their pA_2 values or slopes. The slopes of unity indicate that pirenzepine is acting as a competitive antagonist at a single site. Composite Schild plots for the IgE-producing and control rabbits are illustrated in Figures 10 and 11.

Since there was no significant difference between the IgE-producing and control rabbits in their responses to atropine and pirenzepine, Schild plots were constructed which combined the IgE-producing and control rabbits, and slopes and pA_2 values were calculated for atropine and pirenzepine in peripheral lung strips (Figs. 12 and 13). The group mean slopes and pA_2 values for all rabbits were compared for atropine versus pirenzepine and values are shown in Table 4. Both atropine and pirenzepine competitively inhibit the methacholine contractile response, but pirenzepine is approximately 180-fold less potent than atropine. Thus, in this assay system, pirenzepine is acting as a low-affinity muscarinic receptor antagonist.

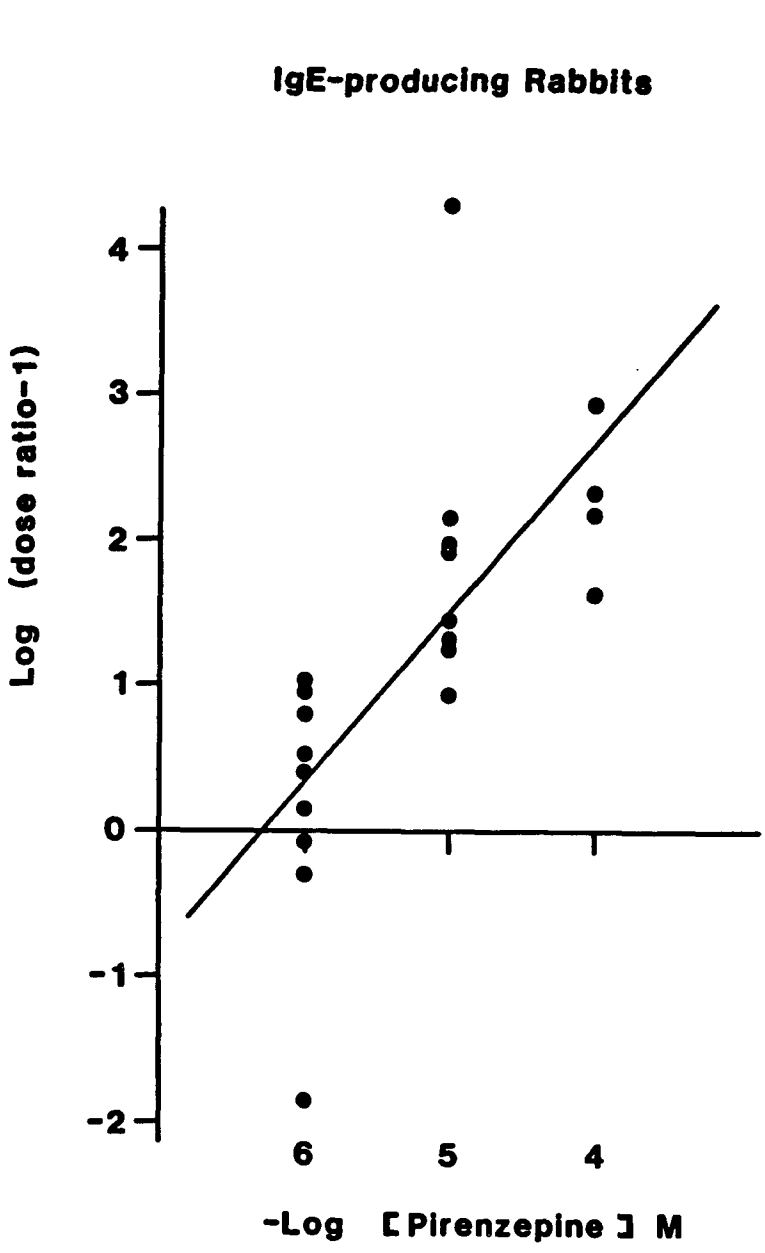


Figure 10. Schild plot for pirenzepine antagonism of methacholine contraction in peripheral lung strips for 10 IgE-producing rabbits. Each point represents one tissue. In some cases, values could not be obtained in each of the 10 rabbits for all concentrations of pirenzepine. A group of only 4 rabbits was used at the highest concentration of pirenzepine. The slope is 1.15 and pA_2 value is 6.32.

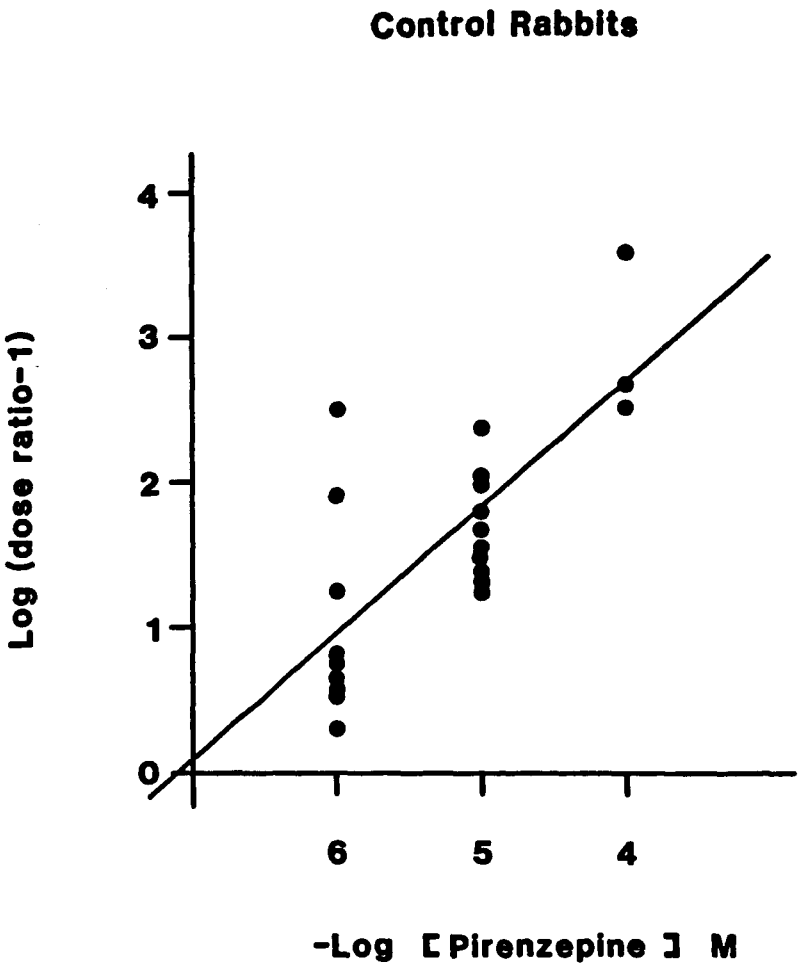


Figure 11. Schild plot for pirenzepine antagonism of methacholine contraction in peripheral lung strips for 10 control rabbits. Each point represents one tissue. In some cases, values could not be obtained in each of the 10 rabbits for all concentrations of pirenzepine. A group of only 3 rabbits was used at the highest concentration of pirenzepine. The slope is 0.87 and the pA_2 value is 7.09.

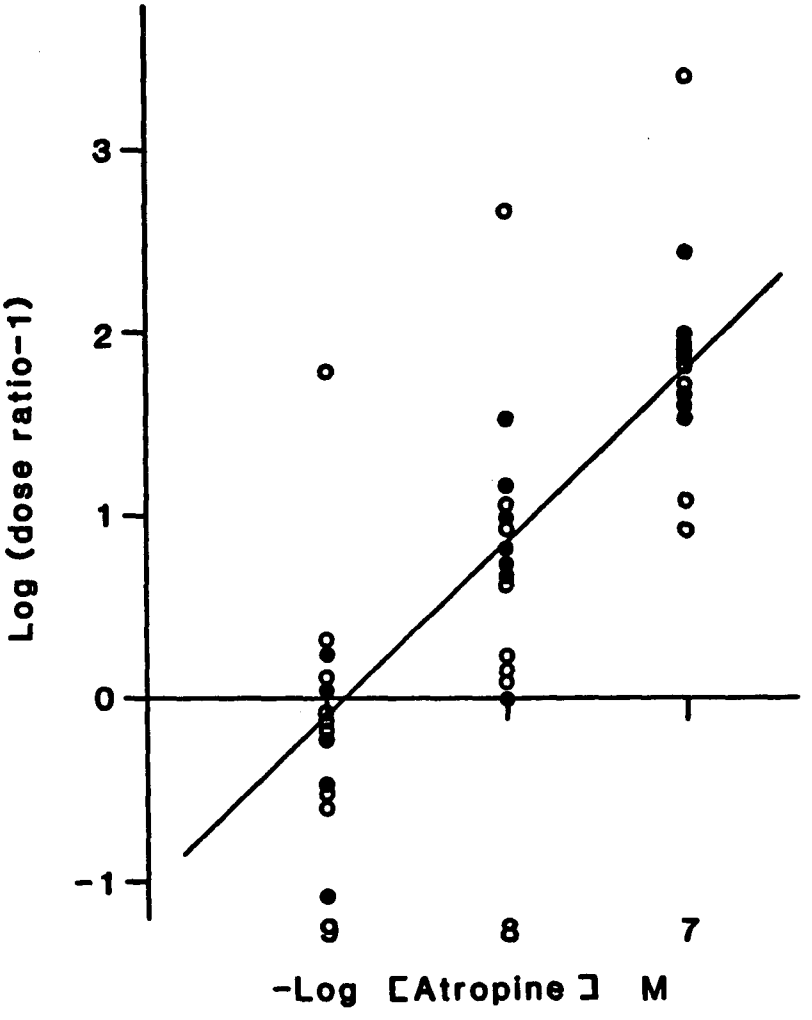


Figure 12. Schild plot for atropine antagonism of methacholine contraction in peripheral lung strips for IgE-producing and control rabbits combined (n=14). Each point represents one tissue. Filled symbols indicate control rabbits and open symbols IgE-producing rabbits. The slope is 0.94 and the pA_2 value is 8.92.

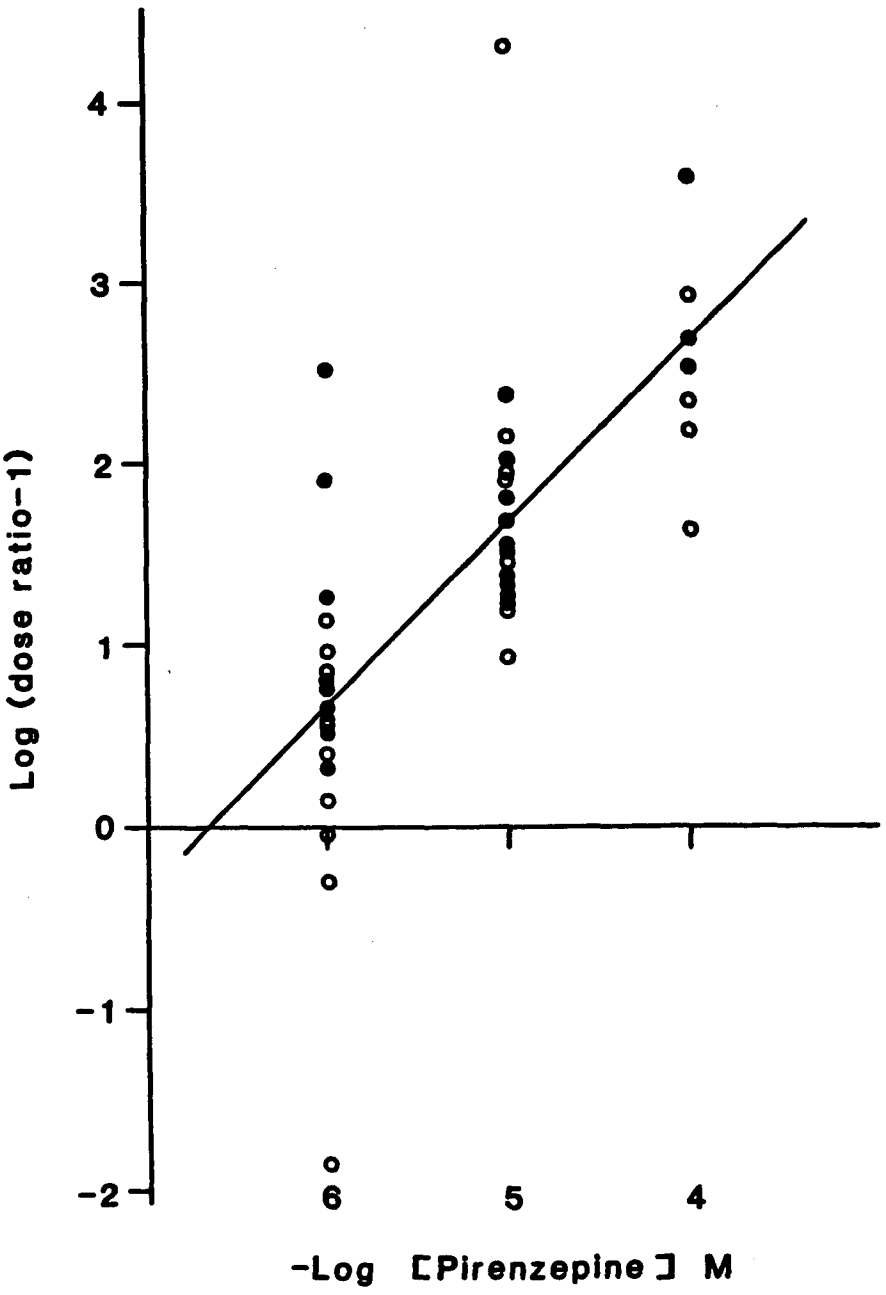


Figure 13. Schild plot for pirenzepine antagonism of methacholine contraction in peripheral lung strips for IgE-producing and control rabbits combined (n=20). Each point represents one tissue. Filled symbols indicate control rabbits and open symbols IgE-producing rabbits. In some cases, values could not be obtained in each of the 20 rabbits for all concentrations of pirenzepine. The slope is 1.01 and the pA_2 value is 6.05.

Atropine and pirenzepine also shifted the methacholine concentration-response curve to the right in intrapulmonary and mainstem bronchial tissues (Figs. 14,15, and 16.). Table 5 indicates that Schild plots and pA_2 values for atropine and pirenzepine in these tissues reveal that atropine has a similar potency among intrapulmonary bronchi and peripheral lung strips and pirenzepine also has a similar potency among all three types of tissues.

In order to assess whether pirenzepine induced any tissue relaxation or unmasked any relaxant activity of methacholine, tissues were precontracted with 10^{-4}M phenylephrine or 10^{-4}M histamine before the cumulative concentrations of methacholine were added. Pirenzepine produced no tissue relaxation in precontracted rabbit peripheral lung strips, nor did it reveal any muscarinic relaxant activity of methacholine (Figs. 17 and 18). As described above, pirenzepine competitively inhibits the methacholine-induced contractile response and maximum responses were seen with 10^{-4} to 10^{-2}M methacholine. The precontracted tone was stable throughout the course of the experiment when neither pirenzepine nor methacholine was added to the tissue.

Responses to Electrical Field Stimulation

Exogenous methacholine is thought to act directly on smooth muscle muscarinic receptors to induce contraction. A second way of assessing muscarinic receptor contractile activity is to electrically stimulate the cholinergic nerves in the airway wall to release acetylcholine and induce contraction. To assess the relative

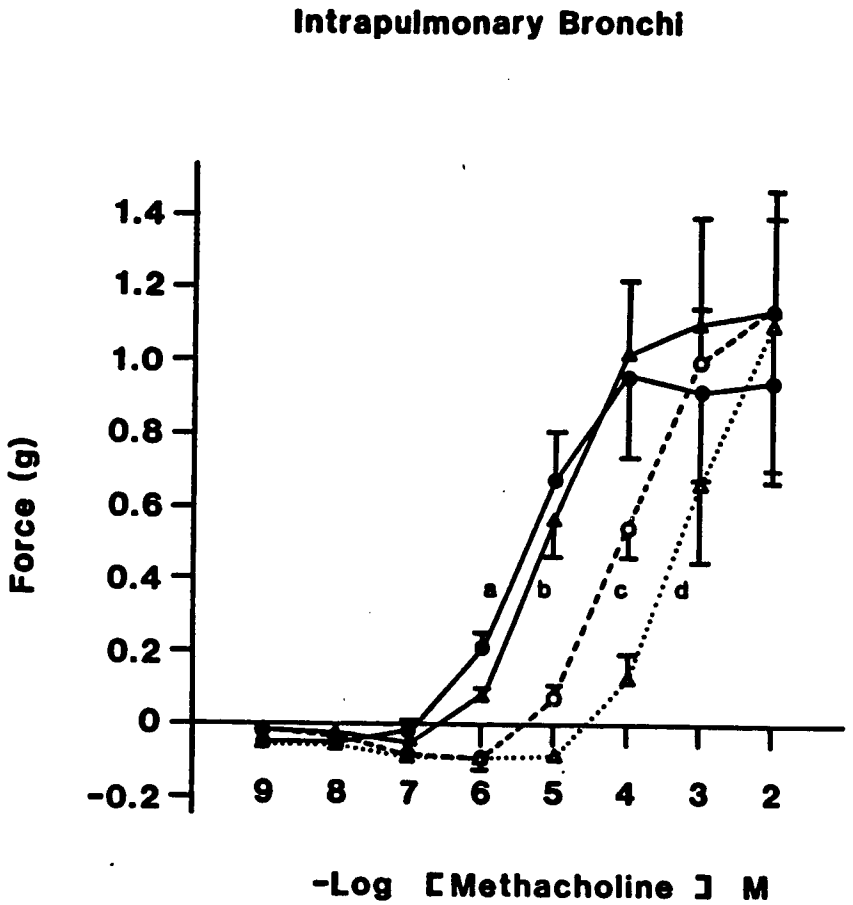


Figure 14. Atropine antagonism of methacholine contractility in intrapulmonary bronchial rings of control rabbits. Values are expressed as means ± S.E.M. Experimental conditions are as in Figure 3. a) No atropine (n=7). b) 10^{-9} M atropine (n=7). c) 10^{-8} M atropine (n=6). d) 10^{-7} M atropine (n=6).

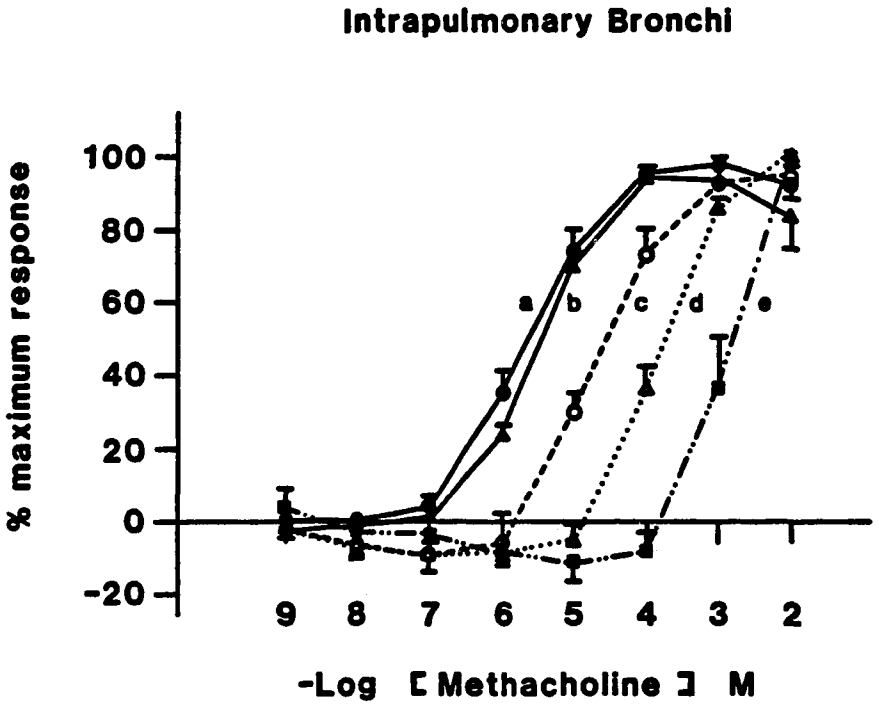


Figure 15. Pirenzepine antagonism of methacholine contractility in intrapulmonary bronchial rings of control rabbits. Values are expressed as means ± S.E.M. Experimental conditions are as in Figure 3. a) No pirenzepine (n=6). b) 10^{-7}M pirenzepine (n=6). c) 10^{-6}M pirenzepine (n=6). d) 10^{-5}M pirenzepine (n=5). e) 10^{-4}M pirenzepine (n=2).

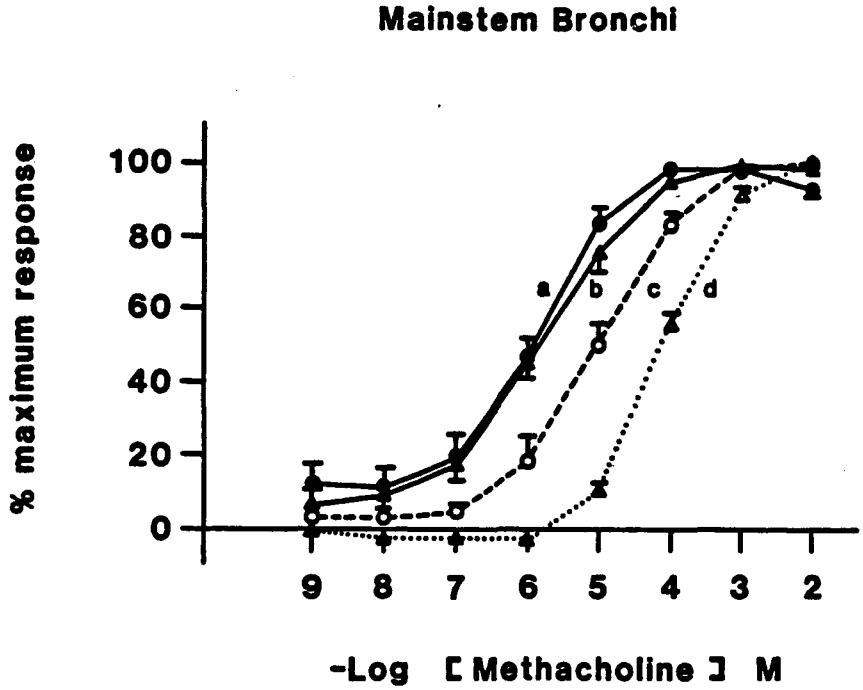


Figure 16. Pirenzepine antagonism of methacholine contractility in mainstem bronchial rings of control rabbits. Values are expressed as means ± S.E.M. Contractile force was measured isometrically in rings under 2.0g baseline tension. Methacholine was added cumulatively at 5 min. intervals. a) No pirenzepine (n=6). b) 10^{-7}M pirenzepine (n=6). c) 10^{-6}M pirenzepine (n=6). d) 10^{-5}M pirenzepine (n=5).

Table 5. Means ± S.E.M. for Schild Slopes and pA_2 Values for Atropine and Pirenzepine Antagonism of Methacholine Contractile Activity in Intrapulmonary and Mainstem Bronchi.

	n	Schild Slope	pA_2
Intrapulmonary Bronchi			
Atropine	7	1.15±0.10	9.16±0.22
Pirenzepine	6	1.17±0.16	7.06±0.30
Mainstem Bronchi			
Pirenzepine	5	1.04±0.21	6.89±0.25

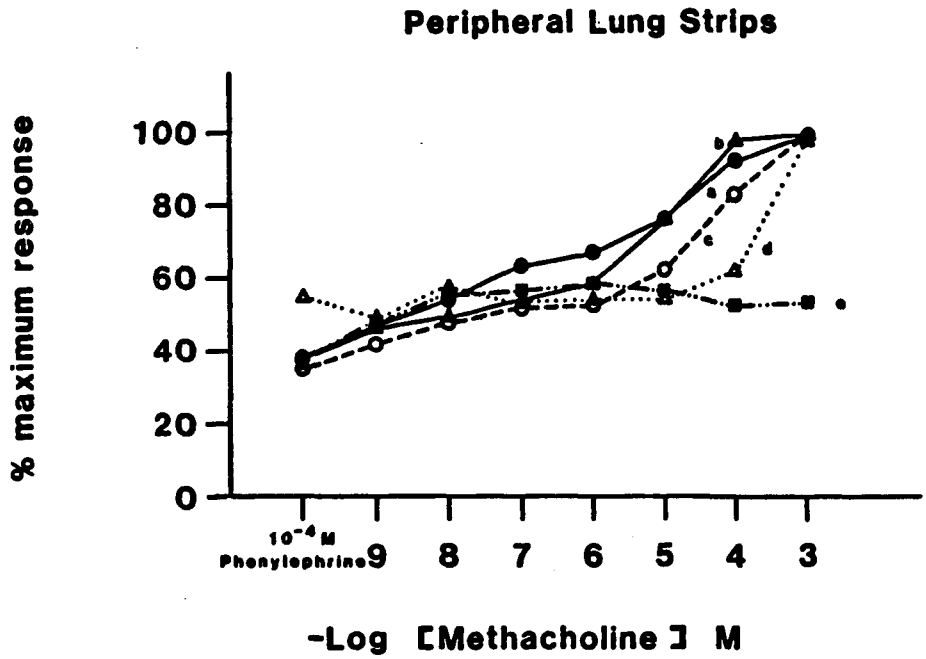


Figure 17. Control rabbit peripheral lung strip concentration-response curves after precontraction with phenylephrine. Control rabbit peripheral lung strips in the presence of pirenzepine were precontracted with 10^{-4}M phenylephrine followed by cumulative concentrations of methacholine at 5 min. intervals. Tissues are from one animal. a) No pirenzepine. b) 10^{-7}M pirenzepine. c) 10^{-6}M pirenzepine. d) 10^{-5}M pirenzepine. e) No pirenzepine and no methacholine.

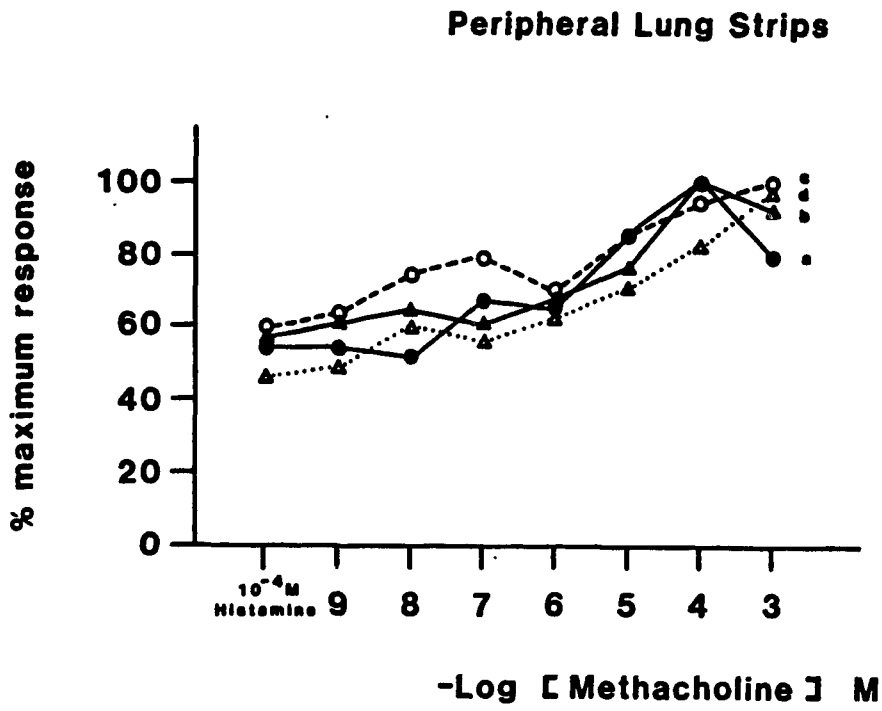


Figure 18. Control rabbit peripheral lung strip concentration-response curves after precontraction with histamine. Control rabbit peripheral lung strips in the presence of pirenzepine were precontracted with 10^{-4}M histamine, followed by the addition of cumulative concentrations of methacholine at 5 min. intervals. Tissues are from one animal. a) No pirenzepine. b) 10^{-7}M pirenzepine. c) 10^{-6}M pirenzepine. d) 10^{-5}M pirenzepine.

potencies of pirenzepine and atropine in antagonizing this endogenous contractile activity, a field stimulation protocol was established for rabbit bronchial rings.

Stimulation-response curves were constructed to determine optimal stimulator settings of voltage and frequency, with duration arbitrarily set at 0.2 msec. A voltage of 25V was chosen because a maximum response plateau was reached at 20-40V (Fig. 19). A frequency of 16 Hz was selected for both mainstem and intrapulmonary bronchi because it gave approximately a one-half maximal response (Figs. 20 and 21). We wanted a one-half maximal response rather than maximal because we wanted to be on the ascending portion of the stimulation-response curve for subsequent inhibition studies. The maximum g responses at the highest frequency used (64 Hz) were approximately 0.2g for intrapulmonary bronchi, whereas mainstem bronchi gave a maximum response of about 3.0g. Even though the maximum g responses differ, the half-maximal response is generated at approximately 16 Hz for both types of tissues.

Atropine and pirenzepine both inhibited the electrical field stimulated contractile response in intrapulmonary and mainstem bronchial tissues. Percent inhibition curves were constructed which compared atropine to pirenzepine (Figs. 22 and 23). In the intrapulmonary bronchi tissues the IC50 for atropine was 3.7×10^{-9}M and the IC50 for pirenzepine was 2.3×10^{-7}M, thereby making their differences in potency 160-fold. In the mainstem bronchial tissues the IC50 for atropine was 5.1×10^{-9}M and that for pirenzepine was 3.8×10^{-7}M, yielding a similar potency difference, with pirenzepine

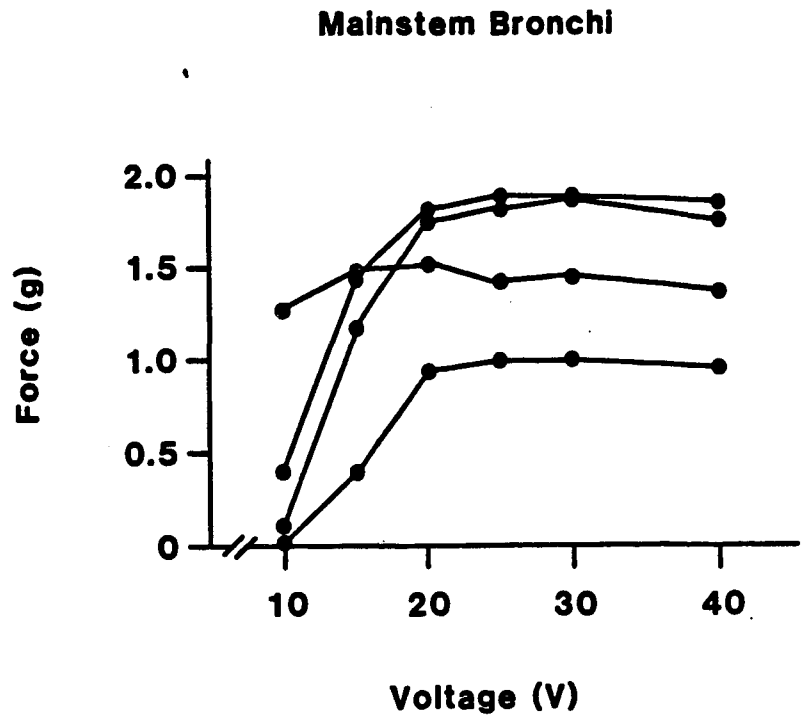


Figure 19. Electrical field stimulation voltage-response curve for mainstem bronchi. Curves are from four separate tissues from one animal. Maximum g responses are reached between 20 to 40 V.

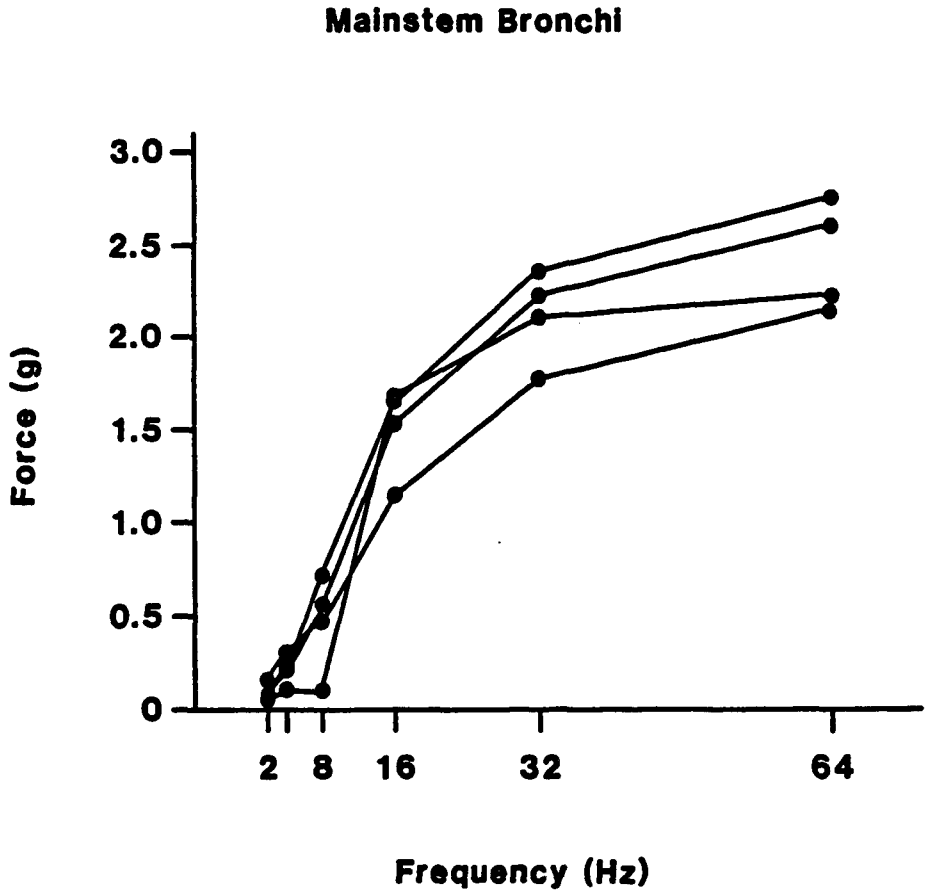


Figure 20. Electrical field stimulation frequency-response curve for mainstem bronchi. Curves are from four separate tissues from one animal. The half-maximal response is reached at approximately 16 Hz.

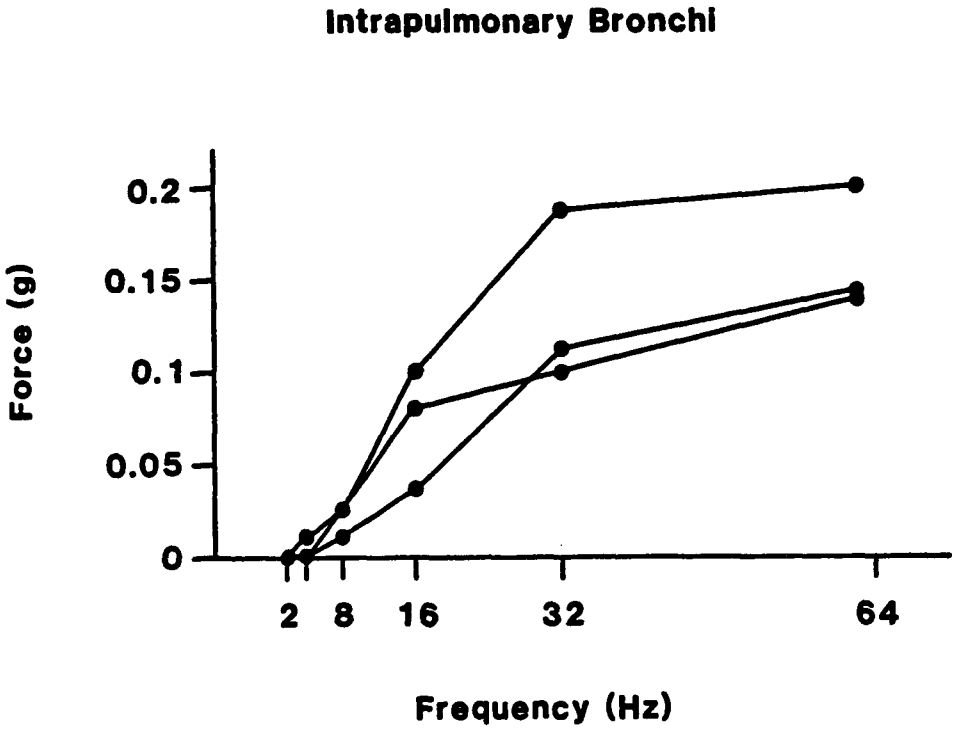


Figure 21. Electrical field stimulation frequency-response curve for intrapulmonary bronchi. Curves are from three separate tissues from one animal. The half-maximal response is reached at approximately 16 Hz.

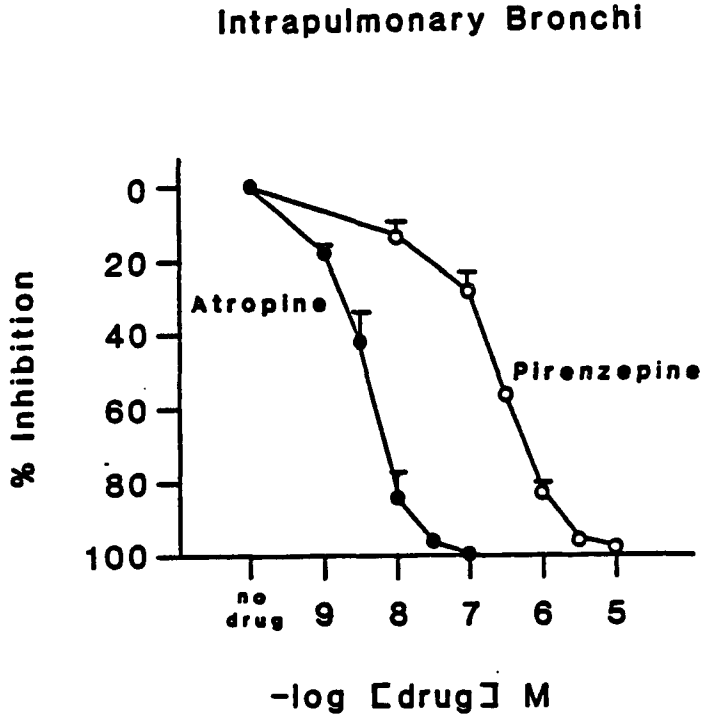


Figure 22. Electrical field stimulation percent inhibition curves for atropine (n=5) and pirenzepine (n=5) in intrapulmonary bronchi. Values are calculated as percent inhibition of the response before the tissues were treated with the antagonist. The IC50 for atropine is 3.7×10^{-9}M and the IC50 for pirenzepine is 2.3×10^{-7}M. (Mean $\pm$ S.E.M.).

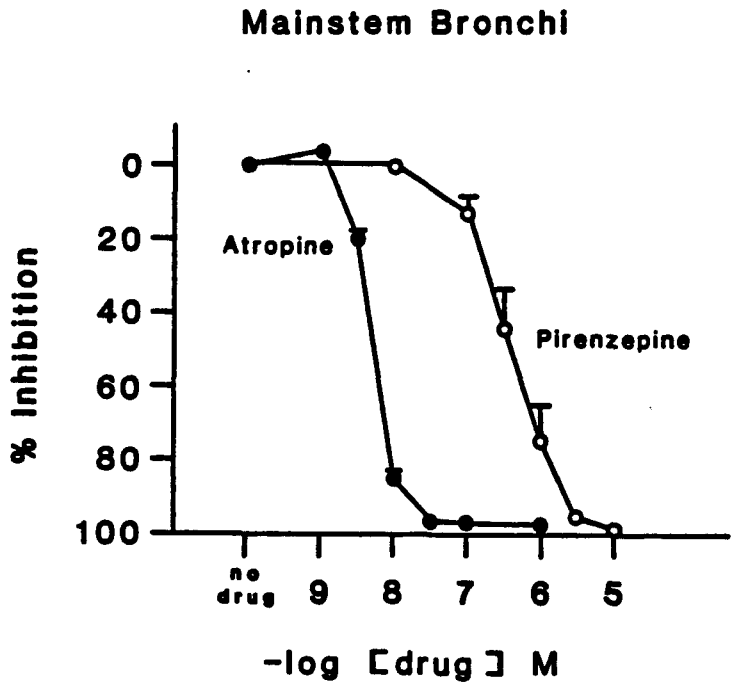


Figure 23. Electrical field stimulation percent inhibition curves for atropine (n=4) and pirenzepine (n=4) in mainstem bronchi. Values are calculated as percent inhibition of the response before the tissues were treated with the antagonist. The IC50 for atropine is 5.1×10^{-9}M and the IC50 for pirenzepine is 3.8×10^{-7}M. (Mean $\pm$ S.E.M.).

approximately 130-fold less potent than atropine. These data indicate that there are pirenzepine low-affinity type muscarinic receptors in both types of tissues.

Relaxation Responses of Bronchial Smooth Muscle to Isoproterenol

Although airway tissues from IgE-producing and control rabbits did not differ in their contractility to methacholine, it was possible that they might differ in adrenergic relaxation. To test this possibility, isoproterenol relaxation studies were performed with intrapulmonary and mainstem bronchi. After being precontracted with a concentration of methacholine which was known to produce an approximately half-maximal response ($3x10^{-6}M$), cumulative concentrations of isoproterenol were added to the tissue baths. Values are expressed as percent reversal of the methacholine-induced tension. One-hundred percent reversal is equivalent to relaxation back to the baseline before the addition of the methacholine. In the intrapulmonary bronchi tissues (Fig. 24) there was no significant difference in relaxation between the IgE-producing (n=8) and control (n=6) rabbits. Both groups relaxed to the same extent, that being approximately 140-150 percent reversal. In the mainstem bronchi (Fig. 25) there was also no significant difference in relaxation between the IgE-producing (n=8) and control (n=6) rabbits. Tissues in both groups relaxed to about the same extent. Intrapulmonary bronchi tissues relaxed approximately 50 percent more than the mainstem bronchi tissues, showing that the intrapulmonary bronchi possess an inherent tone not present in the mainstem bronchi.

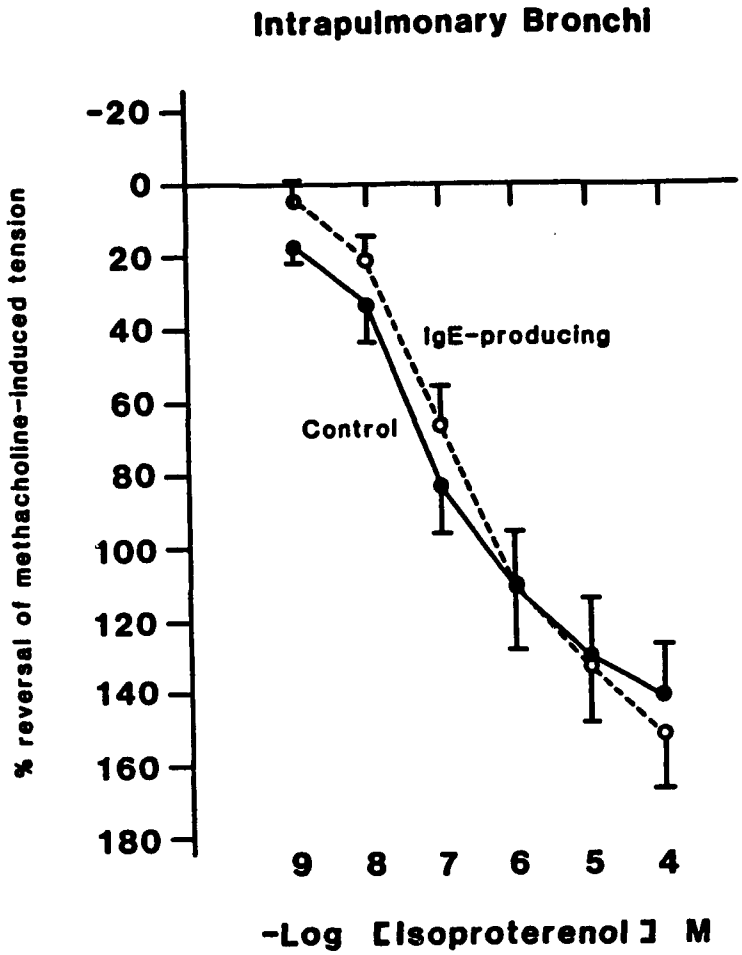


Figure 24. Intrapulmonary bronchi isoproterenol relaxation in IgE-producing (n=8) and control (n=6) rabbit tissues. Values are expressed as percent reversal of the methacholine-induced tension. There is no significant difference in relaxation between the two groups ($p<.05$). Values are expressed as means $\pm$ S.E.M.

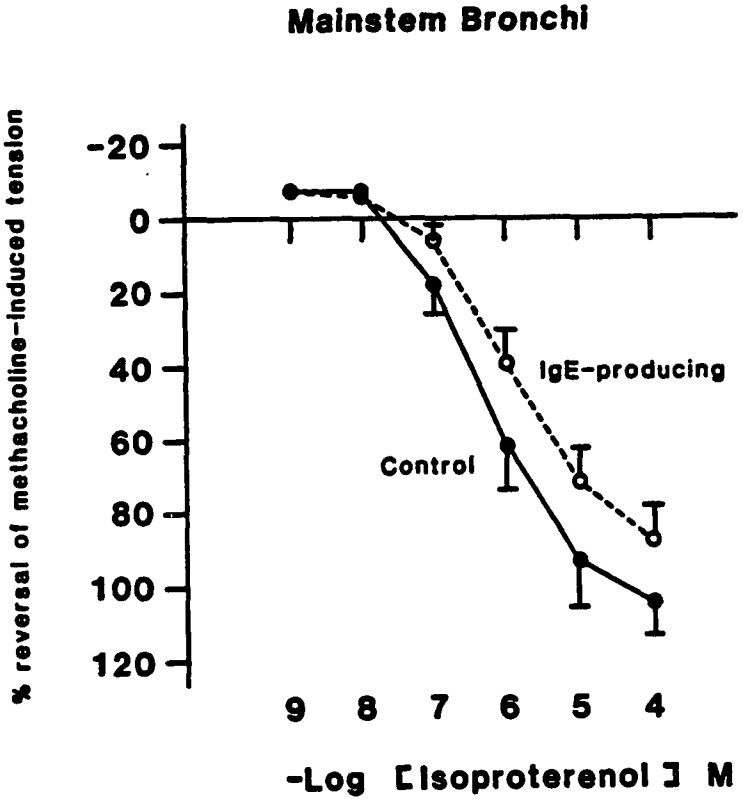


Figure 25. Mainstem bronchi isoproterenol relaxation in IgE-producing (n=8) and control (n=7) rabbit tissues. Values are expressed as percent reversal of the methacholine-induced tension. There is no significant difference in relaxation between the two groups ($p<.05$). Values are expressed as means $\pm$ S.E.M.

CHAPTER 4

DISCUSSION

Asthmatic patients have been shown to possess an increased bronchial responsiveness to cholinomimetic agents. These patients generally also exhibit elevated serum IgE levels. The rabbit model of IgE-mediated allergy of Pinckard et al. (1972) was used here to determine whether IgE production alters airway reactivity *in vitro*. The information presented in this thesis indicates there is no discernable effect of elevated serum IgE levels on the reactivity of isolated airway smooth muscle to methacholine. The muscarinic receptors which mediate methacholine-induced airway smooth muscle contraction were also investigated and found to be of the subtype with low affinity for pirenzepine (putative M_2 receptor).

Contractile Responses to Methacholine

A study of the contractile responses of rabbit airway tissues to methacholine established that there was no significant difference between IgE-producing and control rabbits as to either the concentration of methacholine needed to produce a 50 percent maximal contraction (EC50) or in the force generated by a maximal contraction.

Therefore, the airway hyperreactivity to inhaled methacholine or histamine (Reed, 1974) observed in asthmatic or allergic individuals is not observed in the isolated airway smooth muscle from IgE-producing rabbits. These data contrast with the *in vivo* cholinergic responsiveness seen in the pupillary muscle of the IgE-producing rabbits as shown by Halonen and Kaliner (1984).

The absence of hyperreactivity of airway tissues in allergic animals was also noted by Antonissen et al. (1979). This group used a canine model of allergic asthma to study the role of changes in contractile properties of airway smooth muscle in the pathogenesis of asthma. They used an immunization procedure similar to that of Pinckard et al. (1972), but used ovalbumin instead of HRP as the sensitizing antigen. They used electrical stimulation (which produces endogenous neural stimulation) and cholinergic agonists (carbachol, which acts directly on the smooth muscle) on tracheal smooth muscle strips to show contractile ability. The sensitized strips showed no significant hyperexcitability or hyperreactivity to either of these stimuli. To contrast this, Rinard et al. (1979) used dogs that bronchoconstrict to aerosolized ascaris antigen and showed that *in vitro* tracheal rings of these dogs were hyporesponsive to the beta-adrenergic agonist isoproterenol in relaxing tracheal smooth muscle contracted by methacholine.

One theory of hyperresponsiveness in asthma is that there may be an exaggerated vagal reflex response to stimulation of irritant receptors present in the airways leading to an enhanced release of

acetylcholine at the neuroeffector junction. This possibility would not be detectable *in vitro* nor does it explain hyperreactivity to cholinergic agonists which are thought to act directly on smooth muscle muscarinic receptors. A second possibility is that there is an enhanced sensitivity of smooth muscle to the effects of cholinergic agonists. Another theory, by Szentivanyi (1968), is that the cholinergic hyperresponsiveness is due to a decrease in beta-adrenergic responsiveness.

The results from the studies presented here show that detectable cholinergic hyperresponsiveness in isolated rabbit airway smooth muscle is not acquired with the production of an IgE antibody response. The possibility remains however, that airway responsiveness may be demonstrated *in vivo*.

Relaxation Responses to Isoproterenol

The beta-adrenergic theory of the atopic abnormality in bronchial asthma as proposed by Szentivanyi (1968) states that these individuals have cholinergic hyperreactivity because they have hyporesponsive beta-adrenergic receptors and therefore possess a decreased ability to bronchodilate when airways are constricted. The results of the experiments presented here demonstrated that there is no significant difference between the control and sensitized rabbits in their abilities for isoproterenol, a beta-adrenergic agonist, to relax a methacholine-induced tension of either mainstem or intrapulmonary bronchial tissues. Thus, in our rabbit model the

beta-adrenergic receptors do not seem to be "desensitized" such that they have a decreased response to beta-agonists when the tissues are under cholinergic-induced tension.

These results differ from those of Rinard et al. (1979) who showed that the ability of isoproterenol to induce relaxation of trachealis smooth muscle was significantly impaired in tissues from dogs that bronchoconstrict to aerosolized ascaris antigen as compared to dogs that do not respond to antigen. In our experiments the observation that the intrapulmonary bronchial tissue relaxed to nearly 150 percent reversal of the methacholine-induced tension but the mainstem bronchial tissue relaxed to approximately 90-100 percent, reveals that the intrapulmonary bronchi possess an inherent baseline tone not present in the mainstem bronchi.

Atropine Antagonism

Atropine, a classical muscarinic antagonist, can competitively inhibit the cholinergic muscarinic contractile response in isolated airway tissues (Simonsson et al., 1967, Fleisch and Calkins, 1976, Drazen and Schneider, 1978, Nadel, 1980, Parry and Heathcote, 1982, Del Tacca et al., 1984). In this study atropine has been shown to competitively inhibit the methacholine contractile response in rabbit airway tissue. The potency of atropine in the IgE-producing vs. control rabbit airway tissues is not significantly different when compared using pA_2 values or slopes. The pA_2 values and slopes for atropine are also very similar among the different types of

tissues used. The fact that the slopes are very close to unity indicate that there is a homogeneous population of receptors at these sites and that atropine is acting competitively.

The results in these experiments agree very closely with other experiments in other types of tissues as to the potency of atropine. Functional studies with isolated rabbit and rat tracheal strips by Fleisch and Calkins (1976) have shown the atropine pA_2 values to be 9.28 and 9.18 and the Schild plot slopes to be -1.04 and -1.01, respectively. Our experiments for rabbit peripheral lung and intrapulmonary bronchi have shown the atropine pA_2 values to be 9.02 and 9.16 and the Schild slopes to be -0.95 and -1.15, respectively. Studies by Szelenyi (1982) with isolated guinea pig ileum smooth muscle contraction have shown the pA_2 value of atropine in these tissues to be 8.72 and the Schild slope 1.07. Also in agreement are studies by Del Tacca et al. (1984) who in guinea pig gastric circular smooth muscle strips found the atropine pA_2 value to be 8.52 and the Schild slope to be -1.09. All of these results indicate that atropine has the same potency for muscarinic receptors in these tissues, having a pA_2 value near 9 and a slope near unity. These results thus show the competitive antagonism of atropine for muscarinic receptors and imply a homogeneous population of receptors.

Pirenzepine Antagonism

Recently pirenzepine, a putative M_1 subtype muscarinic antagonist, has been used in both ligand binding (Hammer, 1980, Hammer et al., 1980, Watson et al., 1982, Hammer and Giachetti, 1982, Birdsall

and Hulme, 1983) and functional (Heathcote and Parry, 1980, Heathcote and Parry, 1982, Del Tacca, 1984) studies and has been shown to have different affinities for muscarinic receptors in different tissues. Radiolabeled ligand binding studies by Hammer et al.(1980) using a classical muscarinic antagonist, N-methylscopolamine (NMS), and pirenzepine have shown that NMS binds to muscarinic receptors of sympathetic ganglia, sublingual gland, parotid gland, stomach wall, and atria with little variation in potency, but pirenzepine binds to these different tissues with a wide range of potencies. In functional studies done by Heathcote and Parry (1980), it was found that the dose of atropine needed to inhibit gastric acid secretion in the rat and the dose needed to increase pupil diameter of the mouse eye had very similar ED50 values, mg/Kg i.v., but for pirenzepine greater than three times the ED50 was needed to increase pupil diameter than was needed to inhibit gastric acid secretion.

Recent studies by my colleagues have demonstrated the presence of both high and low affinity pirenzepine receptors in homogenized rabbit lung preparations using radioligand binding techniques. Also, Bloom et al. (unpublished) have recently demonstrated <u>in vivo</u> that pirenzepine can inhibit bronchoconstriction induced by vagal stimulation and is only 17-fold less potent than atropine. This receptor could be present on the smooth muscle cell, on the postganglionic nerve endings (inhibiting acetylcholine release) or in the parasympathetic ganglia (modulating transmission of excitatory nerve impulses).

The results from this investigation show that pirenzepine acts as a competitive muscarinic antagonist to inhibit methacholine-induced smooth muscle contraction, but is approximately 180-fold less potent than atropine when comparing pA_2 values. As with atropine, there was also no difference in pirenzepine potency between IgE-producing and control rabbits. Also, pirenzepine appears to have the same potency in the different types of airway tissues studied, with pA_2 values near 7. Since pirenzepine is markedly less potent than atropine at these smooth muscle muscarinic receptors in the bronchial and peripheral lung tissue, it appears that the receptors mediating the contractile response are of the pirenzepine low-affinity (M_2) subtype.

The in vitro functional studies wherein muscarinic agonists are added to stimulate isolated airway smooth muscle contraction shows the ability of exogenous agents to stimulate contraction. Electrical field stimulation, which stimulates postganglionic cholinergic nerves, can also be used to show tissue contraction via the release and action of endogenous acetylcholine. Therefore, in this investigation electrical field stimulation was used to determine whether there may be high-affinity pirenzepine muscarinic receptors on the postganglionic neurons even though the muscarinic receptors on the smooth muscle are low-affinity.

The studies done here examining inhibition of the endogenously stimulated airway contractile response with atropine and pirenzepine inhibition have shown that these two antagonists differ in potency

similarly to that seen with the exogenously applied muscarinic agonist. In intrapulmonary bronchial tissues atropine is about 160-fold more potent than pirenzepine and in mainstem bronchial tissues it is about 130-fold more potent than pirenzepine. This is quite similar to the 180-fold difference seen with exogenously applied stimuli. Therefore, both the muscarinic receptors on the smooth muscle and those in the postganglionic fibers at the smooth muscle (if present) are low-affinity receptors for pirenzepine.

In summary, it has been shown that cholinergic hyperresponsiveness is not seen in the isolated airways of the IgE-mediated allergic rabbit model. Also, we have shown that the pirenzepine high-affinity muscarinic receptors present in the pathway of vagally induced bronchoconstriction do not appear to be on the rabbit airway smooth muscle cells or on the nerve terminals at the smooth muscle.

APPENDIX A

MEANS AND STANDARD ERRORS

I. Methacholine concentration-response curves

a) Control Rabbits

Tissue	Pretreatment	[MCh]	Mean (g)	SEM	n
Intra-pulmonary Bronchi	None	10^{-9}	-.018	.016	15
		10^{-8}	-.007	.021	15
		10^{-7}	.056	.036	15
		10^{-6}	.357	.073	15
		10^{-5}	.789	.115	15
		10^{-4}	1.103	.176	15
		10^{-3}	1.129	.119	15
		10^{-2}	1.240	.209	15
Peripheral Lung Strips	None	10^{-9}	.005	.003	10
		10^{-8}	.007	.004	10
		10^{-7}	.010	.003	10
		10^{-6}	.019	.004	10
		10^{-5}	.036	.005	10
		10^{-4}	.053	.007	10
		10^{-3}	.055	.008	10
		10^{-2}	.047	.005	10

b) IgE-producing Rabbits

Tissue	Pretreatment	[MCh]	Mean (g)	SEM	n
Intra-pulmonary Bronchi	None	10^{-9}	-.033	.012	9
		10^{-8}	-.054	.015	9
		10^{-7}	-.015	.027	9
		10^{-6}	.321	.050	9
		10^{-5}	.657	.080	9
		10^{-4}	.856	.099	9
		10^{-3}	.804	.102	9
		10^{-2}	.789	.111	9

Tissue	Pretreatment	[MCh]	Mean(g)	SEM	n
Peripheral Lung Strips	None	10^{-9}	-.001	.001	10
		10^{-8}	.001	.002	10
		10^{-7}	.002	.002	10
		10^{-6}	.011	.002	10
		10^{-5}	.028	.004	10
		10^{-4}	.054	.010	10
		10^{-3}	.052	.011	10
		10^{-2}	.043	.010	10

II. Methacholine response with atropine

a) Control Rabbits

Tissue	Pretreatment	[MCh]	Mean(g)	SEM	n
Intra-pulmonary Bronchi	None	10^{-9}	-.048	.008	7
		10^{-8}	-.052	.012	7
		10^{-7}	-.029	.019	7
		10^{-6}	.211	.038	7
		10^{-5}	.673	.134	7
		10^{-4}	.962	.226	7
		10^{-3}	.923	.248	7
		10^{-2}	.953	.283	7
Intra-pulmonary Bronchi	10^{-9}M atropine	10^{-9}	-.021	.011	7
		10^{-8}	-.024	.014	7
		10^{-7}	-.053	.010	7
		10^{-6}	.070	.023	7
		10^{-5}	.559	.097	7
		10^{-4}	1.017	.209	7
		10^{-3}	1.108	.295	7
		10^{-2}	1.136	.338	7
Intra-pulmonary Bronchi	10^{-8}M atropine	10^{-9}	-.018	.019	6
		10^{-8}	-.035	.020	6
		10^{-7}	-.079	.026	6
		10^{-6}	-.097	.033	6
		10^{-5}	.069	.035	6
		10^{-4}	.541	.083	6
		10^{-3}	.997	.153	6
		10^{-2}	1.144	.260	6

Tissue	Pretreatment	[MCh]	Mean(g)	SEM	n
Intra-pulmonary Bronchi	10^{-7}M atropine	10^{-9}	-.045	.009	6
		10^{-8}	-.052	.011	6
		10^{-7}	-.084	.005	6
		10^{-6}	-.090	.007	6
		10^{-5}	-.091	.010	6
		10^{-4}	.126	.065	6
		10^{-3}	.663	.217	6
		10^{-2}	1.113	.415	6
Peripheral Lung Strips	None	10^{-9}	.003	.002	6
		10^{-8}	.004	.001	6
		10^{-7}	.006	.002	6
		10^{-6}	.012	.002	6
		10^{-5}	.028	.004	6
		10^{-4}	.044	.005	6
		10^{-3}	.042	.005	6
		10^{-2}	.034	.004	6
Peripheral Lung Strips	10^{-9}M atropine	10^{-9}	.000	.002	6
		10^{-8}	-.002	.002	6
		10^{-7}	.002	.003	6
		10^{-6}	.007	.003	6
		10^{-5}	.026	.007	6
		10^{-4}	.044	.009	6
		10^{-3}	.038	.009	6
		10^{-2}	.028	.010	6
Peripheral Lung Strips	10^{-8}M atropine	10^{-9}	.001	.002	6
		10^{-8}	-.001	.001	6
		10^{-7}	-.004	.002	6
		10^{-6}	-.001	.002	6
		10^{-5}	.005	.002	6
		10^{-4}	.031	.010	6
		10^{-3}	.041	.010	6
		10^{-2}	.033	.008	6

Tissue	Pretreatment	[MCh]	Mean(g)	SEM	n
Peripheral Lung Strips	10^{-7}M atropine	10^{-9}	-.001	.001	6
		10^{-8}	.000	.002	6
		10^{-7}	.005	.003	6
		10^{-6}	.005	.003	6
		10^{-5}	.007	.002	6
		10^{-4}	.014	.002	6
		10^{-3}	.037	.007	6
		10^{-2}	.049	.004	6

b) IgE-producing Rabbits

Tissue	Pretreatment	[MCh]	Mean(g)	SEM	n
Peripheral Lung Strips	None	10^{-9}	.000	.001	8
		10^{-8}	-.002	.002	8
		10^{-7}	.000	.003	8
		10^{-6}	.007	.004	8
		10^{-5}	.021	.002	8
		10^{-4}	.048	.010	8
		10^{-3}	.044	.008	8
		10^{-2}	.029	.008	8
Peripheral Lung Strips	10^{-9}M atropine	10^{-9}	-.001	.001	8
		10^{-8}	-.001	.002	8
		10^{-7}	-.003	.001	8
		10^{-6}	.007	.002	8
		10^{-5}	.015	.002	8
		10^{-4}	.049	.010	8
		10^{-3}	.052	.009	8
		10^{-2}	.041	.010	8
Peripheral Lung Strips	10^{-8}M atropine	10^{-9}	-.001	.001	8
		10^{-8}	-.001	.001	8
		10^{-7}	-.001	.002	8
		10^{-6}	-.001	.002	8
		10^{-5}	.005	.002	8
		10^{-4}	.022	.002	8
		10^{-3}	.036	.003	8
		10^{-2}	.033	.004	8

Tissue	Pretreatment	[MCh]	Mean(g)	SEM	n
Peripheral Lung Strips	10^{-7}M atropine	10^{-9}	-.004	.002	7
		10^{-8}	-.005	.002	7
		10^{-7}	-.005	.004	7
		10^{-6}	-.003	.004	7
		10^{-5}	-.002	.004	7
		10^{-4}	.010	.005	7
		10^{-3}	.037	.010	7
		10^{-2}	.059	.016	7

III. Methacholine response with pirenzepine

a) Control Rabbits

Tissue	Pretreatment	[MCh]	Mean (% max.)	SEM	n
Mainstem Bronchi	None	10^{-9}	11.99	5.50	6
		10^{-8}	11.37	5.26	6
		10^{-7}	19.30	6.64	6
		10^{-6}	46.51	5.59	6
		10^{-5}	83.27	4.31	6
		10^{-4}	98.33	1.18	6
		10^{-3}	98.08	1.24	6
		10^{-2}	92.40	1.52	6
Mainstem Bronchi	10^{-7}M pirenzepine	10^{-9}	6.61	4.00	6
		10^{-8}	9.22	4.03	6
		10^{-7}	17.16	4.26	6
		10^{-6}	46.30	5.35	6
		10^{-5}	75.57	5.83	6
		10^{-4}	94.77	1.96	6
		10^{-3}	99.43	0.56	6
		10^{-2}	97.92	1.23	6
Mainstem Bronchi	10^{-6}M pirenzepine	10^{-9}	2.71	1.35	6
		10^{-8}	2.94	2.03	6
		10^{-7}	4.52	2.50	6
		10^{-6}	18.57	6.56	6
		10^{-5}	50.52	5.31	6
		10^{-4}	83.63	2.96	6
		10^{-3}	97.78	1.10	6
		10^{-2}	100.00	0.00	6

Tissue	Pretreatment	[MCh]	Mean (% max.)	SEM	n
Mainstem Bronchi	10^{-5}M pirenzepine	10^{-9}	-1.39	0.40	5
		10^{-8}	-2.45	0.30	5
		10^{-7}	-3.28	0.53	5
		10^{-6}	-3.64	1.10	5
		10^{-5}	10.16	2.21	5
		10^{-4}	56.00	2.76	5
		10^{-3}	91.38	1.82	5
		10^{-2}	100.00	0.00	5
Intra-pulmonary Bronchi	None	10^{-9}	0.57	1.39	6
		10^{-8}	-0.97	2.79	6
		10^{-7}	4.02	3.03	6
		10^{-6}	35.45	5.64	6
		10^{-5}	73.51	5.89	6
		10^{-4}	94.46	2.59	6
		10^{-3}	97.34	2.24	6
		10^{-2}	91.73	3.58	6
Intra-pulmonary Bronchi	10^{-7}M pirenzepine	10^{-9}	-1.68	0.65	6
		10^{-8}	-0.58	2.07	6
		10^{-7}	1.19	2.42	6
		10^{-6}	23.53	2.63	6
		10^{-5}	70.49	2.24	6
		10^{-4}	93.72	2.29	6
		10^{-3}	93.07	2.57	6
		10^{-2}	83.43	8.66	6
Intra-pulmonary Bronchi	10^{-6}M pirenzepine	10^{-9}	-1.28	0.98	6
		10^{-8}	-5.94	3.93	6
		10^{-7}	-8.85	5.42	6
		10^{-6}	-6.32	8.88	6
		10^{-5}	29.71	5.40	6
		10^{-4}	72.80	7.10	6
		10^{-3}	92.22	3.83	6
		10^{-2}	93.70	3.28	6
Intra-pulmonary Bronchi	10^{-5}M pirenzepine	10^{-9}	-2.14	2.59	5
		10^{-8}	-6.51	2.69	5
		10^{-7}	-8.96	2.78	5
		10^{-6}	-8.94	3.14	5
		10^{-5}	-6.02	5.05	5
		10^{-4}	36.31	5.70	5
		10^{-3}	86.04	2.19	5
		10^{-2}	100.00	0.00	5

Tissue	Pretreatment	[MCh]	Mean (% max.)	SEM	n
Intra-pulmonary Bronchi	10^{-4}M pirenzepine	10^{-9}	4.20	4.21	2
		10^{-8}	-2.89	0.19	2
		10^{-7}	-3.92	1.78	2
		10^{-6}	-7.63	3.47	2
		10^{-5}	-11.55	5.26	2
		10^{-4}	-8.45	5.36	2
		10^{-3}	36.11	13.89	2
		10^{-2}	100.00	0.00	2

Tissue	Pretreatment	[MCh]	Mean(g)	SEM	n
Peripheral Lung Strips	None	10^{-9}	.005	.003	10
		10^{-8}	.007	.004	10
		10^{-7}	.010	.003	10
		10^{-6}	.019	.004	10
		10^{-5}	.036	.005	10
		10^{-4}	.053	.007	10
		10^{-3}	.055	.008	10
		10^{-2}	.047	.005	10
Peripheral Lung Strips	10^{-7}M pirenzepine	10^{-9}	.000	.001	9
		10^{-8}	.003	.002	9
		10^{-7}	.007	.003	9
		10^{-6}	.009	.003	9
		10^{-5}	.025	.003	9
		10^{-4}	.043	.005	9
		10^{-3}	.050	.007	9
		10^{-2}	.043	.008	9
Peripheral Lung Strips	10^{-6}M pirenzepine	10^{-9}	.000	.002	10
		10^{-8}	-.001	.002	10
		10^{-7}	.001	.003	10
		10^{-6}	.004	.003	10
		10^{-5}	.015	.004	10
		10^{-4}	.034	.005	10
		10^{-3}	.045	.005	10
		10^{-2}	.041	.006	10

Tissue	Pretreatment	[MCh]	Mean(g)	SEM	n
Peripheral Lung Strips	10^{-5}M pirenzepine	10^{-9}	.000	.002	9
		10^{-8}	.000	.002	9
		10^{-7}	-.001	.002	9
		10^{-6}	.002	.003	9
		10^{-5}	.009	.003	9
		10^{-4}	.022	.004	9
		10^{-3}	.045	.005	9
		10^{-2}	.051	.006	9
		10^{-1}	.063	.015	2
Peripheral Lung Strips	10^{-4}M pirenzepine	10^{-9}	.002	.002	3
		10^{-8}	-.001	.003	3
		10^{-7}	.002	.006	3
		10^{-6}	.006	.009	3
		10^{-5}	.006	.009	3
		10^{-4}	.010	.009	3
		10^{-3}	.014	.009	3
		10^{-2}	.041	.013	3
		10^{-1}	.030	.008	2

b) IgE-producing Rabbits

Tissue	Pretreatment	[MCh]	Mean(g)	SEM	n
Peripheral Lung Strips	None	10^{-9}	-.001	.001	10
		10^{-8}	.001	.002	10
		10^{-7}	.002	.002	10
		10^{-6}	.011	.002	10
		10^{-5}	.028	.004	10
		10^{-4}	.054	.010	10
		10^{-3}	.052	.011	10
		10^{-2}	.043	.010	10
Peripheral Lung Strips	10^{-7}M pirenzepine	10^{-9}	-.004	.002	10
		10^{-8}	-.003	.002	10
		10^{-7}	.002	.003	10
		10^{-6}	.011	.004	10
		10^{-5}	.031	.010	10
		10^{-4}	.064	.015	10
		10^{-3}	.066	.014	10
		10^{-2}	.053	.012	10

Tissue	Pretreatment	[MCh]	Mean(g)	SEM	n
Peripheral Lung Strips	10^{-6}M pirenzepine	10^{-9}	-.002	.002	9
		10^{-8}	-.002	.002	9
		10^{-7}	.000	.003	9
		10^{-6}	.004	.002	9
		10^{-5}	.015	.003	9
		10^{-4}	.038	.008	9
		10^{-3}	.049	.010	9
		10^{-2}	.042	.007	9
Peripheral Lung Strips	10^{-5}M pirenzepine	10^{-9}	-.001	.002	8
		10^{-8}	-.002	.003	8
		10^{-7}	.002	.003	8
		10^{-6}	.002	.004	8
		10^{-5}	.006	.004	8
		10^{-4}	.016	.006	8
		10^{-3}	.039	.011	8
		10^{-2}	.045	.012	8
		10^{-1}	.031	.004	5
Peripheral Lung Strips	10^{-4}M pirenzepine	10^{-9}	.000	.004	4
		10^{-8}	.001	.003	4
		10^{-7}	-.002	.004	4
		10^{-6}	-.002	.006	4
		10^{-5}	.000	.005	4
		10^{-4}	.008	.003	4
		10^{-3}	.029	.009	4
		10^{-2}	.073	.018	4
		10^{-1}	.058	.013	3

IV. Isoproterenol Relaxation Study

a) Control Rabbits

Tissue	[Isoproterenol]	Mean (% max.)	SEM	n
Intrapulmonary Bronchi	10^{-9}	17.35	4.30	6
	10^{-8}	32.94	10.53	6
	10^{-7}	82.71	13.57	6
	10^{-6}	110.80	17.51	6
	10^{-5}	130.26	16.17	6
	10^{-4}	141.39	14.63	6
Mainstem Bronchi	10^{-9}	-7.83	0.85	6
	10^{-8}	-7.40	1.41	6
	10^{-7}	18.00	8.22	6
	10^{-6}	61.96	11.99	6
	10^{-5}	93.74	12.03	6
	10^{-4}	105.08	8.49	6

b) IgE-producing Rabbits

Tissue	[Isoproterenol]	Mean (% max.)	SEM	n
Intrapulmonary Bronchi	10^{-9}	4.22	5.36	8
	10^{-8}	21.05	6.44	8
	10^{-7}	66.47	10.61	8
	10^{-6}	110.26	14.23	8
	10^{-5}	133.13	15.16	8
	10^{-4}	151.77	14.96	8
Mainstem Bronchi	10^{-9}	-8.14	1.02	8
	10^{-8}	-6.50	1.70	8
	10^{-7}	6.33	4.83	8
	10^{-6}	39.41	8.83	8
	10^{-5}	72.29	9.62	8
	10^{-4}	87.88	9.56	8

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