THE UNIVERSITY OF MANITOBA

LOCAL PARASYMPATHETIC AND MUSCULAR MECHANISMS FOR THE HYPERRESPONSIVENESS OF TRACHEAL SMOOTH MUSCLE FROM A CANINE MODEL OF ASTHMA

BY

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WHO REPEATEDLY ENCOURAGED ME

BY SAYING

"SEVENTEEN YEARS UNIVERSITY

AND YOUR FATHER'S STILL

SMARTER THAN YOU!"

(MISS YOU DAD)

ABSTRACT

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In an attempt to elucidate causative mechanisms of bronchospasm at the level of the airway smooth muscle, physiological and pharmacological studies were conducted on isolated muscles from a canine model exhibiting allergic bronchospasm. Mongrel dogs were actively sensitized in vivo to ragweed pollen extract, after which they reacted cutaneously to injected ragweed extract with wheals and flares and reacted to the inhalation of aerosolized ragweed extract with increased airways resistance. Human allergic asthmatics show similar responses when they are either naturally or clinically challenged.

The mechanism for increased pulmonary responsiveness in ragweed sensitized dogs was investigated using in vitro methods. Tracheal smooth muscle (TSM) strips were dissected from sensitized (S) and littermate control dogs (C) and suspended in tissue baths to maintain homeostatic conditions and record isometric force development under a variety of conditions. STSM showed a generalized hyperreactivity (upward shift of the dose-response relationship) to histamine, serotonin, acetylcholine, increased $[K^+]_{out}$ and increased reactivity to prolonged supramaximal 60 Hz electrical field stimulation when compared to CTSM. STSM also showed hypersensitivity (leftward shift of the dose-response relationship) to histamine, acetylcholine and increased $[K^+]_{out}$ and a leftward shift in electrical stimulus response relationships.

Electrical stimulation of STSM revealed that atropine sensitive spontaneous baseline activity could be induced and that prolonged stimulation could maintain plateau isometric tension. Neither of these phenomena could be observed in CTSM unless eserine, (10^{-7} M) an acetylcholinesterase inhibitor was present. These results indicated that the sensitization process produced either an increase in the basal release of acetylcholine or a decrease in acetylcholinesterase activity.

Atropine not only abolished spontaneous baseline activity of STSM but also shifted the dose-response relationships of STSM to the right for histamine and increased $[K^+]_{out}$. In order to decide between these alternatives more directly, STSM labelled with [^{14}C] - choline showed increased basal release and increased histamine induced release of ^{14}C- choline compared to littermate CTSM. A direct measurement of acetylcholinesterase activity showed a 20 % decrease in STSM. Histamine had no further effect on STSM cholinesterase activity but reduced CTSM cholinesterase rates to sensitized levels.

These results were further substantiated by the observations 1) that STSM and CTSM showed no difference in reactivity to acetylcholine in the presence of eserine or to carbamylcholine (which is not hydrolyzed readily by acetylcholinesterase) and that there was a leftward shift in the dose-response relationship to acetylcholine, 2) that atropine affected the Schultz-Dale response by inhibiting that portion of it which is produced through the mechanism of a presynaptic histamine stimulation of acetylcholine release (from degranulating mast cells), 3) that in

the presence of atropine the dose-response relationship to increased $[K^+]_{out}$ was not different between STSM and CTSM since voltage sensitive Ca^{++} channels should be unaffected by the sensitization process and 4) that with increased acetylcholine in the neuromuscular synapse, the resting membrane potential of STSM was slightly depolarized in comparison to CTSM.

These results indicate a role for the parasympathetic nervous system in the etiology of allergic bronchospasm in the dog. Increased parasympathetic activity through increased release of the neurotransmitter acetylcholine or decreased cholinesterase activity may parallel local reflex bronchospasm and may explain, in part, the reduction in allergic bronchospasm after atropine administration.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF TABLES

INTRODUCTION

A) GENERAL INTRODUCTION AND THEORIES OF ASTHMA

Human asthma is characterized by increased central and peripheral airways resistance and a decreased dynamic compliance causing the lungs to become stiffer. This increase in resistance is due to three main factors. The first is that asthmatics are extremely sensitive to physical (cold or exercise), chemical (pollens and noxious gases) and pharmacological (leukotrienes and histamine) stimuli and this sensitivity manifests itself in increased contraction of the airway smooth muscle of the tracheobronchial tree. The smooth muscle of the airways is under the control of the parasympathetic nervous system with acetylcholine being the neurotransmitter, (Richardson, 1979). The second factor which increases airways resistance is the secretion of mucus which can occlude bronchioles and alveoli. The secretion of mucus is also under the control of the parasympathetic nervous system. The third factor is edema of the mucosa which increases the turbulence of airflow. These obstructive mechanisms result in alterations in lung mechanics such that there is a reduction in vital capacity and increases in residual volume and functional residual capacity.

The mechamism by which the airways of asthmatics become more sensitive to a variety of agents has been investigated intensively for the past 20 years. The research has been directed towards the four main theories of the causation of asthma.

The first theory put forward by Szentivanyi (1968) stated

that the beta-adrenergic receptors which sparsely populate the tracheobronchial smooth muscle was the principle inhibitory mechanism and when stimulated caused the airways to relax, but if there should be a reduction in the number of beta-adrenergic receptors on the airway smooth muscle or a blockade of them, their ability to relax the airway would be reduced. This would leave the parasympathetic nervous system unopposed. However, Zaid and Beall (1966) could not show increased histamine sensitivity in healthy subjects given beta-receptor blockers. In addition, the data used to substantiate this theory were taken from patients already taking beta-adrenergic agonists (McNeill, 1964).

The second theory resulted from the findings of Crema et al. (1968) and Burnstock (1972) that there existed a non-cholinergic, non-adrenergic inhibitory nervous system in the gut, and since the airways had arisen from the same embryological origin it should be present there as well. Coburn and Tomita (1973) found it in guinea pig airways. Richardson and Beland (1976) later found evidence for it in humans. They went on to suggest that this nervous system was the major inhibitory system since only an extremely sparse adrenergic innervation could be found eventhough adrenergic receptors were present in the human airways. However, the foundation for a non-cholinergic, non-adrenergic inhibitory system in humans is indirect and inconclusive. It is based on blocking known relaxant systems, inducing tone, and then stimulating electrically and showing the response can be blocked by tetrodotoxin. The neurotransmitter has yet to be identified although vasoactive intestinal peptide (VIP) may be a candidate

(Dey et al., 1981), and the nerves have yet to be found histologically. Furthermore this inhibitory system has not been demonstrated in humans in vivo. In fact its presence in vivo has only been shown in cats (Irvin et al., 1980) and guinea pigs (Chesrown et al., 1980). Nevertheless, a defect or dysfunction in this system would again leave the parasympathetic nervous system unopposed and could result in the various airway obstructions. The importance of this inhibitory system in asthma however, has not been established.

The third theory states that mediators (namely histamine and slow reacting substances of anaphylaxis - SRS-A, now known to be leukotrienes C_4 and D_4) are released from mast cells after the antigen - antibody reaction on their surface. These released mediators then diffuse to the airway smooth muscle and cause bronchoconstriction (Austen, 1973; Gold, 1973; Lichtenstein, 1973). Investigations in vivo have demostrated that LTC_4 and LTD_4 have selective actions on the small airways of the lung, cause a minor fall in specific airway conductance, but preferentially reduce lung compliance (Drazen et al., 1980). Weiss et al., (1982) reported, that unlike histamine, LTC_4 showed no signs of acting on central airways and asthmatics showed a hyperreactivity to histamine but not LTC_4 (Griffin et al., 1983). Thus the release of these transmitters would have preferential sites of action with histamine affecting central and the leukotrienes peripheral airways.

Widdicombe (1977) combined the classical theory of asthma (antigen-antibody reaction and release of mediators) with Nadel's (1965) irritant receptor-bronchoconstrictor reflex theory - the

fourth major theory of asthma. Nadel proposed that asthma may be due to an irritant receptor-bronchoconstrictor reflex and that the hyperirritability observed in asthmatic airways might be due to a lowering of the threshold, or an increase in the response of certain pulmonary vagal receptors. The stimulation of these receptors would result in a reflex bronchoconstriction through the parasympathetic nervous pathway which innervates the tracheobronchial tree. Widdicombe suggested that this system interacts with IgE - mediated antigen-antibody reactions, in that ccchemical mediators released by the mast cells act on the nervous receptors within the bronchial epithelium to produce a reflex bronchoconstriction (Gold et al., 1972 (a); Richardson et al., 1973; Gold, 1975).

Recently, a non-cholinergic, non-adrenergic excitatory nervous system has been proposed since the presence of immunoreactive substance P has been found in a fine network of nerves supplying the airways of guinea pigs (Nelsson et al., 1977) and rabbit (Tanaka and Gruustein, 1983). Substance P causes airway constriction in these animals, however, Grunstein et al. (1984) have demonstrated that the constriction is mediated in part through acetylcholine and proposed a neuromodulatory role of substance P for acetylcholine release at a site distal to the airway parasympathetic ganglia.

The cause of bronchospasm has been attributed to many trigger mechanisms set off by a variety of non-specific stimuli. The focus ultimately is the airway smooth muscle. Isolated in a tissue bath, airway smooth muscle is cut off from central reflexes and blood-borne neuromodulatory agents and offers an

excellent opportunity to study local mechanisms that might be aberrant in asthma. The latter include the parasympathetic neuromuscular junction, the smooth muscle cell membrane and receptors, excitation-contraction coupling, and contractile proteins. These areas are discussed below.

B) THEORIES OF ASTHMA IN RELATION TO THE ISOLATED AIRWAY SMOOTH MUSCLE PREPARATION

As previously discussed there are four major theories concerning the etiology of asthma. The beta-adrenergic blockade theory (Szentivanyi, 1968) and the non-adrenergic , non-cholinergic inhibitory nervous system theory (Richardson and Bouchard, 1975; Richardson and Beland, 1976), due to insufficient evidence, have given way to the last two major theories which Widdicombe (1977) has interrelated, i.e., the irritant receptor-bronchoconstrictor reflex theory (Nadel, 1965) and the classical theory of antigen-antibody reaction leading to anaphylactic mediator release and bronchoconstriction (Austen, 1973; Gold, 1973; Lichtenstein, 1973).

The major inhibitory system in humans and guinea pigs was thought to be mediated via beta-adrenergic receptors. Szentivanyi (1968) proposed that if beta-adrenergic receptors were blocked or lacking on airway smooth muscle cells in asthmatic individuals, it would leave the predominating contractile parasympathetic nervous system unopposed leading to the hyperreactivity displayed by airways in asthma. However, the majority of the experiments conducted to support the theory were carried out on patients already on beta-agonist treatment

(McNeill, 1964) and it was further shown that the beta-blockade failed to increase airway sensitivity to histamine in non-asthmatic subjects (Zaid and Beall, 1966). More recently Bergen and Kroeger (1979) have shown a potential role for alpha-adrenergic receptor activation in allergic bronchospasm from a sensitized canine model of asthma. Sensitized tracheal smooth muscle contracted in response to low doses of exogenous norepinephrine or to tyramine-induced release of endogenous norepinephrine in vitro. Control tissues did not respond unless basal tension was increased with histamine, serotonin, acetylcholine or high K^+ (Bergen and Kroeger, 1980).

The non-adrenergic inhibitory nervous system as exhibited in guinea pig (Coburn and Tomita, 1973) and human (Richardson and Beland, 1976) airways may be the major inhibitory system in these species. It has not been found in the dog. A defect in this inhibitory system, as in the case of the adrenergic beta-receptors could leave the parasympathetics unapposed and lead to the hyperreactivity of airway smooth muscle observed in asthma. Again, evidence for this theory, including the possible nature of the neurotransmitter, is lacking. In addition, the relatively small potential for relaxing the airways is offset by the predominating contractile nature of the cholinergic parasympathetic pathway present in man, dog, and other species in vivo and in vitro. (Up to 95% of canine airways response to electrical field stimulation can be blocked by the acetylcholine antagonist atropine in low concentrations - 10^{-8} M).

The classical theory for the cause of asthma contends that IgE antibodies are attached to the surface of mast cells which

are found throughout the airways. The antibodies are produced in response to exposure to a specific antigen. When an asthmatic person is exposed to the antigen it may diffuse into the airway tissue containing the antibody covered mast cells. The antigen-antibody reaction causes the degranulation of the mast cells which release mediators such as histamine and leukotrienes. These mediators migrate to the smooth muscle of the airways and initiate the bronchospasm (Austen, 1973; Gold, 1973; Lichtenstein, 1973). The mast cells also release mediators of the inflammatory response such as platelet activating factor and eosinophil chemotactic factor of anaphylaxis.

Widdicombe (1977) has modified the original irritant receptor-bronchoconstrictor reflex theory (Nadel, 1965) to include the antigen-antibody reaction and the release of mediators. Nadel proposed that airway hyperirritability might be due to a lowering of the threshold, or an increase in the response of certain vagal receptors in the lung. The stimulation of these receptors by ozone, sulphur dioxide, smoke, or anaphylactic mediators released from mast cells after the antigen-anitbody reaction (Widdicombe, 1977) was proposed to result in a reflex bronchoconstriction via the parasympathetic nervous pathway which innervates the airways.

The importance of the parasympathetic nervous system in the etiology of asthma is becoming more evident. Direct receptor binding assays have demonstrated a high density of muscarinic receptors in tracheal smooth muscle from animals (Murlas et al., 1982; Cheng and Townley, 1982) and in the smooth muscle of the large airways (Barnes et al., 1983). However, the density of

these receptors decreases as the airways become smaller so that terminal bronchioles are almost devoid of muscarinic receptors (Barnes et al., 1983 a,b). In an asthmatic attack, it is the smooth muscle of the central airways that is most affected by the rapid onset reflex bronchoconstriction as opposed to the predominantly leukotriene mediated slow phase of the peripheral airways. Because of the small total cross-sectional area of the central airways compared to the peripheral airways, central airway bronchospasm is the major cause for increased airways resistance. Thus the response of the lungs to antigen challenge can be modified and attenuated by cutting or cooling the vagus nerves (Mills and Widdicombe, 1970; Gold, 1975) or with the administration of atropine - a specific antagonist for the parasympathetic neurotransmitter acetylcholine (Gold et al., 1972b; Drazen and Austen, 1975; Gold, 1975).

This phenomenon has not been as well established in the human as it has in the dog (Gold et al., 1972b). Evidence has suggested that it is present in the human but may be secondary to mediator release, hypoxemia or abnormal breathing patterns during an attack of asthma (Gold, 1976). The persistence of bronchoconstriction after inhibition of the vagal reflex mechanism indicates that vagal reflexes cannot be responsible for the entire sequence of events in an asthmatic attack. Mitchell and Bouhuys (1976) suggested an interaction between histamine and cholinergic stimulation of airway smooth muscle in the human. An interaction such as this may explain the observations of diminished or blocked airways response to histamine after vagal blockade.

C) THE DOG AS A MODEL OF ALLERGIC BRONCHOSPASM AND ASTHMA

A canine model of allergic bronchospasm (Kepron et al., 1977) was selected to study the pharmacological properties of airway smooth muscle and the influence of the parasympathetic nervous system on these properties after sensitization. Several reasons influenced this choice. Patterson (1969) has shown that dogs can display a natural reaginic hypersensitivity to pollen and are well suited to a study of allergic asthma. This hypersensitivity is similar in many respects to pollen induced asthma in humans (Patterson, 1960). Ragweed sensitized dogs show immediate and late phase cutaneous responses, an immediate airway response after the inhalation of ragweed antigen aerosol (Chung et al., 1985), and increased airway responsiveness to inhaled histamine and methacholine (Mapp et al., 1983).

The immune reaction in the dog is mediated through the IgE class of immunoglobulins which is the same as in the human (Kessler et al., 1974). Also, the sensitization procedure of Kepron et al. (1977) which uses puppies initially immunized within 24 hrs of birth, produces sensitized dogs possessing a high titre specific to the sensitizing antigen. In this respect, the model used in this study, is quite unlike the adult Ascaris model that is more usually employed. Chiesa et al., (1975) have shown increased airways resistance upon specific antigen aerosol challenge to allergic dogs similar to that observed in human asthma. Antonissen et al. (1980) have shown a direct link between antigen challenge and histamine release upon mast cell degranulation in the dog, however, the importance of histamine in

human asthma has still not been established (Brown et al., 1977).

Nevertheless, the sensitized dog is the only animal model which possesses pathological, physiological and immunological changes similar to the human disease. Hirshman et al., (1980) using inbred Basenji-Greyhounds have suggested a genetic link as well. The availability of the ragweed model and the familiarity of this laboratory with the canine airway smooth muscle were associate factors in choosing this model.

The guinea-pig model has failed to show either acute or chronic lung volume changes (Pare et al., 1977) upon sensitization and has been shown to produce a predominance of IgG immunoglobulins unlike the human or dog model. Isolated human tissue would be the best model for human asthma. However, the experience in this laboratory is that human tracheal samples are not readily available and the time post-mortem to acquisition of the tissue usually ranges from 12 to 24 hours. Lung samples from surgical removals are not frequent enough to rely on this source and the chances of obtaining asthmatic lung samples are less likely.

The major short-coming with the canine model is the lack of a non-adrenergic, non-cholinergic inhibitory system which is present in guinea-pigs (Coburn and Tomita, 1973) and has been postulated for humans (Richardson and Beland, 1976) and is the predominant inhibitory system in these species. Defects of this system are known for the gastrointestinal tract in humans where it plays a role in sphincter relaxation and peristalsis (Daniel and Sarna, 1978; Wood, 1975). However, in mammals the non-

adrenergic inhibitory system is secondary to the predominant cholinergic system which overrides the inhibition (Richardson, 1981). In addition, evidence for changes in the non-adrenergic system in asthma is lacking due to a paucity of information concerning the neurotransmitter involved. Therefore, sensitized canine airway smooth muscle was used as a model of allergic bronchospasm. Its availability and similarity in pathological, physiological and immunological changes with the human disease were of prime importance.

D) TRACHEAL SMOOTH MUSCLE (TSM)

a) Utility of TSM as a model of airway smooth muscle generally.

The airways are not merely passive conduits through which air flows to and from the alveoli. All the airways contain smooth muscle which, under the influence of neural and hormonal mechanisms, can control the diameter (to produce a balance between anatomical dead space and airway resistance) and wall tension and thus regulate the general and regional distribution of air to the lungs (Widdicombe and Nadel, 1963) as well as stabilize the airways (Olsen et al., 1967 a,b).

Cervical TSM is under the same neural and hormonal tone control mechanisms as the rest of the central airways. Stimulation of the vagus nerve results in wide-spread airway narrowing including the trachea (Woolcock et al., 1969), and, in asthmatic patients, intravenous histamine has been shown to constrict both large and small airways (Newball, 1974). In general, TSM appears to be mechanically similar to smooth muscle

of airways down to at least the sixth generation of bronchi (Hawkins and Schild, 1951; Nadel, 1973) and responds to inhalation of agents such as sulphur dioxide and dust similar to resistance units (Nadel, 1973; Permutt, 1971) probably through the irritant receptor bronchoconstrictor reflex since bronchospasm can be abolished by sectioning the vagus nerve or atropine treatment in healthy human subjects. This also suggests that post-ganglionic cholinergic pathways are also involved in the human response. These observations suggest that tracheal smooth muscle is a good model for the study of normal and disease states such as allergic bronchoconstriction and asthma.

b) Properties of canine tracheal smooth muscle.

Certain anatomical and biophysical properties of canine TSM render it very suitable for characterizing the responses to neural and chemical stimulation. In the transverse axis of the trachealis muscle all the fibres run parallel and greater than 75% of the tissue is muscle as assessed by histological (Stephens et al., 1969) and biochemical (Stephens and Wrogemann, 1970) methods. The muscle, under normal in vitro conditions, exhibits no spontaneous rhythmical electrical or contractile activity (Stephens and Kroeger, 1970) and at its optimal length has a relatively small resting tension (Stephens, 1975).

Canine TSM is predominantly activated by cholinergic neural inputs in response to electrical stimulation in vitro (Stephens and Kroeger, 1970). Direct electrical stimulation or through the parasympathetic nerve net results in graded depolarizations with

accompanying graded contraction without any action potentials on the TSM. Kirkpatrick (1975) has reported histamine-induced depolarization of bovine TSM with slow oscillations in membrane potential and concomitant fluctuations in contractile activity. The latter were dependent upon extracellular calcium concentrations. Graded responses of canine TSM to K^+ and tetraethylammonium (TEA), a K^+ channel blocker which produces rhythmic electrical and mechanical responses in TSM, have been shown to be dependent on extracellular calcium (Stephens et al., 1975; Stephens, 1976). Calcium channel blockers also inhibit action potentials recorded in canine TSM after treatment with TEA (Suzuki et al., 1976; Kroeger and Stephens, 1975). These studies point out the importance of extracellular calcium in the maintenance of TSM tone and suggest an important role for a calcium current in the development of action potentials by this tissue.

Canine TSM responds to electrical field stimulation (EFS) with contraction elicited via the parasympathetic nerve net in the preparation, with acetylcholine being the neurotransmitter. Blocking acetylcholine receptors in TSM with atropine results in almost complete inhibition of the response to supramaximal EFS. It also responds directly to acetylcholine, histamine, serotonin and prostaglandin application and can be relaxed by the beta-agonists isoproterenol and nor-epinephrine. Antonissen et al. (1979) have demonstrated a Schultz-Dale reaction; i.e., antigen-antibody reaction with the subsequent release of mediators of anaphylaxis in TSM from an ovalbumin sensitized canine model of asthma.

E) TIME-COURSE OF ALLERGIC BRONCHOSPASM

In allergic asthmatic patients, inhalation of specific antigens results in an early phase or immediate bronchoconstrictor response. This early response peaks at approximately 15 minutes and abates spontaneously after an hour or so. This may be followed by a late phase response from 3 to 8 hours after antigen exposure (Robertson et al., 1974), which peaks at 8 to 12 hours and may take several days to abate (Pepys and Hutchcroft, 1975).

The mediators of the early response in the human are numerous (Wilson and Galant, 1974) and are released from degranulating mast cells after the antigen-antibody reaction on the mast cell surface. The primary mediators of anaphylaxis in the human are histamine and slow-reacting substances of anaphylaxis (SRS-A) - now known to be leukotrienes C_4 and D_4 (Samuelsson et al., 1980). Following allergen exposure asthmatic airways show a non-specific hyperreactivity to a variety of agents which may be the result of the exposure to allergic mediators (Cockcroft et al., 1977; Cartier et al., 1982). The importance of the early phase mediator release in producing the long-term generalized hyperreactivity which perhaps leads to the late phase response is illustrated by the observation that sodium cromoglycate (prevents degranulation of mast cells) inhibits both the early and late phase reactions to antigen challenge in human asthmatics (Kang et al., 1976). Cutaneous responses in human allergy follow approximately the same time course as the airway

responses (Umemoto et al., 1975).

Sensitized dogs have been shown to possess some of the characteristics of human allergic asthma (Chung et al., 1985). Cutaneous responses in the dog parallel the human, however, airway responses have only been seen in the immediate or early phase after the inhalation of antigen aerosol with associated increased responsiveness to inhaled histamine and methacholine (Mapp et al., 1983). The absence of an airway late phase response may be species specific for the dog or may be suppressed due to anesthesia with barbiturates (Chung et al., 1985) since late-phase bronchoconstriction has been demonstrated only in conscious humans (Robertson et al., 1974), rabbits (Shampain et al., 1982) and sheep (Abraham et al., 1983).

F) CHEMICAL MEDIATORS OF THE ASTHMATIC RESPONSE

Many chemical mediators are released from sensitized lungs after antigen challenge. The major mediators are histamine and metabolites of arachidonic acid. However, chemicals such as platelet activating factor (PAF) and eosinophil chemotactic factor of anaphylaxis (ECF-A) are also released.

PAF aggregates platelets with the resultant release of serotonin (5-HT) - a potent airway smooth muscle constrictor and activator of reflex bronchoconstriction. PAF has been shown to be released from rabbit (Henson, 1970; Siraganian and Osler, 1971) and human (Benveniste, 1974) cell suspensions and from human lung fragments (Bogart and Stechschulte, 1974) by IgE dependent mechanisms. ECF-A is released after the antigen-

antibody reaction (Kay et al., 1971) from mast cells (Wasserman et al., 1973) and attracts eosinophils to the sites of inflammation in the lungs. Eosinophils may contain a histaminase and an arylsulphatase which break down the anaphylactic mediators histamine and SRS-A (leukotrienes C_4 and D_4) released from the mast cells (Gold, 1976).

Histamine is released from mast cells upon their degranulation, and causes constriction of isolated airways through H_1 receptors. H_2 receptors exist in sheep (Eyre, 1973) and cats (Eyre, 1973; Moengwyn-Davies, 1968) and produce bronchodilation. The guinea pig possesses both H_1 and H_2 receptors but no in vivo or in vitro physiological role has been found for the H_2 receptors (Brink et al., 1982). Similar results have been observed in humans (Michaud et al., 1981). Histamine is the major mediator released in dogs (Meyers et al., 1973; Gold et al., 1977) and has a potent direct contractile effect on canine tracheal smooth muscle which possesses only H_1 agonist receptors (Antonissen et al., 1980).

Histamine can have direct or indirect effects (through the vagal reflex pathway) on airway smooth muscle. In spontaneously breathing guinea-pigs that were not anesthetized, Drazen and Austen (1975) showed that cholinergic blockade with atropine blocked lung resistance and compliance changes to low doses of histamine. At higher doses the effects of histamine effects were resistant to atropine blockade. Vagotomy also attenuated histamine-induced mechanical changes in lungs of spontaneously breathing anesthetized guinea pigs (Mills and Widdicombe, 1970). Vagotomy or cholinergic blockade with atropine also effectively

blocked increases in airway resistance upon injection of histamine into bronchial arteries of dogs (Wasserman, 1975).

In human asthmatics plasma levels of histamine are elevated and, during asthmatic episodes, correlate with decreased pulmonary function (Simon et al., 1977). Antihistamine therapy (Levy and Seabury, 1947; Criep and Aaron, 1948) has failed to demonstrate significant improvement in pulmonary function. The inability to deliver adequate doses of antihistamines before contraindications develop is also a problem. In addition, antihistamines have been shown to release histamine (Arunlakshana, 1953; Frik-Holmberg, 1972) and contract airway tissue (Hawkins, 1955; Hawkins and Schild, 1951) following aerosol treatment. The role of histamine in the etiology of human asthma, therefore, cannot be dismissed on these grounds.

Histamine is a potent initiator of arachidonic acid metabolism through the activation of phospholipase A_2 (Burka and Paterson, 1980; Grodzinska et al., 1975). Arachidonic acid can be further metabolized to form prostaglandins and leukotrienes which have profound effects on airway tone in guinea pigs, humans, and dogs. The leukotrienes C_4 and D_4 have been identified as SRS-A (Samuelsson et al., 1980) and are released from mast cells after degranulation due to the antigen-antibody reaction, in addition to being manufactured after the fact (Wasserman et al., 1973).

Arachidonic acid liberated from phospholipids can follow the cyclooxygenase metabolic pathway to produce the prostaglandins which have profound effects on vascular (McGiff and Nasjletti, 1973) and extravascular (Goldberg and Ramwell, 1975; Wilson,

1974) smooth muscles. Prostaglandin F_{2alpha} is a potent constrictor of airway smooth muscle in guinea pigs (Ono, 1979) and man (Ghelani et al., 1980) whereas prostaglandin E_2 generally relaxes airway smooth muscle from most species in vitro including guinea pigs (Schneider and Drazen, 1980) dogs (Krell, 1978) and man (Karim et al., 1980). In the dog, prostaglandin F_{2alpha} has been shown to augment the response of tracheal and bronchial airways to vagal stimulation without any independent induction of active tone (Leff et al., 1985).

Arachidonic acid may also be metabolized along its lipoxygenase pathway to produce leukotrienes. SRS-A - leukotrienes C_4 and D_4 - which constrict guinea pig (Piper et al., 1981) and human airways (Dahlen et al., 1980) has long been known as an important mediator of anaphylaxis. SRS-A has been shown to have little or no effect in rat, cat, or dog (Krell et al., 1981) when applied exogenously, however Tesarowski et al., (1981) and Tesarowski and Kroeger (1982) have produced indirect evidence for a contractile effect of endogenously derived SRS-A in the dog.

Other metabolites of the arachidonic acid cascade such as prostacyclin, the thromboxanes, endoperoxides, and prostaglandin and leukotriene intermediates have many and varied physiological effects, (See review by Anderson, 1985) including both contractile and relaxant effects on the airways; a systematic discussion of these metabolites, however, is beyond the scope of this manuscript.

G) POTENTIAL SITES OF INVOLVEMENT IN AIRWAY HYPERREACTIVITY

After noting the many similarities between canine and human allergic bronchospasm (Patterson, 1960; Patterson, 1969; Booth et al., 1970) and then taking into account the few specific differences - the dog's lack of a non-adrenergic inhibitory system and its failure to respond to exogenously applied SRS-A - it is apparent that the sensitized dog is a good model for the study of asthma. Without ignoring the potential genetic predisposition of dogs to airway hyperirritability (Hirchman et al., 1984) and the loss of the central vagal reflex pathway with in vitro studies, the responses of the isolated canine airways, and in particular the tracheal smooth muscle, correlate well with in vivo responses to antigen aerosol bronchial challenge (Rubinfeld et al., 1982). While studies have shown a centrally mediated bronchoconstrictor reflex (Mills and Widdicombe, 1970; Gold, 1975) local reflex bronchospasm could not be ruled out (Shore et al., 1985).

Local mechanisms can best be investigated in the isolated tissue in an organ bath. Possible sites of local reflex involvement may be associated with ganglia, adrenergic nerves present in the trachealis preparation, or pre- or post-junctional abnormalities in the cholinergic parasympathetic nerve net which predominates in canine tracheal smooth muscle. At the smooth muscle membrane level there are the possibilities of involvement of receptor operated and/or voltage sensitive calcium channels as well as the electrogenic sodium pump (Souhrada and Souhrada, 1984). Beyond the membrane, the handling of calcium by the cell, contraction coupling (Stephen et al., 1984), cyclic AMP

levels (Rinard et al., 1979) and contractile and regulatory proteins may be affected. Techniques and tools are available to investigate these areas.

a) Genetic factors

Data regarding a familial basis for some types of non-specific airway hyperresponsiveness in humans is increasing (Konig and Godfrey, 1973; Sibbald et al., 1980; Townley and Bewtra, 1983), however, a high proportion of these findings may be due to common environmental factors such as air pollution and respiratory infection although a dominant inheritance for low IgE cannot be ruled out (Marsh et al., 1974).

Hirshman et al. (1984) conclude from data obtained from the offspring of spontaneously hyperreactive Basenji-greyhound dogs that familial rather than environmental factors are important for the development of the non-specific airway hyperresponsiveness observed in her model of asthma (Hirshman et al., 1980). Whether or not the Basenji-greyhounds were reactive to Ascaris suum antigen, their airways were hyperresponsive to both methacholine and citric acid aerosols. This non-specific airway hyperresponsiveness could be partially inhibited by atropine administration (Hirshman and Downes, 1981) but they concluded that the major component of the antigen-induced bronchoconstriction was not cholinergically mediated in their model.

b) The role of the irritant receptor-bronchoconstrictor reflex in asthma.

Gold et al., (1972b) unilaterally challenged, with Ascaris antigen, one lung of sensitized dogs and showed bilateral bronchoconstriction which was inhibited by cooling the vagus nerve of the challenged lung. These elegant experiments led to the conclusion that a major component of acute antigen induced canine asthma is vagally mediated after stimulation of pulmonary irritant receptors. Mills and Widdicombe (1970) have shown similar results in the guinea-pig. In man, Bouhuys et al., (1960) have shown that the bronchmotor actions of histamine can be partially or completely blocked by atropine or hexamethonium, again suggesting a reflex mechanism.

The irritant receptor is likely located within the airway epithelium (Mortola et al., 1975) and can be stimulated by mediators of anaphylaxis such as histamine (Sampson and Vidruk, 1975) noxious gases (Nadel et al., 1965) and exercise (Simonsson et al., 1967). Viral infections which damage airway epithelium (Dixon et al., 1979) or increased mucosal membrane permeability (Hogg et al., 1979) may increase exposure or sensitivity of the receptors to these stimuli. A significant number of individuals show an atropine-sensitive increase in airway resistance after respiratory tract infections. Histamine (Loring et al., 1978; Douglas et al., 1973) and serotonin (Hahn et al., 1978; Sheller et al., 1982) have been implicated to act at the level of the ganglia or central nervous system to augment the bronchoconstriction produced by vagal stimulation suggesting a possible role for these amines on vagal activity, acetylcholine release from the parasympathetic nerve terminals, or in producing

changes in the smooth muscle itself.

c) The role of the sympathetic nervous system

From the time of Szentivanyi's (1968) proposal that the defect in asthma was a blockade of beta-adrenergic relaxant receptors in the airways, a functional role for these sympathetic neurons in canine airway smooth muscle in vivo (Cabezas et al., 1971) and in vitro (Russell, 1980) has been suggested in the mediation of bronchodilation. In the dog, this is the major inhibitory system, whereas in the human the non-adrenergic inhibitory system is dominant (Richardson and Beland, 1976), although a role for alpha-adrenergic receptor mediated tone has been demonstrated in human tracheal and bronchial strips from patients with pneumonia or chronic obstructive airway disease (Richardson and Beland, 1976).

Russell (1980) showed that a propranolol - sensitive relaxation of canine airways from the trachea to bronchi of 1.5 mm diameter could be produced in response to exogenous norepinephrine on a histamine-induced contraction and Cabezas et al. (1971) produce bronchodilation by stimulating thoracic sympathetic nerves where bronchomotor tone was already present. Pre-existance of tone was necessary to demonstrate these relaxations.

A bronchoconstrictor action of the sympathetic nervous system via alpha-adrenergic receptors has been suggested (Adolphson et al., 1971) in humans and guinea pigs. Pandya (1977) has demonstrated alpha-adrenergic stimulated contraction of canine tracheal smooth muscle superimposed on acetylcholine

induced tone without beta-adrenoreceptor blockade. This was in contrast to the results of most other investigators (Castro de la Mata et al., 1962; Fleisch et al., 1970; Patel and Kerr, 1973) who had to block beta receptors in order to demonstrate alpha-adrenergic contractile activity. Pandya stated that this difference is due to the age of the dogs used since he used neonates (1-25 days) whereas others have used adult dogs. He also demonstrated a progressive loss of alpha-adrenergic induced response with increasing age. However, Bergen and Kroeger (1979, 1980) have demonstrated native alpha-adrenergic induced contractions (without beta-blockade) to low doses of norepinephrine or to tyramine-induced release of endogenous norepinephrine in canine tracheal muscle with tone elevated by histamine, serotonin, acetylcholine or increased potassium. They also demonstrated a contraction of ovalbumin sensitized tracheal strips in response to norepinephrine or tyramine without any preinduced tone whereas littermate control dogs, without pre-induced tone, remained quiescent. These results suggest that the sensitization procedure may increase alpha-adrenergic sensitivity or responsiveness or that increased endogenous basal tone was present in the sensitized tracheal preparations prior to alpha-adrenergic stimulation. The source of this increased basal tone is unknown.

d) Parasympathetic innervation of the airways

The parasympathetic nervous system plays a predominant role in the regulation of airway tone. Suzuki et al., (1976) showed dense cholinergic and few adrenergic nerve fibres in canine

tracheal muscle and demonstrated manifold greater responses in this tissue to electrically-induced cholinergic stimulation compared with adrenergic excitation through alpha-adrenoceptors in vitro. In vivo, electrical stimulation of vagal motor nerves causes bronchoconstriction (Colebatch and Halmagyi, 1963; Olsen et al., 1965). In animals and healthy humans a mild degree of resting tone is maintained in the airway via the parasympathetics (Widdicombe, 1966) and cutting or cooling the vagus nerves (Hoppin et al., 1978) or the administration of atropine in animals (Severinghaus and Stupfel, 1955) and humans (Hoppin et al., 1978; de Troyer et al., 1979) causes bronchodilation.

In patients with airway disease acetylcholine (Curry, 1947) and methacholine (Laitinen, 1974) are potent bronchoconstrictors and acetylcholine receptor antagonists can effectively promote bronchodilation (Cavanaugh and Cooper, 1976; Chamberlain et al., 1962; Herxheimer, 1959). These observations indicated a potential role for parasympathetic overactivity in asthma. As discussed previously the irritant receptor-bronchoconstrictor reflex would be part of this overactivity, however, this reflex cannot explain all of the airway bronchoconstriction.

Serotonin, in doses which cause no bronchoconstrictor response itself, markedly augments the response to stimulation of vagal motor nerves in dogs (Hahn et al., 1978) but not exogenously applied acetylcholine (Sheller et al., 1982) suggesting a specific potentiating action of mediators on the vagal motor pathways and production of hyperreactivity (Boushey and Holtzman, 1985). However, despite ganglionic blockade, increased bronchoconstrictor response to the muscarinic agonist

methacholine has been demonstrated (Holtzman et al., 1980) and indicates an increased sensitivity of the muscle itself to muscarinic stimulation.

The role of histamine and the parasympathetic nervous system in allergic bronchospasm may be similar to that observed for serotonin. Mitchell and Bouhuys (1976) have observed supra-additive effects of histamine and methalcholine in healthy human subjects, and suggest that vagal blockade interferes with the histamine-cholinergic interaction at the level of the airway smooth muscle. Hulbert et al. (1985) have suggested a similar local effect of histamine on cholinergic activity in the guinea pig. Shore et al. (1983) demonstrated that in the dog, neostigmine (a cholinesterase inhibitor) augmented the histamine dose-response relation in vitro whereas tetrodotoxin or atropine (Shore et al., 1985) reduced tension developed in response to low doses of histamine but not high doses. They concluded that histamine either accelerates the release of, or interacts supra-additively with spontaneously released acetylcholine at the smooth muscle level in the dog.

e) <u>The parasympathetic neuromuscular synapse</u>

Cholinergic nerve terminals have been identified on the basis of small agranular vesicles (Burnstock, 1970) and by histochemical techniques (Suzuki et al., 1976) which showed acetylcholinesterase diffusely distributed between and on canine tracheal muscle cell membranes. Richardson and Ferguson (1980) have shown the presence of ganglia in the airway muscle and adjacent to it. Hexamethonium (a ganglion-blocker) can block

stimulation through the vagal nerve fibres which suggests the involvement of ganglia in the parasympathetic pathway. Richardson and Ferguson also showed that the ganglia receive cholinergic and adrenergic fibres and, depending on the species, non-adrenergic inputs. Post ganglionic vagal fibres branch out with multiple neurotransmitter-containing varicosities. No true nerve terminals have been observed. Innervation of the airways has been described as "en passage" (Burnstock, 1970).

Stimulation of the parasympathetic nerves releases acetylcholine onto the airway smooth muscle membrane where, after attaching to muscarinic receptors on the smooth muscle cells, it is rapidly broken down by acetylcholinesterase to acetate and choline. The acetate is usually lost to the circulation but 50% of the choline is recovered by the nerve for the resynthesis of new transmitter (Burnstock, 1979). Electrical stimulation of the isolated tracheal muscle preparation also causes the release of acetylcholine (Colebatch and Halmagyi, 1963). The accompanying muscle contraction can be blocked by atropine but not by hexamethonium, thus demonstrating that the contraction is mediated by post ganglionic cholinergic fibres. Russell (1978) has shown similar results in the in vitro canine tracheal preparation.

There is evidence for a spontaneous or basal release of acetylcholine in the guinea pig in vitro (Carlyle, 1963), in the human in vivo (Vincent et al., 1970) and dog in vivo (Severinghaus and Stupfel, 1955) since atropine, vagal cooling or vagotomy can produce relaxation of in vitro muscle strips or bronchodilation in human subjects and anesthetized dogs. In the

isolated rabbit airway substance P was found to increase the basal release of acetylcholine (Grunstein et al., 1984). Tetrodotoxin did not effect the contraction due to substance P administration; however, atropine blocked and neostigmine enhanced the response. This supported the notion of accelerated release of acetylcholine from the neuromuscular junction upon administration histamine in isolated canine tracheal muscle (Shore et al., 1983).

Acetylcholine produces a depolarization of tracheal smooth muscle cell membrane after being released by the nerve varicosities and attaching to muscaric receptors. Depolarization by acetylcholine is not required for contraction (Coburn, 1979), since calcium channel blockers (which prevent the entrance of external calcium through voltage sensitive calcium channels) had little effect on contractions induced by acetylcholine (an example of pharmaco-mechanical coupling). In contrast, calcium channel blockers inhibited contractions stimulated by increased $[K^+]_{out}$ or serotonin which require electromechanical coupling and external calcium.

Drugs which inhibit acetylcholinesterase such as neostigmine or eserine (physostigmine) potentiate the responses of and increase sensitivity to acetylcholine of the rat vas deferens for example (Westfall et al., 1974). However, no change was noted in the carbachol dose-response relationship. (It should be borne in mind that carbachol a cholinoceptor agonist which is not hydrolyzed by cholinesterase.)

Many agents (Silver, 1974) can inhibit cholinesterase

activity. Organophosphates used in nerve gases or insecticides (Fonnum et al., 1984) potentiate the contractions of rat bronchial muscle stimulated either electrically or by acetylcholine. This potentiation is blocked by atropine. Ozone has been shown to increase the reactivity of the airways (Holtzman et al., 1983; Murlas and Roun, 1985) with increased responses to acetylcholine (Golden et al., 1978). Ozone may have many effects such as airway epithelial inflammation or irritant-receptor stimulation but it is interesting to note that this gas has been shown to decrease acetylcholinesterase concentrations in red blood cells (Goldstein et al., 1968).

Prostaglandins E_2 and F_{2alpha} have been shown to inhibit acetylcholinesterase activity in cat brain slices in a dose dependent manner (Grbovic and Radmanovic, 1981) and potentiate gross behavioral changes. Recently, Leff et al. (1985) have demonstrated that prostaglandin F_{2alpha}, while inducing no tone of its own, is able to augment responses of canine tracheal and bronchial airways to vagus nerve stimulation and to reduce the threshold stimulus for maximal contraction in situ. Orehek et al., (1975) have demonstrated this phenomenon in vitro in the dog. Histamine has been shown to stimulate arachidonic acid metabolism with the production of prostaglandins in the dog (Anderson et al., 1979; Tesarowski et al., 1981; Tesarowski and Kroeger, 1982) and in subhuman primates (Krzanowski et al., 1980). It is then possible that histamine-stimulated prostaglandin synthesis could cause the partial inhibition of acetylcholinesterase and potentiate responses to acetylcholine, histamine, and electrical stimuli in sensitized models of

allergic bronchospasm.

f) Muscle membrane receptors

The onset of bronchoconstriction is initiated at the level of the smooth muscle cells by an increase in the sarcoplasmic calcium concentration. The source of this calcium is either from within the cell or from extracellular sources. Tracheal smooth muscle cells contain a sparse network of sarcoplasmic reticulum and therefore rely to a greater extent on extracellular calcium for force generation. However, the neurotransmitter acetylcholine can apparently mobilize an intracellular pool of calcium (Kroeger and Stephens, 1971) and these acetylcholine-stimulated canine tracheal muscle can contractions persist for several hours in a calcium-free environment containing EGTA.

External calcium for contraction can enter the muscle cells via receptor-operated or voltage-sensitive calcium channels. (Bolton, 1979) The tonic phase of acetylcholine contractions is due to calcium entry through receptor-operated calcium channels (Coburn, 1977). In canine tracheal smooth muscle serotonin and potassium have been shown to elicit contractions through voltage-dependent mechanisms (Coburn, 1979). Antonissen (1978) has demonstrated a labile phasic response to histamine in a calcium-free environment indicating that some calcium may be released from intracellular stores to cause contraction in canine tracheal smooth muscle. This pharmacomechanical coupling is akin to that seen for acetylcholine.

Bolton et al. (1981), working with the guinea-pig ileum, have suggested that histamine and muscarinic receptors open the

same calcium channels in smooth muscle to produce depolarization although muscarinic receptors can open more. Histamine and methacholine (a muscarinic agonist) contract airway smooth muscle of many species including humans (Schild et al., 1965) in vitro. In guinea pigs, the constrictor effects of histamine are potentiated by methacholine and by stimulation of cholinergic nerve fibres to the isolated trachealis (Finch et al., 1974). Shore et al. (1983) have shown that histamine and acetylcholine interact supra-additively in isolated canine tracheal smooth muscle and have suggested that release of acetylcholine may participate in the bronchoconstricting action of histamine even when the vagus nerves have been sectioned or cooled thus interrupting the irritant receptor-bronchoconstrictor reflex. Mitchell and Bouhuys (1976) concluded that the reflex theory cannot account for this potentiation of histamine responses by constant levels of vagal nerve stimulation in isolated trachea-nerve preparations. They hypothesized an interaction between physiological levels of cholinergic stimuli and histamine at the level of the airway smooth muscle cells since this could explain why in isolated tissues atropine reduces and physostigmine intensifies histamine-induced airway constriction (Douglas et al., 1973).

Alpha-adrenergic receptor activation may be involved in the asthmatic process by increasing calcium entry into the airway smooth muscle cells (Bergen and Kroeger, 1979; 1980). After blockade of cholinergic receptors by atropine, electrical field stimulation and alpha-adrenergic agonists could not cause

contraction of dog tracheal muscle in vitro. However, after precontraction of airway smooth muscle by histamine or serotonin, both electrical field stimulation (which releases norepinephrine from sympathetic nerve terminals) and alpha-agonists produced a contractile response that could be inhibited by alpha-adrenergic antagonists (Barnes et al., 1983). Similar results were obtained in isolated in situ tracheal preparations in living dogs (Leff and Munoz, 1981) suggesting that alpha-responses could become activated by histamine and serotonin both in vitro and in vivo.

Both histamine and serotonin cause depolarization of airway muscle membrane potential (Coburn, 1977; Kirkpatrick, 1975; Suzuki et al., 1976) and this depolarization may increase the likelihood of alpha-receptor activation, calcium entry, and contraction (Barnes et al., 1983). When histamine and serotonin produced a similar increase in muscle tension, no difference in the magnitude of the alpha-responses could be observed. However, acetylcholine activation to the same initial muscle tension could not produce alpha-adrenergic responses of the magnitude seen for the other spasmogens. Acetylcholine does depolarize airway muscle membrane (Coburn, 1979; Farley and Miles, 1977). Spasmogens which activate voltage-sensitive calcium channels are able to activate the alpha-adrenoceptor response and thus increase the influx of calcium in vascular muscle (Van Miel et al., 1981). This activation is blocked by calcium channel antagonists which effectively block voltage-sensitive channels. In the canine trachea, calcium antagonists abolish serotonin and potassium induced contractions but have little inhibitory effect on acetylcholine-induced contractions; i.e, receptor-operated

calcium entry (Coburn, 1977). These results may explain exaggerated alpha-adrenergic responses of human airways in asthma (Patel and Kerr, 1973; Prince et al., 1972; Snashall et al., 1978) since asthma has been associated with increased histamine release (Findlay and Lichtenstein, 1980). Alpha-adrenergic antagonists have been shown to prevent bronchoconstriction induced by histamine (Kerr et al., 1970), allergen (Patel et al., 1976) and exercise (Barnes et al., 1981).

Calcium channel blockers such as verapamil and D-600 have been considered as antiasthmatic drugs since they have been shown to inhibit histamine, and leukotriene induced contractions in the guinea pig (Cheng and Townley, 1983; Weiss et al., 1983) and to inhibit contractions to low doses of acetylcholine but not higher doses (Coburn, 1977) in canine trachea. It has been established that histamine mobilizes a loosely bound, verapamil-sensitive calcium pool (extracellular) whereas acetylcholine mobilizes a tightly bound verapamil insensitive calcium pool (intracellular) (Anderson et al., 1983; Farley and Miles, 1978). However, calcium channel blockers were not effective in preventing bronchoconstriction in asthmatics that were challenged with either histamine or methacholine (Patel, 1981; Patel and Al-shamma, 1982).

The role of beta-adrenergic receptors has been discussed previously in this manuscript (p. 1, 5).

g) <u>The electrogenic sodium pump.</u>

Souhrada and Souhrada (1984) have suggested, on the basis of their work with an immunologically sensitized guinea pig model

that the sodium-potassium pump is affected by the sensitization process. Their data show that sensitization shifts the resting membrane potential of the airway smooth muscle in a hyperpolarizing direction by 10 millivolts and they suggest that this hyperpolarization is due to stimulation of the electrogenic sodium pump. The guinea pig has been shown to produce IgG predominantely (Pare et al., 1977). The scheme in which Souhrada and Souhrada interpret these findings is by analogy to IgE mediated hyperpolarization of macrophages, where the attachment of immunoglobulins to the airway muscle cell membrane may increase the permeability to sodium and thus stimulate the sodium-potassium pump to re-establish equilibrium. Furthermore, they suggest that this increase in sodium permeability may increase the influx of calcium through Na^{+} - Ca^{++} exchange and thus cause the guinea pig airway muscle to become more responsive to stimulation. One would then expect membrane resistance to be decreased with increased ion permeability, however, Souhrada and Souhrada have not investigated this possibility.

A systematic investigation of membrane properties of sensitized airway muscle has not been carried out in the dog or in man. Evidence put forth by Fleming (1980) shows that in the vas deferens of the guinea pig supersensitivity produced by denervation results in a depolarization of the smooth muscle membranes due to a loss in electrogenic pumping. The increased sensitivity of the airways to a great variety of stimuli directly on the smooth muscle or indirectly through irritant receptors would indicate a depolarized membrane and deserves investigation.

h) Calcium homeostasis.

An increase in free cytoplasmic calcium could account for the increased reactivity of airway smooth muscle to a variety of nonspecific stimuli - a characteristic of asthmatic airways reactivity.

Abnormal calcium handling could lie at a variety of levels of smooth muscle organization including increased permeability to calcium ions, abnormal control of phosphorylation and dephosphorylation of myosin light chains or other key molecules of the contractile apparatus, or defective calcium resequestration mechanisms.

Weiss and Viswanath (1979) found increased sensitivity of resting isometric tension to extracellular calcium following in vitro anaphylaxis of guinea pig tracheal muscle. This would suggest increased calcium entry and the use of calcium channel blockers in allergic bronchospasm or other smooth muscle disorders (Schwartz et al., 1984). However, calcium channel blockers were ineffective in preventing bronchoconstriction in asthmatic patients challenged with histamine or methacholine (Patel, 1981; Patel and Al-Shamma, 1982).

Since airway smooth muscle is relatively poor in sarcoplasmic reticulum, calcium removal after bronchoconstriction is probably across the sarcolemma back into the extracellular space. The involvement of the sodium-potassium pump in this process as it pertains to sensitization in guinea pig airways has been suggested (Souhrada and Souhrada, 1984). Inhibition of the sodium-potassium pump with low concentrations of ouabain induced contraction of guinea pig tracheal rings (Kolbeck et al., 1981),

increased airway resistance and decreased pulmonary compliance in the dog (Marco et al., 1968) when given in vivo, and has been shown to potentiate contractions due to endogenous constrictors (Fleming, 1980).

Smooth muscle contraction is a calcium dependent process, initiated by increased cytosolic free calcium concentration. The free calcium ions bind to calmodulin (from Hartshorne and Persechini, 1984). This calcium-calmodulin complex interacts with myosin light chain kinase to activate the enzyme which then phosphorylates the light chains of myosin. The phosphorylation of myosin allows the formation of an active actomyosin complex initiating the contractile process. As long as the intracellular calcium concentration remains above threshold the myosin remains in the phosphorylated state and tension is maintained. When the calcium level is reduced the myosin light chain kinase is inactivated, the myosin dephosphorylates and the muscle relaxes. All these steps represent potential loci of regulation of the balance between contraction and relaxation (Hartshorne and Siemankowski, 1981; Contri and Adelstein, 1980) and, thus, the initiation and maintenance of bronchospasm in airway smooth muscle. Silver and Stull (1984) have shown a direct correlation between the extent of myosin light chain phosphorylation and the development of isometric force in bovine tracheal smooth muscle. However, this point is controversial. Dillon et al. (1981) find that the development of isometric force, especially approaching and including the plateau of the response, is associated with dephosphorylation of the myosin light chain. Perhaps the link is

obtained in data reported by Chatterjee and Murphy (1983). They show that tension maintenance does require Ca^{++} but in concentrations which are only very slightly greater than resting. They suggest that the binding of Ca^{++} that occurs in this phase is to a locus on the heavy chain.

i) Cyclic-AMP and the regulation of airway smooth muscle tone.

Bronchomotor tone is ultimately regulated by the availability of cytosolic free calcium to the contractile machinery of airway smooth muscle. Sequestration of calcium whether intra- or extracellularly results in relaxation whereas increased calcium augments muscle contraction. Intracellular cyclic nucleotides modulate contraction by controlling intracellular calcium availability (Andersson and Nilssen, 1977). Increased production of 3,5-cyclic adenosine monophosphate (cAMP) is promoted by activation of the enzyme adenylate cyclase which itself requires Ca^{++}-calmodulin and a protein kinase system. Increased cAMP leads to sequestration of calcium and relaxation. Beta-adrenergic agonists promote the activation of adenylate cyclase to increase cAMP and may as well have a possible direct effect on calcium sequestration (Schultz, 1977). Alpha-adrenergic and cholinergic stimulation, on the other hand, inhibit cAMP synthesis.

Calcium (through the Ca^{++}-calmodulin complex) and cAMP exert opposing actions on the activity of myosin light chain kinase mediated by calmodulin and on a cAMP-dependent protein kinase respectively. In addition, cAMP degradation by phoshodiesterase is stimulated by calcium and mediated by calmodulin in smooth

muscle (Wolff and Bostrom, 1979). The production of cAMP, in turn, requires micromolar concentrations of calcium but is inhibited by high calcium levels.

Calcium ionophores increase cytoplasmic calcium concentrations in smooth muscle, as indicated by contraction. In the guinea pig trachea (Creese and Denborough, 1980a), calcium ionophores increase cAMP levels. They also increase cAMP levels in human monocytes (Stolc, 1980) and rat peritoneal macrophages (Gemsa et al., 1979). All these responses are inhibited by indomethacin indicating the involvement of prostaglandins (such as PGE_2). Prostaglandins of the E-series have been shown to increase cAMP levels in airway smooth muscles (Murad and Kimura, 1974; Creese and Denborough, 1981). Other bronchoconstrictor agents such as acetylcholine and histamine, which elevate intracellular calcium in airway smooth muscle also elevate cAMP in the guinea pig trachea (Creese and Denborough, 1980b). These effects are also mediated by prostaglandins.

Rinard et al. (1979) showed evidence that cAMP basal levels in the "asthmatic" dog are depressed compared to "non-asthmatic" dogs. The addition of the beta-adrenergic agonist isoproterenol increased cAMP levels in both populations in a parallel fashion at all doses of the drug even though the degree of relaxation in the nonasthmatic tissues was greater. They concluded that a lesion at the beta-adrenergic receptor level as suggested by Szentivanyi (1968) was unlikely since cAMP stimulation was not impaired.

H. STATEMENT OF THE PROBLEM.

The parasympathetic nervous system has been implicated as a contributing factor in the etiology of asthma. It is responsible for maintaining airway tone and normal mucus secretion in normal individuals. The major theories of asthma state that either through inhibitory nervous system dysfunction (adrenergic or non-adrenergic), which would leave the parasympathetics unapposed, or through the irritant receptor-bronchoconstrictor reflex, the parasympathetic nervous system plays a major role.

Mills and Widdicombe (1970) and Gold (1975) showed that the pulmonary response to antigen challenge could be altered by vagotomy. Gold and his co-workers (1972b), as well as Drazen and Austen (1975), also observed altered pulmonary responses after atropine administration.

While these studies indicated a possible central reflex, local reflex bronchospasm could not be ruled out. The sources of a possible local lesion have been outlined above. The present studies, using a ragweed-sensitized canine model of allergic bronchospasm, have investigated these sources in vitro. The objectives of these studies were 1) to characterize the pharmacological responses of airway smooth muscle from ragweed sensitized dogs and to compare these responses with those from airway smooth muscle non-sensitized littermate dogs which would act as the age, environmental and genetic control population, 2) to determine the direct role of the parasympathetic nervous system on airway smooth muscle pharmacology and physiology at the local level in the ragweed dogs by observing the effects of the

muscarinic blocker atropine - on the pharmacological responses of sensitized and control airway muscle to bronchospastic agents such as histamine, serotonin and ragweed pollen extract and to potassium depolarization, 3) to elucidate the mechanism of the phenomena of spontaneous baseline activity and prolonged isometric plateau force maintenance that have been observed in airway smooth muscle in vitro from an ovalbumin sensitized canine model of asthma (Antonissen et al., 1979) and 4) to investigate pharmacologically the cholinergic "neuromuscular junction" in airway muscle and to determine the possible involvement of presynaptic (acetylcholine release), synaptic (acetylcholine-esterase activity), and postsynaptic (membrane receptors and resting membrane potential) foci in the sensitization process.

The hyperreactivity and hypersensitivity of airway smooth muscles to a variety of mediators of bronchoconstriction observed in other models of asthma in vitro may be related to one or more of these possible sites. A lesion in any of the loci directly or indirectly referred to above would tend to increase the cytosolic concentration of calcium in airway smooth muscle and thus influence the activity of cyclic nucleotides and actomyosin crossbridges.

Thus, the present experiments were designed to determine the general influence of the parasympathetic nervous system on the reactivity and sensitivity of airway smooth muscle in a canine model of asthma in order to better understand the locally induced bronchospasm. In addition, since mucus secretion in the lungs is stimulated by parasympathetic branches of the vagus nerve, a possible insight into excess mucus secretion, resulting in

bronchiolar and alveolar occlusion in asthma, may be gained.

METHODS

a) Active in vivo sensitization procedure

Mongrel puppies were sensitized to ragweed pollen extract using an adaptation of the method developed by Pinckard et al. (1972) to elicit the production of IgE antibodies and anaphylactic sensitivity (Kepron et al., 1977) to ragweed extract. IgE antibody production was induced by injecting 0.5 mg of ragweed extract mixed with 30 mg of $Al(OH)_3$ intraperitoneally into mongrel dogs within 24 hours of birth. Booster injections were repeated weekly for eight weeks and thereafter biweekly using the same dosage of allergen. Littermate control dogs followed the same regimen of injection with the $Al(OH)_3$ adjuvant alone. Serum IgE antibody titres were measured by passive cutaneous anaphylaxis (PCA) in non-sensitized dogs.

For the present studies tracheal smooth muscle was obtained from sensitized dogs (6 months to 1 year old) whose PCA titres were greater than or equal to 256. That is, serum samples of these sensitized dogs when diluted 256 times still produced cutaneous anaphylaxis (inflammation and discolouration of the injection site). A more sensitive dog would have a greater dilution factor. In vivo antigenic provocation of sensitized dogs by aerosol inhalation produced marked increases in specific airflow resistance when antibody titres were greater than 64 (Kepron et al., 1977) for this sensitization procedure.

In vitro antigenic provocation of isometrically mounted sensitized tracheal muscle with ragweed extract produced results similar to other models of allergic bronchospasm (Antonissen et al., 1979) in that sensitized muscle strips produced a sustained contraction whereas tracheal muscle from littermate control dogs

failed to respond. The specificity of the ragweed antibody-antigen reaction was demonstrated by a lack of cross-reactivity with other antigenic-potential proteins such as ovalbumin or bovine serum albumin.

b) Tissue dissection.

i) Trachealis muscle was dissected from the cervical tracheae of littermate control and ragweed sensitized mongrel dogs anesthetized with 30 mg/kg sodium pentobarbital by intravenous injection. The tracheae were removed and then an intracardiac injection of saturated potassium chloride was used to kill the animal. The tracheae were immersed in ice-cold oxygenated Krebs-Henseleit solution to preserve them from the effects of hypoxia until individual muscle strips were dissected, tied and mounted in tissue organ baths. Tissues thus prepared required less than 2 hours equilibration time in the muscle baths.

The musculus transversus tracheae (or trachealis muscle) was cleaned of the ventrally lying tunica fibrosa of the paries membranaceous which connects the incomplete dorsal ends of the cartilaginous canine tracheal rings (fig. 1) after anterior bisection and eversion of the two cartilaginous ends (fig. 2). Fig. 2 shows the completeness of separation of these two layers.

Parallel fibered strips of the trachealis muscle (TSM) were dissected such that the weight of the muscle strip per cm. at optimal muscle length rarely exceeded 10 mg. in order to prevent hypoxia in the innermost cells of the TSM preparations (Stephens et al., 1975). One end of the muscle was tied with a loop of 000

surgical silk to a rigidly clamped aerating tube at the bottom of a muscle bath. The other end was tied by a short length of 000 surgical silk to an isometric force transducer (either a Grass FT 0.03 or a Gould-Stathem UC-3) mounted on a rack and pinion, thus enabling the muscle to be stretched to its optimal length and held there isometrically. Output of the force transducers was amplified and recorded on a 4-channel Gould 2400 Brush recorder. The compliance of the system was negligible over the range of forces applied to it.

The TSM strips were bathed in mammalian Krebs-Henseleit solution of the following composition (in mM): NaCl, 115; $NaHCO_3$, 25; NaH_2PO_4, 1.38; KCl, 2.51; $MgSO_4 \cdot 7H_2O$, 2.46; $CaCl_2$, 1.91; and dextrose, 5.56. The baths were aerated with an O_2-CO_2 mixture that maintained a P_{O_2} of 600 Torr, a P_{CO_2} of 40 Torr, and a pH of 7.40 at a temperature of 37^{o} C. Where varying potassium concentrations were needed, the KCl concentration was adjusted accordingly, substituting on a equimolar basis for NaCl.

ii) Some studies herein used TSM strips from the lower trachea, primary (main stem) intrapulmonary bronchial rings and third generation (tertiary) bronchial rings from littermate control and ragweed sensitized dogs. These airway smooth muscle preparations were obtained from the lower tracheae and lungs excised through a thoracic opening. These dogs (already anesthetized) died by the ensuing rapid exsanguination.

The TSM strips from the lower trachea were dissected and mounted as described above. The primary and tertiary intrapulmonary airways were dissected in narrow rings and mounted in the organ baths as rings since cartilaginous plaques are found

Figure 1: A single intact canine tracheal ring. The incomplete cartilaginous ring is closed dorsally by the paries membranaceous. Cutting through the cartilage ventrally and everting the end facilitates further dissection (see fig. 2).

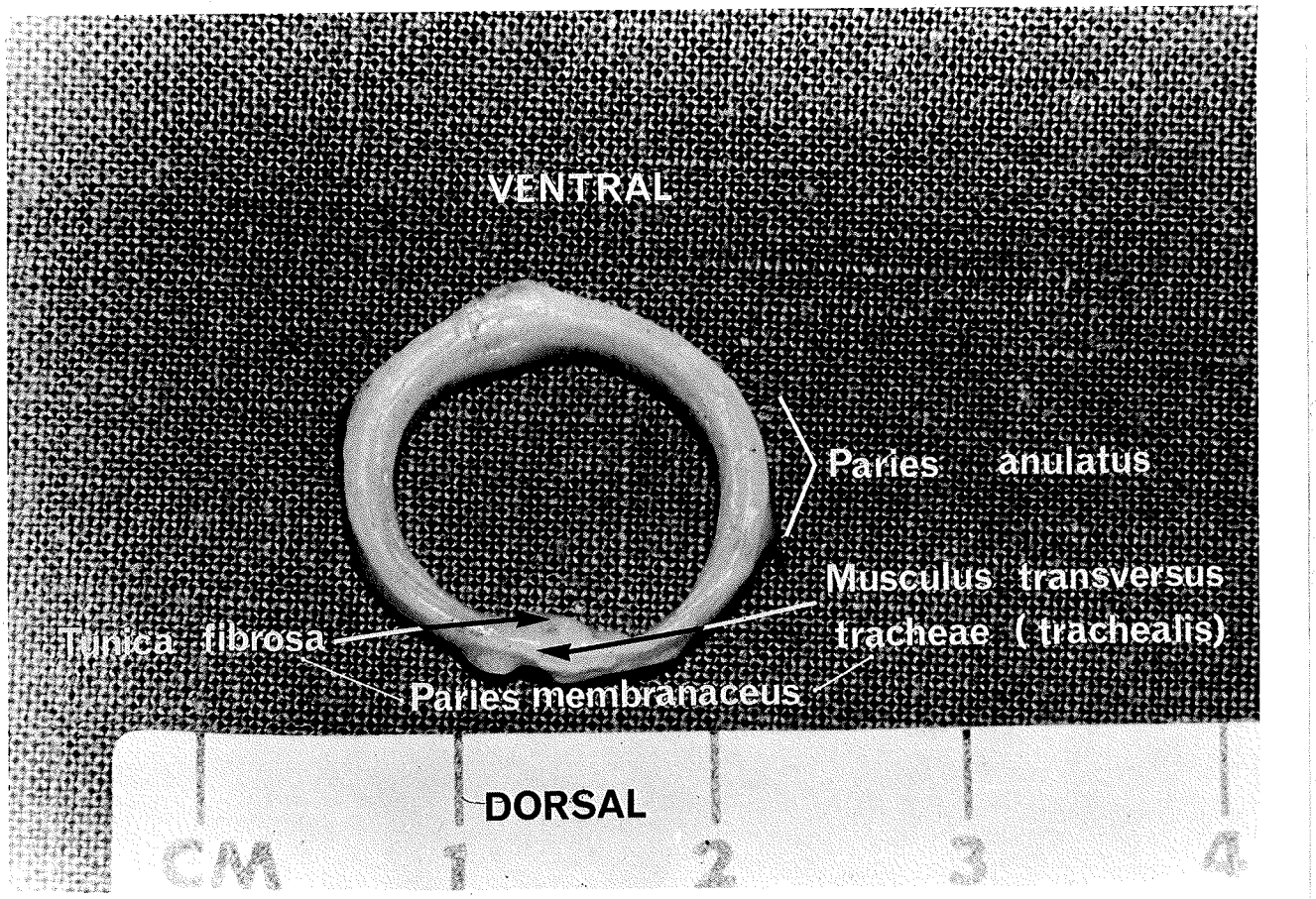
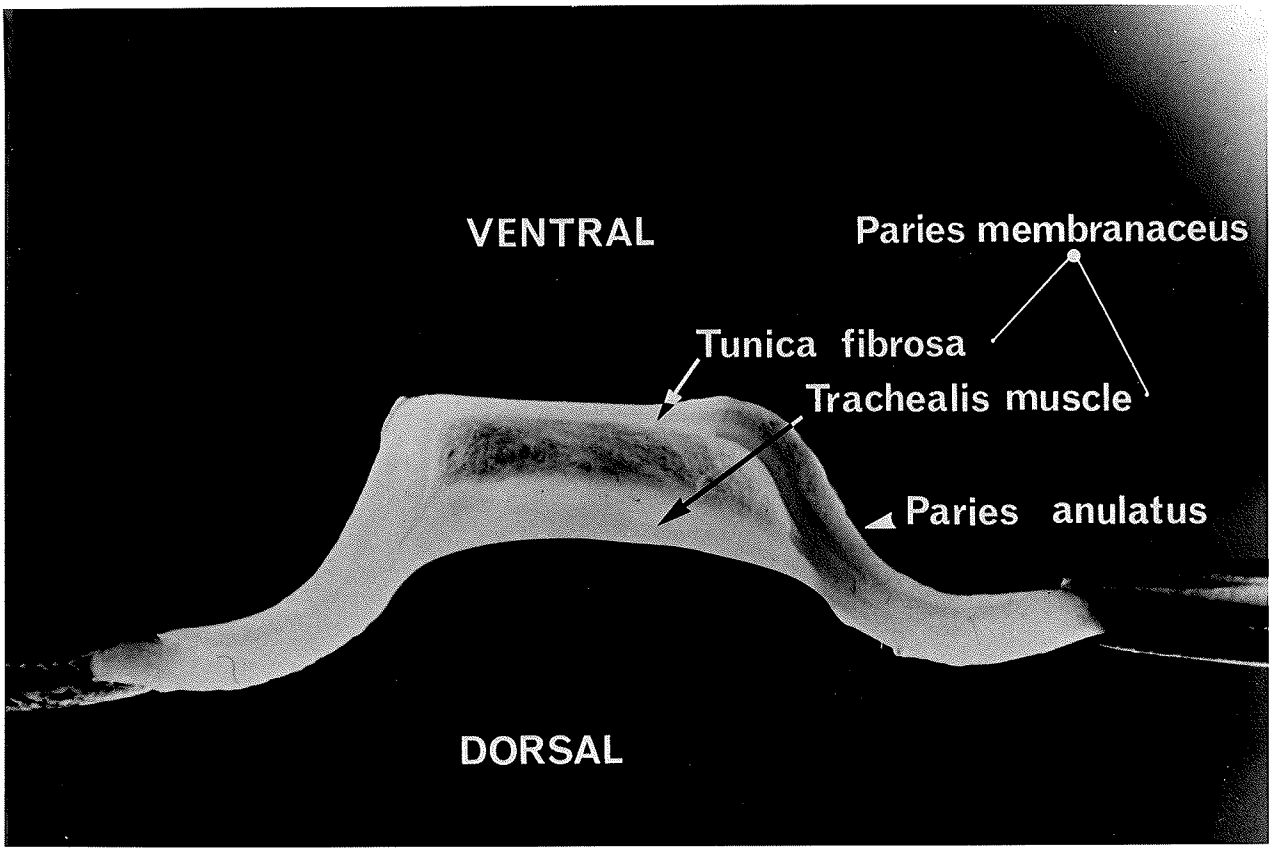


Figure 2: Eversion of a ventrally cut single tracheal ring causes the trachealis muscle and its overlaying tunica to separate somewhat. The tunica fibrosa is carefully removed and the ends of the trachealis muscle can then be tied with 000 silk for mechanical studies.



randomly throughout these airways and make further dissection difficult.

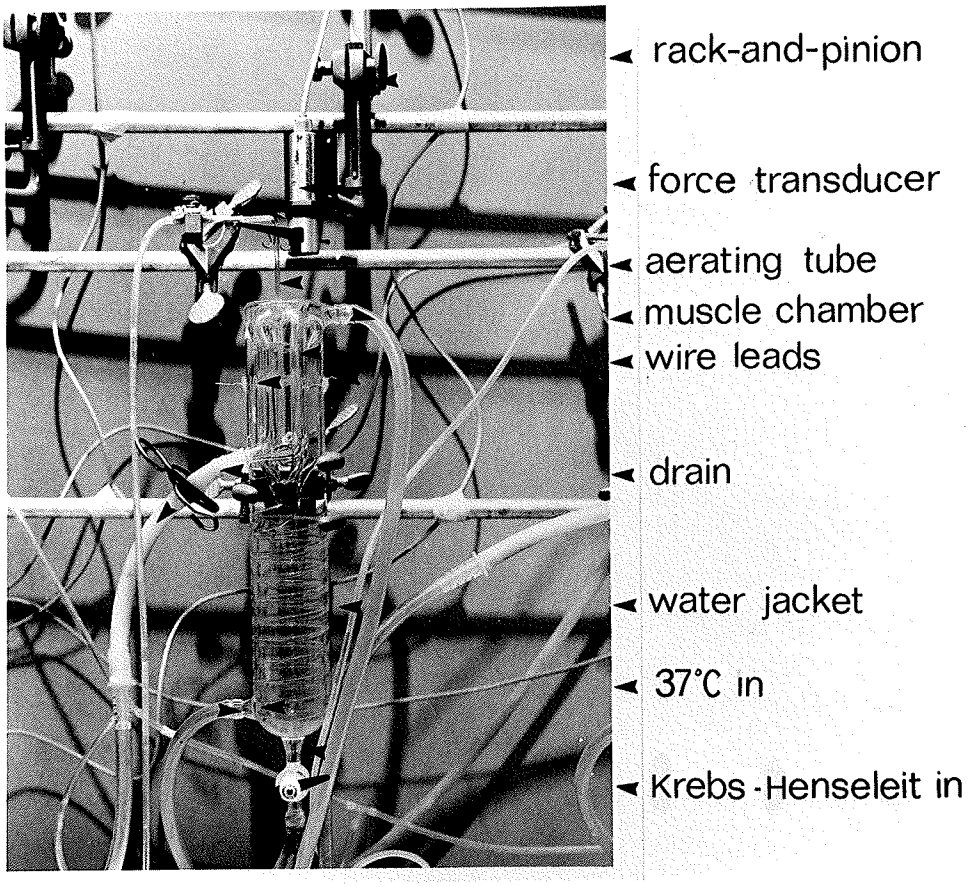
For studies in which the airway smooth muscle (ASM) preparations were electrically or pharmacologically stimulated the muscles were stretched to their appropriate optimal length - that length at which maximum active tension is elicited. Previous experience in this laboratory has shown that for the size of the tissues used in most of the experiments a resting tension of 0.6 to 0.8 g stretches the TSM to optimal length. Generally, a limited length-tension curve was delineated from which optimal length was identified.

The tissue baths in which the muscle strips were mounted contained a fixed volume (10 cc) of mammalian Krebs-Henseleit solution at 37^{o} C and aerated with 95% O_2 and 5% CO_2. The airway muscles were allowed to equilibrate for not less than 90 minutes to re-establish ionic equilibrium. During this time the muscles were stretched periodically and stimulated with 127 mM K^{+} solutions to establish suitable resting tensions and accelerate the equilibration process. Multiple banks of identical muscle baths, of a style similar to that pictured in fig. 3, were employed and control and sensitized ASM randomly placed in them to minimize the effects of time and statistical non-randomness.

c) Passive in vitro sensitization of control TSM.

TSM strips from a control dog (not necessarily a littermate) were dissected and suspended in tissue baths as described above. To the 10 ml of Krebs-Henseleit solution in the tissue bath 5 ml of plasma (obtained after centrifuging the blood

Figure 3: Single muscle bath of the type used in these studies. The bath is water-jacketed to maintain the temperature of 37° C. Fresh Krebs-Henseleit solution is fed through the pre-warming coil at the bottom of the apparatus and spills over into the bath through 4 portals to ensure proper rinsing. The drain allows quick removal of Krebs-Henseleit solution. Platinum plate electrodes are permanently positioned in the bath as the wire leads pass through the sealed water jacket. Drugs are introduced through the top of the calibrated muscle chamber. The muscle is held in the bath by being tied with a short loop of 000 silk to a ridgedly clamped aerating tube through which the Krebs-Henseleit is bubbled with 95% O_2 - 5% CO_2 carbogen. The other end is tied to the force transducer suspended above the bath on a rack - and - pinion to facilitate length changes of the muscle.



from the sensitized and littermate control dogs at 10,000 x g for 12 minutes) from the sensitized dog was injected into one bath and 5 ml of plasma from the littermate control was injected into a second muscle bath. A third TSM strip received 10^{-6} M serotonin (5-HT), while a fourth strip acted as a time control. Serotonin acted as a physiologic control since TSM, incubated in plasma, produces a methysergide sensitive tonic contraction for the duration of the plasma incubation of 1 hour. Methysergide is an antagonist of serotonin. After the 1 hour incubation all TSM strips were rinsed several times with fresh Krebs-Henseleit solution before any further studies were carried out.

Only TSM strips incubated in plasma from sensitized animals manifested a contractile response to challenge with ragweed extract (Schultz-Dale reaction) indicating that at least the mast cells present in the preparation had been sensitized to the ragweed antigen. Serum, tone, and time control muscle strips remained quiescent with antigen challenge.

d) Electrical stimulation.

Electrical stimulation of ASM preparations was effected from a constant voltage 60Hz AC source via rectangular platinum plate electrodes. At supramaximal voltages (a further increase in stimulus voltage produced no further increase in response of the tissues), this field stimulated method produced a current density of approximately 400 milliamps/cm^2. The TSM strips were usually tetanized every 5 minutes for the duration of the experiment. This regimen has provided optimal conditions for recovery and equilibration of TSM from repeated electrically induced tonic

contractions (Stephens, 1975).

Stimulus duration was the minimum necessary to elicit the maximal active contractile responses (usually 10-12 seconds) under normal conditions.

e) Dose-response curves.

Both cumulative and non-cumulative dose-response studies were performed in these studies, depending on the experimental protocol. All drug concentrations expressed in the results are final bath concentrations. Stock solutions of drugs were made at a concentration which was 10^3 greater than the desired bath concentrations and usually dissolved in 0.1 N HCl to preserve the stock solutions. These procedures allowed minimal volume and pH changes in the muscle baths.

After the addition of an agonist drug to the bath sufficient time was allowed for responses to stabilize before the next dose was added in the case of cumulative dose-response studies. In non-cumulative studies, after the responses had reached a plateau, the bath volume was exchanged 3 - 5 times and at least 30 minutes was allowed before the next dose of agonist was added. This was especially important with the agonist histamine to minimize contact time and, thus, tachyphylaxis.

When TSM strips were pretreated with an antagonist such as atropine, a 30 minute period was allowed before the addition of agonist drugs or increasing K^+ concentrations to allow antagonist-receptor equilibration.

f) Data presentation and analysis.

Electrical and pharmacologic responses of TSM were expressed in grams or kilograms of force per square centimeter of cross-sectional area (Kg/cm^2) of the muscle to normalize the data (unless otherwise stated). Cross-sectional area of the muscle was estimated by measuring its length at optimal length, its blotted wet weight and, assuming that the specific gravity of the tissue is 1.0 and that the TSM strip is a cylinder, dividing the weight in grams by its length in cm. This normalization eliminates the possibility of overestimating hyperreactivity of sensitized TSM should there have been any hyperplasia of the tracheal muscle as a result of the sensitization process.

Statistical means and standard errors of the mechanical responses were determined for each stimulus voltage or dose when plotting the response curves. The data were analyzed depending on the experimental design by either parametric or non-parametric tests or both. The tests used in this study were either paired-t tests, unpaired t-tests, Duncan's (1955) new multiple range tests for three or more groups of data, sign-tests, or the Wilcoxon matched pair test. In all cases statistical significance was determined when a p value of less than 0.05 was obtained.

g) Agonists and antagonists.

A list of the drugs used to elicit agonist or antagonist responses or to block or promote certain physiological or pharmacological processes are listed in the first column of Table 1. The second column contains the abbreviations used in this manuscript for these drugs. The third column briefly describes

the prominant actions of each drug. All drugs were purchased from the Sigma Chemical Company, St. Louis, Mo., except ^{14}C-labelled-choline (New England Nuclear, Montreal) FPL55712 (Fisons Pharmaceuticals), KCl - Fisher Scientific (Winnipeg), and phentolanine (Giba-Geigy). The ragweed pollen extract was a gift from Pharmacia (Sweden).

h) Radiolabelled choline efflux experiments.

Tracheal smooth muscle strips were mounted in organ baths (as above). The strips were of similar length and weight as were used in the dose-response studies. They were incubated for 1 hour with ^{14}C-choline in the Krebs-Henseleit solution during which time supramaximal electrical field stimulation (approx. 10 V, 60 Hz, approx. 12 sec. duration) was applied through platinum plate electrodes every 5 minutes. This method increases neurotransmitter turnover thereby facilitating the uptake of ^{14}C-choline into the nerve varicosities found in the TSM preparation. The concentration of ^{14}C-choline used was 10^{-6} M with a specific activity of 2 - 5 mCi/mol. Choline rather than acetylcholine was used since nerve endings take up choline rather than acetylcholine to resynthesize transmitter.

Eserine was used (added at the end of the incubation period) in these experiments to prevent the breakdown of ^{14}C-acetylcholine released and thus the reuptake of ^{14}C-choline. TSM strips from sensitized and littermate control dogs were stimulated by histamine (10^{-4} M) or RWX (.15% w/v). Simultaneous isometric tension measurements were made throughout the incubation and sampling periods.

After the incubation period a collection period began.

Table 1 AGONISTS AND ANTAGONISTS

DRUG	ABBREVIATION	ACTION
Acetylcholine	ACh	muscarinic and nicotinic receptor agonist
Atropine	ATRO	muscarinic receptor antagonist
Carbamylcholine (Carbachol)	CCh (CARB)	muscarinic and nicotinic receptor agonist (not susceptible to cholinesterase breakdown)
[^{14}C]-choline		acetylcholine precursor
Eserine	ESER	acetylcholinesterase inhibitor
FPL55712		leukotriene C_4 and D_4 (SRS-A) antagonist.
Hexamethonium	HEXA	ganglionic blocker
Histamine	HIST	H_1 and H_2 receptor agonist
5-Hydroxytryptamine	5-HT	smooth muscle agonist
Isoproterenol	ISO	beta-adrenoceptor agonist
Phentolamine	PHENT	alpha-adrenoceptor antagonist
Potassium	K^+	increased $[K^+]_{out}$ depolarizes excitable membranes
Propranolol	PROP	beta-adrenoceptor antagonist
Pyrilamine maleate	PYR-MAL	H_1 receptor antagonist
Ragweed pollen extract	RWX	crosslinks with RWX antibody on mast cells to release HIST.

Every 3 minutes the entire organ bath was drained (10 ml volume),the radiolabelled Krebs-Henseleit collected and the bath volume replenished with fresh non-labelled Krebs-Henseleit. Thirteen collection intervals were made. The first 3 were discarded since they represented primarily extracellular label and were not used in the calculation of rate coefficients. Of the remaining 10 collections, specific stimuli to be tested were given during the seventh collection interval. It was found that a stable basal rate of ^{14}C-choline release was attained by the fifth and sixth intervals.

After the sampling period, a 1 ml aliquot of each sample collected was transferred into a scintillation vial containing 7 ml of Beckman, HP scintillation cocktail and radioactivity was measured with a Beckman LS-350 scintillation spectrometer. Counting efficiency was greater than 90% for ^{14}C. After the last sample the muscles were removed and solubilized in scintillation vials in 300 microlitres of NCS (Amersham/Searle) tissue solubilizer. Scintillation fluid was added to the solubilized muscles (3 days solubilization at 50^{o} C in a shaker bath) and radioactivity in counts per minute (CPM) determined as for the previous samples. The rate of release of ^{14}C from the TSM strips was normalized by expressing the release as a rate coefficient (Shanes and Bianchi, 1959). The rate coefficient was obtained from the following equation:

$$\frac{\text{CPM released/minute/sample}}{\text{CPM remaining in tissue at the start of the collection period for this sample}} \times 100$$

The rate coefficient results are expressed as a % of tissue radioactivity released per minute.

The data were plotted as rate coefficient vs collection period time to graphically display the effects of treatment on radioactivity overflow assuming that the radioactivity is neurotransmitter. Therefore, an increase in rate coefficient indicates an increase in neurotransmitter release. Efflux curves were obtained by calculating means for samples 3, 4, 5 and 6 for control and sensitized TSM strips and plotting the values obtained vs sample interval time. Linear regression analysis gave the slope and intercept values for the two groups of data as well as an indication of basal release rates for control and sensitized tissues. From the slope and the intercept theoretical value of rate coefficients could be calculated for samples 7, 8, 9 and 10. The actual rate coefficient obtained from the equation above was then compared with the theoretical using a one sample t-test.

i) Acetylcholinesterase activity determinations.

Control and sensitized smooth muscle from cervical tracheae were dissected free of connective tissue and separately minced in volumes of phosphate buffer (pH = 7.8) that were proportional to the wet weight of the isolated sheets of muscle. The tissues were minced with scissors and homogenized at 4^{o} C using Kontes Duall 22 glass homogenizers. Samples of homogenate (200 microlitres) were placed in cuvettes containing 2.6 ml of phosphate buffer at 25^{o} C.

Cholinesterase determinations were made using a method

described by Ellman et al. (1961) in which they used acetylthiocholine (5 mmol/litre) as the substrate for the endogenous cholinesterase enzyme. The thoicholine produced reacted with 5,5 - dithiobis(nitrobenzoic) acid or DTNB (final concentration 0.25 mmol/litre) in the cuvette to produce 2-nitro-5 mercapto-benzoate, the rate of production of which was read at 405 nanometers wave length on a Pye-Unicam SP 1800 Ultraviolet Spectrophotometer.

A typical experiment used 2.6 ml of phosphate buffer with 100 microlitres DTNB solution to a final concentration of 0.25 mmol/litre. To the cuvette 200 microlitres of homogenate was added and the spectrophotometer readout was allowed to stabilize before the addition of the acetylthiocholine substrate. The absorbances at 405 nanometers were read on a strip chart recorder and the best fit line to the slope was used to calculate the change in absorbance per minute. Thus the rate of production of 2-nitro-5 mercapto-benzoate is directly proportional to cholinesterase activity. All experiments were paired and the results expressed as a percent of the rate in control tissues. Drugs such as eserine, histamine, and prostaglandins were added to the cuvette in appropriate concentrations before the substrate was added and allowed to equilibrate with the homogenate or plasma. The rates of change in absorbance per minute were calculated with these drug interventions and expressed as a percent of the original control value. The first and last runs with substrate alone were compared for both control and sensitized samples to be sure that time was not an uncontrolled variable on the samples reactivities.

j) Ultramicroelectrode determination of resting membrane potentials.

Intracellular records of membrane potentials of tracheal smooth muscle cells from sensitized and control dogs were made by impalement of cells with conventional microelectrodes (Ling and Gerard, 1949) pulled from Pyrex capillary tubes using a David Kopf two-stage vertical puller. The oven coil temperature and solenoid pull were adjusted so as to produce tips of no greater than 0.5 micron diameter with a sharply tapered shank to increase physical strength. The electrodes were placed in a beaker of filtered 2 M KCl which had been heated to boiling and then equilibrated in a vacuum chamber overnight.

The microelectrodes were fastened to a 1 cm long Ag-AgCl wire soldered to a 6 cm length of thin, flat, Z-folded foil which was in turn mounted on a WPI electrode holder clamped to an X-Y-Z micromanipulator. The electrode in turn was coupled to a WPI 750 high impedance, direct-coupled amplifier. The output was recorded on tape and monitored simultaneously on a Tektronix D12 dual beam oscilloscope and Gould Brush 280 chart recorder. The circuit was completed by a salt bridge (2 M KCl in agar) indifferent electrode in the recording chamber connected to the amplifier ground input. Dynamic calibration was accomplished by inserting a Bioelectric Instruments Inc. CA5 calibrator in series with the salt bridge and the amplifier.

Strips of sensitized or control trachealis were dissected and tied as described earlier for pharmacologic experiments. However, the muscle strips were mounted in a plexiglass bath of a design modified from that of Abe and Tomita (1968). The muscle

is placed in an open horizontal, water-jacketed tissue bath with inports at either end for Krebs-Henseleit solution entry (total flow 3 ml/min). Two Ag-AgCl stimulating electrodes are positioned in the bath such that one is at one end and the other splits and separates the muscle bath into approximately 3 and 5 ml chambers. The central electrode is insulated with Araldite epoxy resin on the side facing the other stimulating electrode and with celluloid facing the recording side of the bath (fig. 4). This central electrode has a small ovoid hole through which the muscle strips were suspended with one end of the muscle tied to a glass hook in the stimulating bath and the other end to a Statham UC-3 force transducer with a UL-5 lever adapter after looping the 000 silk around a small pulley. Resting tension was applied as described earlier. The output of the force transducer was also monitored on paper and oscilloscope and recorded on tape (Philips Analog-7 tape recorder).

An independent pair of Ag-AgCl wires placed in the stimulating portion of the bath to monitor stimulus intensity and this signal was also recorded on tape. Krebs-Henseleit was propelled by gravity from an overhead reservoir. Suction ports maintained the solutions in the recording and stimulating baths at an optimal level.

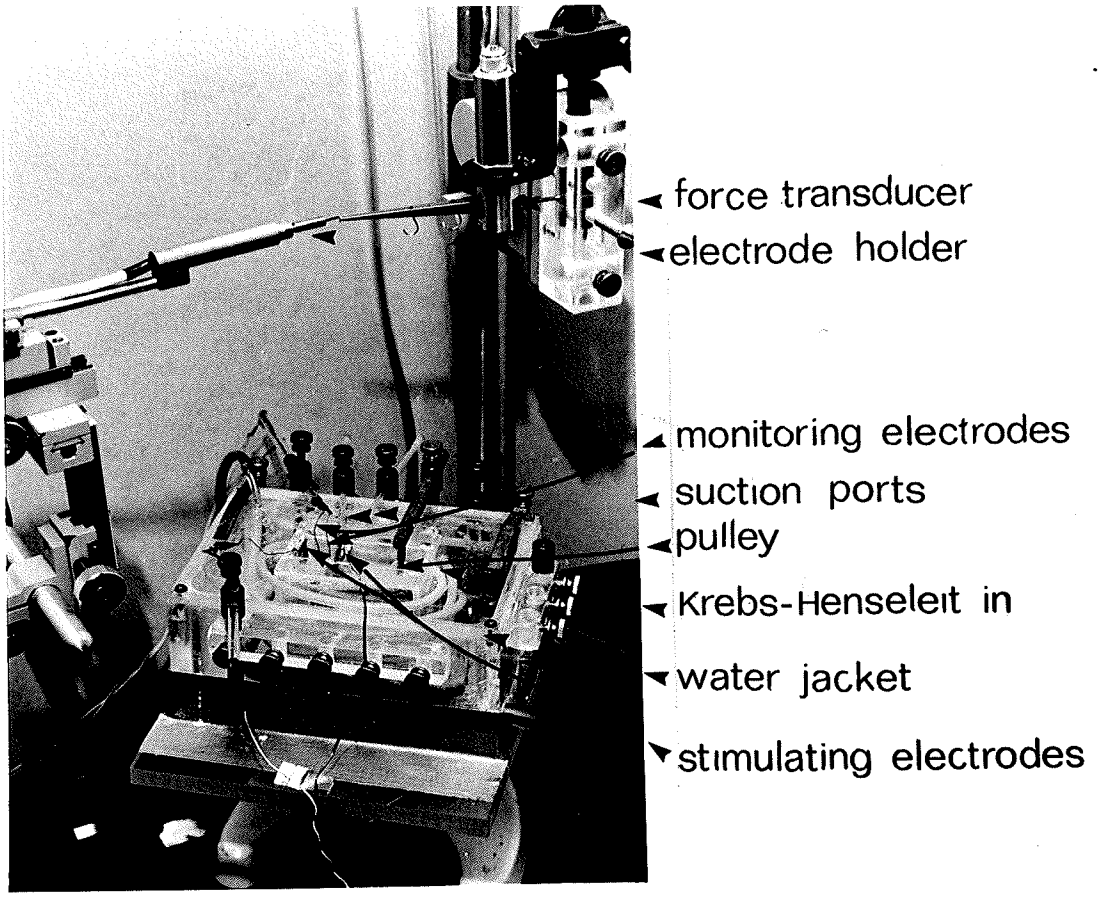
The "floating" electrode assembly was positioned above the muscle and lowered into the solution. The impedance of the electrode was checked electrically and the electrode was used only if the impedance was greater than 20 megohms. This "floating" assembly helps to prevent contraction-induced extrusion of the microelectrode once it has penetrated a cell and

allows simultaneous recording of electrical and mechanical activity (Marshall and O'Brien, 1967).

The TSM strips were stimulated by a Grass S-44 constant voltage (output resistance = 250 ohms) stimulator. The output of the stimulator was isolated from ground by a WPI photon-coupled isolation unit. Membrane potential and stimulus intensity were calibrated with a dynamic calibrator. The outputs of all channels were fed through a flutter compensation unit (bandpass 0 to 500 Hz) before being displayed on the oscilloscope and Gould recorded and saved on the Philips tape recorder. The entire experimental area was electrically isolated in a Faraday type shielded room.

The electrode was advanced toward the tissue until a sudden step change in potential, as displayed on the recording systems, indicated that a cell had been impaled. (The electrode was lowered carefully until small amplitude electrical activity signaled muscle surface contact. Cell impalement was finally achieved by manual table vibration - short taps. The criteria for acceptable impalements were: 1) an instantaneous increase in negative voltage, 2) the voltage achieved held steady for at least 10 to 15 seconds, 3) upon withdrawl of the electrode the initial baseline was regained and 4) the electrode impedance was unchanged.

Figure 4: Modified Abe and Tomita type tissue bath for stimulating and recording of membrane potentials from TSM cells. A description of the bath is given in the text.



RESULTS

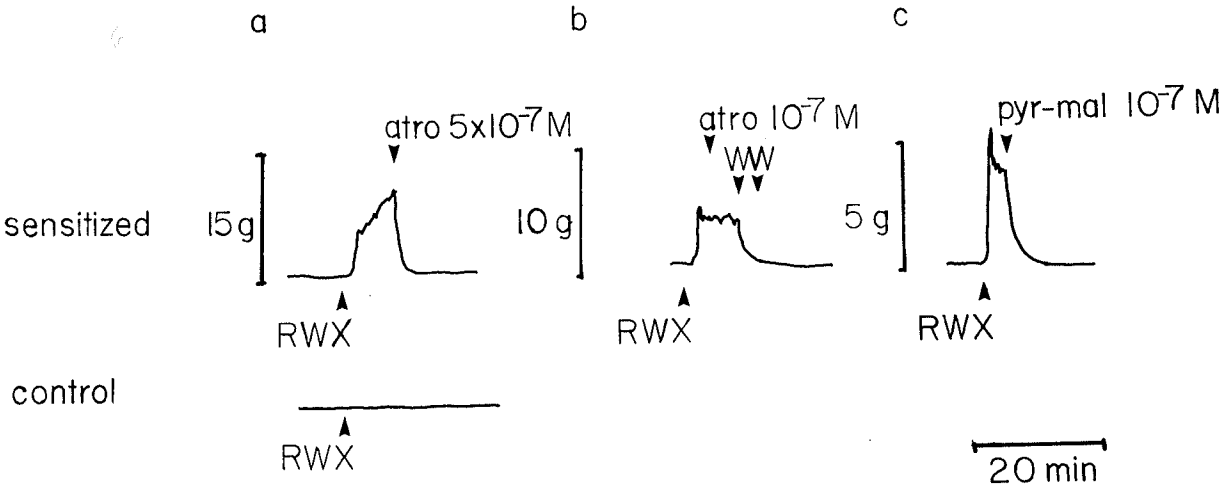
a) Schultz-Dale Reaction.

As a prerequisite to all subsequent studies, separate strips of sensitized TSM (both active and passive) were challenged in vitro with 0.3 mg/ml RWX (final concentration). Control strips were similarily challenged. Only the sensitized TSM developed active tension in response to the sensitizing antigen - known as the Schultz-Dale response (Fig. 5); TSM strips from littermate control dogs were always quiescent. This in vitro response to RWX could be elicited only once per sensitized muscle tested. A second challenge issued up to 18 hours after the first failed to provoke a contraction in viable TSM.

The Schultz-Dale reaction could be inhibited (up to 95%) by pre-equilibrating in vivo sensitized TSM with the H_1 receptor antagonist pyrilamine-maleate in a concentration of 10^{-7} M. The reaction was specific for RWX in that other proteins such as ovalbumin or bovine serum albumin given in the same manner did not produce any active tension in the sensitized TSM preparation. Antihistamines in much greater concentrations have been shown to have local anesthetic actions (Halpern, 1942). A direct anaesthetic action of antihistamines on smooth muscles is not prominent; mild spasmogenic effects on the bowel, uterus and bladder may be seen (Douglas, 1967). Presumably, the antigen-antibody reaction that takes place on the mast cell surface membrane and causes degranulation releases histamine to elicit the mechanical response through H_1 receptors on the smooth muscle cells.

However, atropine (10^{-7} M) - an acetylcholine receptor antagonist - also significantly reduced the degree of tension

Figure 5: The effect of ragweed pollen extract (RWX) on sensitized and littermate control TSM. Only sensitized tissues showed a Schultz-Dale response represented by contraction in response to the sensitizing antigen. Control TSM strips were always quiescent. a) Schultz-Dale response from an in vivo sensitized muscle strip. b) Schultz-Dale response from an in vitro sensitized TSM strip. Atropine does not relax the contraction. W indicates washout. c) Schultz-Dale response from another in vitro sensitized TSM strip showing the rapid relaxation after treatment with pyrilamine-maleate.



development during the Schultz-Dale reaction (Fig. 6a and b). In addition, atropine (10^{-7} M) given after pyrilamine-maleate (10^{-7} M) or alone at the plateau of the tension response to antigen challenge (Fig. 6c and d) produced reversals of the contractions in the in vivo actively sensitized TSM. However, atropine was not effective in relaxing the Schultz-Dale response of in vitro passively sensitized TSM (Fig. 5b), whereas pyrilamine-maleate caused an immediate relaxation to baseline tension (Fig. 5c). These results indicated a potential local role for the parasympathetic neural elements present in the TSM preparation for the allergen induced bronchospasm observed in in vivo ragweed sensitized dogs.

b) Histamine dose-response relationships.

i) Effects on in vivo actively sensitized TSM.

The major mediator of anaphylaxis released from degranulating mast cells after the antigen-antibody reaction in dogs is histamine (Meyers et al., 1973; Gold et al., 1977). Therefore, histamine dose-response studies were performed (Fig. 7) comparing the in vivo sensitized TSM with TSM from littermate controls. The sensitized TSM strips (filled stars) exhibited statistically significant hypersensitivity (a shift to the left of the dose-response curve) and hyperreactivity (greater maximal active tension) to doses of histamine when compared with control (filled circles) values. Sensitized TSM developed a maximum active tension of 1.882 kg/cm^2$\pm$0.087 S.E. (n=8) compared with controls (n=6) of 1.151 kg/cm^2$\pm$0.253. The effective doses of histamine that produced 50% of these maxima (ED_{50}) were

Figure 6: Schultz-Dale responses from 4 in vivo ragweed sensitized TSM. Muscle (a) was pre-exposed to 10^{-7} M atropine (ATRO) and produced 0.373 kg/cm^2 of tension in response to ragweed (RW) challenge compared with 1.512 kg/cm^2 in (b) a sensitized TSM strip from the same dog that was not blocked with atropine. Atropine, injected into the muscle bath at the plateau of the Schultz-Dale reaction either in tandem with pyrilamine-maleate (PYR-MAL) 10^{-7} M (c) or alone (d), had a profound relaxing effect.

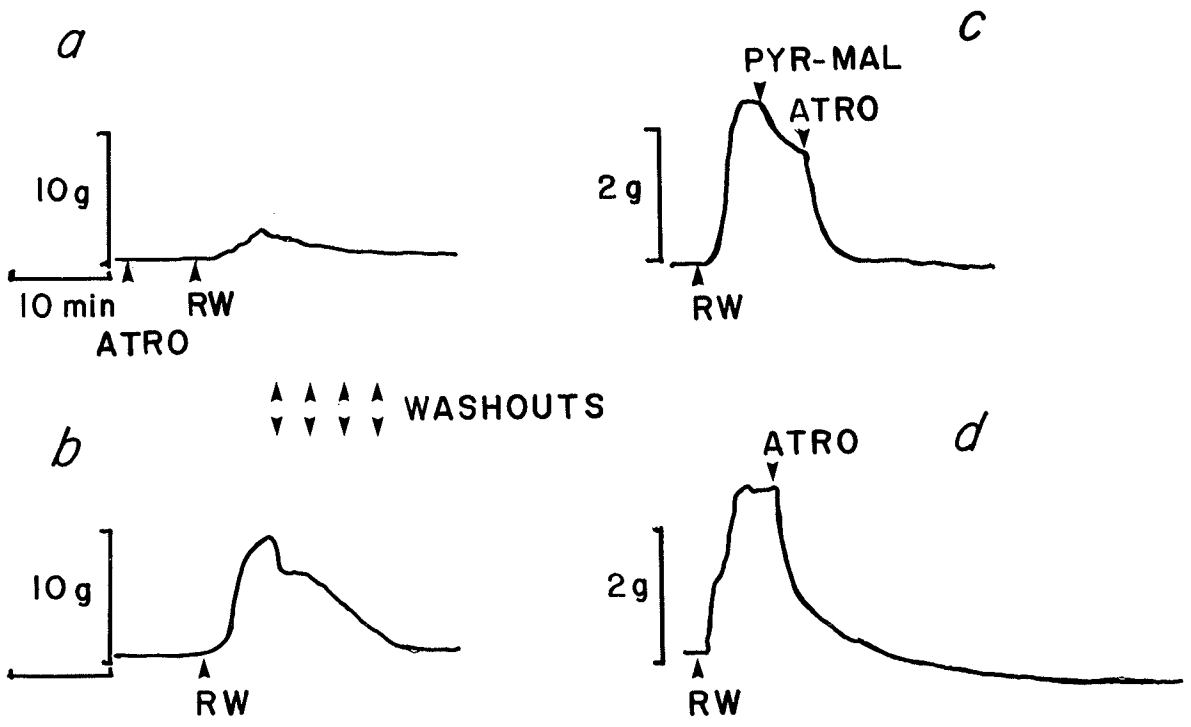
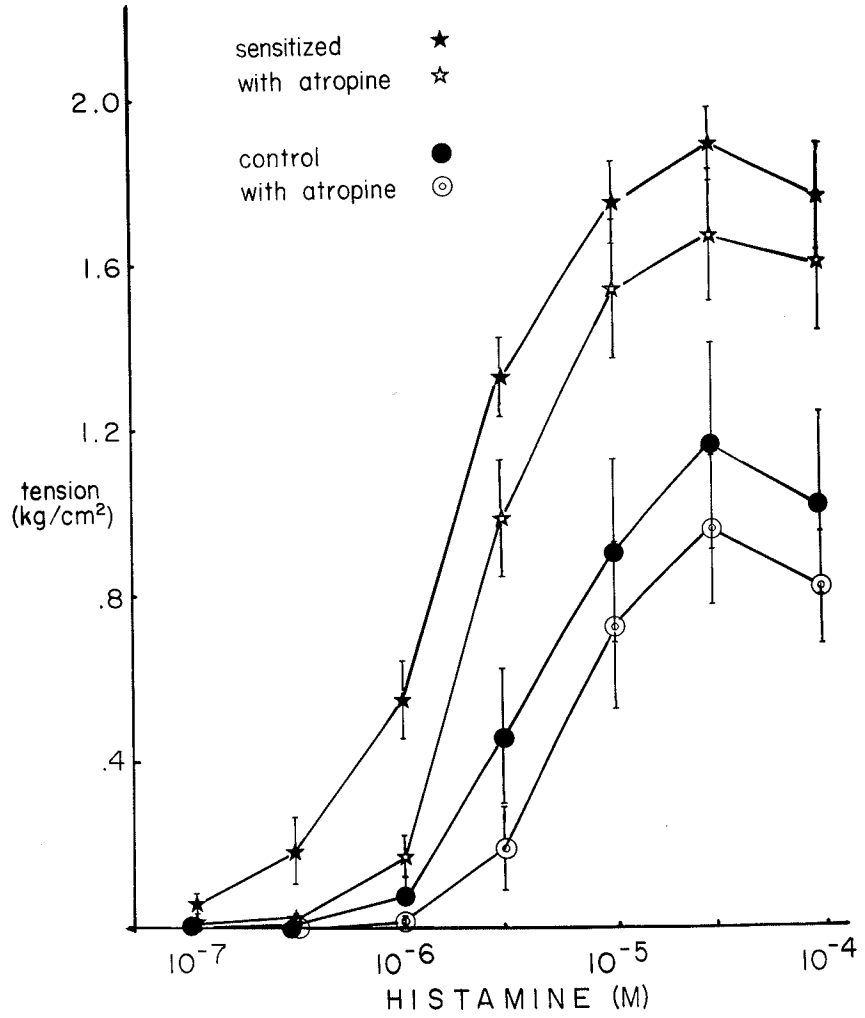


Figure 7: Histamine dose-response curves for TSM strips from in vivo ragweed sensitized (stars, n=8) and littermate control (circles, n=6) dogs in the presence (open symbols) and absence (filled symbols) of 10^{-7} M atropine. Sensitized TSM were hyperreactive (upward shift) and hypersensitive (leftward shift of the dose-response curve) to histamine. In the presence of atropine however only the hyperreactivity remained. Atropine had no significant effect on TSM from littermate controls. The data is presented numerically in Table 2.



statistically different with $1.86 \times 10^{-6} \pm 0.24$ M for sensitized TSM and $5.54 \times 10^{-6} \pm 1.35$ M for control TSM, indicating a hypersensitivity of sensitized TSM to histamine in vitro.

In the presence of 10^{-7} M atropine, both sensitized (open stars) and control (open circles) TSM demonstrated small, statistically non-significant decreases in reactivity to histamine. The hyperreactivity of the sensitized TSM (1.661 ± 0.164 kg/cm^2, n=8) persisted when compared with control TSM either in the presence ($.955 \pm .178$ kg/cm^2, n=6) or absence (1.151 ± 0.253 kg/cm^2) of 10^{-7} M atropine.

Sensitized TSM, however, demonstrated a significant rightward shift in the dose-response curve as indicated by ED_{50} values in the presence of atropine, $2.91 \times 10^{-6} \pm 0.42$ M, compared with $1.86 \times 10^{-6} \pm 0.24$ M in the absence of atropine when statistically analyzed using a paired-t or a Wilcoxon matched pair test. Values for ED_{50} in atropine-treated sensitized TSM were significantly less than control values. Control values failed to demonstrate any statistically significant shift ($5.54 \times 10^{-6} \pm 1.35$ M in the absence compared to $5.30 \times 10^{-6} \pm 0.50$ M in the presence of atropine).

The numerical values of the responses to the dose range of histamine from the 4 curves in Figure 7 are presented in Table 2 in order to further document the effect of atropine on the reactivity of the sensitized TSM to low doses of histamine. In the dose range from 10^{-7} M to 10^{-6} M the atropine treated sensitized TSM strips did not differ in their responses from

Table 2 A numerical representation of the dose-response curves from Figure 7.

HISTAMINE	SENSITIZED		CONTROL	
DOSE (M)		ATROPINE		ATROPINE
10^{-7}	.056 ± .027	*.007 ± .005	*.004 ± .003	* Ø
3×10^{-7}	.183 ± .080	*.022 ± .011	*.010 ± .007	*.002 ± .002
10^{-6}	.545 ± .099	*.168 ± .050	*.077 ± .049	*.017 ± .011
3×10^{-6}	1.319 ± .095	.979 ± .142	*.453 ± .166	*.191 ± .098
10^{-5}	1.739 ± .103	1.530 ± .169	*.900 ± .233	*.719 ± .201
3×10^{-5}	1.882 ± .087	1.661 ± .164	*1.151 ± .253	*.955 ± .177
10^{-4}	1.752 ± .128	1.594 ± .175	*1.013 ± .224	*.813 ± .137

maximum responses in kg/cm^2 for each dose

* indicate maximum tensions produced at each dose are statistically different from sensitized TSM strips without atropine.

either the control or atropinized control values, but were significantly different from zero indicating higher threshold values as well. At doses of histamine greater than 10^{-6} M the responses of atropine treated TSM were not different from the non-atropine treated sensitized TSM but were significantly different from both control groups (atropine present and atropine absent).

ii) Effects on in vitro passively sensitized TSM.

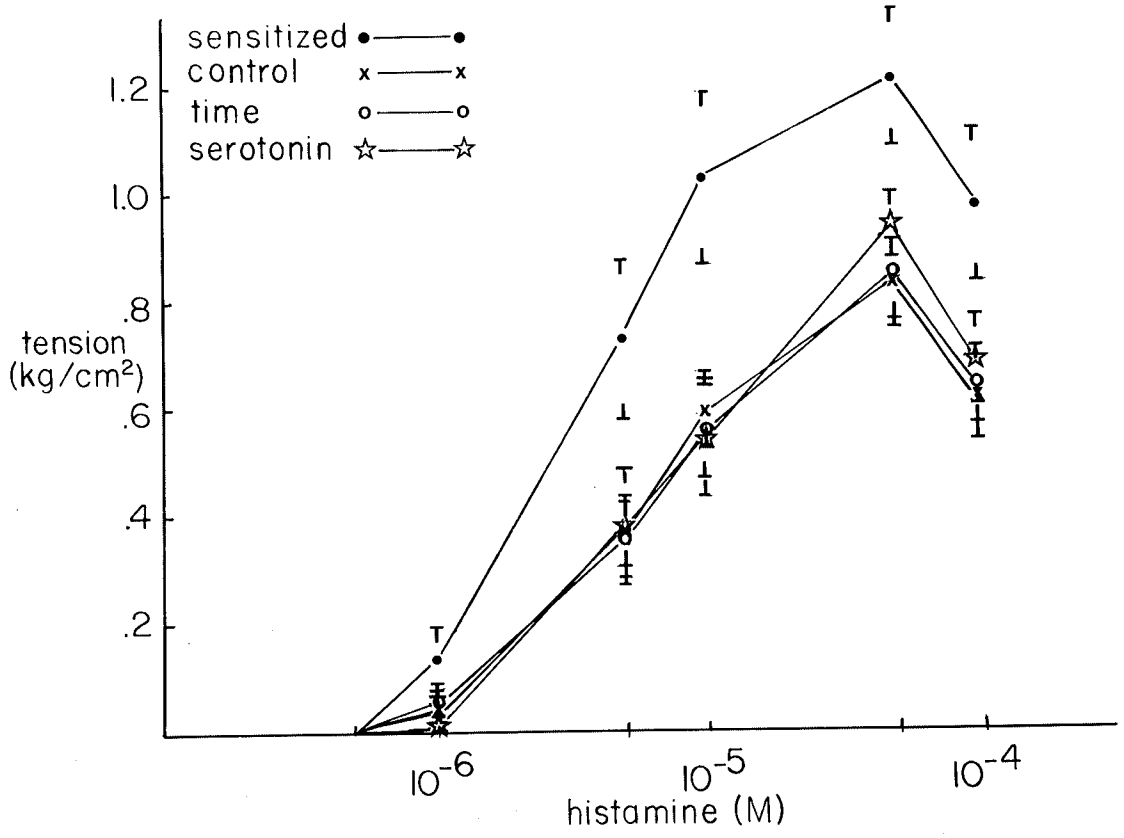
The effect of in vitro sensitization on non-cumulative histamine dose-response curves is shown in Figure 8. Tracheal muscle strips incubated in sensitized serum (n=9), showed a maximum contractile response to histamine of 1.204$\pm$.127 kg/cm^2 with a mean ED_{50} of 4.38 x 10^{-6} $\pm$0.52 M. This response was significantly different from the time control group (0.838$\pm$0.087 kg/cm^2 n=7) and the groups incubated in control serum, (0.825$\pm$0.081 kg/cm^2, n=7) and 5-HT (0.925$\pm$.065 kg/cm^2, n=8). However, the ED_{50}'s for these control groups; 5.67 x 10^{-6} $\pm$0.71 M, 5.92 x 10^{-6} $\pm$1.04M, and 4.00 x 10^{-6} $\pm$3.55 M respectively, were not significantly different from the in vitro sensitized TSM group. In vitro passive sensitization produced TSM strips that were hyperreactive but not hypersensitive to histamine.

c) $\underline{K^+}$ dose-response relationships.

Increasing the external K^+ concentration depolarized TSM cells by activating voltage sensitive calcium channels and thereby causing contraction.

The dose-response relationships for increasing concentrations of external K^+ on TSM strips from in vivo

Figure 8: Histamine dose-response curves from TSM sensitized in vitro (filled circles, n=9) and time control muscles (open circles, n=7) control serum incubated muscles (x's, n=7) and 5-HT incubated muscles (stars, n=8). Muscles incubated with serum from ragweed sensitized dogs were hyperreactive to histamine. No leftward shift in the curve could be observed based on mean ED_{50} values, indicating similar sensitivities.



sensitized (stars) and littermate control dogs (circles) are presented in Figure 9. Sensitized TSM developed significantly greater maximum active tension (filled stars) to 120.3±8.7% (n=8) of littermate control values (filled circles) - 99.4±0.6% (n=7) at 84 mM K^+. The ED_{50} for sensitized TSM was 22.4 ±0.66 mM and was significantly less than the control value of 25.2 ±1.02 mM; i.e. sensitized TSM strips displayed both a hyperreactivity and hypersensitivity to K^+.

However, in the presence of 10^{-7} M atropine the maximum responses of sensitized and control TSM were not significantly different from each other (98.4±7.0% (n=7) vs 90.9±5.4% (n=8) respectively) nor were they significantly different from control values in the absence of atropine. The ED_{50} values (24.1 ± 0.72 mM vs 25.4 ±0.79 mM respectively) were also similar; i.e., sensitized TSM in the presence of atropine was neither hyperreactive nor hypersensitive to K^+ compared to sensitized TSM in the absence of atropine.

d) Serotonin dose-response relationships.

Serotonin (5-HT) is a naturally occurring amine that produces contraction in TSM by opening voltage sensitive calcium channels.

Serotonin dose-response relationships for TSM strips from n=7 ragweed sensitized (stars) and n=5 littermate control (circles) dogs are shown in Figure 10. Significant hyperreactivity was observed at 5-HT concentrations greater than 3×10^{-7} M in sensitized TSM using an unpaired t-test. Sensitized TSM strips showed an ED_{50} of 3.96×10^{-7} ±0.72 M which

Figure 9: Cumulative K^+ dose-response curves for TSM strips from in vivo ragweed sensitized (stars, n=8) and littermate control (circles, n=7) dogs in the presence (open symbols) and absence (filled symbols) of 10^{-7} M atropine. The values are expressed as a percent of control maximum K^+ induced tension values (1.98 ± 0.145 kg/cm^2) and are plotted against the log of the concentration of K^+ in the modified Krebs-Henseleit solution. Sensitized TSM strips were hypersensitive (leftward shift) and hyperreactive (upward shift of the dose-response relationship) to $[K^+]_{out}$. Both the hypersensitivity and hyperreactivity however were abolished by atropine.

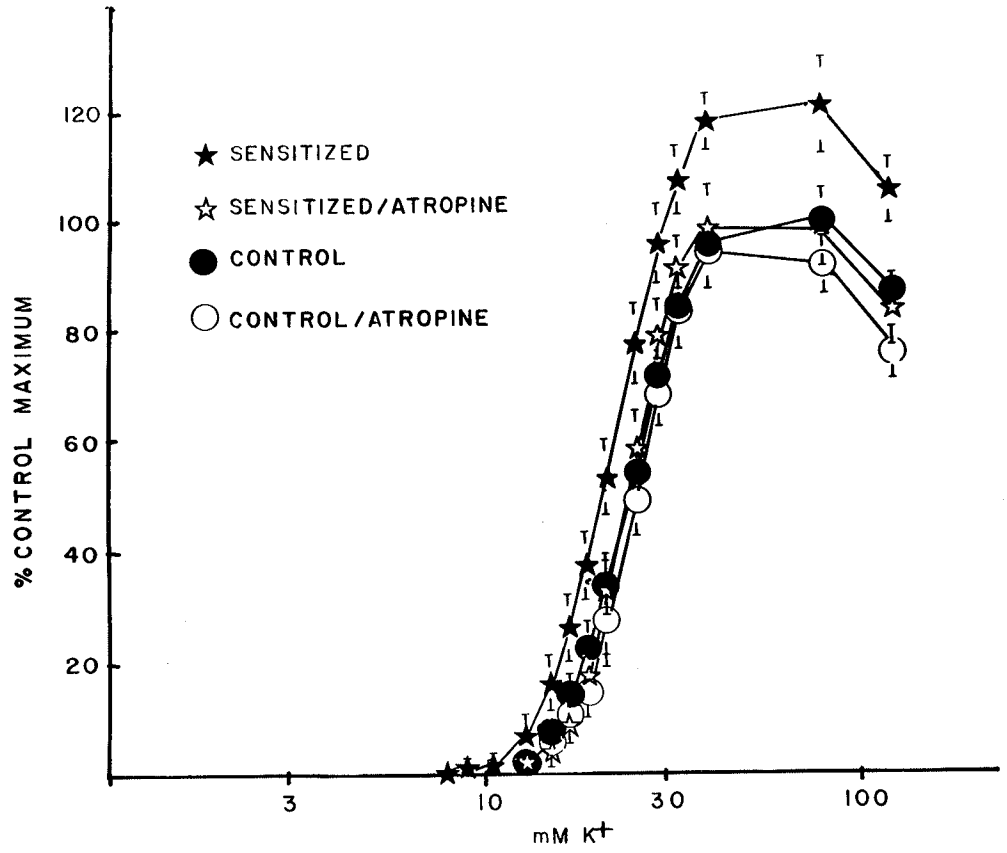
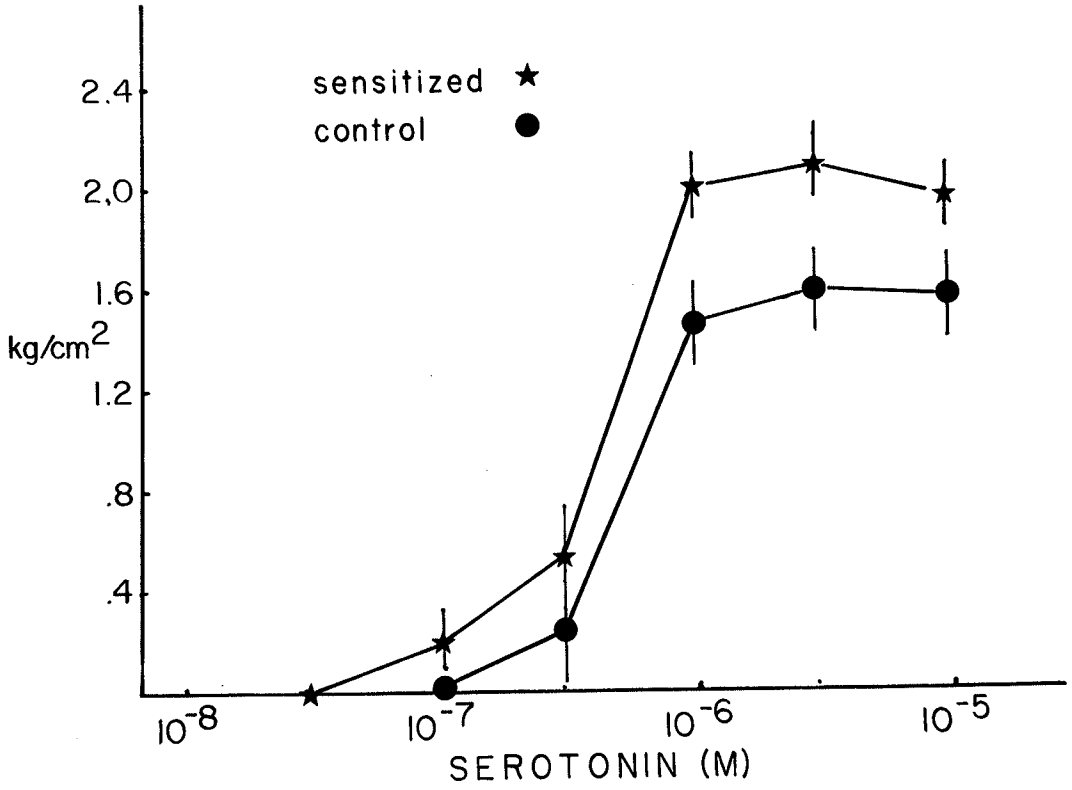


Figure 10: Serotonin dose-response curves from n=7 in vivo ragweed sensitized (stars) and n=5 littermate control (circles) dogs. Sensitized TSM were hyperreactive to serotonin which produces contraction in TSM by allowing calcium to enter through voltage sensitive channels.



was not significantly different from control TSM 5.38×10^{-7} ± 0.62 M.

e) Acetylcholine dose-response relationships.

Acetylcholine dose-response curves for TSM strips from n=8 ragweed sensitized (stars) and n=6 littermate control (circles) dogs are presented in Figure 11. Significant hyperreactivity was observed for sensitized TSM at acetylcholine concentrations greater than 10^{-7} M using an unpaired t-test. There were also significant differences in the ED_{50} values between sensitized and control TSM - ($4.08 \times 10^{-7} \pm 1.02$ M and $7.95 \times 10^{-7} \pm 1.41$ M respectively).

f) Electrical stimulus-response and related studies.

Sensitized and control TSM tissues were stimulated from an alternating current source at varying voltage strengths and their responses in kg/cm^2 plotted graphically in Figure 12. Sensitized and control TSM produced equal maximal tension responses at the higher voltages (2.004 ± 0.154 kg/cm^2 and 1.868 ± 0.119 kg/cm^2 respectively), however, sensitized TSM displayed a significant leftward shift in threshold responses as indicated by the manifold increased responses at the lower voltages. The responses within each 0.5 volt segment were compared using an unpaired t-test and from 1.5 to 3.0 volts sensitized TSM produced significantly greater tensions compared to TSM from littermate control dogs. An analysis was performed to calculate the voltages at which each muscle produced 50% of it maximum isometric tension (EV_{50}). Sensitized values were significantly shifted to the left (2.12 ± 0.14 volts) compared to control values (2.64 ± 0.10 volts) indicating a hypersensitivity to 60 Hz

Figure 11: Acetylcholine dose-response curves for TSM strips from n=8 in vivo ragweed sensitized (stars) and n=6 littermate control (circles) dogs. Sensitized TSM were hyperreactive to acetylcholine as indicated by the upward displacement of the dose-response curve with respect to the control curve. Sensitized TSM also showed a hypersensitivity to acetylcholine (leftward shift) as indicated by the significant differences in mean ED_{50} values between sensitized and control TSM strips ($4.08 \times 10^{-7} \pm 1.02$ M and $7.95 \times 10^{-7} \pm 1.41$ M respectively).

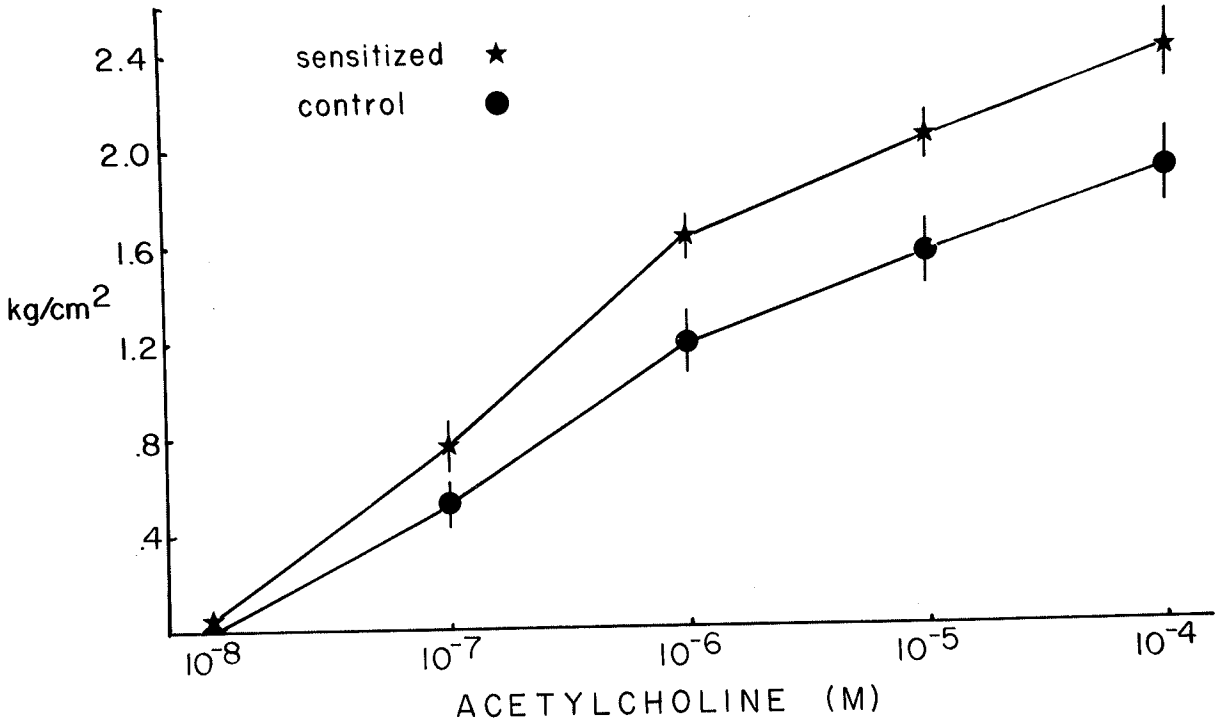
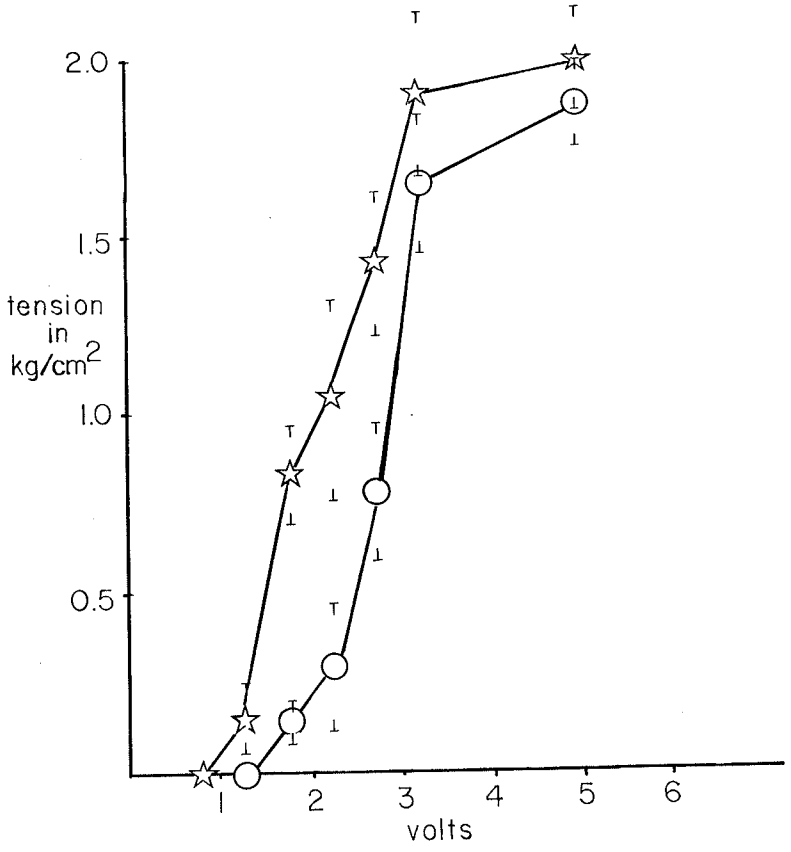


Figure 12: Electrical 60 Hz stimulus-response curves from n=5 sensitized (stars) and n=5 control (circles) TSM tissues. The data are plotted as the mean responses for each 0.5 volt segment of the abscissa. At voltages greater than 3.5 volts a single mean for each (sensitized and control) group is plotted at 5 volts. Sensitized TSM displayed no hyperreactivity of electrically induced tetani at voltages greater than 3.0 with a mean maximum response of 2.004 ± 0.154 kg/cm^2 for sensitized and 1.868 ± 0.119 kg/cm^2 for control strips. However, sensitized TSM showed a significant leftward shift of its stimulus-response relationship compared with the control curve as indicated by a significant difference in voltage to produce 50% of the maximum force (2.12 ± 0.14 volts vs 2.64 ± 0.10 volts respectively). This indicated a hypersensitivity of sensitized TSM to 60 Hz electrical stimulation.



electrical stimulation by sensitized TSM.

The accumulation of stimulus-response data was continually hampered by the presence of spontaneous baseline activity (SBLA) in a great number of TSM strips from ragweed sensitized dogs. Control TSM strips did not display this phenomenon. These observations were very similar to those noted by Antonissen et al. (1979) in an ovalbumin-sensitized canine model of allergic bronchospasm which showed SBLA in sensitized muscles only and also a prolonged isometric tension plateau at supramaximal voltages. These two phenomena were investigated in the ragweed model of asthma.

i) Spontaneous baseline activity (SBLA).

The upper trace in Figure 13 presents a representative mechanogram of a ragweed sensitized TSM strip stretched to its optimal length and supramaximally electrically tetanized at 5 minute intervals. Between tetani, the sensitized TSM displays SBLA which is not seen in TSM from littermate controls (middle trace) similarly tetanized.

The origin of this SBLA was investigated by introducing various interventions (Fig. 14). In the upper lefthand mechanogram of a sensitized TSM stimulated as described above, the SBLA could be "washed out" (W) indicating a possible extracellular source of SBLA stimulation. SBLA activity returned with time suggesting a slow build up of some agonist.

The lower lefthand trace showed that electrical stimulation

Figure 13: The upper trace is a representative set of mechanograms of a ragweed sensitized TSM strip tetanized at 5 minute intervals. Between tetani the sensitized TSM displays SBLA which is not seen in muscle from littermate controls (middle trace) similarly tetanized.

Common to control TSM is a partial relaxation from plateau force even before the tetanizing voltage is terminated as can be seen in the middle trace. However, sensitized TSM shows a prolonged contraction which is not diminished until after the stimulus is discontinued (upper trace).

The lower trace shows the effect of a low dose (10^{-7} M) of acetylcholine (ACh) on a control strip. After the initial rapid rise in isometric force, the muscle shows phase activity much like the SBLA observed in sensitized TSM in the upper trace.

(W) indicates washout of the drug from the muscle bath.

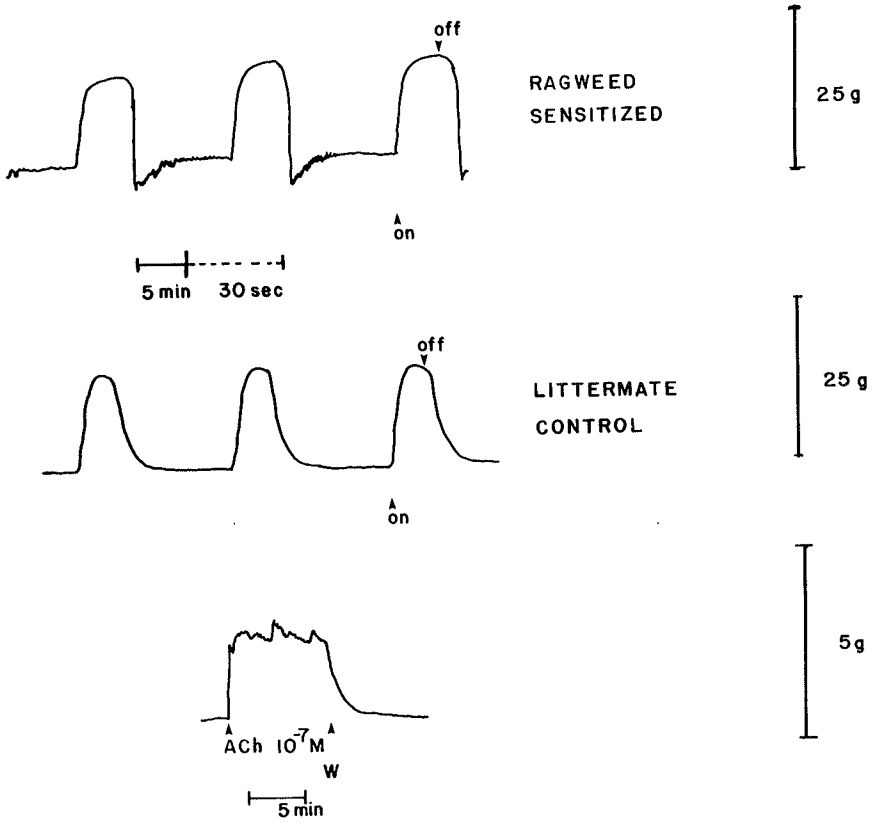


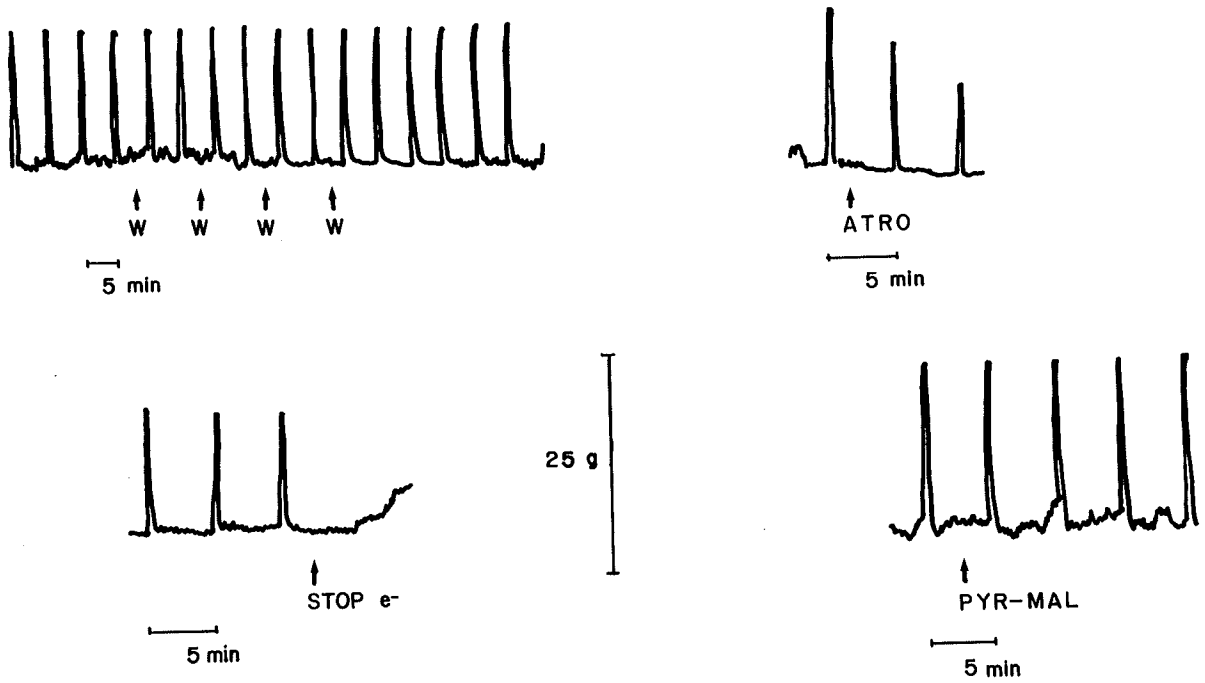
Figure 14: These mechanograms of electrically tetanized sensitized TSM show typical results obtained by various interventions.

The upper lefthand trace shows that with repeated washouts (W), the SBLA could be eliminated. However, after another 3 or 4 tetani without washout the SBLA returned indicating that the agent causing SBLA could be removed and probably arose from the preparation itself.

The lower lefthand trace shows that if the 5 minute interval tetanization was stopped (stop e-), SBLA continued. This indicated that it was not the continual isometric stimulations that perpetuated the SBLA.

The upper righthand trace shows the effect of atropine (10^{-7} M) on the mechanogram from another sensitized TSM displaying SBLA. In addition to an eventual reduction to 5% of the original maximum isometric force with electrical stimulation, atropine caused a cessation of the SBLA indicating a cholinergic source for this phenomenon. (Electrically induced contractions of TSM from the dog are normally neurally mediated.)

The lower righthand trace shows that with the addition of pyrilamine maleate (10^{-7} M), (an H_1 histamine receptor antagonist) the SBLA continues, indicating that histamine is not involved.



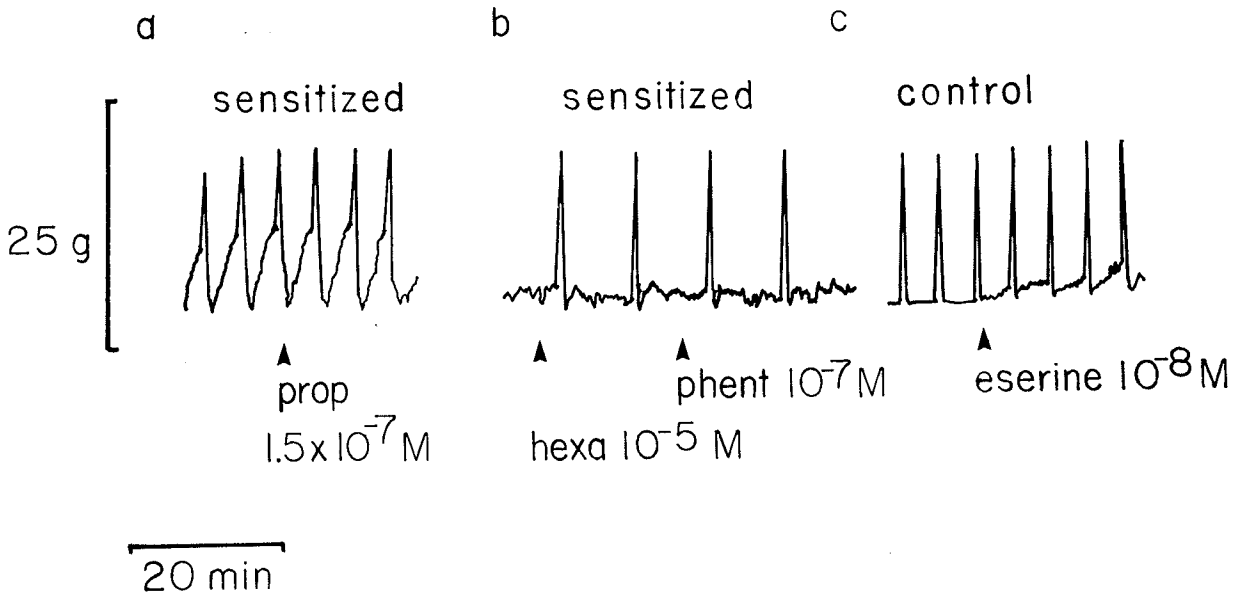
was not necessary for the continuation of the SBLA. In addition, the slowly rising resting tone after the cessation of 5 minute interval tetanization indicated that continual tetanization may be preventing an even greater rise in the resting tone. Looking back to Figure 13 (upper trace), after the stimulus is turned off at the peak of the tetanus, tension falls to the original resting baseline tone before SBLA is reactivated and tone once again rises. This relaxation to baseline was not affected by propranolol (1.5×10^{-7} M) a beta-adrenoceptor blocker (Fig. 15a).

The lower righthand panel in Figure 14 shows that the SBLA is not being activated by histamine or through histamine H_1 receptors since the antagonist pyrilamine-maleate (10^{-7} M) has no effect on it.

Evidence for a cholinergic component can, however, be adduced from the upper righthand mechanogram from a sensitized TSM strip which shows SBLA can be inhibited by the acetylcholine receptor antagonist atropine (10^{-7} M). Atropine also inhibits electrically stimulated contractions as can be observed in this trace. Eventually isometric force is reduced to 5% of initial with this dose of atropine (since electrically induced contractions of TSM from the dog are normally neurally mediated).

These results indicated a cholinergic source for SBLA. In this regard, the lower trace in Figure 13 shows the effect of a relatively low dose of acetylcholine (10^{-7} M) on control TSM. After the initial rapid rise in isometric force, the muscle becomes spontaneously active, much like the SBLA seen in sensitized TSM. At higher dose of acetylcholine, the mechanogram

Figure 15: a) The relaxation of sensitized TSM to baseline even in the presence of SBLA was not affected by the beta-adrenoreceptor blocker propranolol (1.5×10^{-7} M) indicating that beta-receptor activation is not necessary for relaxation. Also, SBLA was not augmented by beta-receptor blockade.
b) SBLA of sensitized TSM was not sensitive to alpha-receptor blockade (10^{-7} M phentolamine) or to the ganglionic blocker hexamethonium (10^{-5} M) eliminating the possibilities of an alpha-adrenergic neural or ganglionic source for the SBLA observed.
c) SBLA similar to that observed for sensitized TSM could be initiated in control TSM by low doses of eserine (10^{-8} M). At this low dose of anticholinesterase contractile force in response to electrical tetanization is only slightly increased over initial control values.



remains smooth.

Other drug interventions were utilized to test the possibilities of adrenergic neural or alpha-adrenergic receptor involvement or a ganglionic source of the SBLA observed in sensitized TSM. As can be seen in Figure 15b, phentolamine (10^{-7} M) - an alpha receptor antagonist - had no effect on SBLA nor did the ganglionic blocker hexamethonium (10^{-5} M).

ii) Prolonged isometric force plateau.

To investigate the second phenomenon of prolonged force plateau in sensitized TSM, the stimulus duration was prolonged to 75 sec. and the resultant isometric mechanograms compared with control TSM similarly tetanized (Fig. 16).

The upper trace is a continuous recording using muscle from a littermate control dog and shows spontaneous relaxation to approximately 60% of the maximum initial force even during supramaximal tetanic stimulation.

The lower trace is from a ragweed sensitized muscle. Some SBLA is seen. In addition, the prolonged duration tetanus does not show the relaxation observed in the control mechanogram. The force remaining at the point where the stimulus is terminated is approximately 90% of its initial maximum.

Several control and sensitized TSM strips were stimulated in this manner and analysis revealed statistical differences between the prolonged mechanograms of (n=6) littermate control and (n=7) ragweed sensitized TSM.

Because of the prolonged stimulation (in comparison with the electrical stimulus-response studies), sensitized TSM were able

Figure 16: In order to investigate the prolonged force plateau observed in sensitized TSM, the stimulus duration was prolonged to 75 sec. and the resultant mechanograms from sensitized TSM were compared to control TSM similarily tetanized.

The upper trace is from a littermate control TSM and shows spontaneous relaxation to approximately 60% of the maximum initial force even during supramaximal tetanic stimulation. The downward arrow indicates termination of the stimulus. Note the change in recording speed.

The lower trace is from a ragweed sensitized TSM. Some SBLA can be observed. In addition, the prolonged tetanus does not show the spontaneous relaxation observed in the control mechanogram. The force remaining at the point where the stimulus is turned off is approximately 90% of its maximum.

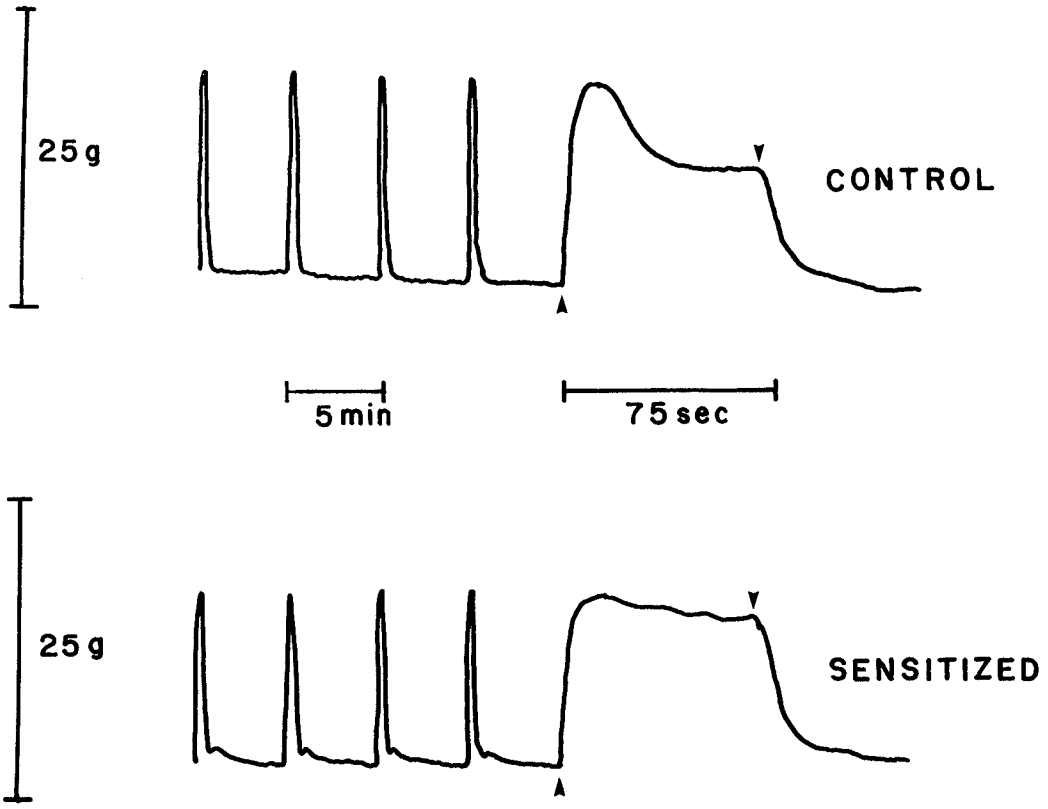


Table 3 Analysis of mechanical characteristics of ragweed-sensitized and littermate control dogs.

	LITTERMATE CONTROLS	RAGWEED SENSITIZED
INITIAL MAXIMUM FORCE	1514 g/cm^2 $\pm$ 122	*1865 g/cm^2 $\pm$ 122
RATE OF FORCE DEVELOPMENT	4.23 g/sec $\pm$0.49	4.18 g/sec $\pm$0.35
TIME TO MAXIMUM FORCE	11.6 sec $\pm$ 1.4	*14.7 sec $\pm$ 0.7
PROLONGED DURATION FORCE	939 g/cm^2 $\pm$ 137	*1599 g/cm^2 $\pm$ 116
% INITIAL FORCE	60.7 % $\pm$ 4.8	*85.8 % $\pm$ 3.5
1/2 RELAXATION TIME	4.25 sec $\pm$0.74	5.33 sec $\pm$0.87
n	6	7

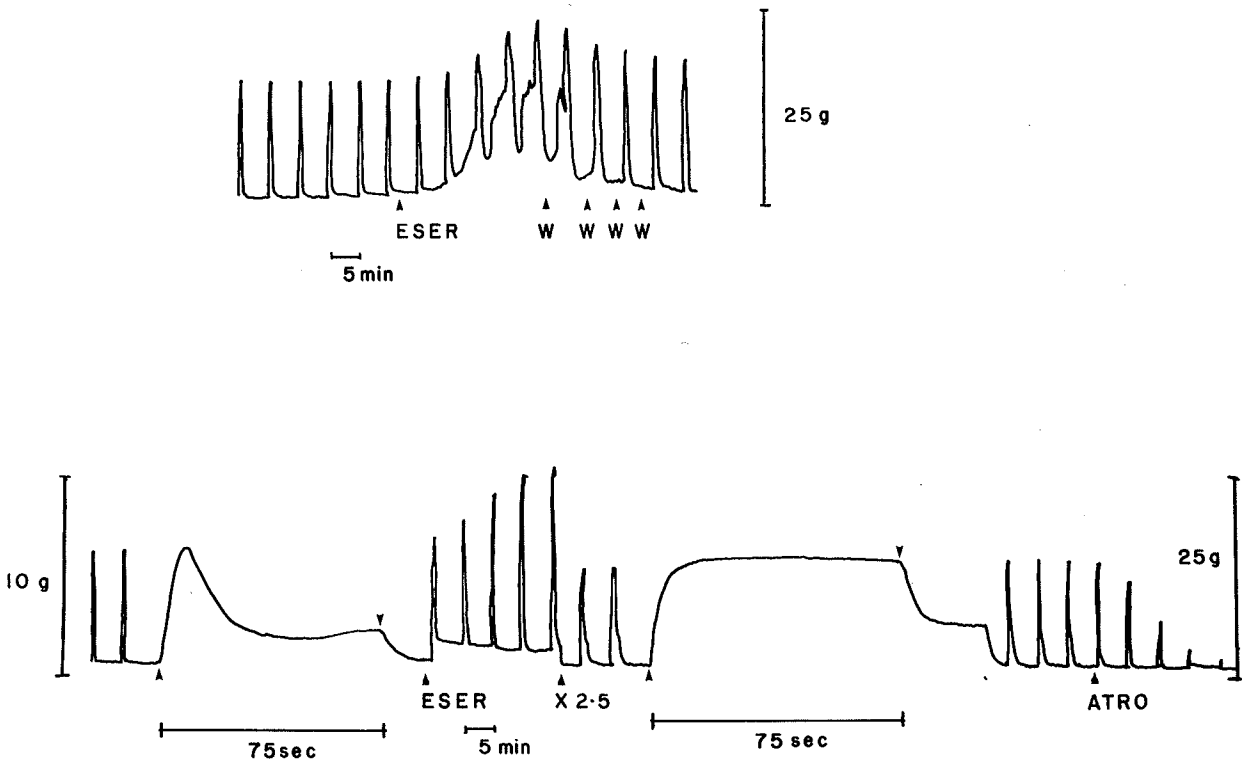
* denotes statistical difference ($p < 0.05$, student t-test) from control mean.

to develop greater isometric forces than did control TSM (1.865 ± 0.122 vs 1.514 ± 0.122 kg/cm^2). This 23% increase in active force production (Table 3) could be attributed to the 27% increase in the time required for sensitized TSM to reach maximum force (14.7 ± 0.7 sec.) compared with control TSM (11.6 ± 1.4 sec) since the maximum rates of force development in both preparations were similar. Also, the active force remaining at the end of the prolonged duration tetani was significantly greater in sensitized strips either when expressed in g/cm^2 or as a percent of the respective initial maximal active force. However, time to half relaxation was unaffected by the sensitization procedure indicating that mechanisms of relaxation and, therefore, beta-adrenoreceptors were not involved.

These results indicated that either sensitized TSM releases more acetylcholine (basally or upon stimulation) or that there is a reduction in the rate of degradation of the neurotransmitter by its cholinesterase at the neuromuscular junction - i.e. inhibition of acetylcholinesterase activity. One cannot rule out at this point the possibilities of a prolongation of excitation-contraction coupling or of phosphorylation of myosin light chains. However, from the data concerning time to half relaxation (Table 3), and the relaxation observed in the presence of SBLA (Figure 13) or cholinesterase inhibitor (Fig. 17), it would appear that relaxation mechanisms are normal in sensitized muscles. Therefore, the cause of the prolongation of the tetanus would appear to be at a loci prior to or at excitation of the smooth muscle membrane.

Figure 17: Control TSM strips were subjected to cholinesterase inhibition with the addition of eserine while being tetanized every 5 min. Increased maximum force and SBLA can be observed in the upper trace after the addition of 10^{-7} M eserine to the bath. Both the increased force and SBLA were diminished with repeated washouts (W).

In the lower trace, two tetani of prolonged duration were induced in a control TSM, before and after the addition of 3×10^{-8} M eserine. In addition to the large increase in isometric force production (after eserine treatment the sensitivity of the recorder was attenuated 2.5 - fold), a prolongation of the active force plateau can be observed.



iii) Production of SBLA and prolonged isometric force plateaus in control TSM.

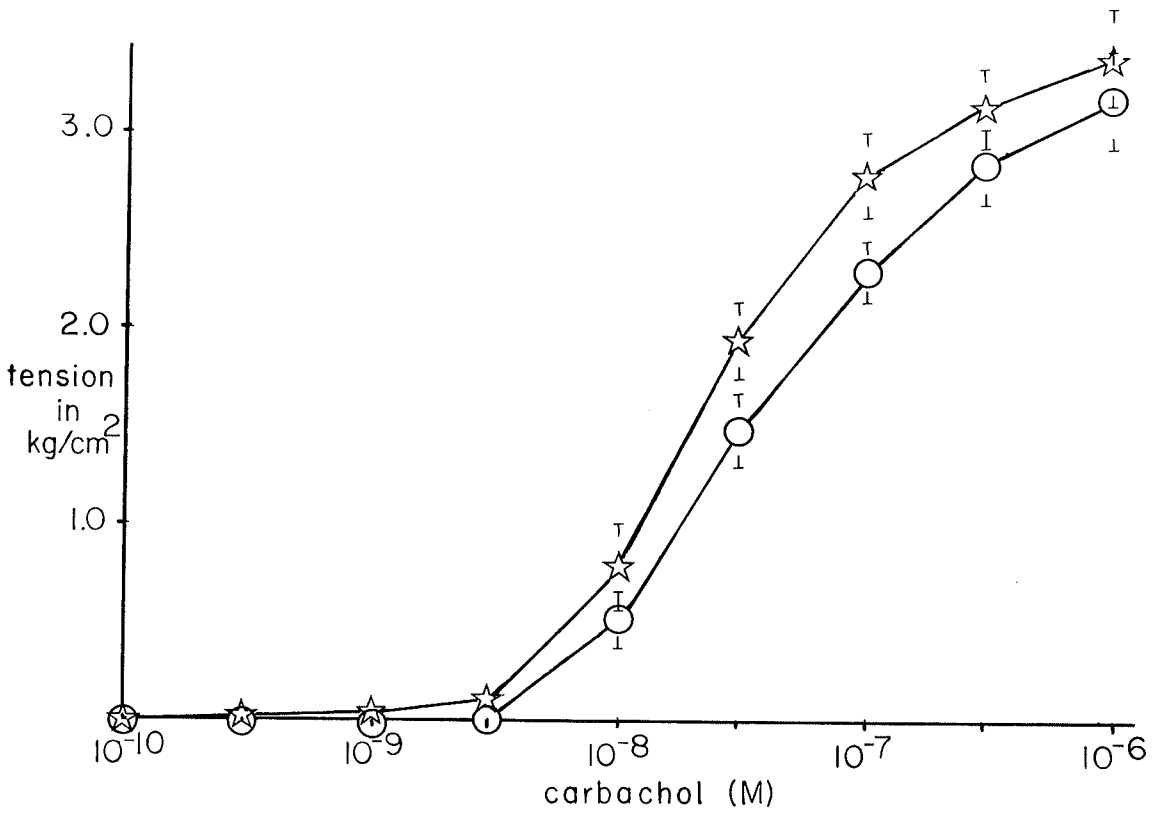
To investigate the possibility that a reduced cholinesterase activity could lead to the SBLA and mechanical changes observed in sensitized TSM, littermate control strips were subjected to cholinesterase inhibition by the addition of eserine (physostigmine) while being supramaximally tetanized from a 60 Hz source every 5 min. The results can be seen in Figure 17. Increased maximum force and SBLA can be observed in the upper trace after the addition of 10^{-7} M eserine to the muscle bath. Relaxation at the cessation of the stimulus appears similar to that previously described in Figure 13 for sensitized TSM and seems unaffected by cholinesterase inhibition. Both the increased force and SBLA were diminished by repeated washouts with fresh Krebs-Henseleit solution.

In the lower trace, two tetani of prolonged duration were induced in a control TSM strip before and after the addition of 3×10^{-8} M eserine. In addition to the large increase in isometric force production (after eserine the sensitivity of the recorder was attenuated 2.5 - fold), a prolongation of the active force plateau was observed. This was similar to that which was observed in sensitized TSM described in Figure 16.

iv) Carbachol dose-response relationships.

Carbachol (carbamylcholine) is an acetylcholine receptor agonist that is broken down only very slowly by acetylcholinesterase. The dose-response relationships obtained for sensitized (stars) and control (circles) TSM strips to carbachol are presented in Figure 18. Sensitized and control

Figure 18: Carbachol dose-response curves for n=6 sensitized (stars) and n=7 control (circles) TSM strips showed equivalent maximum responses to the agonist (3.394 ± 0.235 kg/cm^2 vs 3.167 ± 0.258 kg/cm^2 respectively). The ED_{50} values were 2.66×10^{-8} M$\pm$0.47 and 4.52×10^{-8} M$\pm$1.26 respectively, however, statistical analysis could not determine a difference although the threshold dose of carbachol for sensitized TSM was displaced to the left by 1 log unit. At 10^{-9} M and 3×10^{-9} M carbachol the responses of sensitized TSM were significantly greater than control values and control responses were not significantly different from zero.



tissues were equireactive to carbachol with mean maximum responses of 3.394 ± 0.235 kg/cm^2 (n=6) and 3.167 ± 0.258 kg/cm^2 (n=7) respectively at 10^{-6} M agonist. The ED_{50} values were $2.66 \times 10^{-8} \pm 0.47$ M and $4.52 \times 10^{-8} \pm 1.26$ M respectively but were not significantly different. However, the threshold dose of carbachol for sensitized TSM was shifted significantly leftward by one log unit. At 10^{-9} M and 3×10^{-9} M carbachol the responses of sensitized TSM were significantly greater than control TSM responses and the control responses were not significantly different from zero.

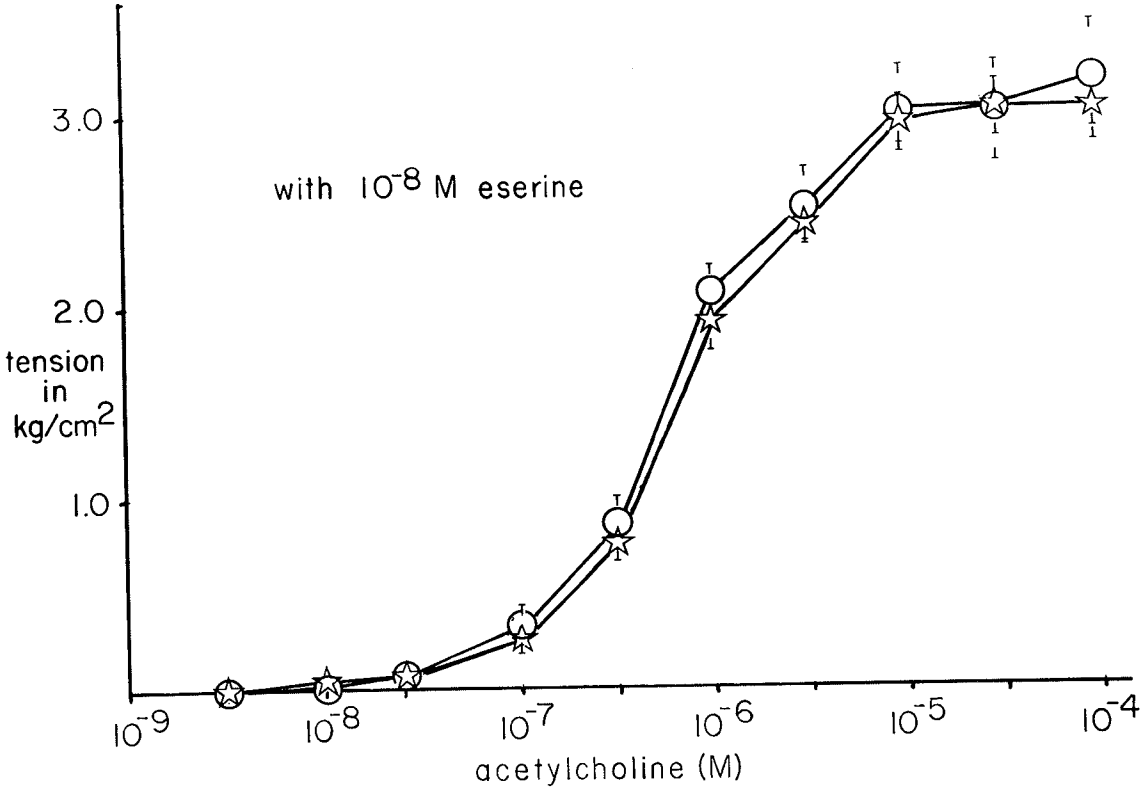
v) Acetylcholine dose-response relationships in the presence of eserine.

Acetylcholine dose-response curves were determined once again for sensitized and control TSM but this time in the presence of 10^{-8} M eserine to prevent the breakdown of acetylcholine by its cholinesterase. Acetylcholine was now equipotent in producing responses of 3.002 ± 0.149 kg/cm^2 (stars) and 2.984 ± 0.265 kg/cm^2 (circles) for sensitized and control TSM strips respectively at 3×10^{-5} M agonist (Fig. 19). In addition, no significant difference was observed in the ED_{50} values ($6.93 \times 10^{-7} \pm 0.98$ M (n=5) and $6.49 \times 10^{-7} \pm 0.87$ M (n=6) respectively) and no differences in threshold doses of acetylcholine could be detected.

g) Ragweed challenged vs non-challenged sensitized TSM TSM responses to K^+.

Souhrada and Souhrada (1981) have suggested significant changes in airway smooth muscle membrane properties with sensitization and challenge with specific antigen in the guinea

Figure 19: Acetylcholine dose-response curves for n=5 sensitized TSM strips (stars) and n=6 control TSM strips (circles) in the presence of 10^{-8} M eserine. No significant differences could be detected in either reactivity, sensitivity or threshold doses.

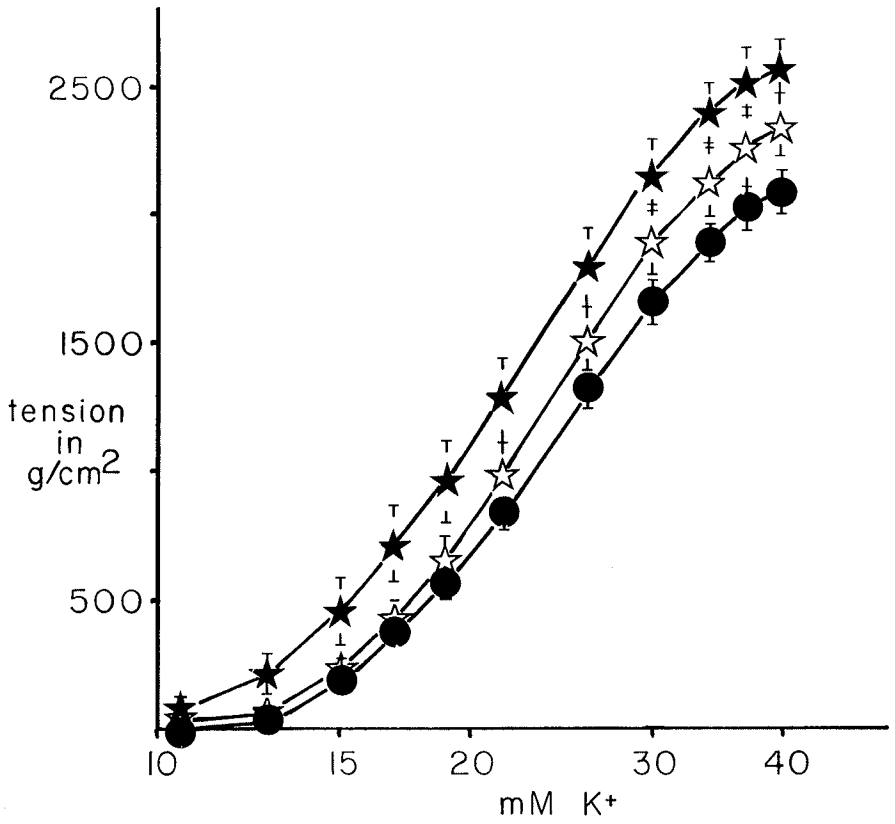


pig. They have shown that challenged sensitized tissues are depolarized some 10 mV from control tissues and sensitized non-challenged tissues are hyperpolarized 5 mV from control values. This 15 mV difference (depolarization) between challenged and non-challenged airway smooth muscle could account for the increased sensitivity to many agonists observed in canine airway smooth muscle.

To investigate this possibility ragweed sensitized and littermate control TSM were subjected to increasing concentrations of external K^+. One-half of the sensitized tissues were challenged with ragweed pollen extract. One hour after the Schultz-Dale reaction and thorough washout, K^+ dose-response relationships were determined for control, sensitized and sensitized - challenged TSM. The results are shown in Figure 20. Sensitized TSM produced the greatest responses 2.568 ± 0.136 kg/cm^2. This response was significantly greater than the control TSM response of 2.080 ± 0.084 kg/cm^2 (circles, n=28). Challenged TSM produced 2.339 ± 0.153 kg/cm^2 of tension but this value was not different statistically from either control or sensitized TSM using a multiple range test (Duncan, 1955).

A similar pattern of results was found for ED_{50} values with; sensitized, 21.0 ± 1.0 mM; sensitized/challenged, 23.0 ± 0.5 mM; and control 24.2 ± 0.6 mM. Sensitized and control TSM showed significant differences but sensitized/challenged TSM were not significantly different from either. At the lower concentrations of external K^+ (9.4-15.9 mM) sensitized TSM showed statistically greater responses compared to either sensitized/challenged or control TSM indicating a leftward shift in the threshold

Figure 20: K^+ dose-response curves for n=12 sensitized TSM strips (filled stars), n=12 sensitized TSM strips challenged with ragweed prior to the dose-response experiment (open stars), and n=28 control TSM strips (circles) were determined. Sensitized TSM maximum responses (2.568 ± 0.136 kg/cm^2) were significantly greater than control values (2.080 ± 0.084 kg/cm^2) but not greater than responses from in vitro challenged sensitized TSM (2.339 ± 0.153 kg/cm^2). A similar pattern of significance was found for ED_{50} values (21.0 ± 1.0 mM vs 24.2 ± 0.6 mM vs 23.0 ± 0.5 mM respectively).



concentration of external K^+ for that group of data.

h) [^{14}C]-choline release experiments.

The previous studies indicated the possibility that histamine may be acting pre-synaptically to augment the release of acetylcholine in ragweed sensitized canine TSM. To test this hypothesis, control and sensitized TSM strips were incubated with [^{14}C]-choline and stimulated electrically every 5 minutes for 1 hour to promote neurotransmitter turnover and the uptake of [^{14}C]-choline into the parasympathetic neural elements in the TSM preparation. Once taken up by the nerves, [^{14}C]-choline should then be a substrate for acetylcholine and the rate of loss of neurotransmitter may then be measured.

After the incubation period, electrical stimulation was suspended, 10^{-8} M eserine was added to prevent acetylcholine breakdown and thus interfere with re-uptake of [^{14}C]-choline, and washouts of the tissue baths were collected every 3 minutes for 18 minutes (i.e. 6 sample collections). Samples 1 and 2 with their high radioactive counts were discarded because of their non-linearity with subsequent counts (CPM) from samples 3 to 6 and because the CPM in samples 1 and 2 probably represent [^{14}C] release from an extracellular interstitial compartment.

The rate coefficient of [^{14}C] loss for each individual muscle - control (n=15) and sensitized (n=44) - was calculated for each collection period. The mean rate coefficient was calculated for the 9, 12, 15 and 18 minute collection periods for control and sensitized TSM strips. The mean rate coefficients were plotted against time and linear regression analysis was employed to determine the equations of the lines and thus enable

estimates of expected values for samples collected at 21, 24, 27 and 30 minutes to be calculated. Control data fitted the equation $y=-.032\ x\ +1.347$ with a coefficient of determination (r^2) of .669 while sensitized rate coefficients fitted the equation $y=-.042\ x\ +1.210$ ($r^2=.881$).

Before samples 7-10 were collected the tissues were treated with either histamine (10^{-5} M) or ragweed pollen extract (0.3 mg/ml final bath concentration). Isometric tension was recorded simultaneously throughout the experiments. Samples 7-10 were then collected and the actual rate coefficients compared to the calculated expected values. The results are shown graphically in Figure 21.

Control (dashed lines) and sensitized estimates of [^{14}C] release (rate coefficients) are plotted as straight lines. The actual rate coefficient values for control (open circles) and sensitized (open stars) after histamine (10^{-5} M) stimulation are also shown. The estimated values were used as hypothetical means and the data points for each of samples 7 through 10 for sensitized and control TSM compared against their respective hypothetical mean using a one sample t-test.

The results for control samples indicated that even though sample 7 (time=21 minutes) showed an increase in rate coefficient for [^{14}C]-choline release after histamine stimulation, this increase was not significant ($t=1.538$) nor were the increases in rate coefficient for samples 8 ($t=0.400$), 9 ($t=1.358$), and 10 ($t=1.270$) for n=10 experiments. However, monitoring mechanical activity, histamine (10^{-5} M) did produce a sustained contraction (Fig. 22).

Figure 21: Control (filled circles) and sensitized (filled stars) estimates of [^{14}C] release (measured as rate coefficients) are plotted as straight lines. The actual rate coefficient values for control (open circles) and sensitized (open stars) after 10^{-5} M histamine stimulation are also shown plotted against the time the sample was taken. Only sensitized TSM showed significant [^{14}C] release after histamine indicating release of significant amounts of acetylcholine.

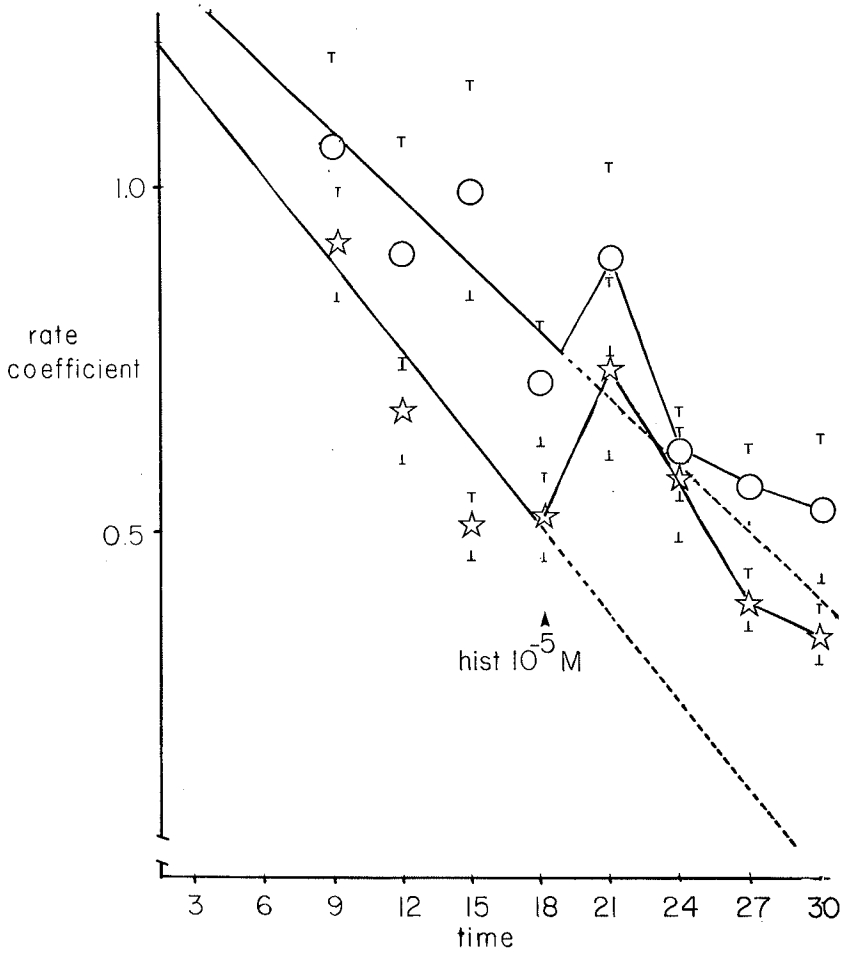
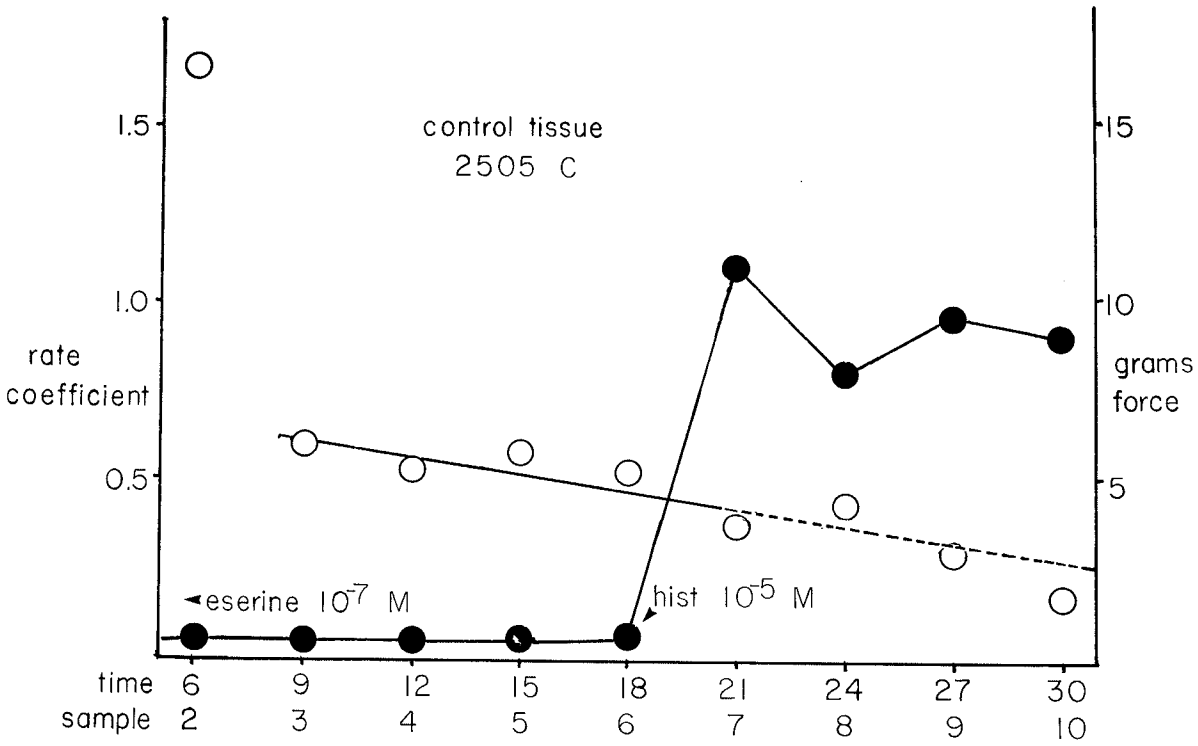


Figure 22: A similar plot of expected (dashed lines) and actual (open circles) control tissue [^{14}C] rate coefficient as in Figure 21 with a typical mechanogram superimposed (filled circles). Eserine (10^{-8} M) was used to prevent reuptake of [^{14}C]-choline by the parasympathetic nerves in the preparation. Histamine (10^{-5} M) produced a strong contracture, however no significant increase in rate coefficient was determined indicating an inability for histamine to release acetylcholine in littermate control TSM.



Sensitized samples 7 through 10 (for n=25 experiments) were all significantly greater than their respective theoretical means with t values ranging from 2.497 for sample 7 to 9.278 for sample 10. A typical mechanical trace for a sensitized TSM is shown in Figure 23. Note, after the introduction of eserine, that SBLA begins and that the contraction to 10^{-5} M histamine is sustained even after washouts 7 - 10.

Ragweed pollen extract was substituted for histamine in 14 experiments with ragweed sensitized TSM. The results are shown in Figure 24 along with a typical mechanical record. Sample 7 was not statistically different from the hypothetical mean (t=1.084), however samples 8 (t=2.626), 9 (t=3.876) and 10 (t=4.084) were significantly different. These results followed the mechanical profile in which there is a 3-5 minute time delay between the introduction of RWX and the initiation of the mechanical response.

If no agonist was added (n=5) the rate coefficients followed the estimated means. The addition of 23 mM K^+ produced a 500% increase in $[^{14}C]$ overflow (n=2) with a concomitant rise in resting tone in the muscles tested.

These results indicated that histamine or ragweed pollen extract can release significant quantities of acetylcholine which can contribute to the overall bronchospasm associated with asthma.

i) Acetylcholinesterase activity estimates.

Previous results indicated that reduced activity of acetylcholinesterase may play a role in the hypersensitivity

Figure 23: A plot of theoretical (dashed lines) and actual (open stars) sensitized tissue [^{14}C] rate coefficient as in Figure 21 with a typical mechanogram superimposed (filled stars). Note that after the introduction of 10^{-8} M eserine SBLA begins and that the contraction to 10^{-5} M histamine is sustained even after washouts 7-10, analogous to the sustained high rate coefficient for [^{14}C] loss from the sensitized TSM strips.

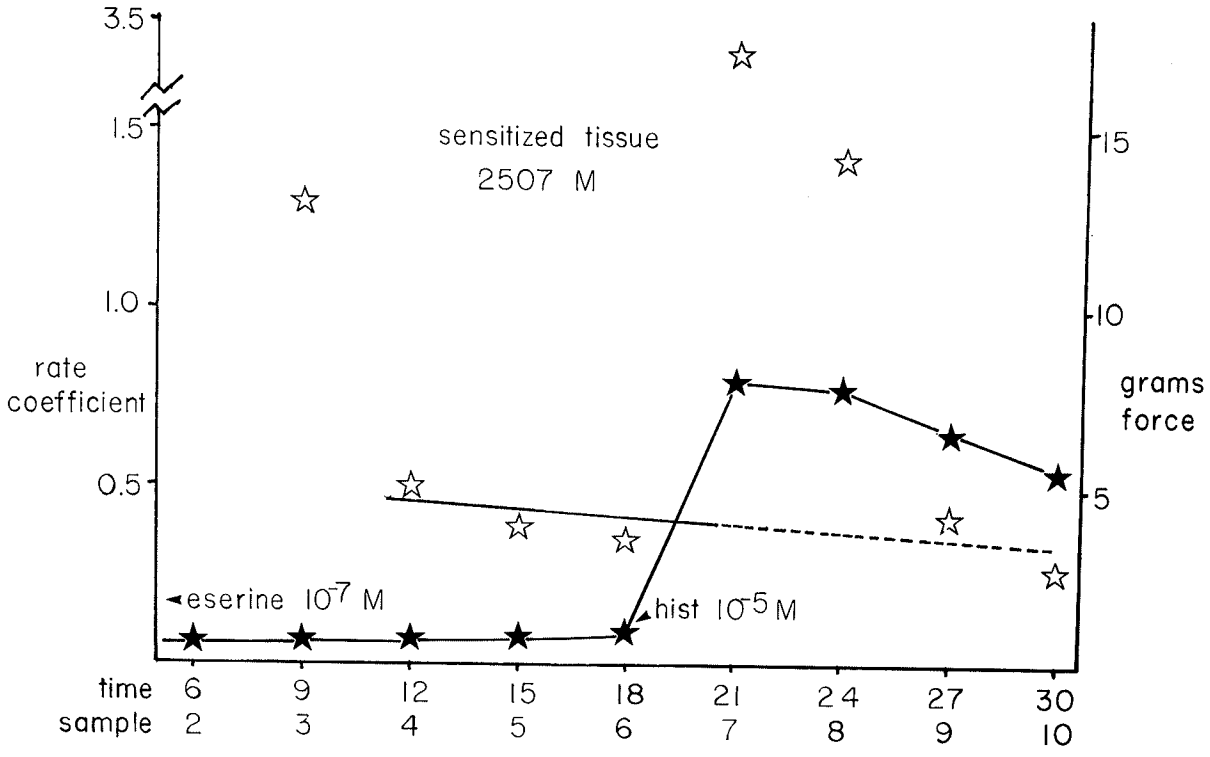
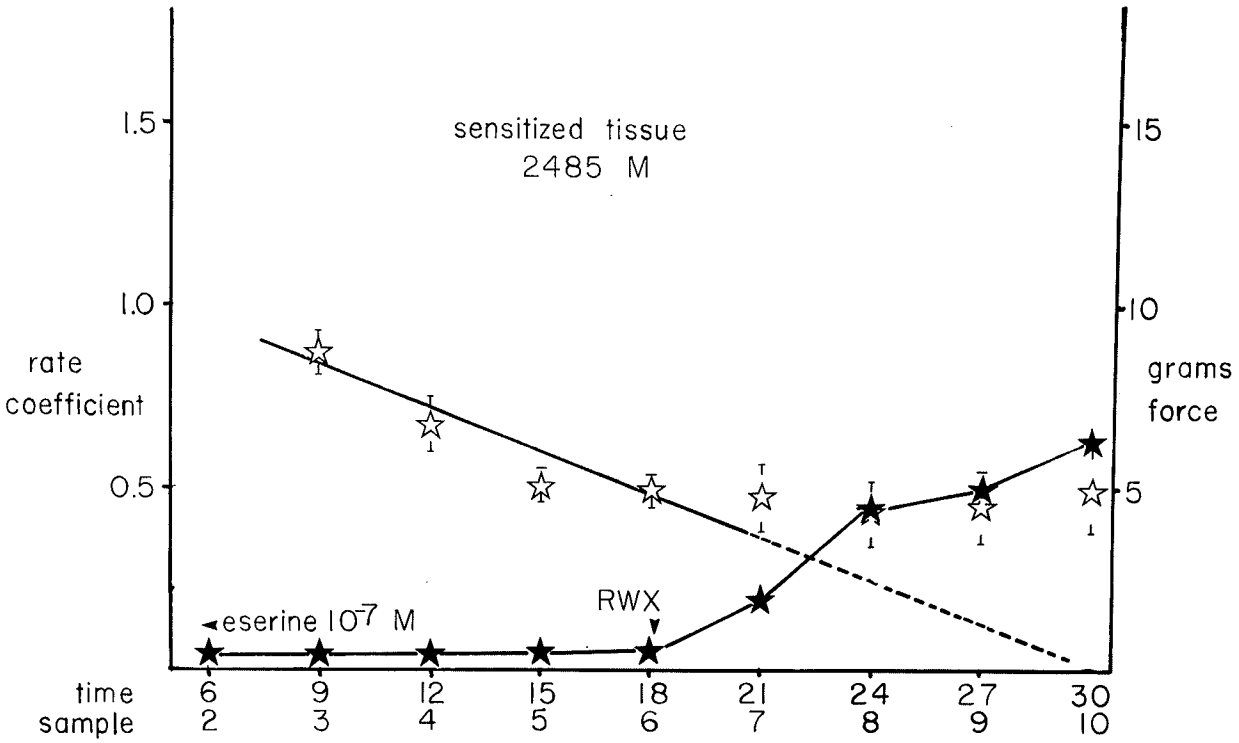


Figure 24: RWX was substituted for histamine in n=14 experiments with ragweed sensitized TSM. The theoretical (dashed lines) and measured (open stars) rate coefficients were plotted against sample time with a typical mechanical record of the Schultz-Dale response superimposed (filled stars). After a time delay peculiar to the Schultz-Dale reaction statistical differences from the expected mean were observed for [^{14}C] release. These results followed the mechanical profile suggesting RWX stimulation of sensitized TSM releases acetylcholine from the parasympathetic nerves in the preparation.



and hyperreactivity of sensitized canine TSM strips to histamine and increased external K^+. To test this hypothesis more directly cholinesterase activity was measured in control and sensitized TSM homogenates using the method of Ellman et al. (1961) which has been described previously. Typical results are shown for control homogenates in Figure 25. The increase in absorbance with time is indicated by the slopes of the spectrophotometric traces where the greater the slope - the greater the rate of production of 2-nitro-5-mercaptobenzoate - the greater the cholinesterase activity. It can be observed that the cholinesterase inhibitor eserine (10^{-7} M) almost completely inhibits cholinesterase activity and that 10^{-4} M histamine depresses cholinesterase activity in control homogenates.

Table 4 presents the data accumulated from paired experiments which determined control and sensitized tissue homogenate cholinesterase activities under various experimental conditions or treatments. The data are expressed as a percent of initial control determinations (absorbance units per minute) on any given day since all experiments were paired littermate control to sensitized dog. This standardization method protected the data from large deviations from the mean which could be contributed by genetic factors between litters, the age of substrates and reagents used in the determinations, and day to day variability in the procedures.

Homogenates of tissues from sensitized animals (n=20) consistently showed reduced cholinesterase activity when compared with littermate controls. This reduction was significant when analyzed using Duncan's (1955) new multiple range test.

Figure 25: Superimposed typical results are shown for control homogenates of TSM for absorbance vs time. The increase in absorbance with time is indicated by the slopes of the spectrophotometric traces where the greater the slope - the greater the rate of production of 2-nitro-5 mercapto-benzoate - the greater the cholinesterase activity. Eserine (10^{-7} M) almost completely inhibits cholinesterase activity and histamine (10^{-4} M) depresses cholinesterase activity in this control homogenate.

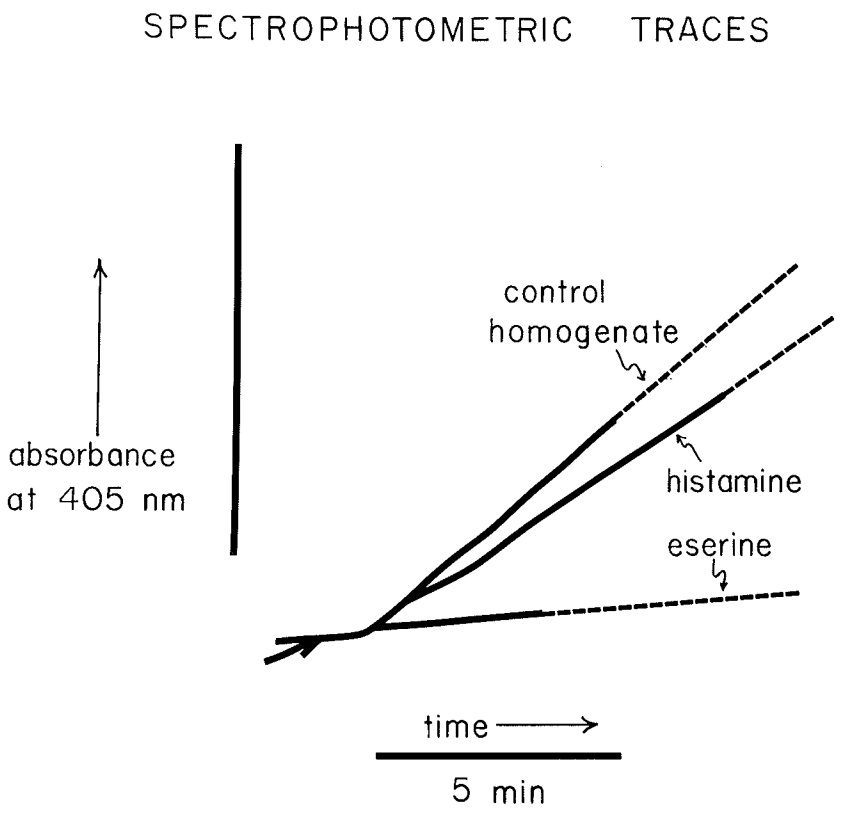


Table 4: Control and sensitized TSM homogenate cholinesterase activity data derived from spectrophotometric analysis such as in Figure 25 with substrate alone or in the presence of 10^{-4} M histamine or 10^{-7} M eserine.

	SUBSTRATE ALONE	10^{-4} M HISTAMINE	10^{-7} M ESERINE
CONTROL	98.5 ± 2.4 (20)	81.1 ± 11.6 (8)	8.6 ± 2.4 (6)
SENSITIZED	82.0 ± 4.3 (20)	75.2 ± 8.6 (6)	5.7 ± 2.8 (5)

- values are expressed as a percent of initial control values (substrate alone) (n)

Histamine (10^{-4} M) significantly reduced control cholinesterase activity (n=8), but had no significant effect on sensitized TSM homogenates (n=6) cholinesterase activity. Eserine (10^{-7} M) was equipotent in reducing both sensitized and control homogenate cholinesterase activity to 5.7±2.8% (n=5) and 8.6±2.4% (n=6) of control values respectively. Because of the natural colour of ragweed pollen extract and its high absorbance at 405 nm, it unfortunately could not be used in these experiments because of the low signal to background noise that it produced in the spectrophotometric cuvette.

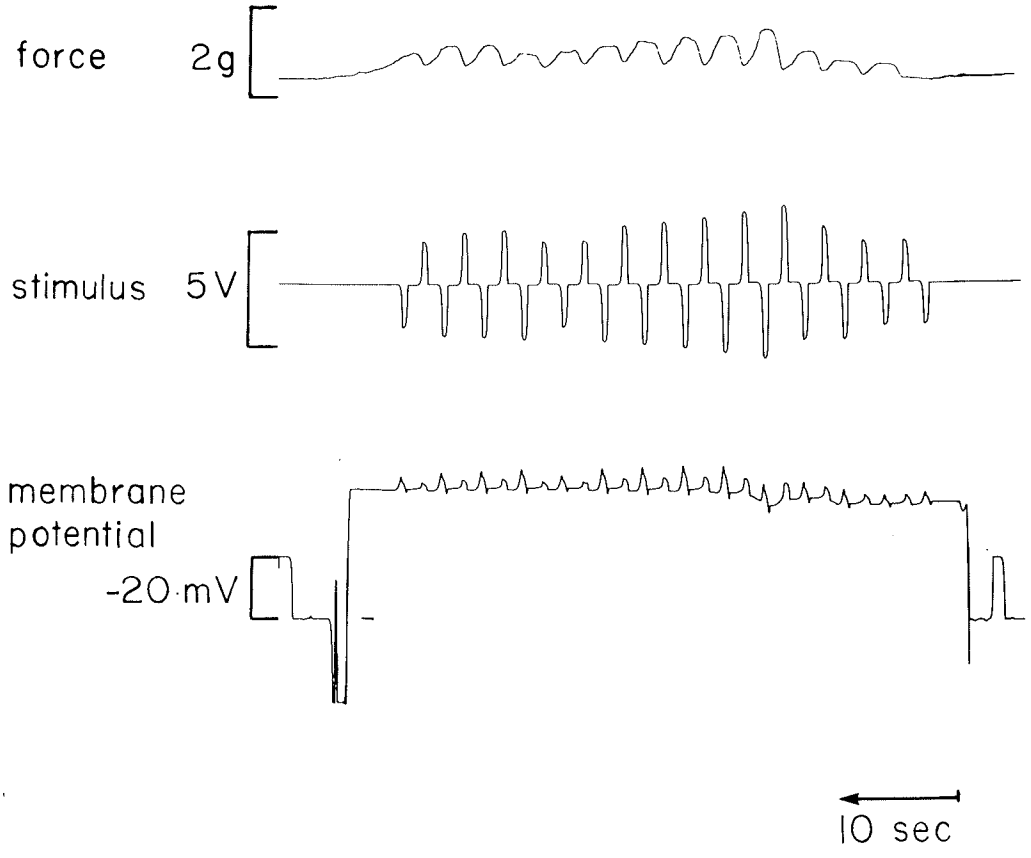
j) Resting membrane potentials. (RMP)

The data presented above indicate that the sensitization process may reduce cholinesterase activity and further suggest that the increased histamine levels associated with allergic bronchospasm may play a role in this reduction. With both a possibility for increased basal release and a decreased deactivation rate for acetylcholine, it seemed likely that there could be an increased concentration of this neurotransmitter at the neuromuscular junctions in sensitized TSM strips which in turn may be responsible for the SBLA seen only in sensitized preparations. One would suspect then that there should be changes in the RMP of sensitized TSM since acetylcholine is known to depolarize canine TSM. Therefore RMP's were determined for control and sensitized TSM using Ling-Gerard type floating microelectrodes.

A typical intracellular recording is shown in Figure 26. This recording met the criteria for a successful impalement. The

Figure 26: A typical intracellular recording of the membrane potential of a control TSM cell showing the criteria for a successful impalement; 1) a sharp negative deflection in potential, 2) the potential attained (-41.5 mV) remains steady for several seconds, and 3) upon withdrawal of the microelectrode the original baseline was attained. Two 20 mV calibrating pulses can be observed before and after the impalement.

Superimposed on the membrane potential are fluctuations caused by alternating depolarizing and hyperpolarizing stimuli. Concomitant mechanical activity is also shown.



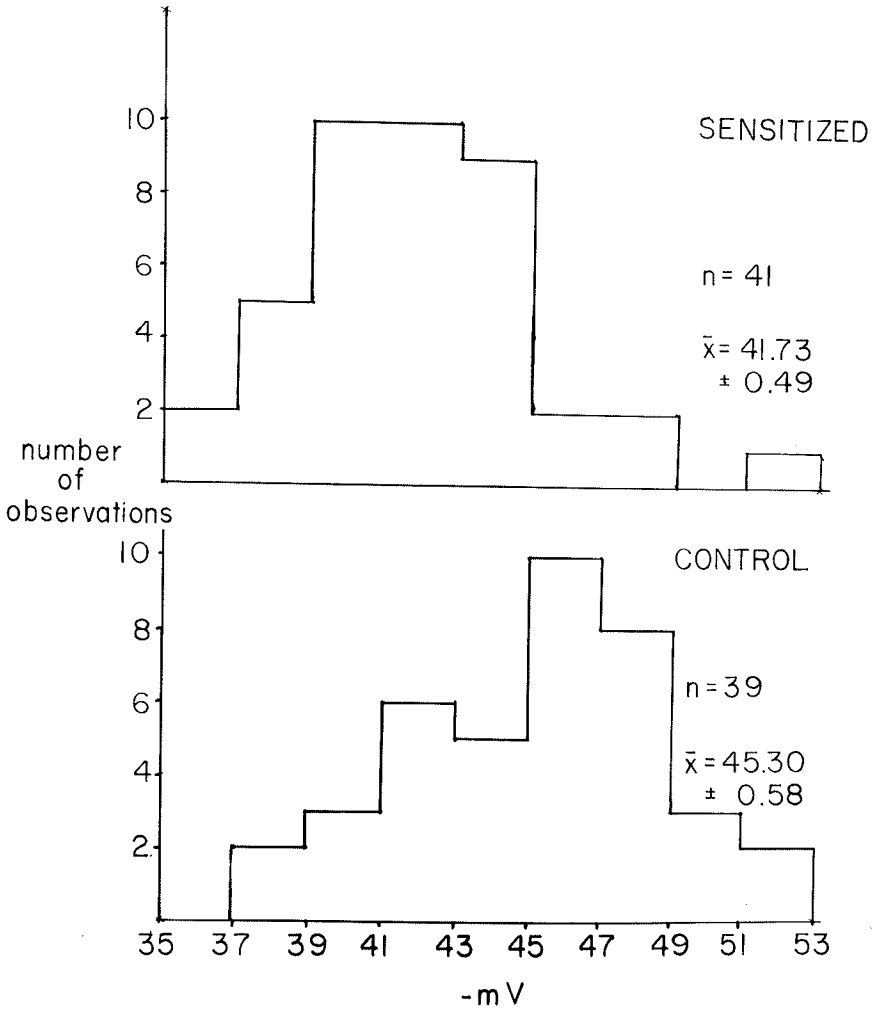
impalement is indicated by a sharp negative deflection in potential. The potential attained remains steady for several seconds. (Superimposed on the membrane potential (-41.5 mV) are fluctuations in the RMP caused by the alternating depolarizing and hyperpolarizing stimuli. Concomitant mechanical activity is also shown.) The final criterion for a successful impalement is also met in that upon withdrawal of the microelectrode the original baseline was unchanged. Two 20 mV calibrating pulses can be observed before and after the impalement.

The results of this study are summarized in Figure 27. For 39 impalements of 7 TSM strips from 7 littermate control dogs the mean RMP was 45.30$\pm$0.58 mV. For 41 impalements of 7 TSM strips from 7 ragweed sensitized dogs the mean RMP was 41.73$\pm$0.49 mV. The sensitized TSM cells were significantly depolarized (t=4.714) compared to control TSM RMP. The two histograms in Figure 27 indicate that both populations of cells had RMP's that were normally distributed throughout the range of the data.

k) A survey of reactivity at different loci along the airway.

Strips of upper and lower trachealis muscle and rings of main stem (primary) and third generation (tertiary) bronchial muscle from sensitized and control canine airways were assessed for their reactivity to 127 mM K^+, 10^{-4} M histamine, 10^{-4} M acetylcholine, 10^{-5} M serotonin, and 75 second sustained supramaximal 60 Hz electrical stimulation. Due to the great variability in responses to low doses of these agonists, probably a result of the degrees to which different segments of the airways are infiltrated with connective tissue, full dose-

Figure 27: A histogram comparison of the RMP's recorded from sensitized and littermate control TSM cells. The mean of the sensitized RMP was significantly depolarized compared to control RMP values using an unpaired t-test. Both sets of data were normally distributed.



response curves were not performed. All preparations were stimulated by these maximal doses of all the agonists with a one hour re-equilibration between agents during which time several rinses of fresh Krebs-Henseleit solution were made. The responses to electrical stimulation were recorded at least one hour after the beginning of a 5 minute tetanus interval regimen.

The results from upper and lower trachea are expressed in gm/cm^2 whereas those for the primary and bronchial rings are expressed as a percentage of the response of that tissue to 127 mM K^+ for normalization purposes. Upper and lower TSM responses are also expressed as a percentage of their respective responses to 127 mM K^+ for comparison. The results are shown in Table 5 and include data from a normal "street" population of dogs acquired from the local pound.

i) Responses to 127 mM K^+.

Consistent with the data of fig. 9, the sensitized upper trachea showed significantly increased reactivity to elevated external K^+ producing 2115 ± 181 g/cm^2 of force compared to 1836 ± 63 g/cm^2 for control upper TSM. However, no significant difference could be detected in the response of lower TSM with control and sensitized values being 1924 ± 89 g/cm^2 and 2022 ± 43 g/cm^2 respectively. No comparison was possible from tissues below this level because of a lack of method for normalizing the responses. The responses of primary and tertiary control and sensitized bronchi were considered as 100% for comparison of other agents.

ii) Responses to 10^{-4} M histamine.

The sensitized airways showed significant increases in reactivity to histamine throughout. Sensitized upper TSM

Table 5 Responses of control and sensitized muscles to maximal doses of contractile agonists and electrical stimulation at various loci of the upper respiratory tree.

CONTROL	127 mM K^+	10^{-4} HIST	10^{-5} 5-HT	10^{-4} ACh	e^- MAX	e^- 75 sec
UPPER TRACHEA	1836±63 (100%) n=25	1406±110 (76±5) n=25	1810±109 (99±5) n=26	2335±107 (127±4) n=25	1894±79 (103±3) n=15	1497±121 (80±5) n=14
LOWER TRACHEA	1924±89 (100%) n=26	1358±135 (71±6) n=26	1732±89 (92±4) n=26	2348±134 (123±5) n=26	2042±120 (100±3) n=15	1779±135 (86±3) n=15
PRIMARY BRONCHUS	(100%) n=24	(93±10) n=23	(71±8) n=23	(136±11) n=24	(119±6) n=13	(68±5) n=13
TERTIARY BRONCHUS	(100%) n=23	(113±12) n=22	(97±10) n=23	(156±13) n=23	(113±5) n=13	(64±6) n=13
SENSITIZED						
UPPER TRACHEA	2115±181* (100%) n=8	2060±192* (103±7)* n=8	2244±249* (106±8) n=8	3041±289* (143±3)* n=8	2049±128 (103±3) n=6	1877±153* (95±5)* n=6
LOWER TRACHEA	2022±93 (100%) n=8	1876±168* (92±5)* n=8	2043±204 (102±9) n=8	2870±129* (142±3)* n=8	2055±140 (103±3) n=6	1857±141 (93±4) n=6
PRIMARY BRONCHUS	(100%) n=8	(137±13)* n=8	(105±7)* n=8	(197±22)* n=8	(118±8) n=6	(74±3) n=6
TERTIARY BRONCHUS	(100%) n=8	(150±13)* n=8	(138±7)* n=7	(196±9)* n=8	(121±8) n=6	(88±5)* n=6

All asterisks indicate significant differences at $p<.05$.

produced 2060±192 g/cm^2 of force compared to 1406±110 g/cm^2 for control upper TSM for a single dose response. Sensitized lower TSM produced 1876±168 g/cm^2 compared to lower control TSM values of 1358±135 g/cm^2. Sensitized primary and tertiary bronchi produced 137±13% and 150±13% of their respective 127 mM K^+ contractions to this same dose of histamine compared to control values of 93±10% and 113±12% respectively.

iii) Responses to 10^{-5} M serotonin.

Sensitized airways showed results similar to those using serotonin in comparison with control airways throughout. However, as can be seen in Table 5, no significant difference could be detected in the responses of lower TSM from control and sensitized dogs, although the trend in the data suggests such a difference may exist. The variability of the responses from sensitized animals is probably the cause of the non-significance of this difference.

iv) Responses to 10^{-4} M acetylcholine.

The mechanical responses of all levels of sensitized airways studied were statistically hyperreactive to acetylcholine in comparison with control airways. Sensitized upper TSM produced 3041±289 g/cm^2 of tension in response to a single maximal dose of acetylcholine compared with 2334±107 g/cm^2 in control upper TSM. Sensitized lower TSM produced 2870±129 g/cm^2 compared with 2348±134 g/cm^2 in control lower TSM. The primary and tertiary bronchi from sensitized airways were also hyperreactive to exogenously applied neurotransmitter producing 197±22% and 196±9% of the force produced by 127 mM K^+ respectively. Control primary

and tertiary bronchi only produced 136±11% and 156±13% of their respective 127 mM K^+ control contractions by comparison.

v) Responses to 60 Hz electrical stimulation.

Sensitized and control airways showed no significant differences in their respective responses to supramaximal electrical stimulation of 10 to 15 sec. duration as can be seen from the data presented in Table 5. However, when the stimulus was prolonged to 75 sec., significant differences were observed in upper TSM and tertiary bronchi. Sensitized upper TSM maintained a force of 1877±153 g/cm^2 compared to 1497±121 g/cm^2 for control upper TSM, and sensitized tertiary bronchi maintained a force equal to 88±5% of their responses to 127 mM K^+ whereas control tertiary bronchi could only maintain 64±6% of the tension they produced to 127 mM K^+. Neither the lower TSM nor the primary bronchi of sensitized dogs showed any significant differences in their responses to prolonged electrical stimulation compared with control values.

1) Systemic anaphylaxis.

Since Kong and Stephens (1981) reported a Schultz-Dale type of reaction in pulmonary veins from actively (in vivo, ovalbumin) sensitized dogs, it was apparent that the airways were not the only smooth muscle containing conduits that would contract in response to antigen challenge. It would be of particular interest to determine the effects of antigen challenge on those other smooth muscles that are cholinergically innervated. Therefore a cursory examination of other smooth muscles was undertaken from dogs sensitized to ragweed pollen extract.

i) The airways.

All levels of the airways investigated reacted to antigen challenge with mechanical responses although they varied in intensity, duration, and overall shape of the contracture. Figure 28 shows some typical responses from several levels of canine airways including parenchymal strips from ragweed sensitized dogs. The upper and lower trachea were particularly sensitive to atropine (10^{-7} M) in addition to pyrilamine maleate (10^{-7} M), however, the tertiary bronchus produced a transient response that appeared not to be affected by atropine or pyrilamine maleate. Parenchymal strips produced a small contraction that was unresponsive to atropine, pyrilamine maleate, the leukotriene antagonist FPL55712 (10^{-5} M), the alpha-adrenoceptor antagonist phentolamine (5×10^{-7} M), nor could the contraction be washed out easily. Only isoproterenol, the beta-adrenoceptor agonist, could relax the parenchymal strip preparation.

ii) Systemic anaphylactic reactions.

Figure 29 shows some typical results obtained for other smooth muscles from the ragweed sensitized dog. Responses from a hepatic artery, ureter and longitudinal muscle of the gut are shown. The hepatic artery shows a prolonged transient response to ragweed pollen extract, whereas the ureter responds with the initiation of fast phasic spike-like contractures that eventually cease. Gut muscle responded by increasing the amplitude of spontaneous phasic activity in addition to a relatively large increase in basal tone. The basal tone subsided somewhat,

however, the amplitude of the spontaneous contractions remained elevated.

Figure 28: Typical Schultz-Dale responses from 4 loci of the airways from a ragweed-sensitized dog upon the injection of RWX (final bath concentration 0.3 mg/ml). See text for details.

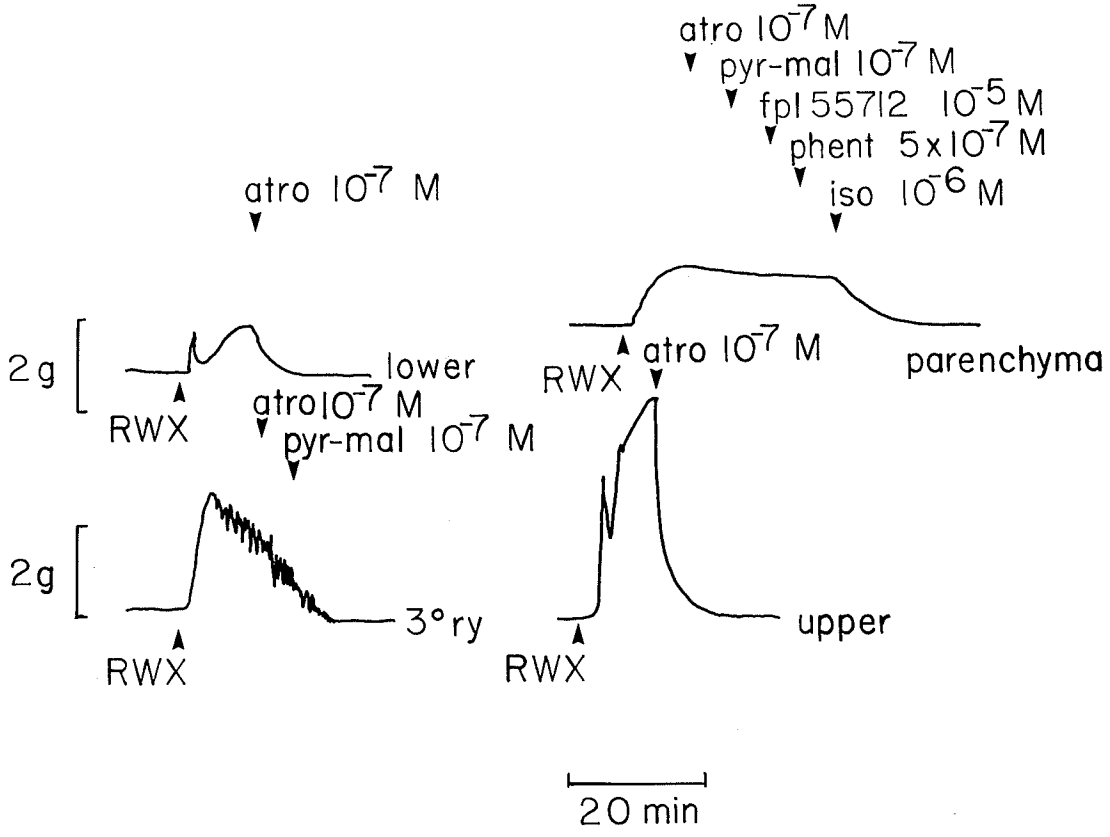
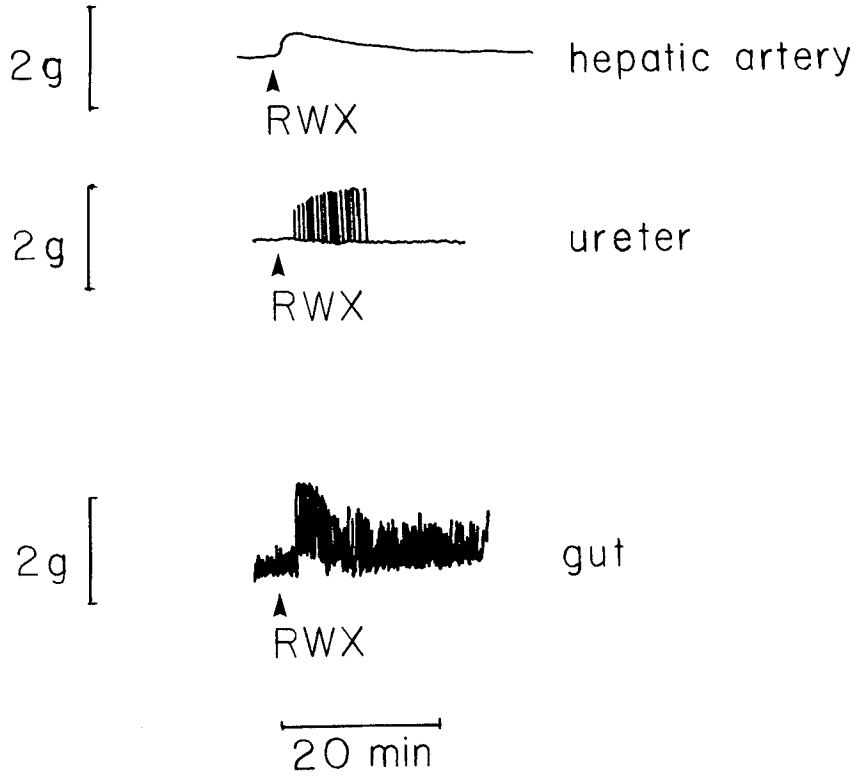


Figure 29: Schultz-Dale responses from the hepatic artery, ureter, and longitudinal gut smooth muscle from a ragweed sensitized dog upon the injection of RWX into the tissue bath.



DISCUSSION

a) Schultz-Dale reaction.

TSM strips from ragweed-sensitized dogs or TSM strips passively sensitized to ragweed pollen in vitro contracted when subsequently challenged with the sensitizing antigen in vitro - the Schultz-Dale reaction. The mechanism by which the contraction is elicited has been presumed to be through the release of mediators of anaphylaxis from degranulating mast cells after the antigen-antibody reaction has taken place on the mast cell surface. The list of potential mediators includes serotonin, platelet activating factor and eosinophil chemotactic factor of anaphylaxis but by far the major mediators released are histamine and slow-reacting substances of anaphylaxis (SRS-A - leukotrienes C_4 and D_4) in the human.

The time course of the Schultz-Dale reaction in this ragweed sensitized canine model of allergic bronchospasm is qualitatively similar to the early phase or immediate bronchoconstrictor response seen in allergic patients after specific antigen challenge. It peaks within a few minutes and abates spontaneously after some time. However, no late phase response is observed in isolated TSM strips.

Ragweed-sensitized strips of canine TSM showed antigen specificity in that a Schultz-Dale response was not elicited when the muscles were challenged with either ovalbumin or bovine serum albumin. Meyers et al. (1973) and Gold et al. (1977) have shown that the major mediator released in dogs is histamine. Pre-incubation of sensitized TSM with the H_1 histamine receptor antagonist pyrilamine-maleate (10^{-7} M) prevented 95% of the contraction induced by antigen challenge suggesting that

histamine is the major mediator. At this dose of antihistamine no non-specific effects have been reported. Antonissen et al. (1980) have shown that in an ovalbumin sensitized canine model, pyrilamine-maleate given at the plateau of tension production after antigen challenge fully relaxed the contraction, however, this was not the case for ragweed sensitization.

Atropine (10^{-7} M) was equipotent in preventing force development upon antigen challenge (Fig. 6) and appeared to be more effective in reversing the Schultz-Dale reaction once it was initiated in in vivo sensitized TSM. These results would suggest that where histamine is still released from degranulating mast cells upon specific antigen challenge its site of action may not only be H_1 contractile receptors on the TSM cell membrane but may also promote the release of acetylcholine from parasympathetic neural elements present in the isolated preparation. Atropine sensitivity of the Schultz-Dale response was observed in only in vivo sensitized TSM strips. Atropine could not reverse the Schultz-Dale response of in vitro sensitized TSM (Fig. 5b) whereas pyrilamine-maleate was a potent relaxing agent for in vitro sensitized Schultz-Dale reactions.

Secondary, slow rises in basal tone associated with SRS-A release from degranulating mast cells were not observed up to 6 hours after challenge of TSM with ragweed pollen extract. This indicated a lack of leukotriene release upon specific antigen challenge or a lack of effectiveness of exogenously administered leukotrienes as Krell et al. (1981) have demonstrated in rats, cats and dogs with exogenously administered SRS-A. However, Krell's findings cannot rule out SRS-A release nor can a lack of

effect of SRS-A be ruled out since Tesarowski et al. (1981) and Tesarowski and Kroeger (1982) have adduced evidence for a contractile effect of endogenously derived SRS-A in the dog.

The Schultz-Dale reaction could be elicited only once per sensitized TSM strip in vitro indicating all mast cells in the preparation were affected by the challenging procedure.

b) Histamine dose-response relationships.

With histamine indicated to be the major mediator of anaphylaxis released upon antigen challenge in this model of allergic asthma it became important to determine its effect upon TSM tone. To this end, dose-response curves for histamine were elicited from a series of TSM strips from ragweed sensitized and littermate control dogs. The data revealed that sensitized TSM was both hypersensitive (leftward shift of the ED_{50} of the dose-response curve) and hyperreactive (greater force production at all concentrations of agonist) to histamine stimulus (Fig. 7).

The hypersensitivity of in vivo ragweed sensitized TSM to histamine was reduced when sensitized TSM strips were preincubated with 10^{-7} M atropine, as demonstrated by the statistically significant rightward shift of the histamine dose-response curve. There was no significant effect of atropine on reactivity. Atropine treated sensitized TSM contracted with the same reactivity to a maximum dose of histamine as untreated sensitized TSM. These results paralleled recent in vivo work by Shore et al. (1985) who showed a similar decreased sensitivity without any apparent change in reactivity to aerosolized histamine of dogs pretreated with atropine. Hulbert et al. (1985)

have shown similar in vivo results in the guinea pig. There was no significant rightward shift in control TSM responses to histamine in the presence of atropine nor any significant change in reactivity.

According to van Rossum (1968) dose-response curves reflect receptor affinity and intrinsic activity of a drug. If two tissues stimulated by the same drug exhibit displaced curves with equal slopes and maximal responses then it can be concluded that the receptor agonist binding must be capable of similar cellular effects but that the receptor affinities for the drug by the two tissues is different. However, the results obtained in this study do not reveal a parallel displacement between sensitized and control TSM histamine dose-response curves. The displaced slope and greater maximal tension to histamine suggest two theoretical possibilities; where the curves obtained coincide with theoretical curves for drugs, 1) with the same affinity constant but with different intrinsic activities or 2) with agonist action in which one curve represents its action on an intact receptor population and the other curve its action after inactivation of a certain fraction of these receptors.

For the data in Fig. 7 the results may be interpreted to mean that sensitized TSM possesses histamine receptors with greater intrinsic activity but the same affinity, or an increased number of histamine receptors with the same affinity and intrinsic activity but which produce an additive cellular action i.e. greater tension development. The second possibility would seem more likely in view of the histamine dose-response data and makes the location of additional receptors critical to the

mechanism of allergic bronchospasm.

The effect of atropine on sensitized TSM (rightward shift in sensitivity without effecting the hyperreactivity) and the lack of any effect on control TSM suggests increased histamine receptors on sensitized TSM cells and on the parasympathetic neural elements in the TSM preparation. Therefore, histamine could cause an increased force production of sensitized TSM contractions via direct stimulation of increased muscle histamine receptors and via acetylcholine release due to stimulation of histamine receptors residing pre-synaptically. From the results in Fig. 7 and Table 2 the release of acetylcholine is most significant in low doses of histamine since atropine has its greatest effect at these doses and may account for the leftward shift (increased sensitivity) of histamine dose-response curves from sensitized TSM.

Alternatively, these results might be obtained if histamine works synergistically with increased basal levels of acetylcholine either through increased neural release or decreased degradation via inhibition of acetylcholinesterase. The increase in neurotransmitter concentration in the synapse could depolarize sensitized TSM cells through receptor operated calcium channels and thereby make available, through voltage sensitive channels, more calcium for histamine induced contraction.

In vitro passive sensitization of control TSM strips with sensitized serum produced muscles that reacted to ragweed challenge and were hyperreactive but not hypersensitive to histamine when dose-response curves were compared to TSM

incubated with control serum, 10^{-6} M serotonin, or to time controls (Fig. 8). The lack of hypersensitivity in sensitized serum incubated TSM may be related to a lack of an intact nerve supply in the isolated TSM or to an insufficient incubation time since in vivo, the sensitization process takes several weeks to months to complete. The possibility of the need for an intact nerve supply is particularly interesting when one considers the different effects of atropine and pyrilamine maleate on the Schultz-Dale reactions of in vivo and in vitro sensitized TSM. In vivo sensitized TSM only partially relaxed in response to pyrilamine-maleate antagonism (Fig. 6c) and were very sensitive to atropine antagonism whereas in vitro sensitized TSM challenged with RWX were unaffected by atropine blockade (Fig. 5b) but were extremely sensitive to the antihistamine (Fig. 5c). Here again, the alteration in the histamine dose-response curve with passive sensitization suggests either increased histamine receptors without changes in affinity/intrinsic activity or increased intrinsic activity of the histamine receptors already present without any effect on affinity.

c) $\underline{K^+}$ dose-response relationships.

The conclusion that histamine or the sensitization process may promote increased acetylcholine release is supported by the responses to K^+. The addition of excess K^+ into the physiological salt solution bathing the TSM not only depolarizes the muscle cell membranes thus producing tension through voltage sensitive Ca^{++} channels, (Coburn, 1979) but also depolarizes the parasympathetic nerve net in the preparation, thereby releasing

acetylcholine. The acetylcholine, if not hydrolyzed by acetylcholinesterase, would be available to produce tension in the muscle through the acetylcholine receptor operated Ca^{++} channels.

In control TSM the amount of acetylcholine released is either insignificant or of a concentration that could be managed (broken down) by acetylcholinesterase since no differences were observed in either sensitivity or reactivity of control TSM strips to K^{+} whether they were in the presence or absence of atropine (Fig. 9). However, in sensitized TSM preparations, atropine had a significant effect on the hyperreactivity and hypersensitivity observed in the presence of elevated K^{+} levels. In the presence of atropine (which specifically blocks the acetylcholine receptor) the sensitized TSM dose-response curve is not different from control curves either in reactivity or sensitivity as indicated by similar maximum responses and ED_{50} values. These findings suggest that the in vivo sensitizing process either induces the basal release of excess acetylcholine or by some mechanism inhibits the rate of breakdown of acetylcholine by its esterase.

d) Serotonin dose-response relationships.

Serotonin may have actions at several foci in the airways and may exert a multiplicity of effects in allergic bronchospasm. It is a potent airway smooth muscle constrictor directly and is an activator of reflex bronchoconstriction. Sheller et al. (1982) observed enhanced vagally stimulated contractions in the presence of serotonin in canine airways in vivo and suggested an

interaction between serotonin and efferent cholinergic fibres to airway smooth muscle as well.

Serotonin stimulates contraction in airway smooth muscle directly through voltage-sensitive calcium channels; (the same mechanism mobilized by increased $[K^+]_{out}$; Coburn, 1979). It was felt that K^+ dose-response relationships could better elucidate possible mechanisms since K^+ is not oxidized in the tissue bath whereas serotonin in particular has a short lifespan in the muscle bath at 37^{o} C at a pH of 7.4 and is quickly oxidized.

e) Acetylcholine dose-response relationships.

Sensitized TSM showed hyperreactivity to acetylcholine and a hypersensitivity as indicated by ED_{50} values compared to control TSM (Fig. 11). These findings are consistent with interpretations that either the same numbers of agonist receptors of equal affinities are present but the efficacy or intrinsic activity is greater or in which there are increased numbers of receptors with the same efficacy and affinity. In view of the data from histamine, K^+, and serotonin dose-response curves, it is likely that the number of acetylcholine receptors is the same between control and sensitized TSM but that the efficacy of those receptors in sensitized TSM may have been increased by the reduced ability of acetylcholinesterase to hydrolyze the neurotransmitter to its inactive metabolites either through reduced enzyme content or reduced activity. This resultant increased intrinsic activity of acetylcholine receptors with cholinesterase inhibition is paralleled clinically where patients

with myasthenia gravis are given the cholinesterase inhibitor neostigmine to improve skeletal muscle tone by increasing the efficacy of the reduced number of receptors they have for the neurotransmitter. However, it is possible that alterations in excitation-contraction coupling or in the contractile proteins of sensitized TSM may manifest themselves by an apparent increase in the efficacy of pharmacological agents. This would be particularly true of agents which mobilize intracellular Ca^{++} stores such as histamine and acetylcholine. Intracellular organelles such as the sarcoplasmic reticulum and mitochondria may play a role in the resequestration of these intracellular stores.

Antonissen et al. (1980) reported no observable differences between ovalbumin- sensitized and littermate control TSM dose-response relationships to carbamylcholine (carbachol). This could indicate that different sensitizing antigens have different pathophysiological effects, but more likely, carbachol, which is broken down only very slowly by acetylcholinesterase and thus offers the same efficacy to acetylcholine receptors, may have masked any differences Antonissen et al. might have observed had they used acetylcholine.

The inability to observe a shift in the threshold dose of sensitized TSM to acetylcholine stimulation may be a result of the cholinesterase enzyme's ability to handle low doses of the neurotransmitter. This would be consistent with the data presented in Fig. 11 in which only at doses greater than 10^{-7} M acetylcholine were significant differences found in that there was an increased reactivity of sensitized TSM compared to control

values.

The results of dose-response studies to histamine, K^+, serotonin, and acetylcholine discussed above are consistent with the possibility of increased acetylcholine at or near its receptor site. Agonists such as K^+, when in the presence of atropine, show no differences in dose-response relationships between sensitized and control TSM. Serotonin and K^+ produce contraction in TSM by allowing entry of Ca^{++} through voltage sensitive channels (Coburn, 1979). Verapamil or D-600 inhibits these channels, prevent Ca^{++} entry and thus inhibit contraction. Therefore, it appears that voltage sensitive Ca^{++} channels are not responsible for the increased reactivity of sensitized TSM to all these agents in the absence of atropine. Acetylcholine may have a bound intracellular store of Ca^{++} at its disposal (Kroeger and Stephens, 1971) but in addition allows Ca^{++} entry through receptor operated Ca^{++} channels (Coburn, 1977). Verapamil or D-600 have little effect on acetylcholine induced contractions. The number of channels (or receptors) appears not to be altered by the sensitization procedure, however, their efficacy may be increased by a reduction in the ability to breakdown acetylcholine by acetylcholinesterase.

Histamine represents a hybrid in terms of its Ca^{++} mobilization activating both receptor operated and voltage sensitive Ca^{++} channels (Antonissen, 1978). The number of histamine receptors appears to be increased both pre- and post synpatically with in vivo sensitization resulting in increased reactivity on the muscle directly and increased sensitivity

through augmentation of the release of acetylcholine from parasympathetic neural elements in the preparation. This would be in addition to any supra-additive effects of histamine and acetylcholine (Mitchell and Bouhuys, 1976; Shore et al., 1983) at the muscle level.

f) Electrical stimulus-response and related studies.

Sensitized TSM showed a leftward shift in mechanical threshold voltage with respect to littermate control TSM. However, the maximum responses at supramaximal voltages were not different when the stimulus duration was held to 10 to 15 sec. which is the normal stimulus duration for control TSM. When the supramaximal stimulus was maintained for a period of 75 sec. significant differences were observed between the tension maintained by sensitized (1.865+0.122 kg/cm^2) and control (1.514+0.122 kg/cm^2) TSM.

Under normal conditions, canine TSM begins to relax even before the supramaximal stimulus is turned off. However, sensitized TSM maintains a prolonged plateau with a slowly rising active tension (Antonissen et al. 1979; present results). During stimulus-response studies the stimulus was turned off when the control tissues had begun to relax and the sensitized tissues had reached the slowly rising plateau stage. Therefore, differences in maximal responses were not observed. However, with prolongation of the stimulus a specific difference was unmasked in that sensitized TSM could maintain a greater force.

It was hypothesized that this increased maintained force was due to a reduction in the rate of acetylcholine breakdown by its

cholinesterase. Relaxation appeared unaffected when an analysis of the isometric mechanograms was performed. The time to 1/2 relaxation was not increased by the sensitization process which argued against Szentivanyi's (1968) beta-receptor blockade theory of asthma.

The electrical stimulus-response studies were plagued by the phenomenon of spontaneous baseline activity (SBLA) in sensitized TSM strips. Not all sensitized TSM displayed SBLA and those that did not were used for the stimulus response study. Those sensitized TSM that began to show SBLA were used to elicidate the origin of it. Control tissues did not display SBLA. SBLA could be "washed out" of the tissue baths with repeated rinsing; continued even after the regularly stimulated (5 min. interval) tetanic stimuli were terminated; were not affected by antihistamines (Fig. 14), alpha-adrenoceptor antagonists or ganglion blockers (Fig. 15b); but were abolished by atropine. These results pointed to post-ganglionic neural acetylcholine as the initiator of SBLA either through increased release or decreased hydrolysis as was hypothesized from the prolonged stimulus data.

To test the hypothesis of reduced cholinesterase activity in sensitized TSM, littermate control TSM were incubated with the cholinesterase inhibitor eserine (10^{-7} M) in an attempt to mimic the SBLA and prolonged tension plateau observed in sensitized TSM. Figure 17 shows typical results of such an intervention. The control muscles became spontaneously active as can be observed by the rise in basal tone between tetani. A concomitant increase in total force production was also observed which may be

eserine dose dependent since 10^{-8} M eserine (Fig. 15a) produced only a slight increase in total force production but produced SBLA much akin to the "naturally occurring" SBLA observed in sensitized TSM also in Fig. 15.

The effect of eserine on control prolonged mechanograms is seen in the lower series of mechanograms of Fig. 17. The initial 75 sec. stimulus appears much like that seen in Fig. 16 for control TSM while the 75 sec. stimulus after eserine treatment appears more like the sensitized mechanogram of Fig. 16. Therefore, inhibition of cholinesterase can induce responses in control TSM which are similar to sensitized tissue, and supports the hypothesis of reduced cholinesterase activity with sensitization.

Antonissen et al. (1979) have shown that no demonstrable differences existed between carbachol dose-response curves in ovalbumin sensitized and littermate control tissues. Carbachol is relatively unaffected by cholinesterase, and therefore should have the same efficacy for the acetylcholine receptor with the same intrinsic activity whether the TSM is from sensitized or control dogs and thus produce the same maximum forces (unlike acetylcholine). Carbachol dose-response curves (Fig. 18) for sensitized and control TSM revealed that this assumption was valid since no significant differences could be observed between maximum responses at or near the maximum doses applied. There was a significant leftward shift in the threshold dose of carbachol which may be accounted for by the additive effect of a low dose of carbachol in addition to the increased availabilty of

acetylcholine hypothesized above.

Acetylcholine dose-response relationships from control and sensitized TSM in the presence of a minimal dose of eserine (10^{-8} M) should then produce results similar to those in the presence of carbachol. The dose-response curves from such a study are shown in Fig. 19. In the presence of eserine, acetylcholine was equipotent in producing maximum responses of 3.002 ± 0.149 kg/cm^2 and 2.984 ± 0.265 kg/cm^2 for sensitized and control TSM respectively. These values were not significantly different from their respective values for carbachol maxima; 3.394 ± 0.235 kg/cm^2 and 3.167 ± 0.258 kg/cm^2.

The ED_{50} values were not significantly different from each other indicating control and sensitized TSM had similar sensitivities to acetylcholine in the presence of eserine.

These studies substantiate the hypothesis that in ragweed sensitized TSM the increased sensitivity and reactivity to various agents is a result of acetylcholinesterase inhibition. The inhibition of the breakdown of acetylcholine causes this neurotransmitter to build up in concentration and produce the effects observed of SBLA and prolonged isometric plateau tension. This is in addition to the hyperreactivity to histamine observed in sensitized TSM which was independent of the hyperreactivity observed to potassium, serotonin and acetylcholine.

The above conclusions does not preclude other possible mechanisms operating in allergic bronchospasm. These studies were done on isolated TSM and were independent of the irritant receptor bronchoconstrictor reflex (Nadel, 1965). Also, these

studies do not preclude the possibility of a histamine and acetylcholine supra-additive interaction at the TSM sarcolemma (Shore et al., 1983) or the possibility of histamine and muscarinic receptors opening the same receptor operated calcium channels (Bolton et al., 1981). Alpha-adrenergic receptor activation may be involved as well in allergic bronchospasm through increasing calcium entry into airway smooth muscle cells (Bergen and Kroeger, 1979;1980) via voltage sensitive calcium channels (Barnes et al., 1983). Beyond excitation of the membrane and the entry of calcium, contraction coupling and contractile proteins may be altered in such a way as to increase the response of the TSM cell to various excitatory stimuli. These areas have been discussed above.

g) Challenged vs non-challenged sensitized TSM.

All of the sensitized tissues used in the studies reported herein were not immunologically challenged (in vitro) previous to electrical or dose-response studies. Other TSM strips were challenged with resultant Schultz-Dale reactions but were not used for further studies. Souhrada and Souhrada (1984) have suggested, from their work with a single immunization sensitized guinea pig model, that the electrogenic sodium-potassium pump is affected by the sensitization process. Their data indicates that after immunization, the airway smooth muscle cells of the guinea pig are hyperpolarized with respect to controls by $\simeq$ 5 millivolts and that after challenge with specific antigen the membrane potential is depolarized by approximately 10 millivolts with respect to control values.

The canine ragweed sensitized model that was used in these

studies received booster injections (mini challenges) every two weeks to increase their PCA titres to a more sensitive (higher) level (Kepron et al., 1977) and according to their calculations, booster injections were in such low concentrations that they could not themselves have acted as inadvertent challenges. However, in the isolated TSM, all sensitized strips tested showed a Schultz-Dale reaction indicating that the mast cells were primed and responded with degranulation upon antigen challenge. The electrical and pharmacological data from the above studies indicate a possible depolarization of canine sensitized TSM. The hypersensitivity of sensitized TSM to increased $[K^+]_{out}$ indicates a shift to the left in the mechanical threshold, again suggesting depolarization with an associated rise in sarcoplasmic calcium. Weiss and Viswanath (1979) found increased sensitivity of resting isometric tension to extracellular calcium following in vitro anaphylaxis of guinea pig tracheal muscle.

In vivo sensitized strips of canine TSM were challenged in vitro and the dose-response relationship to increasing $[K^+]_{out}$ was compared to unchallenged sensitized TSM, and littermate control TSM (Fig. 20). As was observed previously, sensitized TSM were hypersensitive and hyperreactive to K^+ compared to control TSM. No significant differences could be observed between sensitized and challenged sensitized TSM either in reactivity or sensitivity as indicated from ED_{50} data analysis.

These findings, in addition to the results from K^+ dose-response curves in the presence of atropine (that sensitized and control TSM do not differ in the presence of the acetylcholine antagonist) argue against major changes in membrane potential due

to alterations in the electrogenic sodium pump with sensitization and challenge in the canine model of allergic bronchospasm.

h) [^{14}C]-choline release experiments.

The results obtained from histamine dose-response data in the presence and absence of atropine argue for increased release of acetylcholine through presynaptic histamine stimulation in sensitized TSM. To test this hypothesis more directly, sensitized and littermate control TSM were incubated with [^{14}C]-choline. Choline is a precursor for acetylcholine and is retaken up (50%) into the parasympathetic nerves. The uptake of [^{14}C]-choline was promoted by electrical stimulation to increase neurotransmitter turnover. The basal release was measured as a rate coefficient and bath samples were taken every 3 min. for liquid scintillation counting. Control and sensitized TSM were then stimulated by either histamine or ragweed pollen extract and the samples collected after stimulation were compared to expected values (Fig. 21).

Sensitized TSM strips showed significant sustained increases in [^{14}C]-choline release upon stimulation with either histamine or RWX although release by RWX was somewhat delayed, following a time course akin to the Schultz-Dale reaction. Control TSM strips showed no significant increases although a transient rise was noticed. This transient rise may have come about as an artifact of stimulation in that the increase in isometric force produced by histamine may have resulted in a exudation of part of the interstitial space (Krejci and Daniel, 1970) which probably contained significant amounts of label. However, sensitized TSM

showed significant sustained rises in radiolabel release upon either histamine or RWX stimulation.

The addition of 23 mM K^+ in a limited number of trials produced a 5 fold increase in [^{14}C] overflow consistent with K^+ working presynaptically to depolarize neural elements and promote the release of neurotransmitter. These results indicated that histamine or ragweed pollen extract can release significant quantities of acetylcholine which in turn can contribute to the overall bronchospasm associated with asthma. They also substantiate the interpretation of results from histamine dose-response studies. Shore et al. (1983) have suggested that release of acetylcholine may participate in the bronchoconstricting action of histamine even when the vagus nerves have been sectioned thus interrupting the irritant receptor-bronchoconstrictor reflex. Mitchell and Bouhuys (1976) have stated as well that the reflex theory cannot account for potentiation of histamine responses in isolated preparations. Douglas et al. (1973) have demonstrated in isolated tissues that atropine reduces and physostigmine (eserine) intensifies histamine-induced airway constriction in the guinea pig.

i) Acetylcholinesterase activity estimates.

To test the hypothesis that in vivo sensitization results in a decreased cholinesterase activity, control and sensitized TSM homogenates were measured spectrophotometrically for their rates of breakdown of acetylthiocholine substrate by the method of Ellman et al. (1961). Sensitized TSM homogenates consistantly showed reduced cholinesterase activity (82%) compared to

littermate TSM control homogenates. Control enzyme rates could be significantly reduced by preincubating with 10^{-4} M histamine (Table 4) to 81% of normal values and to a rate equal to sensitized enzyme rates. Histamine had no effect on cholinesterase activity in TSM from sensitized animals. Eserine (10^{-7} M) was equipotent in reducing both sensitized and control enzyme rates to below 10%.

Many agents have been found to inhibit cholinesterase including organophosphates (Fonnum et al., 1984) and ozone (Goldstein et al., 1968). Prostaglandins E_2 and F_{2alpha} have also been implicated (Grbovic and Radmanovic, 1981). Histamine has been shown to stimulate prostaglandin formation via arachidonic acid metabolism in the dog (Anderson et al., 1979; Tesarowski et al., 1981; Tesarowski and Kroeger, 1982). The depression by histamine of cholinesterase activity observed in Table 4 may have come about through such a mechanism. The lack of response of sensitized homogenates to histamine may indicate prior elevated levels of prostaglandin synthesis or histamine presence due to the sensitization procedure. (Some preliminary experiments were conducted preincubating control (n=4) and sensitized (n=4) homogenates with PGE_2 and then determining cholinesterase rates. However, due to the large scatter in the data which may be due to the lability of prostaglandins, significant differences could not be determined from control cholinesterase rates, even though the mean values for sensitized and control homogenates were $74.8 \pm 17.5\%$ and $75.8 \pm 15.9\%$ respectively. The trend in this data indicates simularities to

homogenates preincubated with histamine.)

These data support the hypothesis that the sensitization process reduces cholinesterase activity and suggest that the increased histamine levels associated with allergic bronchospasm may play a role in this reduction.

j) Resting membrane potentials (RMP).

A leftward shift in the threshold voltage for mechanical activation of sensitized TSM, the leftward shift in the ED_{50} values for histamine and potassium, and the leftward shift in the threshold dose of carbachol for mechanical activation indicated an increase in the sensitivity of sensitized TSM to a variety of agents and perhaps a depolarized sarcolemmal membrane. The reduced cholinesterase activity in sensitized homogenates and evidence from electrical experiments indicated a possible increase in acetylcholine concentration at the neuromuscular junction in sensitized TSM. From these observations it was hypothesized that there should be a depolarization from the RMP in sensitized TSM compared to control, and that this depolarization could explain the non-specific increase in sensitivity and/or reactivity after in vivo sensitization. To test this hypothesis membrane potentials were determined by standard electrophysiologic techniques.

The results showed that sensitized TSM was significantly depolarized by approximately 3.6 millivolts with respect to littermate control values (Fig. 27). Souhrada and Souhrada (1984) have reported a 5 millivolt hyperpolarization in sensitized guinea pig TSM. This significant discrepancy in the

data may be a result of species difference or the method of immunization. For the guinea pig sensitization is achieved after a single immunizing injection whereas the method used to sensitize the dogs used in this study require multiple injections with possible associated mini-challenges with each immunization (Kepron et al., 1977) although, as discussed earlier, Kepron and his co-workers (1977) calculated that the booster injection would not cause anaphylaxis. Souhrada and Souhrada (1984) report that challenged sensitized guinea pig TSM then shows a net depolarization. Perhaps the multiple injection protocol associated with sensitization of dogs may depolarize canine TSM thus producing the results obtained in this study. However, TSM isolated from these multiple-injected dogs still show a typical Schultz-Dale reaction. This would indicate that up to the point of challenge in vitro the sensitized canine TSM should be hyperpolarized. in vitro challenges were never performed on tissues isolated for electrophysiological studies. This discrepancy remains a moot point.

In view of the data obtained for the sensitized dog intracellular calcium homeostasis could become a factor. The membrane depolarization that was observed, perhaps in conjunction with increased acetylcholine at the neuromuscular junction in sensitized TSM, would allow for an increase in cytoplasmic calcium concentration via receptor-operated calcium channels. Voltage sensitive calcium channels appear unaffected from the data obtained in K^+ dose-response curves in the presence of atropine (Fig. 9) and from the fact that calcium channel blockers

- which block only voltage - sensitive channels - were ineffective in preventing bronchoconstriction in asthmatic patients challenged with histamine or methacholine (Patel, 1981; Patel and Al-Shamma, 1982).

This probable increase in cytoplasmic [Ca^{++}] would lead to calcium-calmodulin binding (from Hartshorne and Persechini, 1984); myosin light chain kinase activation, and phosporylation of the light chains thus allowing the formation of an active actomyosin complex and force production. All these steps are potential sites for the regulation of contraction and relaxation (Hartshorne and Siemankowski, 1981; Contri and Adelstein, 1980) in TSM, and thus the initiation and maintenance of bronchospasm.

Included in this regulation are cAMP levels which oppose the actions of Ca^{++} on the activity of myosin light chain kinase. The production of cAMP requires micromolar concentrations of Ca^{++} but is inhibited by high Ca^{++} levels (Wolff and Bostrom, 1979). Runard et al., (1979) have shown depressed basal cAMP levels in "asthmatic" dogs compared to "non-asthmatic" dogs which argues for increased cytoplasmic Ca^{++} levels in allergic bronchospasm.

k) Reactivity along the airways.

The reactivity to supramaximal stimuli was tested in sensitized and control airways at the levels of the cervical (upper) and thoracic (lower) trachea and the main stem (or primary) and third generation (tertiary) bronchi. The results revealed (Table 5) that, as for upper TSM, all areas tested from sensitized airways were hyperreactive to histamine and

acetylcholine in comparison with control airways and were not hyperreactive to supramaximal electrical stimulation. However, with prolonged (75 sec) stimulation the upper trachea and tertiary bronchi showed significant typical prolonged plateau contractions as observed in earlier studies.

Sensitized bronchi and upper TSM were hyperreactive to serotonin, however, TSM values from the lower trachea were not significantly different from control values although there was a trend in the data (control, 1732±89 g/cm^2; sensitized, 2043±204 g/cm^2) which indicated that with a reduced variability in the data or an increase in the number of experiments significant differences could be found. However, 127 mM K^+, which like serotonin produces contraction via voltage dependent calcium channels, showed no hyperreactivity in TSM from the lower trachea.

The lower trachea and primary bronchus from sensitized airways also failed to sustain developed force with prolonged electrical stimulation as was shown for the upper trachea and tertiary bronchi. The lack of significant differences in the reactivity of lower trachea to K^+, serotonin and prolonged electrical stimulation may be related to a paucity of innervation in this area of the airways or to the fact that the lower trachea is innervated primarily by the recurrent laryngeal branch of the vagus nerve whereas the cervical trachea receives branches of the vagi directly (Miller et al., 1964). The main trunk of the vagus nerves produces several prominent branches to the bronchi as it reaches the root of the lung. Innervation by the recurrent laryngeal branches of the vagi may not be as dense as in other

areas of the airways.

The fact that all areas of the sensitized airways tested were hyperreactive to histamine and acetylcholine suggests that the results obtained for upper trachea and the conclusions drawn from those results may in fact be general manifestations of sensitization throughout the central airways of the allergic canine lung.

l) Systemic anaphylaxis.

All areas of the sensitized airways challenged with RWX showed a Schultz-Dale reaction including lung parenchymal strips (Fig. 28). In addition (Fig. 29), hepatic artery, ureter and longitudinal gut muscle also showed reactions to RWX challenge. Sensitization, therefore is not confined to the airways, at least in the in vivo ragweed sensitized dog model of allergic bronchospasm.

Kong and Stephens (1981) have reported a Shultz-Dale reaction in pulmonary veins from ovalbumin-sensitized dogs. The reaction of the ureter and gut smooth muscle is particularly interesting in view of the fact that these smooth muscles are innervated through cholinergic inputs. In addition it is interesting to note that the lung secretory glands, which also appear to be hyperreactive in an asthmatic attack through the production of large quantities of a viscous mucus, are under the control of a parasympathetic cholinergic input.

m) Summary

This study focussed on the airway smooth muscle and in particular tracheal smooth muscle (TSM), from a ragweed

sensitized canine model of allergic asthma. All experiments were performed on isolated tissues in order that the influence of the irritant receptor bronchoconstrictor reflex be minimized. The data from sensitized isolated TSM was compared to results obtained from non-sensitized littermates which acted as control animals.

In general, ragweed sensitized TSM were hyperreactive and hypersensitive to a number of naturally occuring compounds. They were hypersensitive and hyperreactive to histamine. In the presence of atropine, or in passively in vitro sensitized TSM only hyperreactivity was noted. Sensitized TSM were hyperreactive and hypersensitive to increased concentrations of $[K^+]_{out}$. Both the hyperreactivity and hypersensitivity were abolished by atropine. These results lead to the conclusions that the sensitization process leads to increased numbers of histamine receptors on TSM which accounts for the hyperreactivity observed and that sensitization leads to increases in presynpatic histamine receptors which can augment the release of acetylcholine from the parasympathetic neural elements in the isolated preparation. The latter would account for the atropine sensitive hypersensitivity to histamine observed in sensitized TSM. The atropine sensitive hyperreactivity and hypersensitivity to K^+ also lead to the conclusion of neural involvement in the hyperresponsiveness of sensitized TSM. In addition it argued for normal voltage dependent calcium channels and a normal electrogenic sodium pump.

Sensitized TSM was hyperreactive to acetylcholine but not to carbachol. This argued for a greater efficacy of the

acetylcholine receptor through increased availability of the neurotransmitter in sensitized TSM. The leftward shifts in the threshold voltage from stimulus-response studies and the ED_{50} values from acetylcholine dose-response curves supported this idea in addition to the phenomena of spontaneous baseline activity (SBLA) and prolonged isometric plateau. The source of this increased acetylcholine availability was investigated. Two hypotheses were tested. The first was that there was an increased release of acetylcholine either basally or upon stimulation. The second focussed on the breakdown of the neurotransmitter and, in particular, that acetylcholinesterase activity was reduced in sensitized TSM.

Studies in which [^{14}C]-choline release was measured in sensitized and littermate controls could not confirm any increase in the basal release of neurotransmitter. However, these studies did confirm the ability of histamine to release acetylcholine from neural elements in the sensitized TSM. Ragweed pollen extract (RWX) could also increase the release of acetylcholine and thus gave some indication as to the mechanism of the atropine sensitivity observed in the Schultz-Dale response.

To test the hypothesis of acetylcholinesterase inhibition in sensitized TSM, control TSM strips were incubated with the cholinesterase inhibitor - eserine. Control TSM was then able to mimic the phenomena of SBLA and prolonged isometric plateau force maintenance of sensitized TSM. In the presence of eserine the dose-response curves of control and sensitized TSM were superimposable and the reactivities of both were elevated to those observed for carbachol - an acetylcholine receptor agonist

that is not affected by cholinesterase. Acetylcholinesterase activity was measured directly using a spectrophotometric method and was confirmed to be reduced in sensitized TSM. The ability of the enzyme to breakdown acetylcholine was shown to be histamine sensitive in control TSM; histamine, however, could not further reduce sensitized cholinesterase rates.

Electrophysiological studies showed that sensitized TSM cells were significantly depolarized with respect to control resting membrane potentials. This study tested and confirmed the possibility that an excess of acetylcholine in the neuromuscular junction of sensitized TSM would lead to some depolarization of the cells which could then account for the findings of a general hyperreactivity and hypersensitivity that were atropine sensitive.

The hyperreactivity of sensitized upper and lower trachea, main stem and third generation bronchi to a variety of supramaximal stimuli (electrical and pharmacological) indicated that the results obtained for the upper trachea may be more generalized and could be found throughout the airways. Schultz-Dale challenges of these tissues and lung parenchymal strips indicated the total airway involvement of the allergic bronchospasm. Schultz-Dale challenges of hepatic artery, ureter, and gut smooth muscle showed that conferred sensitivity to a specific antigen was not confined to the airways but may also involve systemic loci.

n) Conclusions

1) Histamine is the major mediator of allergic bronchospasm

in the dog. Sensitized canine airways are hyerreactive to histamine and through the parasympathetic cholinergic neural elements tracheal smooth muscle is hypersensitive to histamine.

2) Histamine can work pre-synaptically to induce the release of acetylcholine and this ability is much increased after active in vivo sensitization. Specific antigens can also release acetylcholine in sensitized tissues probably through histamine release from degranulating mast cells.

3) Sensitized tracheal smooth muscle is hyperreactive and hypersensitive to a variety of agents due to the additive effects of acetylcholine the agent on calcium entry into the sarcoplasm through receptor operated and voltage sensitive calcium channels.

4) Indirect evidence from potassium dose-response experiments indicate that in the presence of atropine (and thus in the presence of voltage dependent calcium channels alone) control and sensitized tracheal smooth muscle are equireactive suggesting normal handling of sodium, potassium and calcium by the sensitized TSM sarcolemma.

5) Sensitized tracheal smooth muscle shows abnormalities in isometric mechanograms which are consistent with increased basal release of the neurotransmitter acetylcholine and/or reduced breakdown of acetylcholine by its cholinesterase. In the presence of cholinesterase inhibitor control muscles can mimic these abnormalities. A direct measurement of cholinesterase activity indicates that either the sensitization procedure or the presence of histamine can significantly reduce the ability of the enzyme to breakdown neurotransmitter.

6) Sensitized tracheal smooth muscles are depolarized with respect to control resting membrane potential, likely as a result of acetylcholine receptor activation due to increased acetylcholine in the neuromuscular junction.

7) Generalized hyperreactivity is not confined to the cervical trachea but maybe found throughout the airways.

8) The parasympathetic nervous pathway, independent of the irritant receptor bronchoconstrictor reflex, plays a role in the etiology of allergic bronchospasm in the dog and suggests that this local effect may participate in the manifestation of human asthma.

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