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ANALYSIS OF AIRWAY RESPONSES TO VAGAL STIMULATION IN GUINEA PIGS

by

ANDREW L. CHOW

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled ANALYSIS OF AIRWAY RESPONSES TO VAGAL STIMULATION IN GUINEA PIGS submitted by ANDREW L. CHOW in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE in PHARMACEUTICAL SCIENCES (PHARMACOLOGY).

.....

Supervisor

.....

.....

Date May 22, 1986

To

my

parents

ABSTRACT

The objective of the work described in this thesis was to determine whether there are presynaptic muscarinic receptors in guinea-pig airways that modulate the release of acetylcholine (ACh) from efferent nerves.

I compared pulmonary responses to vagal stimulation, and to intravenously administered methacholine (MCh), histamine (Hist), and substance P (SP), in groups of guinea pigs, 2, 8, 16, 32, and 64 days after giving them nonylvanylamide ("synthetic capsaicin") (50 mg/kg, s.c.) or vehicle, and in untreated controls. Pulmonary flow resistance (R), and dynamic thoracic elastance (E) were measured in urethane-anesthetized animals while monitoring arterial blood pressure and heart rate. Pulmonary responses to the bronchospastic drugs were similar in all groups of animals at all times post-treatment. In both vehicle-treated and untreated controls, submaximal and maximal electrical vagal stimulation induced increases in R and E. By contrast, maximal nerve stimulation in nonylvanylamide-treated groups induced only small increases and submaximal stimulation was without effect. Atropine (0.1 mg/kg) abolished responses to submaximal stimulation in control groups, and to maximal stimulation in nonylvanylamide-treated groups of animals, and eliminated the bronchospastic effects of MCh. However, in control groups, responses to maximal nerve stimulation were only reduced by this drug. In controls, gallamine potentiated responses to submaximal stimulation, but had insignificant effects on responses to maximal stimulation and to agonists. By contrast, gallamine enhanced responses to maximal stimulation 2-5 times in all nonylvanylamide-treated animals, but had no effect on responses to agonists.

The effects of nonylvanylamide are rapid in onset and long lasting — persisting for at least 64 days. Nonylvanylamide treatment does not alter airway smooth muscle sensitivity to MCh, Hist, or SP. However, responses to vagal stimulation are reduced — the peptidergic component of the response to maximal nerve stimulation is eliminated and the cholinergic pathway is partly inhibited. The latter can be apparently reversed by gallamine or pancuronium. Gallamine's effects could arise via several possible mechanisms, alone or in

combination. Many of these have been ruled out, but one likely possibility is the selective inhibition of presynaptic, ACh release-modulating muscarinic receptors.

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List of Abbreviations

ACh:	Acetylcholine
AMP:	5'-Adenosine monophosphate
Atr:	Atropine
BP:	Blood pressure
C:	Pulmonary compliance
CCK:	Cholecystokinin
CGRP:	Calcitonin gene-related peptide
4-DAMP:	4-Diphenylacetoxy-N-methyl piperidine methiodide
E:	Dynamic thoracic elastance
Gal:	Gallamine
Hist:	Histamine
HR:	Heart rate
-IR:	Immunoreactive
[redacted]v.:	Intravenous
J:	Juxtapulmonary capillary
M:	Maximal
MABP:	Mean arterial blood pressure
MCh:	Methacholine
min:	Minutes
NANC:	Nonadrenergic, noncholinergic
PV:	Pressure volume
R:	Pulmonary flow resistance
s:	Seconds
s.c.:	Subcutaneous
SM:	Submaximal

SPM:	Supramaximal
TLC:	Total lung capacity
Tmax:	Peak response time
TP:	Tracheal pressure
$\dot{V}$:	Flow rate
VIP:	Vasoactive intestinal polypeptide

1. INTRODUCTION

The autonomic nervous system supplies the lungs with efferent and afferent nerves which help regulate smooth muscle tone, submucosal gland secretion, the pulmonary and bronchial vasculature and other cell systems in the airways (Nadel & Barnes, 1984). The densities of these nerves vary from region to region and from species to species (Richardson, 1979).

The vagus, the tenth cranial nerve, innervates many organs in the thorax and abdomen including the heart, lungs, gastrointestinal tract and other abdominal organs. It contains both efferent and afferent fibers. The vagal efferent nerves arise from cell bodies in the dorsal motor vagal nucleus in the medulla, and comprise preganglionic fibers, most of which do not synapse until they reach the many small ganglia lying directly on or in the viscera of the thorax and abdomen. In the lungs, they are situated in peribronchial plexuses near the hila. Thus, preganglionic fibers are very long and the postganglionic fibers quite short. In addition, the vagus nerve carries many afferent fibers from the viscera to the medulla. The cell bodies of these afferent fibers lie mainly in the inferior (nodose) and superior vagal ganglia.

1.1 Efferent innervation

The lungs receive efferent nerves belonging to three nervous systems: the classical sympathetic (adrenergic) and parasympathetic (cholinergic) nervous systems, and a third system that is nonadrenergic and noncholinergic.

1.1.1 Sympathetic nervous system

The sympathetic nervous system constitutes one of the two inhibitory nervous systems known to exist in mammalian tracheobronchial smooth muscle (Richardson, 1979). Sympathetic regulation of airway smooth muscle tone, submucosal gland secretion, and the pulmonary and bronchial vasculature occurs directly via noradrenaline released from

sympathetic nerve endings, and indirectly via circulating catecholamines (mainly adrenaline) from the adrenal medulla. The upper thoracic sympathetic preganglionic fibers terminate in the stellate and superior cervical ganglia. Postganglionic fibers from these ganglia run and enter the lungs at the hila together with the vagus nerves (Nadel, 1980; Nadel & Barnes, 1984). Histochemical studies and electron microscopy have shown that adrenergic nerves pass to the submucosal glands, the bronchial blood vessels, and the parasympathetic ganglia in man (Richardson, 1979; Partanen *et al.*, 1982; Sheppard *et al.*, 1983).

The density of sympathetic (adrenergic) innervation as determined by fluorescent histochemical studies is generally sparse in most species (Mann, 1971), but there is great interspecies variability. Feline airway smooth muscle receives an abundant sympathetic innervation (Silva & Ross, 1974; Dahlström *et al.*, 1966). The lungs of calves also receive an abundant adrenergic innervation — perhaps greater than other species (Hebb, 1969; Mann, 1971). By contrast, the adrenergic innervation of the airway smooth muscle of the guinea pig is found mainly in the central portion of the trachea (Coburn & Tomita, 1973; O'Donnell & Saar, 1973). However, the pulmonary and bronchial vasculatures are densely innervated (O'Donnell *et al.*, 1978).

Despite the great variation in adrenergic innervation, most anatomical studies have shown few, if any, adrenergic fibers in bronchial smooth muscle, and none in bronchiolar muscle (Richardson, 1979). This pattern of adrenergic innervation is consistent in the guinea pig, sheep, cow, and goat (O'Donnell *et al.*, 1978).

In humans, histochemical studies and electron microscopy have failed to demonstrate significant sympathetic innervation of airway smooth muscle (Richardson & Beland, 1976; Sheppard *et al.*, 1983). Studies using electrical field stimulation *in vitro* have yielded results consistent with these findings (Richardson & Beland, 1976). As in other species, there is sympathetic innervation to the submucosal glands, bronchial blood vessels and parasympathetic ganglia. The latter finding suggests that modulation of cholinergic neurotransmission may occur at the ganglionic level (Baker *et al.*, 1982, 1983). β-Adrenergic

blockade has little effect on airway resistance in healthy individuals (Tattersfield *et al.*, 1973), but induces bronchospasm in asthmatic patients (McNeill & Ingram, 1966; Zaid & Beall, 1966), suggesting that the physiological effects of circulating catecholamines are important in regulating airway smooth muscle tone in asthma. Therefore, efferent sympathetic nerves are unlikely to have a major regulatory function in the control of airway smooth muscle tone in human airways.

1.1.1.0 Circulating catecholamines

Although the sympathetic innervation to airway smooth muscle is sparse in most species studied, receptor binding studies have revealed that both α- and β-adrenoceptors are present on the smooth muscle cells in large numbers (Barnes *et al.*, 1983c). Presumably, these receptors are accessible to circulating catecholamines (Nadel & Barnes, 1984; Barnes, 1984b).

α- and β-adrenoceptors are not uniformly distributed on the smooth muscle throughout the tracheobronchial tree (Barnes *et al.*, 1983a,b). A study of ferret airway smooth muscle using autoradiographic localization techniques has shown that the density of β-adrenergic receptors increases from the trachea to the distal bronchioles, and that α-receptors are sparse in the trachea, but that their density increases to equal the density of β-receptors in the peripheral airways (Barnes *et al.*, 1983a). The greater overall density of β-receptors in airway smooth muscle may explain why noradrenaline produces bronchodilation despite its greater potency for α-receptors than β-receptors (Barnes *et al.*, 1984c).

β-Adrenergic receptors mediate bronchodilation and their density in lungs has been found to be higher than in any other tissue in several species (Rugg *et al.*, 1978; Barnes *et al.*, 1980). β_2-Receptors are 4 times as abundant as β_1-receptors in canine tracheal smooth muscle (Barnes *et al.*, 1983d). β_1-Receptors are absent in human airway smooth muscle (Zaagsma *et al.*, 1983; Lofdahl & Svedmyr, 1982). β_1-Receptors mediate relaxation to sympathetic nerve stimulation, whereas β_2-receptors are selectively

stimulated by exogenous agonists and circulating catecholamines (Barnes *et al.*, 1983d). In addition to their relaxant effects on airway smooth muscle (Davis *et al.*, 1982; Zaagsma *et al.*, 1983), β-agonists inhibit antigen-induced release of mediators from human pulmonary mast cells (Peters *et al.*, 1982), selectively increase airway mucus secretion by mucous cells (Nadel *et al.*, 1985; Phipps *et al.*, 1982), and reduce histamine-induced microvascular leakage in airways. This may account for their efficacy in reducing mucosal edema in asthmatics (Persson *et al.*, 1982). β-Agonists may also modulate cholinergic neurotransmission at ganglia (Baker *et al.*, 1982) or at postganglionic nerves (Vermeire & Vanhoutte, 1979). However, Martin and Collier (1985) found that noradrenaline does not appear to modulate cholinergic neurotransmission in canine airways. Relaxation of airway smooth muscle by β-agonists may be brought about by their direct and indirect actions on the smooth muscle.

Both subtypes of α-receptors, α_1 and α_2, are present on canine (Leff & Munoz, 1981) and human (Simonsson *et al.*, 1972; Kneussl & Richardson, 1978) airway smooth muscle. The α_2-subtype predominates on canine muscle (Barnes *et al.*, 1983f). α_1-Receptors are sparse in the large airways of ferrets, but are abundant in bronchiolar smooth muscle (Barnes *et al.*, 1983b). α-Adrenoceptors generally mediate contraction, but their effects can only be demonstrated in diseased human airways or in normal airway smooth muscle strips pretreated with histamine or serotonin (Kneussl & Richardson, 1978; Barnes *et al.*, 1983e). Catecholamine-induced contractions of canine tracheal smooth muscle are mediated by α_2-receptors (Barnes *et al.*, 1983f). α-Agonists also stimulate fluid and mucus secretion from the submucosal glands of several species (Nadel *et al.*, 1985; Phipps *et al.*, 1982). By contrast to β-agonists, α-agonists selectively stimulate serous gland cells (Basbaum *et al.*, 1981) resulting in a watery secretion (Leikauf *et al.*, 1984; Ueki *et al.*, 1980). Recently, α_2-receptors have been claimed to modulate both the excitatory cholinergic (Grundström *et al.*, 1981) and the noncholinergic (Grundström & Andersson, 1982,1985; Grundström *et al.*, 1984)

pulmonary responses to vagal stimulation through a presynaptic α_2-adrenergic mechanism. The α-blockers, phentolamine and thymoxamine, prevent the bronchoconstrictor responses to histamine, allergen and exercise, but these drugs lack pharmacological specificity so these results are difficult to interpret (Barnes, 1984a). Prazosin, a specific α_1-blocker, has no effect on airway tone or on bronchoconstrictor responses to histamine, although there is partial inhibition of exercise-induced bronchoconstriction. This suggests that α_1-receptors do not play an important role in bronchial hyperreactivity (Barnes, 1984a).

1.1.2 Parasympathetic nervous system

The parasympathetic nervous system is the dominant constrictor mechanism in the airways of most species studied, including man and guinea pig (Boushey, 1985; Nadel & Barnes, 1984; Nadel, 1980; Richardson 1979,1983; Rikimaru & Sudoh, 1971; Foster, 1966; Paton & Hawkins, 1958; Carlyle, 1963). Cholinergic innervation is dense in airway smooth muscle and sparse or absent in the pulmonary vasculature. Preganglionic efferent fibers enter the lungs via the vagus nerves and synapse in small ganglia located in the airway wall, from which short postganglionic fibers directly innervate the submucosal glands and the airway smooth muscle as far distally as the terminal bronchioles (Richardson, 1979; Nadel & Barnes, 1984).

Receptor binding (Murlas *et al.*, 1982; Cheng & Townley, 1982) and autoradiographic studies (Barnes *et al.*, 1983c; Basbaum *et al.*, 1983) have demonstrated dense concentrations of muscarinic receptors in smooth muscle from the trachea and the large airways. The density of these receptors decreases with the size of the airways until they are almost absent in the terminal bronchioles (Barnes *et al.*, 1983a,b). Physiologic studies using tantalum bronchography have yielded results consistent with these findings — vagal-nerve stimulation induced changes on the caliber of the large airways with minimal effects in the small bronchioles (Nadel *et al.*, 1971).

Besides mediating bronchoconstriction, the parasympathetic nervous system also regulates mucus secretion by the airway submucosal glands (Murlas *et al.*, 1980) Vagal stimulation *in vivo*, and acetylcholine (ACh) *in vitro*, stimulate mucus secretion in animals (Nadel *et al.*, 1985). Muscarinic receptors are present in high densities in the submucosal glands (Basbaum *et al.*, 1983).

1.1.3 Nonadrenergic noncholinergic (NANC) nervous system

The airways are innervated by both inhibitory and excitatory NANC nerves. In the following text, the term NANC refers to the inhibitory system unless otherwise specified.

1.1.3.1 Inhibitory NANC

Inhibitory NANC nerves that relax the airway smooth muscle were first described in the toad by Campbell in 1971. Similar nerves were discovered shortly after in the guinea-pig trachea, the first mammalian airway shown to contain significant NANC innervation (Coburn & Tomita, 1973; Coleman & Levy, 1974; Bando *et al.*, 1973; Richardson & Bouchard, 1975; Kamikawa & Shimo, 1976). Since then NANC nerves have been described in several species including man (Richardson, 1981; Richardson & Beland, 1976; Davis *et al.*, 1982).

In some species (man and nonhuman primates), the NANC system is the only inhibitory system present in the airways (Richardson & Beland, 1976; Doidge & Satchell, 1982; Middendorf & Russell, 1980). In guinea pigs and cats, it forms one component of a dual inhibitory system (Yip *et al.*, 1981; Diamond & O'Donnell, 1980; Irvin *et al.*, 1980). The dog, a species which is widely used in pulmonary research, does not have significant pulmonary NANC innervation and the adrenergic pathway is the only inhibitory system (Russell, 1980; Kannan & Daniel, 1980).

NANC innervation has been demonstrated *in vitro* in tracheobronchial smooth muscle obtained from guinea pigs (see references above), humans (Richardson & Beland, 1976; Richardson, 1981), baboons (Middendorf & Russell, 1980), and chickens (Bhatia

et al., 1980). It has also been demonstrated in tracheal pouch preparations *in situ* in anesthetized guinea pigs (Yip *et al.*, 1981; Chesrown *et al.*, 1980), and in feline intrapulmonary airways *in vivo* (Diamond & O'Donnell, 1980; Irvin *et al.*, 1980). Yip and coworkers (1981) showed that 60-80% of the maximal relaxation evoked in guinea-pig airways was adrenergic, and 20-40% was attributable to NANC inhibition.

NANC nerves also regulate the secretion of airway mucus in animals (Peatfield & Richardson, 1983; Borson *et al.*, 1982). An inhibitory NANC system in the pulmonary vasculature has been demonstrated in some species (Hamasaki & Said, 1981; Said, 1982), but not in humans or guinea pigs.

Experiments with the NANC system generally involve nerve or field stimulation after cholinergic and adrenergic blockade. The tone of the preparations is often raised artificially by infusing histamine or serotonin i.v.. Atropine eliminates the initial contraction due to excitatory parasympathetic effects and a β-adrenergic blocker such as propranolol partly blocks the relaxation that follows the initial contraction. The relaxant response that remains is thought to be derived from stimulation of NANC inhibitory neurons. It can be blocked by ganglion blocking agents such as hexamethonium (Diamond & O'Donnell, 1980; Irvin *et al.*, 1980), and by tetrodotoxin (Richardson, 1981; Hammarström & Sjöstrand, 1979). Thus, this relaxation is neurally mediated and involves a ganglionic relay.

The lack of a specific antagonist for the NANC system has hindered progress in identifying its neurotransmitter and its physiological role. There is considerable controversy over the chemical nature of the neurotransmitter. Burnstock, who studied this system in the gastrointestinal tract, presented evidence that adenosine triphosphate or a related nucleotide was the likely mediator and named it the "purinergic system" (1972). However, others have suggested that these nerves are peptidergic (Baumgarten *et al.*, 1970; Kamikawa & Shimo, 1976; Kuchii *et al.*, 1973), and evidence increasingly favors this hypothesis (see Barnes, 1984c). Several regulatory peptides, including vasoactive

intestinal polypeptide (VIP), substance P (SP), bombesin, cholecystokinin (CCK), and somatostatin have been identified by radioimmunoassay in the lung tissue of several species (Polak & Bloom, 1982; Håkanson *et al.*, 1983) Some of these peptides are located in nerves within the airways.

Adenosine is a possible purinergic neurotransmitter of the NANC system. Adenosine is formed from the cleavage of 5'-adenosine monophosphate (AMP) by the enzyme 5'-nucleotidase, when the cell is unable to reconvert AMP to high energy containing nucleotides. This occurs when the cell is deprived of nutrients or oxygen, is stimulated excessively or is damaged (Church & Holgate, 1986). However, inhaled adenosine causes bronchoconstriction in asthmatic but not in normal subjects by an undefined mechanism (Cushley *et al*, 1983). Adenosine-induced bronchoconstriction is not mediated by cholinegic reflex mechanisms or by decreased β_2-adrenoceptor responsiveness of the airways (Mann *et al*, 1985). The receptor-mediated actions of adenosine are blocked competitively by methylxanthines, at concentrations which have little effect on phosphodiesterase activity (Fredholm, 1980). Thus, adenosine does not appear to have a role in NANC-mediated relaxation of the airways in either healthy or asthmatic subjects.

The evidence in favor of VIP as a neurotransmitter of NANC nerves in the airway has been reviewed by Barnes (1984c). VIP-ergic neurons have been identified in airway smooth muscle, particularly in the upper airways, around submucosal glands, beneath airway epithelial cells, and in bronchial and pulmonary vessels (Polak & Bloom, 1982; Uddman & Sundler, 1979; Dey *et al.*, 1981). Injection of exogenous VIP induces many of the physiological and pharmacological effects of NANC-nerve stimulation in animals (Ito & Takeda, 1982; Cameron *et al.*, 1983). Inhaled VIP prevents bronchospastic responses to histamine in dogs (Said, 1982) and guinea pigs (Cox *et al.*, 1983), and an infusion of VIP reverses serotonin-induced bronchoconstriction in cats (Diamond *et al.*, 1983). Field stimulation of tracheobronchial preparations releases VIP into the bathing

medium and this release is inhibited by tetrodotoxin (Cameron *et al.*, 1983; Matsuzaki *et al.*, 1980). Prolonged incubation of cat airway smooth muscle with VIP induces tachyphylaxis and reduces the magnitude of NANC-mediated relaxation (Ito & Takeda, 1982). Preincubation with a specific antibody to VIP reduces NANC-mediated relaxation of guinea-pig trachea (Matsuzaki *et al.*, 1980).

Evidence against VIP as a neurotransmitter of NANC nerves in the airways includes the minimal relaxant effect of VIP in isolated human airways, and the weak protective effect of inhaled VIP against histamine-induced bronchoconstriction. (Barnes & Dixon, 1984). However, this could be due to the lack of access of VIP to airway smooth muscle by this route compared with neurally released VIP. Karlsson and Persson (1983) have suggested that VIP is not the only peptidergic inhibitory NANC neurotransmitter in the airways. However, until a specific antagonist is found for the NANC system, this cannot be proved or disproved.

1.1.3.2 Excitatory NANC nerves

Excitatory NANC nerves can be demonstrated in guinea-pig airways after inducing cholinergic blockade with atropine and stimulating the vagi electrically (Lundberg *et al.*, 1983a,b; Martling *et al.*, 1984). Under these conditions, stimulation induces a delayed bronchospastic response, and increases mucosal permeability. These effects can be abolished by an antagonist to SP, but not by ganglion blockers. Lundberg and coworkers suggested that these responses were due to the release of the tachykinin SP via antidromic stimulation of vagal afferents. The excitatory NANC system will be elaborated upon in the section on capsaicin.

1.2 Afferent innervation -

Several review articles have been published on this subject within the last 15 years (Paintal 1973,1977; Widdicombe, 1981; Sant'Ambrogio, 1982; Coleridge & Coleridge, 1984). The lungs are innervated by sensory neurons of spinal and vagal origin.

1.2.1 Sympathetic afferents

Spinal afferents are termed sympathetic afferents because they travel in sympathetic nerve branches to enter the spinal cord (Kostreva *et al.*, 1975). The afferents from pulmonary receptors traverse the right and left upper thoracic white rami communicantes. In dogs, these receptors respond to lung inflation and to pinching of the lung parenchyma, and do not show adaptation. Increases in afferent activity have been observed in some fibers when the pulmonary arteries and veins were stimulated mechanically by probing (Kostreva *et al.*, 1975). The conduction velocities of these afferents suggested that the fibers were of the Aδ type. Uchida (1976) showed that tachypnea can be produced by excitation of afferent cardiac sympathetic nerve fibers in dogs. Stimulation of either sympathetic chain at any thoracic segment resulted in the inhibition of respiration in vagotomized monkeys and dogs (Kostreva *et al.*, 1978). Myelinated spinal afferents are known to mediate the reflex disturbances in the pattern of breathing induced by irritant chemicals (Widdicombe, 1954a; Coleridge *et al.*, 1983). Sympathetic afferents of airway origin, unlike those that innervate the heart, do not seem to be involved in sensing pain. Instead, pain is transmitted by vagal afferents (Morton *et al.*, 1951). The functions of the unmyelinated afferents are not known, however sympathetic afferents do not seem to play a major role in pulmonary defensive reflexes (Coleridge & Coleridge, 1985).

1.2.2 Vagal afferents

Vagal afferents arise from pulmonary sensory receptors and travel to the medulla via the vagi. There are four main types of receptors in the lungs and airways: pulmonary stretch or slowly-adapting receptors (Adrian, 1933), irritant receptors (Mills *et al.*, 1969; Sellick & Widdicombe, 1970,1971) — earlier termed rapidly-adapting receptors (Knowlton & Larrabee, 1946), pulmonary C-fibers or type J (or juxtapulmonary capillary) receptors (Paintal, 1955,1969), and bronchial C-fibers (Coleridge & Coleridge, 1977,1984). Fibers from pulmonary stretch and irritant receptors are myelinated, whereas those from J receptors are

mostly nonmyelinated (Paintal, 1973). In cats, 80% of vagal afferents have been reported to be unmyelinated (Agostini *et al.*, 1957).

Pulmonary stretch receptors are the best known of these receptors. Their sensory endings are thought to be located among smooth muscle cells from the trachea to the bronchioles (Elftman, 1943; Fillenz & Widdicombe, 1972) In dogs and cats, they are located in the walls of the trachea and large bronchi (Widdicombe, 1954b; Miserocchi *et al.*, 1973). Physiologic evidence indicates a far greater extrapulmonary than intrapulmonary concentration of these receptors (Widdicombe, 1981). These receptors are stimulated by distension of the lungs and airways, and are responsible for the Hering-Breuer inflation and deflation reflex, and Head's paradoxical reflex. They have not been demonstrated to have a role in lung disease (Widdicombe, 1985).

Irritant receptors are located in the trachea and larger airways and are densest at the carina and hilus pulmonis (Mortola *et al.*, 1975; Widdicombe, 1954b). Their terminals are found in the airway epithelium and also probably deeper in the airway wall (Sant'Ambrogio, 1982; Widdicombe, 1981). Their nerve fibers are myelinated except for their terminals (Laitinen *et al.*, 1985). The most superficial endings are appropriately positioned less than 1 μm from the airway lumen where they can respond to intraluminal irritation (Das *et al.*, 1978). Various types of stimuli activate these receptors including mechanical irritation of the larynx, trachea, and bronchi (Widdicombe, 1954b; Mills *et al.*, 1969), inhalation of dust (Widdicombe *et al.*, 1962; Kessler *et al.*, 1973), smoke (Sellick & Widdicombe, 1971; Fillenz & Widdicombe, 1972), and gaseous chemical irritants (see Widdicombe, 1981; Nadel, 1980), pneumothorax (Sellick & Widdicombe, 1970), hyperpnea (Sellick & Widdicombe, 1969), pulmonary congestion and edema, anaphylaxis and microembolism (Mills *et al.*, 1969; Sellick & Widdicombe, 1969), and histamine (DeKock *et al.*, 1966). Reflex bronchoconstriction precipitated by these stimuli can be abolished by vagotomy or by atropine (Nadel, 1980).

C-fiber receptors are found throughout the tracheobronchial tree and alveoli. They can be divided into pulmonary and bronchial groups. The classification is based on the

accessibility of injected chemicals to the nerve endings, through either the pulmonary or bronchial circulation (Sant'Ambrogio, 1982; Coleridge & Coleridge, 1984). Pulmonary C-fibers are also known as J receptors, a term coined by Paintal (1973) because of their proximity to pulmonary capillaries. According to Widdicombe (1985), the differences between the bronchial and pulmonary C-fibers are more quantitative than qualitative. The pulmonary receptors are stimulated by pulmonary congestion which increases interstitial fluid volume (Paintal, 1970). They are also stimulated by other experimental interventions that cause lung edema, e.g., injection of alloxan, injection of exogenous stimulants such as phenyldiguanide and capsaicin, and inhalation of irritants such as ammonia, volatile anesthetics, and phosgene (Paintal, 1973).

Bronchial C-fiber endings are found in the large airways, and respond to many of the stimuli that excite J receptors. However, J receptors, unlike bronchial C-fibers, are stimulated by histamine or bradykinin. Bronchial C-fibers do not have significant reflex effects on peripheral vascular resistance, but mediate other reflex effects including bronchoconstriction (Coleridge & Coleridge, 1984).

1.3 Pulmonary mechanics

At any instant in time, the force required to drive air into the lungs and airways is the sum of the inertial component (acceleration of the mass of air), an elastic component (the stretch of the lungs and thorax), and a resistive component (the drag on the air through the viscous passageways). The inertial component is usually small enough to be neglected (Mead, 1956). The 2 parameters measured in our experiments are airway flow resistance (R) and dynamic thoracic elastance (E).

1.3.1 Airway flow resistance

Pressure differences between the airway opening (mouth) and alveoli induce the flow of air into and out of the lungs. Airway resistance is defined as the ratio of pressure

difference to rate of flow.

$$R = P/\dot{V}$$

where P = alveolar pressure — airway opening pressure

$\dot{V}$ = rate of airflow.

Pressure-flow relationships in the lungs are extremely complex since the airways are comprised of irregular branching tubes which are neither rigid nor perfectly circular. The 3 different patterns of flow, laminar, turbulent and transitional, occur in different regions of the lungs under certain conditions. These are summarized in Table 1.1.

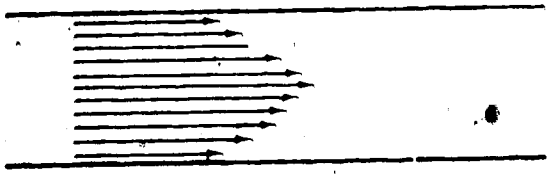
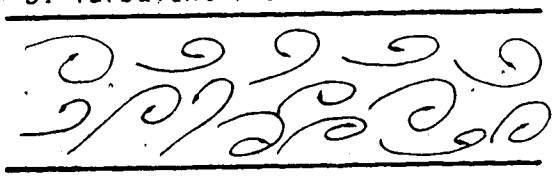
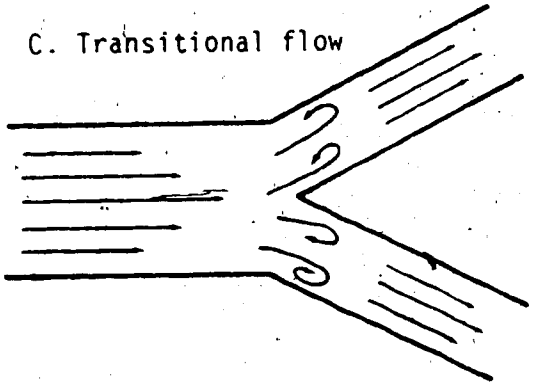
1.3.2 Elastance

This is a measure of the combined elastic properties of the lungs and the elastic recoil properties of the chest wall. Elastance is the reciprocal of compliance. The elastic properties of the lungs can be assessed by plotting the pressure-volume (PV) curves of the lungs during inspiration and expiration. The PV relationship is nonlinear, and the curves during inspiration and expiration do not follow the same course — a behavior known as *hysteresis* (See Fig. 1.1).

The slope of the PV curve, or the volume change per unit pressure change, is known as compliance, i.e., $C = \Delta V/\Delta P$. Since a PV curve is not linear, a single value for its slope is often misleading. The curves show that compliance of the lungs is least at high lung volumes and greatest as the residual volume (the volume of air that remains in the lungs after a maximal expiratory effort) is approached. Thus, compliance will vary depending on the lung volume used and whether it is measured during inflation or deflation. At high expanding pressures, the lungs become less distensible and their compliance is smaller as indicated by the flatter slope of the PV curve. Compliance is also reduced if pulmonary venous pressure is increased and the lung becomes engorged with blood.

At points of zero airflow (end of inspiration or expiration), the intrapleural pressure reflects only the elastic recoil forces and not those associated with airflow. The volume

Table 1.1 Patterns of airflow.

Pattern of airflow	Sites of common occurence	Flow rate
A. Laminar flow 	Small peripheral airways	Slow
B. Turbulent flow 	Trachea	High
C. Transitional flow 	Branches in the tracheo-bronchial tree	Intermediate

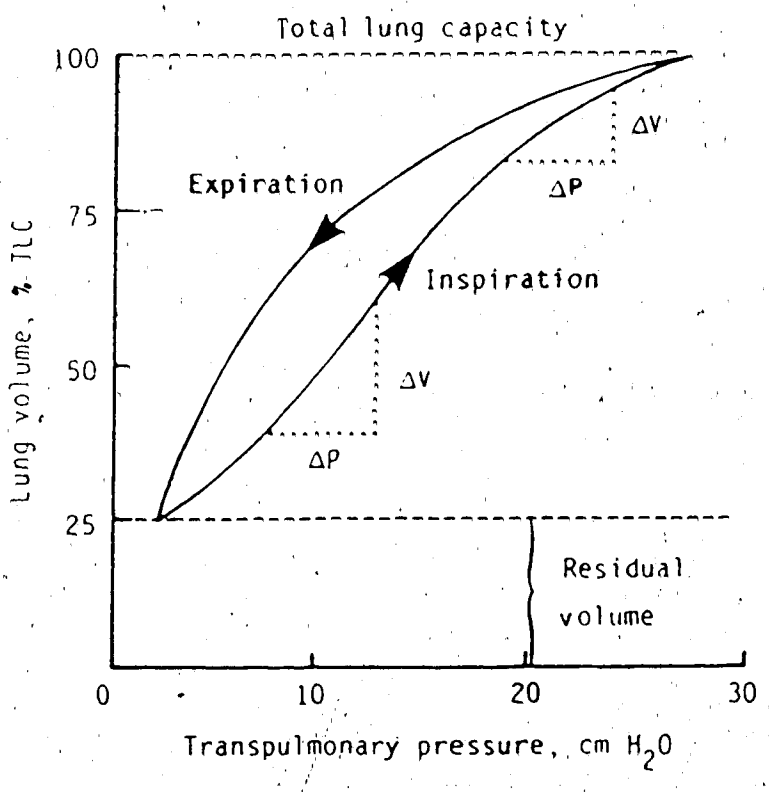


Figure 1.1 Pressure-volume curves of the lung made during inspiration and expiration. The compliance of the lung ($\Delta V/\Delta P$) is greater at low lung volumes than at high lung volumes.

difference divided by the pressure difference at these points is the static compliance. Dynamic compliance of the lungs is the change in the volume of the lungs divided by the change in the alveolar-distending pressure during the course of a breath. Dynamic compliance is about equal to static compliance at low breathing frequencies (Altose, 1980).

In man, the dimensions of the small airways are less than 2 mm in diameter. But in small experimental animals such as guinea pigs, this is the size of the large airways. The regional effects of various pharmacological agents or experimental manoeuvers on the large and small airways of guinea pigs should not be extrapolated to humans without considering this.

In normal subjects, constriction in the large or conducting airways is reflected primarily by an increase in R, and constriction in the small or distensible airways primarily by an increase in E. In practice, though, there is some interdependancy between these 2 parameters.

1.4 Vagal stimulation

A brief historical account of investigations of the vagal innervation of the airways and the effects of vagal stimulation will be given.

1.4.1 History

During the 19th century, experiments on the vagal innervation to the airways were carried out on the excised lungs of freshly killed animals. Because of the crude and insensitive recording methods used, vagal stimulation usually produced little or no change on the tone of the tissue preparations. Any changes induced were very small in magnitude and unconvincing. Dixon and Brodie (1903) reviewed these early experiments. Williams (1840) was the first to examine the action of the vagus on tracheobronchial smooth muscle. By placing a sheet electrode around some excised lungs and applying a current between the electrode and a brass intratracheal tube, he managed to raise the intratracheal pressure by 5 cm H_2O. Other

investigators employed static methods similar to those of Williams with little or no modifications and had similar success. They showed that vagal nerve stimulation increases intratracheal pressure, contracts the bronchi, and even causes an expiratory puff of air from the trachea that, on one occasion, blew out a candle! Einthoven (1892) rhythmically injected air from a syringe into the trachea of curarized and morphinized dogs — a significant technical improvement. During maximal distension, he connected the trachea to a mercury manometer and recorded the maximum pressure attained. A hindrance to the flow of air into the alveoli meant more air retained in the trachea and therefore a higher maximum pressure. However, in all these experiments, the measurements were nonspecific and the cause of any increase in resistance to inflation could not be differentiated into a fall in compliance or an increase in resistance to airflow. Nonspecific methods were used by investigators until Bayliss and Robertson (1939) differentiated between elastance and viscance (or resistance to airflow).

1.4.2 Effects of vagal stimulation

In all species studied, electrical stimulation of the distal ends of the cut cervical vagus nerves results in bronchoconstriction (Colebatch & Halmagyi, 1963; Olsen *et al.*, 1965; Karczewski & Widdicombe, 1969; Green & Widdicombe, 1966; Woolcock *et al.*, 1969a,b; Nadel *et al.*, 1971; Hahn *et al.*, 1978; Gold *et al.*, 1972; Cabezas *et al.*, 1971; Widdicombe, 1963; Lundberg *et al.*, 1983a,b; Fryer & Maclagan, 1984; Blaber *et al.*, 1985). This response can be mimicked by administering cholinergic drugs such as ACh and MCh i.v. It is enhanced by anticholinesterase agents that inactivate cholinesterases in plasma, and prevent the rapid hydrolysis of ACh (Colebatch & Halmagyi, 1963; Olsen *et al.*, 1965). As the responses elicited can be partly (Lundberg *et al.*, 1983a,b; Martling *et al.*,1984), or completely (Nadel, 1980; Fryer & Maclagan, 1984; Blaber *et al.*, 1985), blocked by atropine, it is apparent that they are partially or totally derived from the release of ACh from parasympathetic nerve endings, that, in turn, binds to muscarinic receptors on airway smooth muscle. The strength of the applied stimulus determines the magnitude of the pulmonary responses and the sensitivity of the

responses to atropine. Pulmonary responses elicited by submaximal stimulation can be abolished by atropine, whereas those elicited by maximal or supramaximal stimulation can only be partially reduced by this drug.

In cats during stimulation of the vagi, pulmonary resistance increases within 1 s and reaches a maximum value within 6 s (Olsen *et al.*, 1965). This is accompanied by a concomitant decrease in anatomic dead space. The time course of these events indicates that they are due to contraction of smooth muscle as opposed to mucosal edema or obstruction of the airway lumen by mucus (Nadel, 1980). Bronchoconstriction appears to occur at a lower stimulation threshold than mucus secretion (Olsen *et al.*, 1965). The constriction in response to vagal stimulation occurs from the trachea to large bronchioles. The greatest constriction occurs in bronchi of intermediate size (Woolcock *et al.*, 1969b; Nadel *et al.*, 1971), where the resistance to airflow is greatest (Macklem & Mead, 1977; Ingram *et al.*, 1977). In dogs and cats, stimulation has little effect on airways less than 0.8 mm in diameter (Nadel *et al.*, 1971). However, physiological studies suggest that there are intra- and inter-species differences in the relative contributions of large and small airways (Woolcock *et al.*, 1969b; Gardiner *et al.*, 1974; Douglas *et al.*, 1979). The alveolar ducts and terminal bronchioles are generally unaffected (Olsen *et al.*, 1965; Nadel *et al.*, 1971; Nadel, 1965).

The sites of airway constriction correspond to the distribution of the cholinergic innervation (Hebb, 1969; Fillenz, 1970; Mann, 1971). Unilateral vagosympathetic nerve stimulation in dogs induced increases in resistance that were limited mainly to the homolateral lung (Olsen *et al.*, 1965). A small degree of efferent nerve crossover to the opposite lung exists since a small increase in resistance can be demonstrated in the contralateral lung following the administration of a cholinesterase inhibitor. These studies confirm early observations based on indirect measurements of resistance (Dixon & Brodie, 1903; Daly & Mount, 1951) that suggested that the efferent innervation is largely to the homolateral lung.

Besides causing bronchoconstriction, stimulation of the vagi also produces extrapulmonary effects. Effects on the cardiovascular system are evident by the bradycardia

and hypotension observed during stimulation in all species studied. Stimulation of the vagus nerve in humans has been reported to produce similar effects (Carlsten *et al.*, 1957).

1.5 Capsaicin

The history of, and early findings on, capsaicin were reviewed by Szolcsányi (1984). Several other reviews on this compound have also been published (Buck & Burks, 1983; Fitzgerald, 1983; Monsereenusorn *et al.*, 1982; Nagy, 1982; Virus & Gebhart, 1979). Capsaicin is the principle pungent component of hot peppers of the genus *Capsicum*. It exerts a number of significant pharmacological effects (see Buck & Burks, 1983). Most importantly it has the ability to induce changes in sensory function and to deplete neuronal substance P. These properties make it a very useful pharmacological tool for the study of the afferent innervation. In our experiments, synthetic capsaicin (nonylvanylamide) was used to abolish the excitatory NANC (peptidergic) component of the bronchoconstriction induced by vagal nerve stimulation.

Jancsó, one of the pioneers in the use of capsaicin in laboratory animals, reported in the late forties that topical application of this agent induces an inflammatory irritation followed by a specific and long-lasting desensitization to chemical irritants, including capsaicin itself (see Szolcsányi, 1984). Systemic administration of capsaicin to animals produces similar results which are extremely long lasting and sometimes life-long. In addition, certain sensory functions such as thermal nociception are impaired. By contrast, non-noxious touch, pressure, cold and vibration responses are unaffected by this treatment (Buck *et al.*, 1981; Burks *et al.*, 1985). Capsaicin given i.v. to dogs and cats produces a triad of effects consisting of bradycardia, a fall in blood pressure, and apnea (Bevan, 1962; Coleridge *et al.*, 1965; Makara *et al.*, 1967; Pórszász *et al.*, 1955; Toda *et al.*, 1972; Toh *et al.*, 1955). Since these can be blocked or reduced by bilateral vagotomy or by cooling the vagi, they are mediated reflexly.

The action of capsaicin is selective in that it depletes substance P (SP) from certain sensory neurons (Gamse *et al.*, 1980; Jessell *et al.*, 1978; Nagy *et al.*, 1980) without affecting

SP-containing neurons whose cell bodies are found in the central nervous system (Gamse *et al.*, 1980; Nagy *et al.*, 1980) or in the enteric nervous system (Furness *et al.*, 1982; Holzer *et al.*, 1980; Burks *et al.*, 1985). The neurons and tissues in which depletion occurs include the dorsal cord, dorsal roots, dorsal ganglia, skin, vagus nerve, saphenous nerve, trigeminal nucleus, cornea, sympathetic ganglia, adrenal gland, heart, and cerebral and mesenteric arteries (see Buck & Burks, 1983). Capsaicin also induces a transient reduction in the intraneuronal concentration of other neuropeptides, e.g., cholecystokinin (CCK) (Buck *et al.*, 1983). Papka and coworkers (1981) reported that capsaicin-pretreatment also induces a loss of acetylcholinesterase-containing nerve fibers in the heart. This suggests that capsaicin could also have a toxic effect on cholinergic nerves, though sensory nerves may also contain acetylcholinesterases. Martling and coworkers (1984) found that capsaicin-pretreatment reduced pulmonary responses to vagal nerve stimulation without altering tissue choline-acetyltransferase activity and muscarinic-receptor-binding characteristics.

Certain animal species appear to be more susceptible to capsaicin than others. Guinea pigs have higher levels of SP than rats in their dorsal spinal cord and dorsal root ganglia (Buck *et al.*, 1981) and have been reported to be more sensitive than rats to treatment with capsaicin. (Jancsó-Gábor *et al.*, 1970).

The animal's age is also an important consideration. Administration to neonatal rats causes degeneration of the small (type B) sensory neurons (Jancsó *et al.*, 1977; Jancsó & Király, 1981; Nagy *et al.*, 1981) but adult rats show only limited intracellular changes in small sensory neurons while large (type A) sensory neurons and neurons in the sympathetic ganglia are unaffected (Joó *et al.*, 1969). The effects of capsaicin on neonatal rats are believed to be permanent (Gamse *et al.*, 1979,1980; Jancsó & Király, 1980; Nagy *et al.*, 1981; Scadding, 1980). By contrast, those in adult rats are thought to be reversible (Gamse *et al.*, 1980; Gibson *et al.*, 1982). Unlike rats, adult guinea pigs treated with capsaicin systemically exhibit thermal analgesia and insensitivity to chemical irritants (Buck *et al.*, 1981).

Guinea-pig airways like those of humans, rats and cats, receive sensory innervation containing SP or SP-like immunoreactive material (Nilsson *et al.*, 1977; Wharton *et al.*, 1979; Lundberg *et al.*, 1984). These nerves are found under or within the epithelium, around blood vessels, around local tracheobronchial ganglion cells, and within the bronchial smooth muscle layer (Lundberg *et al.*, 1984). Systemic treatment of guinea pigs with capsaicin results in the almost complete loss of SP-like immunoreactivity from these neurons (Lundberg & Saria, 1983; Lundberg *et al.*, 1983a, 1984) and abolishes the noncholinergic bronchoconstrictor effects of vagal nerve stimulation (Lundberg & Saria, 1982; Martling *et al.*, 1984). The cholinergic component is partially inhibited at the same time (Martling *et al.*, 1984). Why this inhibition occurs is not known, but it does not appear to involve degeneration of the cholinergic nerves.

The structures of capsaicin and 4 of its naturally occuring derivatives are shown in Figures 1.2 and 1.3. The pungency of capsaicin is destroyed by oxidation with potassium permanganate or potassium dichromate, but synthetic capsaicin, which has a saturated side chain, is not readily oxidized (Todd, 1958). Szolcsányi and Jancsó-Gábor (1975, 1976) have analyzed the structure-activity relationships for the pain-producing and the desensitizing properties of a large number of capsaicin congeners. There is a divergence between the structural characteristics required for pungency and those required for desensitization. The acylamide linkage and an optimal alkyl chain length of 10-12 carbon atoms are required for desensitizing activity. Nonylvanylamide, "synthetic capsaicin", (see Figure 1.3, R = $-(CH_2)_7CH_3$) was one of the compounds shown to have similar, albeit slightly weaker, desensitizing properties than capsaicin. This agent was used instead of capsaicin in our experiments due to its greater accessibility and lower cost. We established its cross-reactivity with natural capsaicin in our animal model.

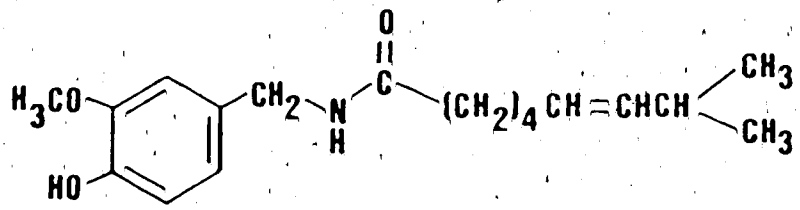
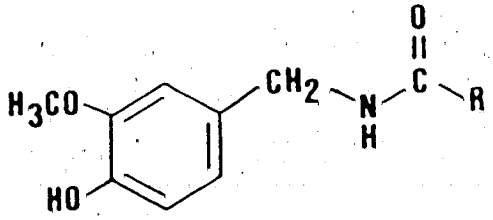


Figure 1.2 The chemical structure of capsaicin



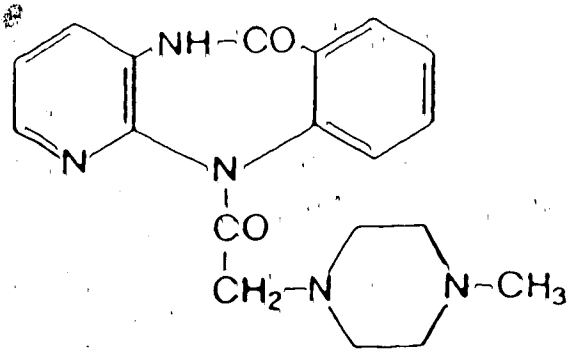
R	COMPOUND
$-(CH_2)_3CH(CH_3)_2$	Norhydrocapsaicin
$-(CH_2)_6CH(CH_3)_2$	Dihydrocapsaicin
$-(CH_2)_5CH=CHCH(CH_3)_2$	Homocapsaicin
$-(CH_2)_9CH(CH_3)_2$	Homodihydrocapsaicin

Figure 1.3 The chemical structures of 4 naturally occuring derivatives of capsaicin.

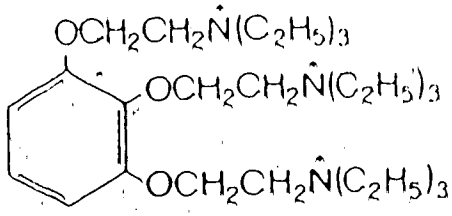
1.6 Muscarinic receptors

The evidence for the existence of muscarinic receptor subtypes has been extensively reviewed in two symposia on muscarinic receptor subtypes (Hirschowitz *et al.*, 1984; Levine *et al.*, 1986). Historically, receptor responses to acetylcholine were subdivided by Dale (1914) into muscarinic and nicotinic types. The concept of muscarinic receptor heterogeneity was first hypothesized by Birdsall and coworkers (1978) to explain anomalies in the binding of cholinergic agonists to brain receptors. Support for this hypothesis came mainly from studies using selective muscarinic agonists. Two years earlier, Barlow and coworkers (1976) investigated 17 potential muscarinic antagonists and reported that 4-diphenylacetoxy-methyl-piperidine methiodide (4-DAMP) discriminated between atrial and ileal muscarinic receptors. They found that the affinity of this agent for receptors in the ileum was 20-fold greater than for receptors in the atria and inferred that muscarinic receptors in heart and smooth muscle were different.

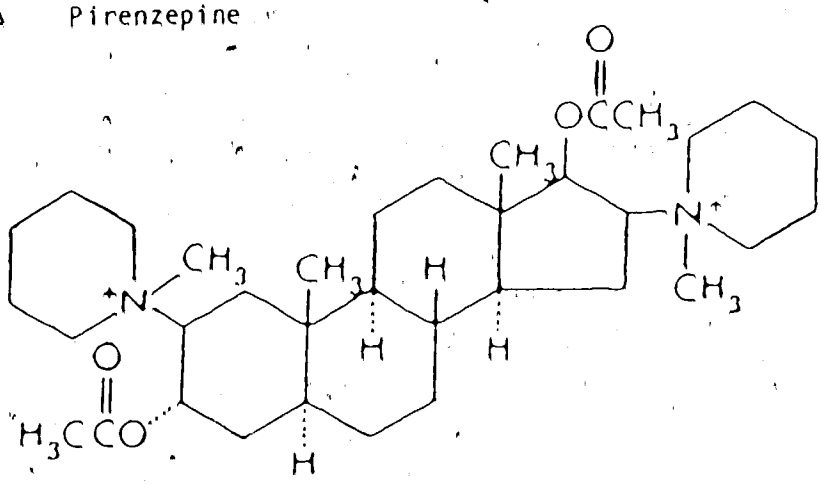
This theory was significantly strengthened by the discovery of novel muscarinic antagonists, e.g., pirenzepine and secoverine, that show selective binding to muscarinic receptor subtypes (Hammer *et al.*, 1980; Birdsall *et al.*, 1980). Pirenzepine is a tricyclic compound (Fig. 1.4) that penetrates the blood brain barrier poorly because of its hydrophilicity (Hammer & Giachetti, 1984). It has been shown to be selective for ganglionic muscarinic receptors compared to smooth muscle receptors, and it markedly decreases gastric acid secretion (Barlow *et al.*, 1981) without affecting gastrointestinal motility (Brown *et al.*, 1980; Jaup *et al.*, 1982). Pirenzepine has been shown to be 17-fold less potent than atropine in inhibiting the increase in airway resistance induced by vagal stimulation, and 260-fold less potent in inhibiting the fall in heart rate during vagal stimulation in guinea pigs (Halonen *et al.*, 1986). Secoverine inhibits gastrointestinal motility at doses that do not affect gastric or salivary secretion (Zwagemakers & Claassen, 1980; Davidson *et al.*, 1983). By contrast, gallamine selectively blocks muscarinic receptors in the heart. The first evidence of muscarinic receptor heterogeneity, from studies with antagonists, was actually provided more than 30



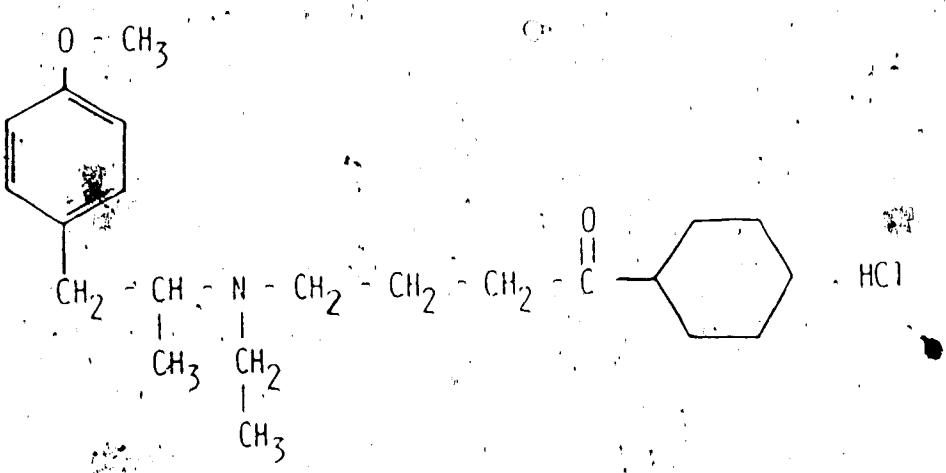
Pirenzepine



Gallamine



Pancuronium



Secoverine

Figure 1.4 The chemical structures of some selective muscarinic antagonists.

years ago when Riker and Wescoe (1951) demonstrated the cardioselective antimuscarinic effects of gallamine. Gallamine is a *tris*-quarternary neuromuscular blocking agent used clinically as a muscle relaxant (see Figure 1.4 for structure). It is not a pure nicotinic antagonist as it also has antimuscarinic properties. In anesthetized cats, bradycardia induced by methacholine or vagal stimulation was inhibited by gallamine, although the hypotension was not changed. Other responses produced by muscarinic receptor activation, such as sweating, salivary secretion and gut motility, were also unaffected. Later, pancuronium, another curare-like neuromuscular blocking agent, was shown to possess a similar cardioselective antimuscarinic action (Saxena & Bonta, 1970). Gallamine's antagonistic action against the cholinomimetics ACh and carbachol, is not competitive (Clark & Mitchelson, 1976). It binds to a second binding site on muscarinic receptors distinct from the conventional muscarinic ligand binding site and modulates the binding of agonists and antagonists to the conventional binding site (Birdsall & Hulme, 1983).

Evidence for muscarinic receptor subtypes has also come from the use of selective muscarinic agonists. These include the oxotremorine analogue McN-A-343, an acetoxypyrrolidine derivative, AHR-602, a spiroquinuclidine compound, and the alkaloid pilocarpine (Caulfield & Straughan, 1983). McN-A-343 is believed to act on the same muscarinic receptor subtype as pirenzepine (Hammer & Giachetti, 1982).

Since Dale's time the list of muscarinic sites has been expanded in 3 respects (Burgen, 1984).

1. There are clearly-defined muscarinic sites on ganglionic neurons which, although not essential for transmission through the ganglia, can be stimulated by muscarinic agonists.
2. In the central nervous system, muscarinic receptors are abundant and muscarinic responses are readily demonstrated.
3. Clear evidence has appeared for the existence of presynaptic muscarinic receptors that can modulate transmitter release.

There is considerable evidence that the cholinergic nerve terminals of the guinea pig myenteric plexus are endowed with inhibitory muscarinic receptors that when activated by various agonists, including ACh, reduce the amount of ACh released in response to electrical stimulation, high potassium concentrations, and the nicotinic agonist DMPP (Fosbraey & Johnson, 1980,1982; Kilbinger, 1984a,b). The pharmacological properties of these presynaptic receptors do not differ from those of postsynaptic receptors located on the longitudinal muscle of the ileum (Halim *et al.*, 1982; Kilbinger, 1984a; Kilbinger & Wessler, 1980).

Since the respiratory tract, like the large and small bowel, is derived from an outpouching of the foregut during embryological development, there is a possibility that presynaptic receptors exist on the nerve terminals in the lungs. Functional evidence of the presence of these receptors in guinea-pig and cat lungs has been reported by Fryer & Maclagan (1984) and Blaber and coworkers (1985) respectively. Maclagan and coworkers (1985) have also demonstrated evidence of these receptors in guinea-pig trachea *in vitro*.

The M_1/M_2 terminology of muscarinic receptors in current use is based upon their relative affinities for the selective antagonist pirenzepine. The M_1-receptor shows a high degree of sensitivity to pirenzepine and exhibits a high affinity for this drug in binding studies. M_1-receptors can be stimulated by McN-A-343. The M_2-receptor shows a low sensitivity and a low binding affinity to pirenzepine. M_1-receptors appear to be homogeneous, but there are indications that M_2-receptors are heterogenous (Birdsall & Hulme, 1983). The M_1/M_2 nomenclature was originally proposed by Goyal and Rattan (1978; Rattan & Goyal, 1984) from their studies of the lower esophageal sphincter of opposums. Stimulation of M_1-receptors decreases lower esophageal sphincter pressure, and stimulation of M_2-receptors increases it. M_1-receptors predominate in neural tissues (autonomic ganglia, discrete brain areas), whereas M_2-receptors are found mainly in peripheral effector organs. Radioligand binding and functional studies have been used to characterize the muscarinic receptors in human tracheal smooth muscle (van Koppen *et al.*, 1985). They have been shown to be of the M_2-subtype because of their low binding affinity for pirenzepine. The order of muscarinic

antagonist potencies is similar to those found in human lung and experimental animal respiratory tissue (Raaymakers *et al.*, 1984; Mita *et al.*, 1982; Beld *et al.*, 1975; Murlas *et al.*, 1982; Barlow & Weston-Smith, 1985).

2. PROPOSED RESEARCH

2.1 Preamble

From the introduction, it is clear that the caliber of the airways is regulated by complex interactions between the different excitatory and inhibitory nervous systems, and by locally and distantly (blood-borne) released mediators. These neurotransmitters and mediators may act directly on airway smooth muscle or indirectly as neuromodulators via receptors on ganglia or on presynaptic nerve terminals. A defect in any one of these mechanisms may upset the balance of this entire system and could result in pathophysiological disorders of the respiratory tract. In the guinea-pig myenteric plexus, the presence of presynaptic muscarinic receptors that modulate ACh-release is well documented. Since the gastrointestinal tract and the respiratory tract have a common embryological origin, similar receptors may also be present on pulmonary neurons. Fryer and Maclagan (1984) and Blaber and coworkers (1985) have presented evidence for the existence of such receptors in guinea-pig and cat airways *in vivo*. There is also evidence supporting the existence of these receptors in guinea-pig trachea *in vitro* (Maclagan *et al.*, 1985). The comparison between the gastrointestinal and respiratory tracts was made earlier in the discussion on the inhibitory NANC system. The absence or incomplete function of this system in the gastrointestinal tract is responsible for human diseases such as Hirschprung's disease (Frigo *et al.*, 1973) and possibly achalasia (Cohen, 1979). The inhibitory neurons for the gastrointestinal tract are located in the myenteric plexus and the latter acts as an integrating system for the control of smooth muscle (Wood, 1975,1981). The structure of the myenteric plexus is very complex as is the neurophysiology of the neurons located within it. The structure of the ganglia in human lung (Richardson & Ferguson, 1979) resembles that of the myenteric plexus (Gabella, 1972). Although neurophysiological studies have not been done on human or animal ganglia, it is likely that the function of the pulmonary ganglia is similar to the function of the ganglia in the myenteric plexus (Richardson, 1981). A possible role of the NANC system in the pathogenesis

of pulmonary diseases such as asthma has been suggested (Barnes, 1984c). With this in mind, it is also possible that a defect in the function of presynaptic muscarinic receptors might be the underlying cause of the bronchial hyperreactivity characteristic of asthma. A decrease in the number or density of these receptors might result in an impaired negative feedback mechanism resulting in exaggeration of responses elicited by irritant stimuli.

2.2 Hypothesis

That presynaptic muscarinic receptors modulate acetylcholine release in guinea-pig airways.

2.3 Objectives

Overall: To demonstrate the functional existence of these receptors and determine their role in pathophysiological conditions of the airways.

1. To characterize the pulmonary responses to vagal-nerve stimulation in normal untreated guinea pigs by using specific agonists and antagonists.
2. To characterize the pulmonary responses to vagal-nerve stimulation in nonylvanylamide-pretreated animals.
3. To study the time course of effects of nonylvanylamide-pretreatment on pulmonary responses to vagal stimulation and to the bronchospastic drugs methacholine (MCh), histamine (Hist), and SP administered intravenously, and compare them to control animals.

2.4 Animal model

The guinea pig was used as the animal model in these experiments because:

1. Its sensitivity to common agonists, peptides, and mediators of asthmatic bronchospasm is similar to that in man.
2. The airway smooth muscle of guinea pigs is abundant and extends up to the alveolar

ducts (Miller, 1947), thus making this species more sensitive to bronchoconstrictor effects.

3. The pattern of its sympathetic, parasympathetic, and NANC innervation is similar to that of man (Richardson, 1979).

4. Most of the drugs used to treat asthma were developed in this species.

3. METHODS AND MATERIALS

3.1 Animal preparation

Hartley strain guinea pigs (Charles River Canada Inc., St. Constant, P.Q.) of either sex, weighing between 400 and 700 g, were anesthetized with urethane (1.25-1.75 g/kg) injected intraperitoneally. A 4-5 cm long PE 240 cannula was inserted about 1.5-2 cm into the trachea in the midcervical region and it was connected via a pneumotach (Fleisch No. 0000) to a Harvard Small Rodent Ventilator for positive pressure ventilation. The animals were ventilated at a tidal volume of 10 ml/kg and at a pump speed of 20 strokes/min. A Validyne MP 45-14 differential pressure transducer connected to the pneumotach was used to monitor flow rate ($\dot{V}$). Intratracheal pressure was monitored using a Grass PT5A pressure transducer connected to a sidearm of the tracheal cannula. Flow rate and pressure signals were recorded on a Grass 7D polygraph. The flow of air into and out of the system was regulated by two separate valves (Kuhnke 65.232 and 65.234). Figure 3.1 shows a schematic drawing of the system.

3.2 Determination of pulmonary flow resistance and elastance

A MINC 23 computer was used to compute breath-by-breath values of pulmonary flow resistance (R) and dynamic thoracic elastance (E) from flow rate and pressure signals using equations derived by Uhl and Lewis (1974). The validity of results obtained with this system has been verified in our laboratories previously (Goel, 1986).

Measurements of R and E were made after allowing equilibration of the animal and adjustment of the system. Animals were given sufficient anesthetic to prevent their breathing against the pump. Experiments were begun after ensuring that values of R and E were constant. Measurements were made as follows: normal ventilation was temporarily interupted to inflate the lungs once with 2 pump volumes by occluding the outlet valve; the animal was allowed 2 min to equilibrate before control measurements of R and E were made; drugs were

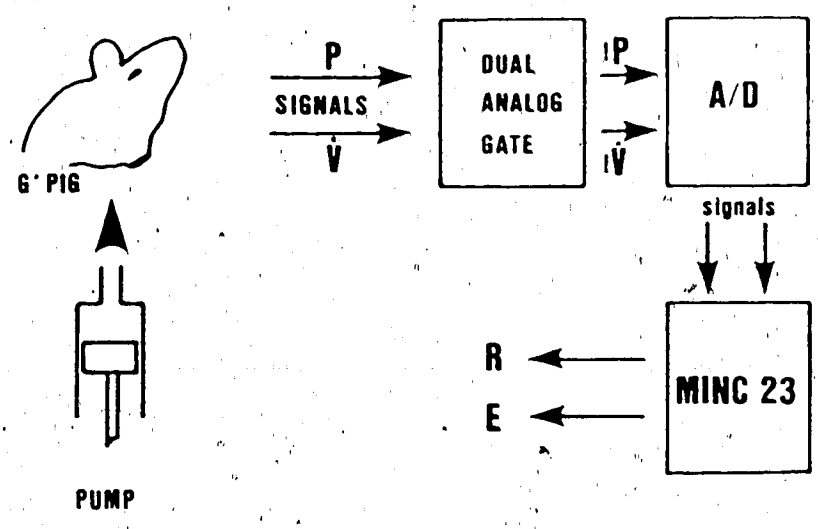


Figure 3.1 Schematic diagram of the system used to measure pulmonary flow resistance (R) and dynamic thoracic elastance (E).

then injected or nerves stimulated while breath-by-breath measurements of R and E were made for up to 5 min. While waiting for R and E values to be computed, the lungs were again inflated once with 2 pump volumes and allowed to equilibrate before the cycle was repeated.

There was a tendency over the course of the entire experiment (2-3 h) for baseline R and E values to increase, but these changes were usually less than 5% of the original baseline values at the start of the experiment.

3.3 Measurement of arterial blood pressure

Arterial blood pressure was monitored from the left carotid artery (anatomical case) via a PE 50 cannula connected to a Statham P23 Dd pressure transducer. Pressure was displayed graphically on a Grass 7D polygraph. The mean arterial blood pressure (MABP) was estimated from the arterial blood pressure tracings. The cannula was flushed periodically with heparinized saline to prevent clotting.

3.4 Heart rate

The heart rate (HR) was determined by running the blood pressure tracings on the polygraph at a fast rate and counting the number of heart beats per unit time.

3.5 Injection of drugs

A PE 50 catheter was inserted approximately 4 cm into the right external jugular vein until the tip was in the superior vena cava or right atrium. It was secured in place and the position of its tip confirmed at necropsy.

3.6 Nerve sections

Bilateral vagotomy was performed in the midcervical region after the nerves were isolated and cleared of connective tissue. For bilateral glossopharyngealotomy, the glossopharyngeal nerves were isolated and sectioned at the level of the jugular foramen.

3.7 Drugs

All drugs except for some injectables, were dissolved in or diluted with 0.9% (normal) saline and injected intravenously. They were injected in volumes less than 0.5 ml whenever possible and the cannulas flushed with 0.2 ml of saline. The jugular cannulas used had dead spaces of about 0.1 ml.

3.7.1 Nonylvanylamide pretreatment

Nonylvanylamide (50 mg) was dissolved in 0.2 ml 95% ethanol and 0.2 ml Tween 80 and diluted with normal saline to 2 ml to give a solution strength of 25 mg/ml. For pretreatment, animals were first anesthetized with pentobarbital sodium (15 mg/kg, i.p.) and given the analgesic/sedative xylazine (2.2 mg/kg, s.c.). The bronchodilator salbutamol (0.05 mg/kg, s.c.) was administered to counteract the bronchoconstriction produced by nonylvanylamide. Nonylvanylamide was given in two subcutaneous injections over two days for a total dose of 50 mg/kg. Initially, nonylvanylamide was injected as a single bolus dose and the mortality rate was about 50%. By giving it over two days in two divided doses, the mortality rate was lowered to about 25%. Deaths occurred within 8 h of injecting nonylvanylamide. Surviving animals were studied 2, 8, 16, 32, and 64 days later. Control animals received the same drugs and the vehicle alone, without nonylvanylamide. Animals were given a test dose of nonylvanylamide (2 and 16 μg/kg) at the start of experiments, and the effects on R and E were measured to confirm the desensitizing efficacy of nonylvanylamide pretreatment. Successfully desensitized animals do not show any significant increases in R and E to nonylvanylamide i.v.

3.7.2 Substance P (SP)-Antagonists

SP-antagonists, dissolved in normal saline, were infused intravenously over 5 or 15 min, using a Sage Instruments infusion pump (Model 249-2) starting within 20 min after the injection of mepyramine (0.1 mg/kg) and atropine (0.1 mg/kg). Responses to vagal stimulation were recorded from 10 min until 60 min (after 5-min infusion) or from 30 min until 90 min (15-min infusions) after the injection of the SP antagonists. For [D-Arg^1, D-Pro^2,D-Trp^{7,9},Leu^{11}]-SP, an additional dose of 0.3 mg/kg was injected immediately prior to injection of an agonist, or electrical stimulation of the vagus nerves (see Lundberg *et al.*, 1983b).

3.7.3 Other drugs

All other drugs were dissolved or diluted with normal saline. The drugs used were: atropine sulfate (B.D.H., Toronto, Ont.), capsaicin (Fluka AG, Buchs), decamethonium bromide (Fluka Chemical Corp., Hauppauge, NY), gallamine triethiodide injection (Rhône-Poulenc Pharma, Montreal, P.Q.), heparin sodium injection (B.D.H.), hexamethonium bromide (K & K Laboratories, Inc., Plainview, NY), mepyramine maleate (Rhône-Poulenc Pharma), mecamylamine hydrochloride (gift from Merck Sharp & Dohme, Kirkland, P.Q.), pancuronium bromide injection (Organon Canada, West Hill, Ont.), pelargonic acid vanillylamide (Fluka AG), pentobarbital sodium (M.T.C. Pharmaceuticals, Hamilton, Ont.), salbutamol sulfate (Allen & Hanburys, Toronto, Ont.), serotonin creatinine sulfate (Sigma Chemical Co., St. Louis, MO), [D-Pro^2,D-Trp^{7,9}]-SP and [D-Arg^1,D-Pro^2, D-Trp^{7,9},Leu^{11}]-SP (Peninsula Laboratories, Belmont, CA), SP (Sigma or Peninsula), urethane (Sigma), and xylazine (Miles Laboratories, Ltd., Rexdale, Ont.).

The pelargonic acid vanillylamide (nonylvanylamide) purchased from Fluka AG was recrystallized from a 2:8 mixture of ethyl acetate:petroleum ether. Recrystallization yielded 2 fractions which were equiactive pharmacologically.

3.8 Stimulation of the vagi

The vagi were isolated in the midcervical region, after carefully separating them from the carotid arteries and clearing them of connective tissue. For unilateral stimulation, one nerve was sectioned and its peripheral end drawn into a shielded platinum electrode and stimulated. The other vagus nerve was either left intact or sectioned. Bilateral stimulation was performed after drawing the cut peripheral ends of both vagus nerves into bipolar shielded platinum electrodes. They were stimulated submaximally (10 Hz, 0.1-0.5 ms, 15 V) and maximally (10 Hz, 5 ms, 15 V) for 15 s with a Grass S44 stimulator via a Grass SIU5 stimulus isolation unit. In nonylvanylamide-pretreated animals, nerves were stimulated at a supramaximal voltage of 50 V because of the small size of their responses to my standard submaximal and maximal parameters. Nerves were covered with cotton pellets soaked with mineral oil to prevent them from drying out.

The times taken to reach the maximal increases in R and E after onset of stimulation were determined in most of the experiments.

3.9 Analysis of data

Whenever possible at least four replicates were obtained in each series of experiments. Student's *t*-test or the Least Significant Difference (L.S.D.) test were used to examine differences. Differences were assumed to be significant at the 5% level.

4. RESULTS

4.1 Characterization of the parasympathetic innervation

4.1.1 Effects of vagal stimulation

Unilateral stimulation of either vagus nerve with the other intact or cut caused small increases in R and E, a fall in MABP, and bradycardia. By contrast bilateral stimulation produced significantly greater increases in both R ($p<0.001$) and E ($p<0.005$) (see Table 4.1), and more pronounced hypotension and bradycardia. Since it was desirable for our experimental purposes to work with large responses, all further stimulations were performed bilaterally. The vagi were stimulated using stimulation parameters that elicited either submaximal or maximal responses.

4.1.1.1 Submaximal vagal stimulation

Submaximal stimulation of the vagi promptly increased values of R and E. Figure 4.1 shows a typical response of an animal to submaximal vagal stimulation. The period of nerve stimulation in this and subsequent figures is indicated by the dark bar marked NS. Increases in R and E reached peak values during or shortly after stimulation before returning to baseline values usually within 2 minutes after stopping stimulation. Percentage increases in R were greater than percentage increases in E, and took less time to reach peak values. HR was slowed and the MABP fell during stimulation, however, they returned to prestimulation values within 30 s.

4.1.1.2 Maximal vagal stimulation

The increases in R and E elicited by maximal vagal nerve stimulation had similar characteristics to those seen after submaximal stimulation. Predictably these responses were larger (Figure 4.2), but they did not differ from submaximal responses in their peak response times (Table 4.2). The longer duration of maximal responses were due mainly to

Table 4.1 Percent increases in R and E induced by uni- and bilateral vagal-nerve stimulation.

	n	% increase	
		R	E
Unilateral	18	27.8 ± 2.8	52.1 ± 6.7
Bilateral	18	153.0 ± 18.5†	99.5 ± 11.6≠

† $p<0.001$

≠ $p<0.005$

Table 4.2 Increases in R and E and the peak response times (Tmax) induced by submaximal and maximal stimulation.

Stimulation	n	R (% increase)	Tmax (s)	E (% increase)	Tmax (s)
Submaximal	12	87.7 ± 18.2	12.27 ± 0.65	43.8 ± 7.9	17.99 ± 0.43
Maximal	12	198.0 ± 20.6†	11.24 ± 1.00	220.4 ± 18.1†	17.68 ± 0.45

† $p<0.001$

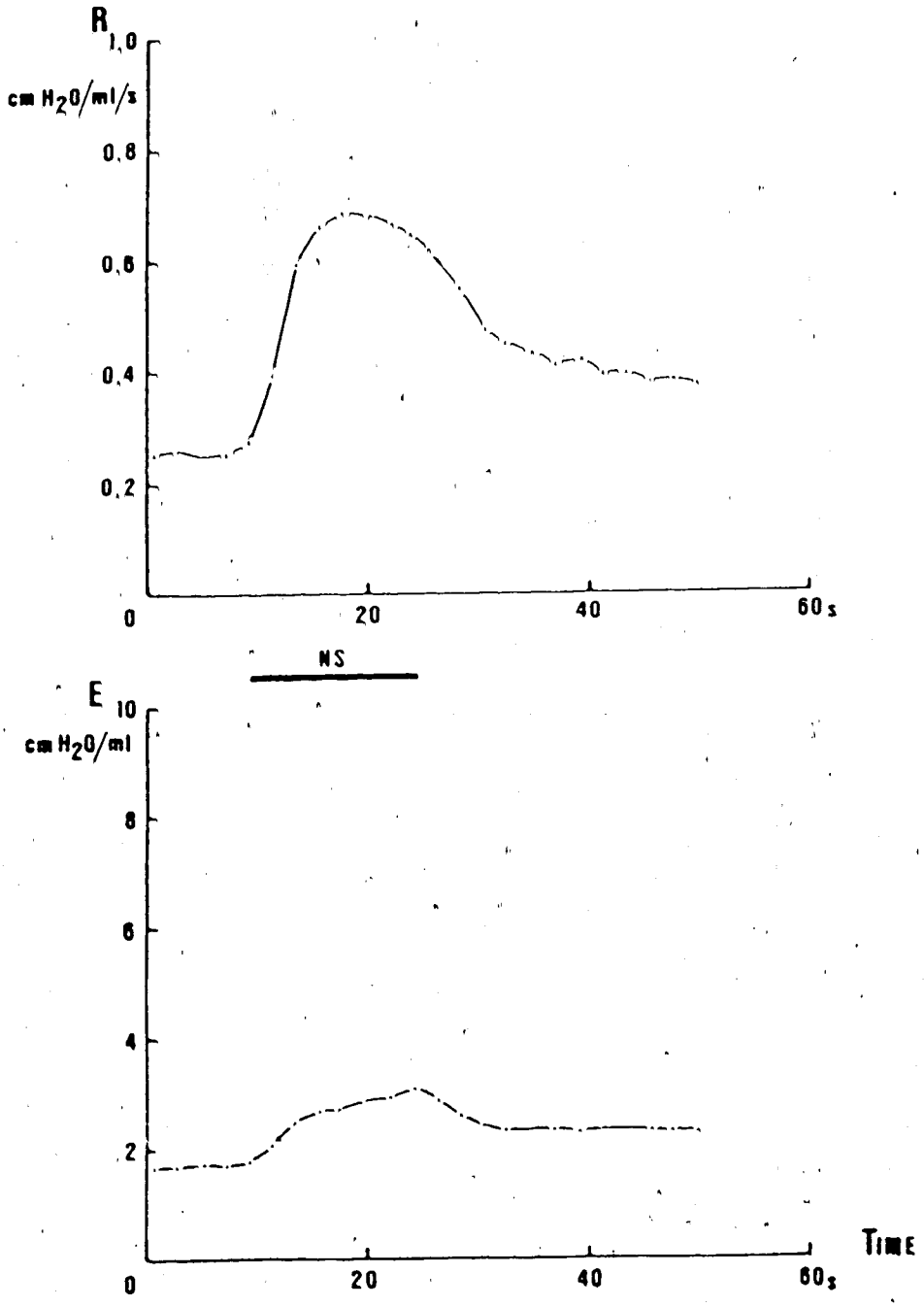


Figure 4.1 Effects of submaximal stimulation on Ṙ and E.

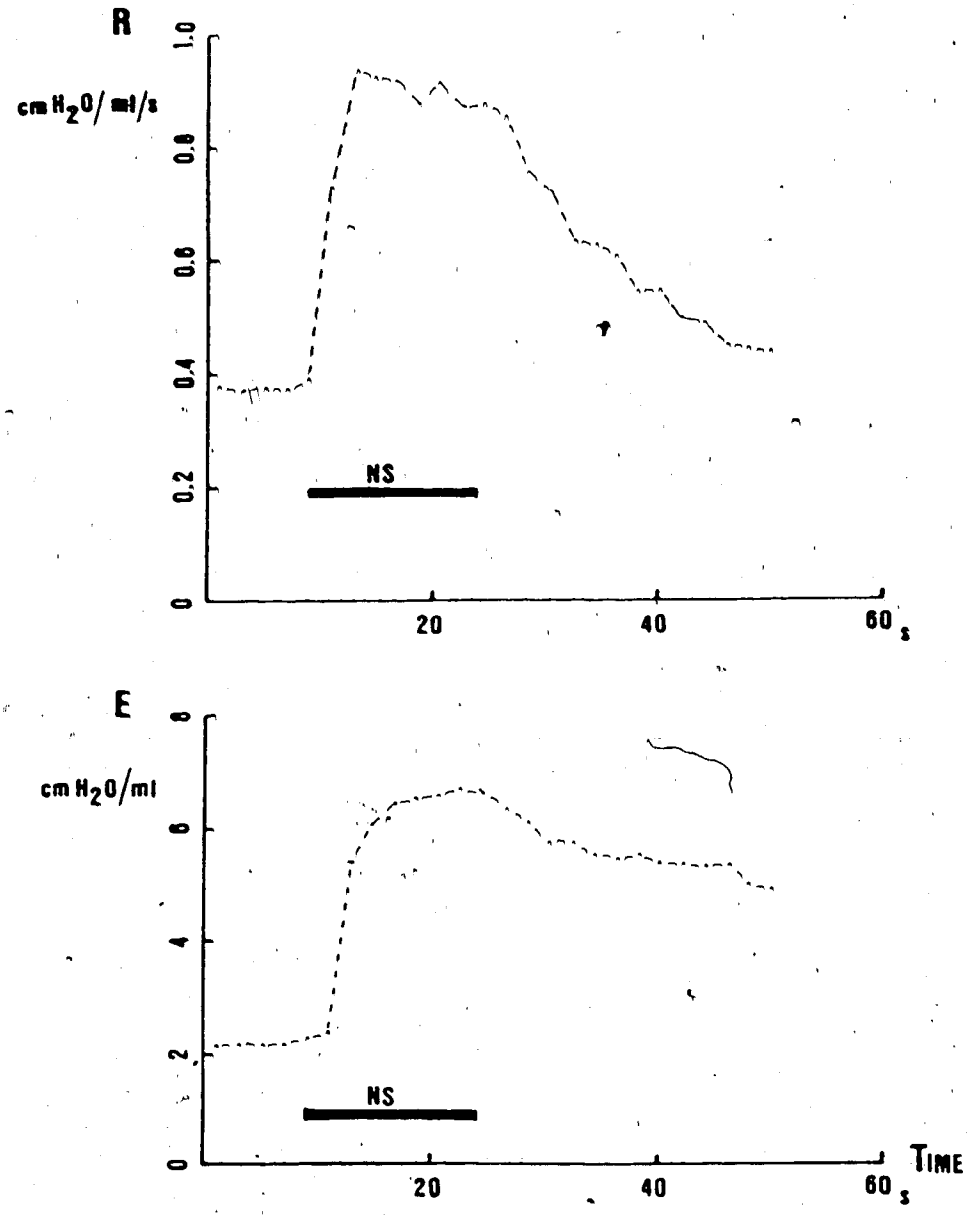


Figure 4.2 Effects of maximal stimulation on R and E.

the longer recovery times to baseline following stimulation. As with submaximal stimulation, the percentage increase in R was greater than the percentage increase in E. than those in E. The stimulus applied was strong enough to cause heart block in many animals for a few seconds during stimulation. Generally, the degree of hypotension and bradycardia was greater than during submaximal stimulation.

4.1.2 Effects of atropine sulfate

Injection of atropine sulfate (0.1 mg/kg) produced a small decrease in MABP but had negligible effects on baseline values of R and E. The bronchoconstrictor responses and the bradycardia induced by submaximal stimulation were abolished by atropine. Atropine significantly reduced, but did not abolish, pulmonary responses to maximal nerve stimulation. These atropine-resistant responses had slower onset times and peaked only after stimulation had ceased. Peak response times were significantly prolonged by atropine for both R and E (see Table 4.3). In all cases, peak R measurements were achieved faster than the respective E values. The dose of atropine administered decreased, but did not abolish, the vagally-induced hypotension. A second dose of atropine (0.5 mg/kg) failed to produce any further changes in R and E. These results are summarized in Figure 4.3.

4.1.3 Effects of SP-antagonists

These agents possess histamine-releasing properties, and were injected only into animals that had been pretreated with atropine and mepyramine. The 2 antagonists to SP used, [D-Pro^2,D-Trp^{7,9}]-SP and [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]-SP induced hypotension on injection. In animals not pretreated with atropine or mepyramine, they induced marked, prolonged bronchospasm. Their effects on vagally-induced pulmonary responses were not identical. Increases in R, but not E, were significantly reduced by [D-Pro^2,D-Trp^{7,9}]-SP (1.0 and 2.0 mg/kg), and, at higher doses of this antagonist, measurements of R fell beyond baseline values during stimulation i.e., bronchodilation occurred in the large airways.

Table 4.3 Peak response times (Tmax) before and after two injections of atropine (0.1 mg/kg and 0.5 mg/kg).

Treatment	Tmax (s)			
	Submaximal		Maximal	
	R	E	R	E
Control	12.21 ± 1.35	17.86 ± 0.52	10.14 ± 1.99	17.13 ± 0.49
Atropine 0.1 mg/kg	17.79 ± 1.71§	67.75 ± 18.63§	21.20 ± 5.08	32.68 ± 3.43#
Atropine 0.5 mg/kg	22.56 ± 7.04	111.50 ± 10.72†	24.75 ± 7.10	60.34 ± 16.80§

† $p<0.001$

§ $p<0.05$

# $p<0.01$

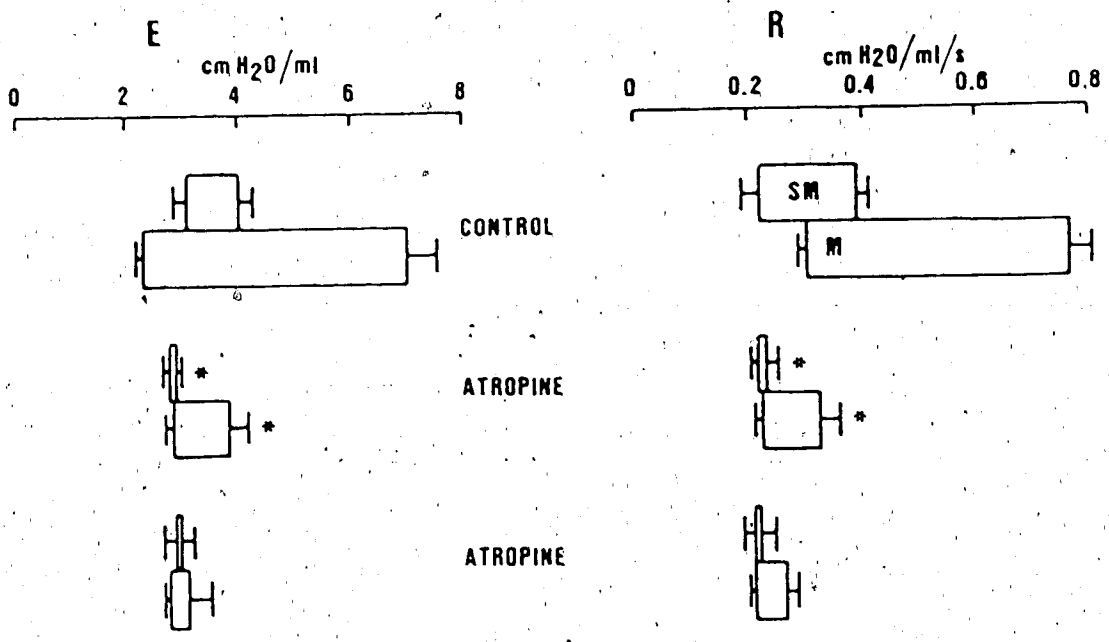


Figure 4.3 Effects of two injections of atropine (0.1 mg/kg and 0.5 mg/kg respectively) on increases in R and E to submaximal (SM) and maximal (M) stimulation. ($^{*}p<0.05$; n=4).

Infusion of [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]-SP significantly reduced the atropine-resistant increases in both R and E induced by maximal stimulation. Bronchodilation was not observed in the large airways with this antagonist. The results are summarized in Figure 4.4.

4.1.4 Effects of autonomic blockers

The effects of ganglionic and neuromuscular blocking agents on vagally-mediated pulmonary responses were investigated in normal guinea pigs. The ganglionic blockers, hexamethonium bromide and mecamylamine hydrochloride, and the neuromuscular blocking drugs, gallamine triethiodide, pancuronium bromide, and decamethonium bromide, were used in this series of experiments. The actions of gallamine and pancuronium were also studied in vehicle- and nonylvanylamide-pretreated guinea pigs, the results of which will be presented in a later section.

4.1.4.1 Hexamethonium bromide

Administration of hexamethonium (5.0 mg/kg) alone increased baseline values of E, decreased those of R, and decreased the MABP. Increases in R and E to submaximal nerve stimulation were virtually abolished by hexamethonium, while those to maximal stimulation were reduced in size. In both cases, the bradycardia and hypotension were abolished. The peak response times were also significantly delayed. This can be seen more clearly from a typical plot of R and E measurements breath-by-breath (Figure 4.5). A second dose of hexamethonium (5.0 mg/kg) abolished increases in R induced by submaximal stimulation without altering the increases in E. Atropine (0.5 mg/kg) reduced the responses further, but these changes were not significant. Responses to maximal stimulation were reduced by the second dose of hexamethonium and also by atropine. However, a hexamethonium- and atropine-resistant response persisted. Figure 4.6 summarizes the results obtained with this agent.

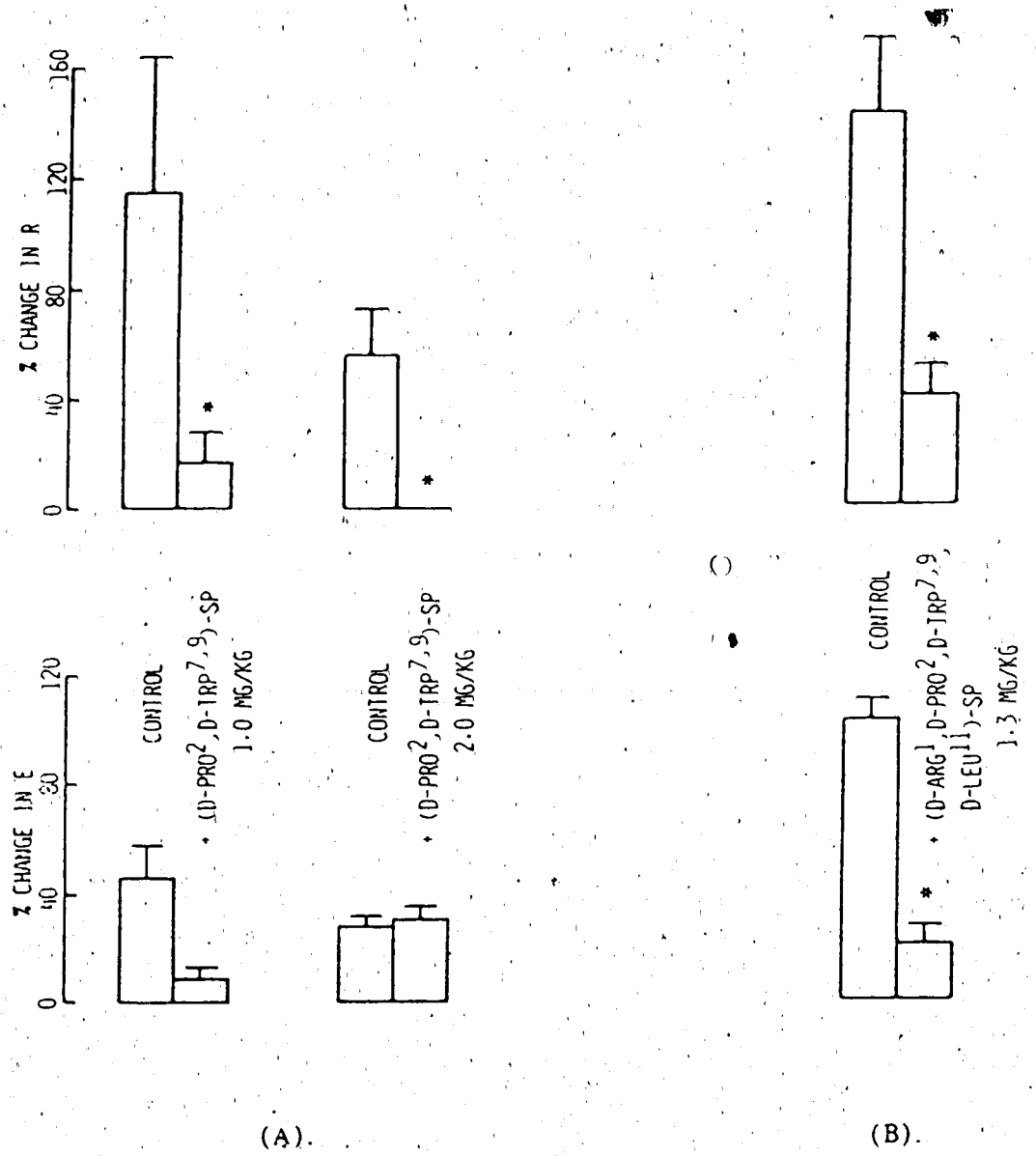


Figure 4.4 Effects of (A). Two injections of [D-Pro^2,D-Trp^{7,9}]-SP (1.0 mg/kg) and (B). [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]-SP (1.0 + 0.3 mg/kg) on increases in R and E induced by maximal stimulation in animals pretreated with atropine and mepyramine. ($^*p<0.05$; n=4).

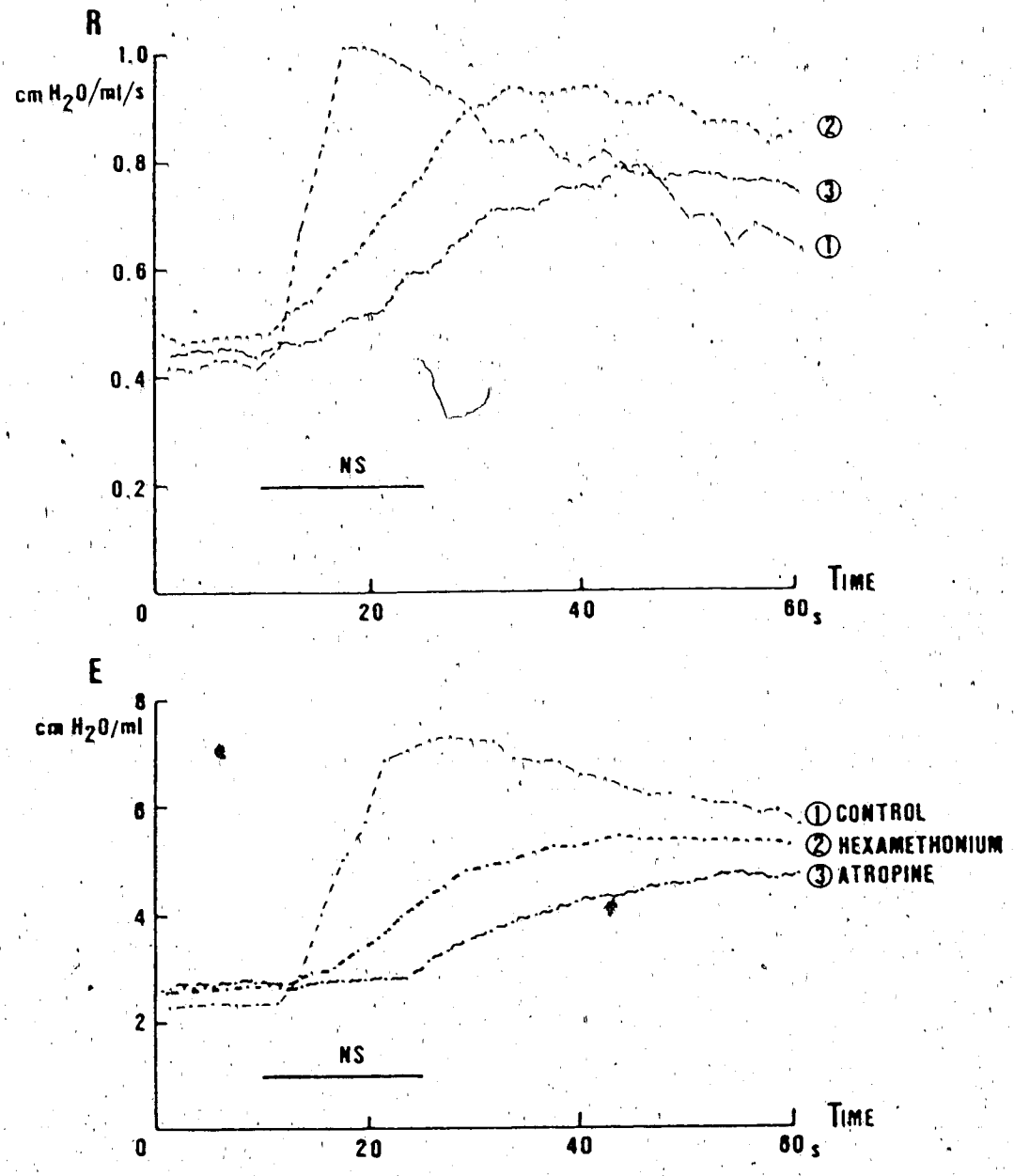


Figure 4.5 Typical effects of hexamethonium and atropine on increases in R and E induced by maximal nerve stimulation.

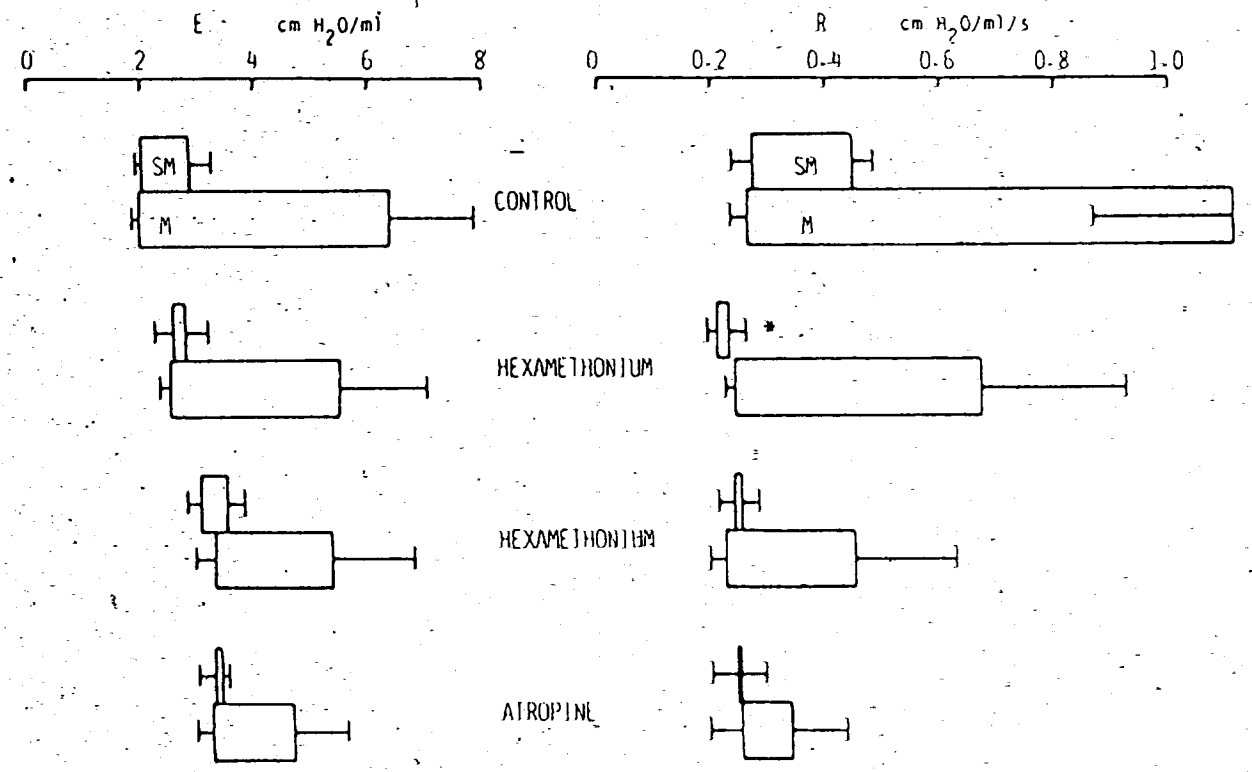


Figure 4.6 Effects of two injections of hexamethonium (5.0 mg/kg) and a subsequent injection of atropine (0.5 mg/kg) on increases in R and E induced by submaximal (SM) and maximal (M) nerve stimulation. (*$p<0.05$; $n=4$).

4.1.4.2 Mecamylamine hydrochloride

After mecamylamine hydrochloride (1.0 mg/kg), baseline measurements of R and E were increased. Increases in R and E to submaximal and maximal vagal stimulation were reduced, but were not significantly different from the control responses (Figure 4.7). The times to maximal response were significantly prolonged after the first dose of 1 mg/kg and increased by a second dose. Atropine (0.5 mg/kg) almost abolished the responses to submaximal stimulation but had insignificant effects on those to maximal stimulation. Atropine also delayed the peak response times in animals given mecamylamine (Table 4.4).

4.1.4.3 Gallamine triethiodide

Gallamine (5.0 mg/kg) alone had minimal effects on baseline measurements of R and E, but caused a fall in MABP. It enhanced responses to submaximal stimulation, but only the increase in E was statistically significant. Responses to maximal stimulation were not altered significantly. In both cases, the increases in E were greater than the increases in R (see Figure 4.8). The concomitant bradycardia was abolished by gallamine, but the fall in MABP was unaltered. Atropine (0.5 mg/kg) abolished responses to submaximal stimulation, but only reduced those to maximal stimulation.

Gallamine did not potentiate responses to vagal nerve stimulation in animals that had been pretreated with either atropine (0.5 mg/kg) or mecamylamine (2.0 mg/kg) (see Figures 4.9 & 4.10).

4.1.4.4 Pancuronium bromide

Results obtained with pancuronium (0.1 mg/kg) were similar to those with gallamine. However, responses to submaximal and maximal stimulation were not increased significantly by this dose of pancuronium. Also, neither the bradycardia nor the hypotension was abolished by pancuronium. Atropine (0.5 mg/kg), given after pancuronium, abolished pulmonary responses to submaximal stimulation, but not those to

Table 4.4 Peak response times (Tmax) before and after two injections of mecamylamine (1.0 mg/kg) and a subsequent injection of atropine (0.5 mg/kg).

Treatment	Tmax (s)			
	Submaximal		Maximal	
	R	E	R	E
Control	14.67 ± 0.48	16.36 ± 0.40	11.54 ± 1.48	17.19 ± 0.28
Mecamylamine 1.0 mg/kg	12.24 ± 1.64	24.20 ± 5.39	19.58 ± 0.46#	33.14 ± 3.92*
Mecamylamine 1.0 mg/kg	15.12 ± 0.48	39.87 ± 4.05#	21.89 ± 2.12*	48.75 ± 5.88#
Atropine 0.5 mg/kg	17.27 ± 4.43	48.48 ± 3.49†	30.23 ± 5.63§	45.92 ± 5.95#

† $p<0.001$

# $p<0.005$

§ $p<0.05$

* $p<0.01$

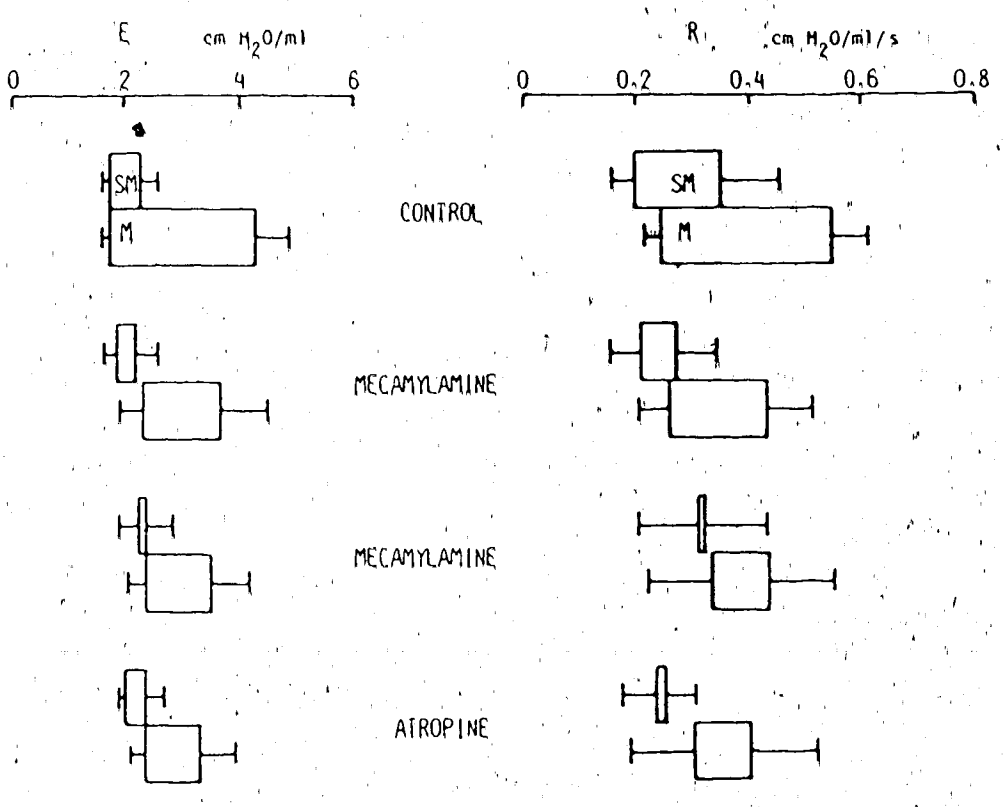


Figure 4.7 Effects of two injections of mecamylamine (1.0 mg/kg) and a subsequent injection of atropine (0.5 mg/kg) on increases in R and E induced by submaximal (SM) and maximal (M) stimulation. (n=4).

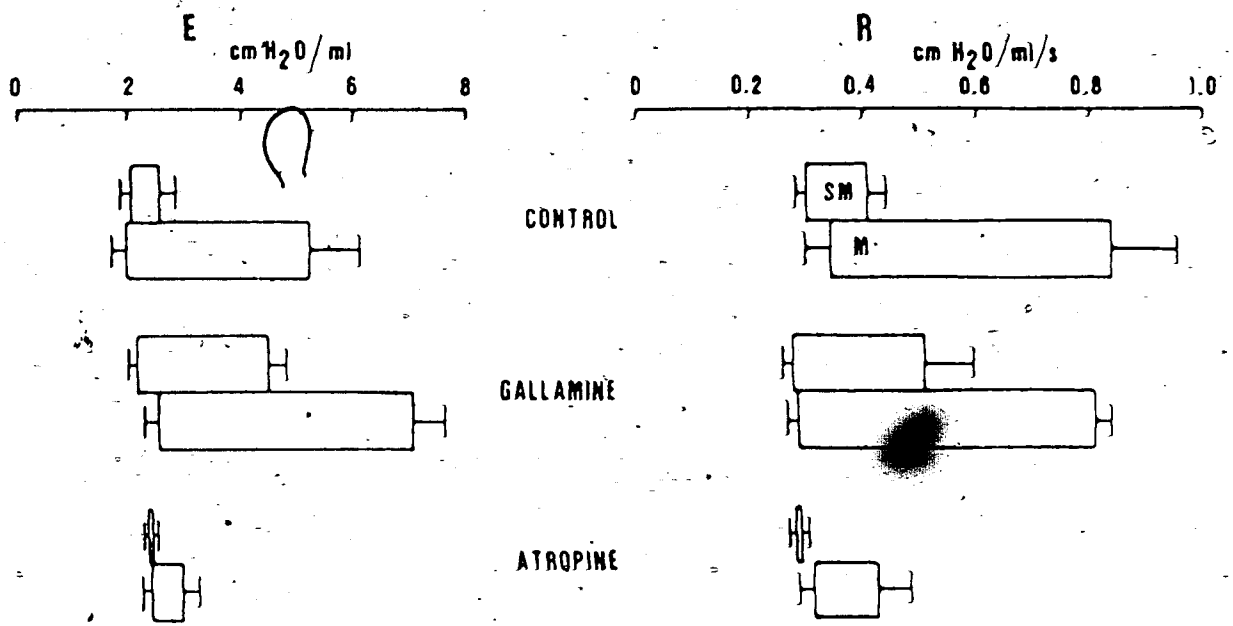


Figure 4.8 Effects of an injection of gallamine (5.0 mg/kg) and a subsequent injection of atropine (0.5 mg/kg) on increases in R and E induced by submaximal (SM) and maximal (M) stimulation. (*$p<0.05$; $n=4$).

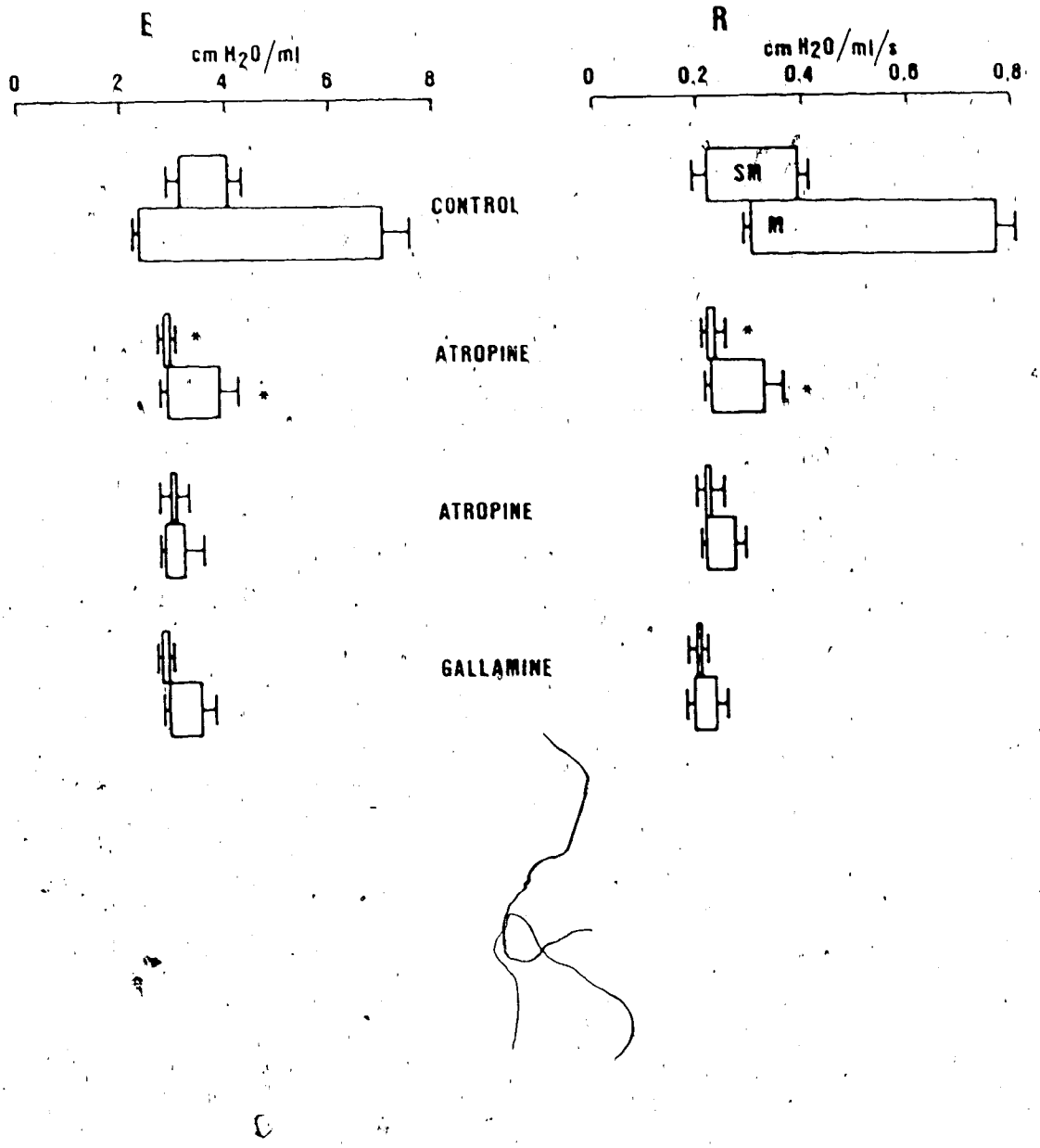


Figure 4.9 Effects of an injection of gallamine (5.0 mg/kg) on increases in R and E after two injections of atropine (0.1 mg/kg and 0.5 mg/kg respectively). (*$p<0.05$; $n=4$).

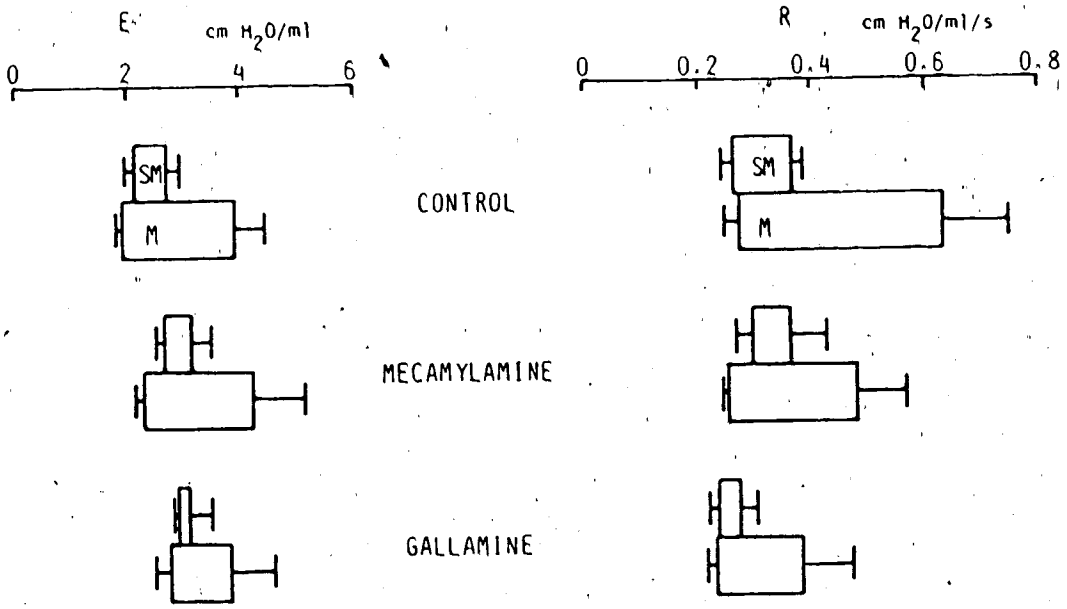


Figure 4.10 Effects of an injection of gallamine (5.0 mg/kg) on increases in R and E after an injection of mecamylamine (2.0 mg/kg). (n=4).

maximal stimulation (Figure 4.11).

4.1.4.5 Decamethonium bromide

Control responses to submaximal and maximal stimulation were similar to those in the gallamine and pancuronium series of experiments. Responses to vagal stimulation were either unchanged or slightly decreased after decamethonium (0.2 mg/kg) but the changes were not significant. As in the other experiments, atropine abolished submaximal responses to vagal stimulation, but only reduced maximal responses. The results are summarized in Figure 4.12.

4.1.5 Effects of methacholine chloride (MCh)

Methacholine (MCh), 2 and 4 μg/kg i.v., induced similar effects to vagal nerve stimulation. MCh increased R and E and caused bradycardia and hypotension. Responses were rapid in onset and reached their maximal size within 30 s after injection. Atropine (0.5 mg/kg) abolished the increases in R and E and eliminated the bradycardia; however, the hypotension was unchanged.

4.2 Characterization of the sympathetic innervation

The effects of the β-adrenoceptor antagonist propranolol on bronchospastic responses induced by vagal stimulation and intravenous MCh were investigated.

4.2.1 Effects of propranolol hydrochloride

The results obtained with propranolol (2.0 mg/kg) are summarized in Figure 4.13. Propranolol alone increased values of both the R and E, however, only the change in R was significantly different from control. Propranolol caused a marked fall in the MABP and the HR. Two out of six animals died after receiving this dose of drug. The times to peak response to submaximal and maximal stimulation were unaltered by propranolol (see Table 4.5). Increases in R and E were not significantly potentiated by propranolol, but they were reduced

Table 4.5 Peak response times (Tmax) before and after an injection of propranolol (2.0 mg/kg) and a subsequent injection of atropine (0.5 mg/kg).

Treatment	Tmax (s)			
	Submaximal		Maximal	
	R	E	R	E
Control	10.96 ± 2.58	18.08 ± 0.89	8.32 ± 0.98	17.80 ± 0.49
Propranolol 2.0 mg/kg	13.48 ± 1.12	18.05 ± 0.30	8.66 ± 0.94	17.81 ± 0.46
Atropine 0.5 mg/kg	48.84 ± 25.11	76.72 ± 13.91*	30.53 ± 6.40§	55.98 ± 5.98†

† $p<0.001$

§ $p<0.05$

* $p<0.01$

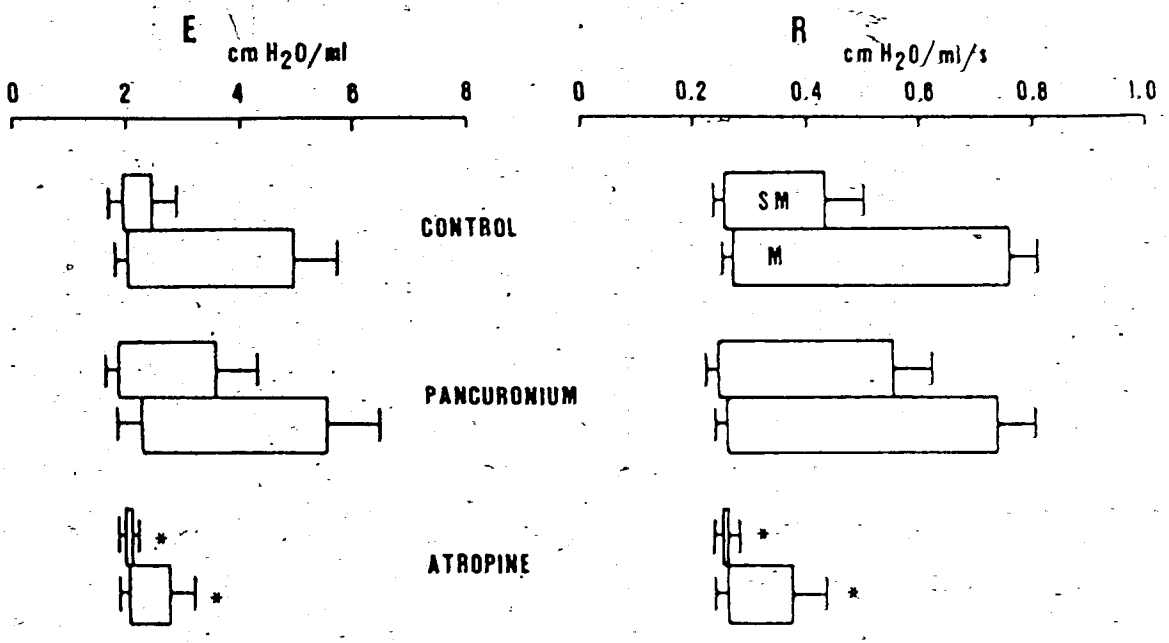


Figure 4.11 Effects of an injection of pancuronium (0.1 mg/kg) and a subsequent injection of atropine (0.5 mg/kg) on increases in R and E induced by submaximal (SM) and maximal (M) stimulation. (*$p<0.05$; $n=4$).

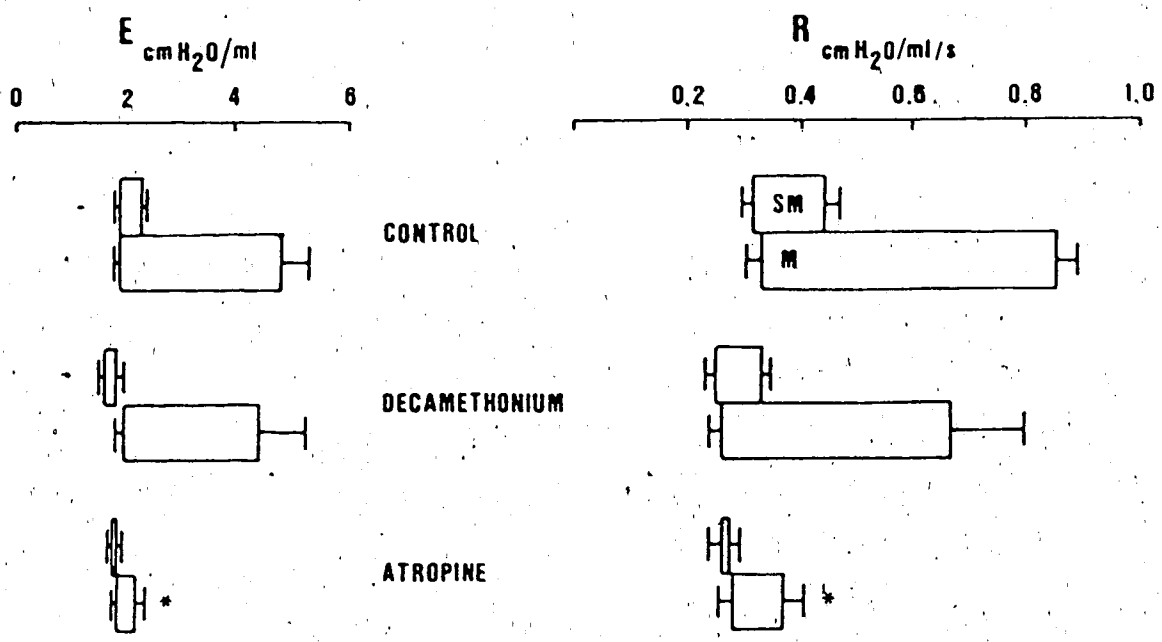


Figure 4.12 Effects of an injection of decamethonium (0.2 mg/kg) and a subsequent injection of atropine (0.5 mg/kg) on increases in R and E induced by submaximal (SM) and maximal (M) stimulation. (*$p<0.05$; $n=4$).

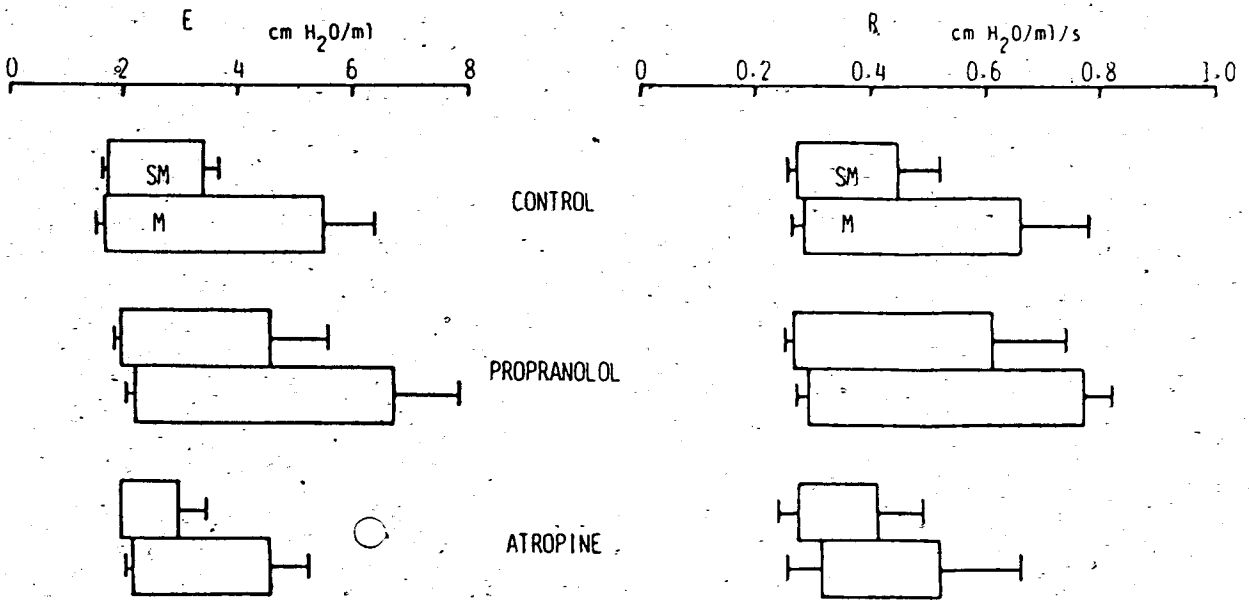


Figure 4.13 Effects of an injection of propranolol (2.0 mg/kg) and a subsequent injection of atropine (0.5 mg/kg) on increases in R and E induced by submaximal (SM) and maximal (M) stimulation. (n=4).

by atropine (0.5 mg/kg) which also significantly prolonged the peak response times. Increases in R and E to submaximal stimulation were not abolished by atropine in these animals.

Injection of exogenous MCh (2 and 4 μg/kg) increased both R and E and decreased HR and MABP concomitantly. The increases in R and E were potentiated by propranolol and abolished by atropine.

4.3 Experiments with nonylvanylamide

The objectives of these experiments were to:

1. Determine the effects of nonylvanylamide pretreatment on responses to submaximal and maximal vagal nerve stimulation 7-8 days after treatment, and compare these responses to those of vehicle-pretreated controls.
2. Examine the effects of gallamine and pancuronium on increases in R and E induced by vagal stimulation in these 2 groups of animals.
3. Try and characterize the inhibitory NANC system in nonylvanylamide-pretreated animals after cholinergic and adrenergic blockade.
4. Determine the time course of the effects on pulmonary responses to vagal stimulation, and to the bronchospastic drugs MCh, Hist, and SP, in animals 2, 8, 16, 32, and 64 days after treatment with nonylvanylamide, and compare these responses to those of vehicle-pretreated controls to the same stimuli. Gallamine was used as a pharmacological tool to enhance the pulmonary responses elicited by vagal nerve stimulation, and the degree of restoration of the responses with time was also investigated.

Test doses of nonylvanylamide (2 and 16 μg/kg i.v.) were injected into animals and the effects on R and E measured to confirm desensitization by nonylvanylamide in all animals.

4.3.1 Effects of vehicle-pretreatment

The pulmonary and cardiovascular effects of nerve stimulation in these animals were similar to those in normal untreated guinea pigs (see Section 4.1.1). Both R and E increased promptly and reached their maximal values during or shortly after stimulation, and HR and MABP were decreased concomitantly. Although responses derived from maximal stimulation were significantly larger than submaximal responses, the peak response times did not differ.

Nonylvanylamide (2 μg/kg i.v.) increased R and E significantly in these animals.

4.3.1.1 Effects of gallamine and pancuronium

Increases in E, but not R, in response to submaximal stimulation were significantly ($p<0.05$) enhanced by gallamine (5.0 mg/kg). Atropine (0.5 mg/kg) abolished these responses. Figure 4.14 shows the typical increases in R and E induced by submaximal stimulation before and after gallamine and atropine respectively. Maximal responses to vagal stimulation were either unchanged or slightly enhanced by gallamine, and only partly reduced by atropine (see Figure 4.15). Bradycardia induced by stimulation was blocked by gallamine; stimulation-induced hypotension was not altered to any appreciable extent. Gallamine also did not alter the times taken to achieve the peak responses, but atropine significantly prolonged them (Table 4.6). The effects of gallamine and atropine on pulmonary responses to vagal stimulation in vehicle-pretreated controls are summarized in Figure 4.16. Figure 4.17 is a tracing of the tracheal pressure (TP) and blood pressure (BP) signals of a vehicle-pretreated animal showing the pulmonary and cardiovascular effects of submaximal and maximal stimulation before and after gallamine and atropine.

Pancuronium (0.1 mg/kg) did not potentiate responses to submaximal or maximal stimulation. It actually decreased the responses slightly. The dose of pancuronium used had minimal effects on the bradycardia and hypotension induced by stimulation. Atropine (0.5 mg/kg) reduced submaximal and maximal responses to stimulation in these experiments. The effects of pancuronium and atropine are summarized in Figure 4.18.

Table 4.6 Effects of an injection of gallamine (5.0 mg/kg) and a subsequent injection of atropine (0.5 mg/kg) on peak response times (Tmax).

Treatment	Tmax (s)			
	Submaximal		Maximal	
	R	E	R	E
Control	13.30 ± 1.30	19.17 ± 0.81	12.61 ± 1.90	18.32 ± 1.17
Gallamine 5.0 mg/kg	9.63 ± 1.62	14.54 ± 1.32§	11.51 ± 2.63	17.12 ± 1.16
Atropine 0.5 mg/kg	31.83 ± 7.33§	50.14 ± 8.48§	35.44 ± 5.79*	45.13 ± 2.85†

† $p<0.001$

§ $p<0.05$

* $p<0.01$

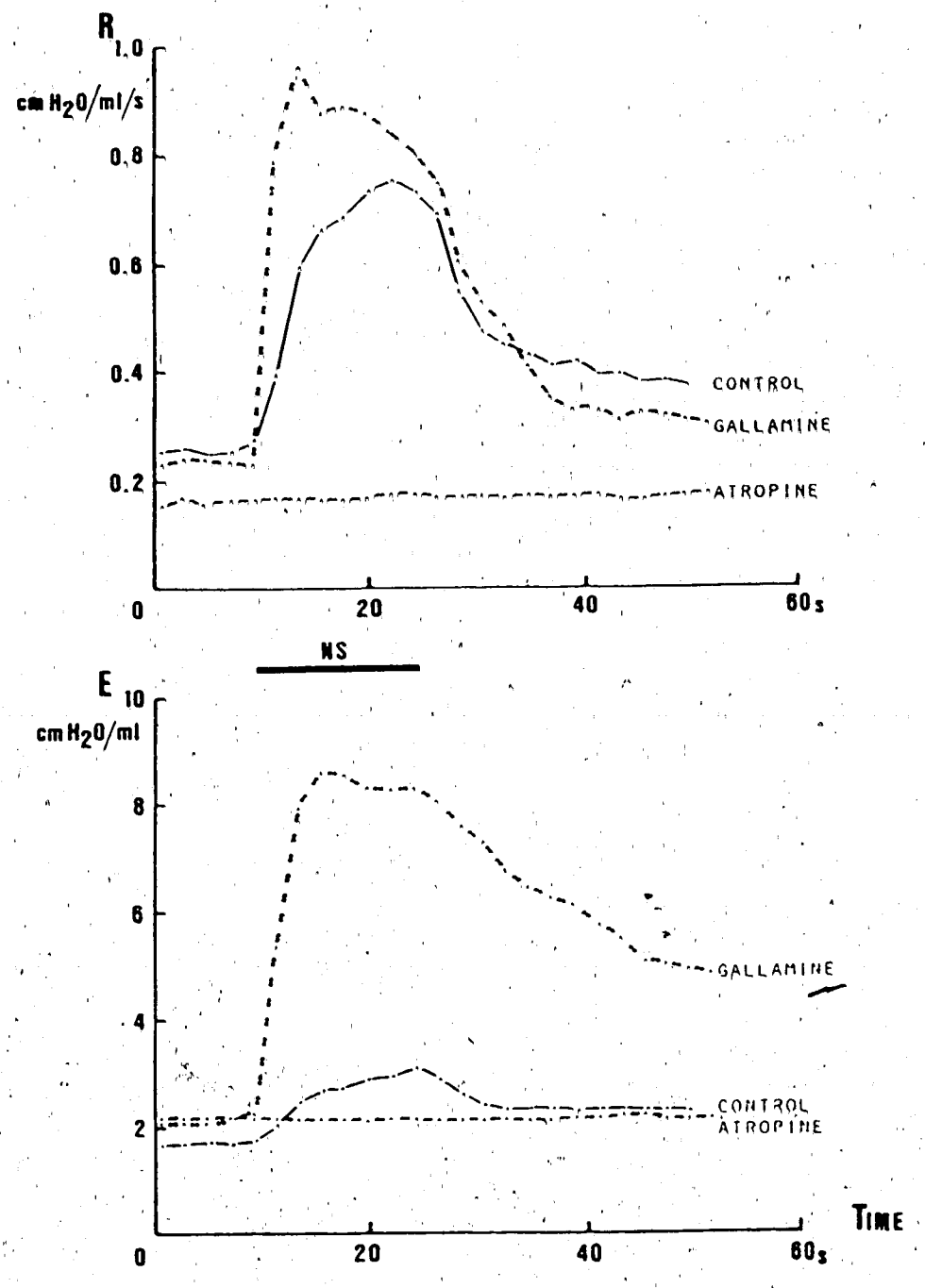


Figure 4.14 Effects of an injection of gallamine (5.0 mg/kg) and a subsequent injection of atropine (0.5 mg/kg) on increases in R and E induced by submaximal stimulation.

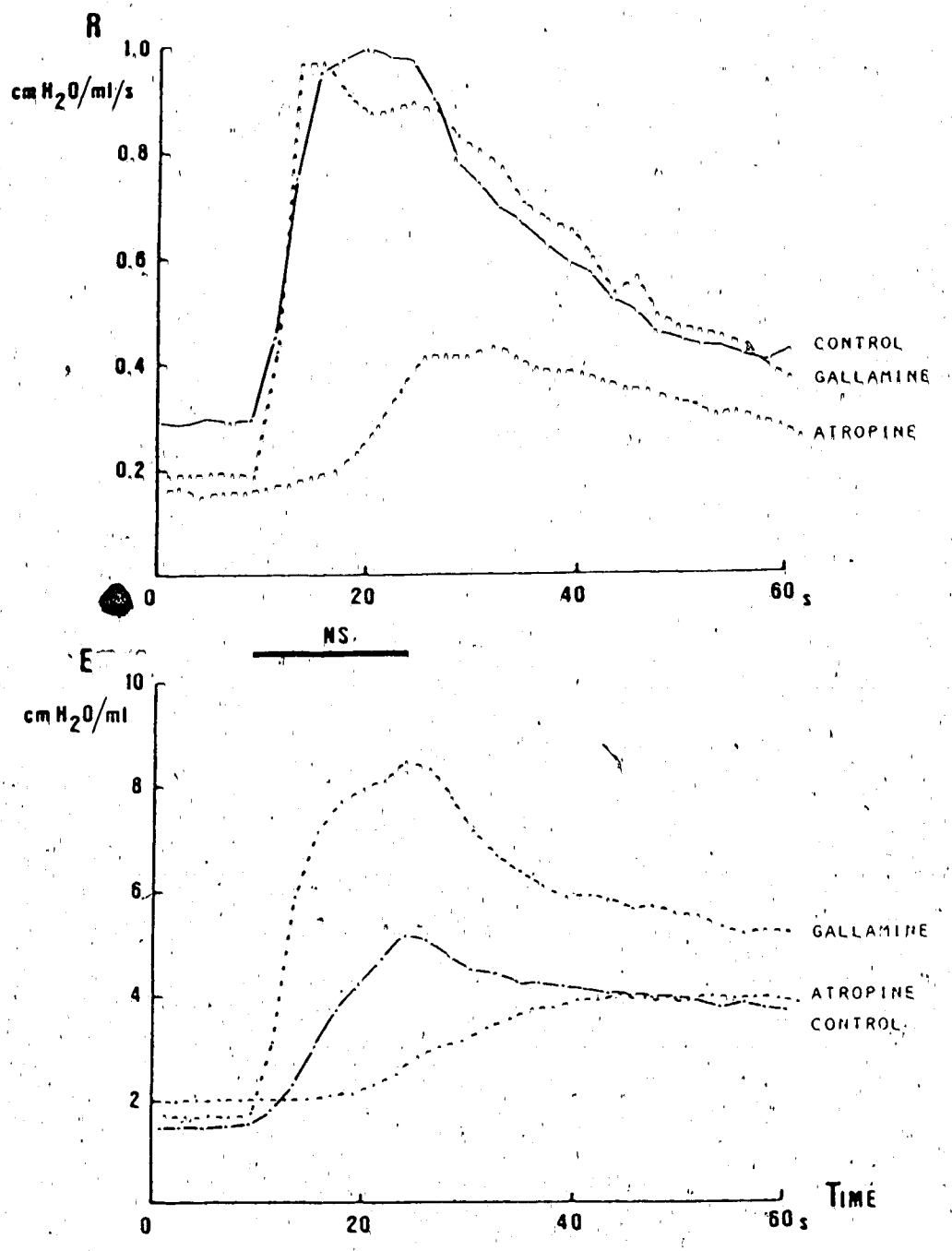


Figure 4.15 Effects of an injection of gallamine (5.0 mg/kg) and a subsequent injection of atropine (0.5 mg/kg) on increases in R and E induced by maximal stimulation.

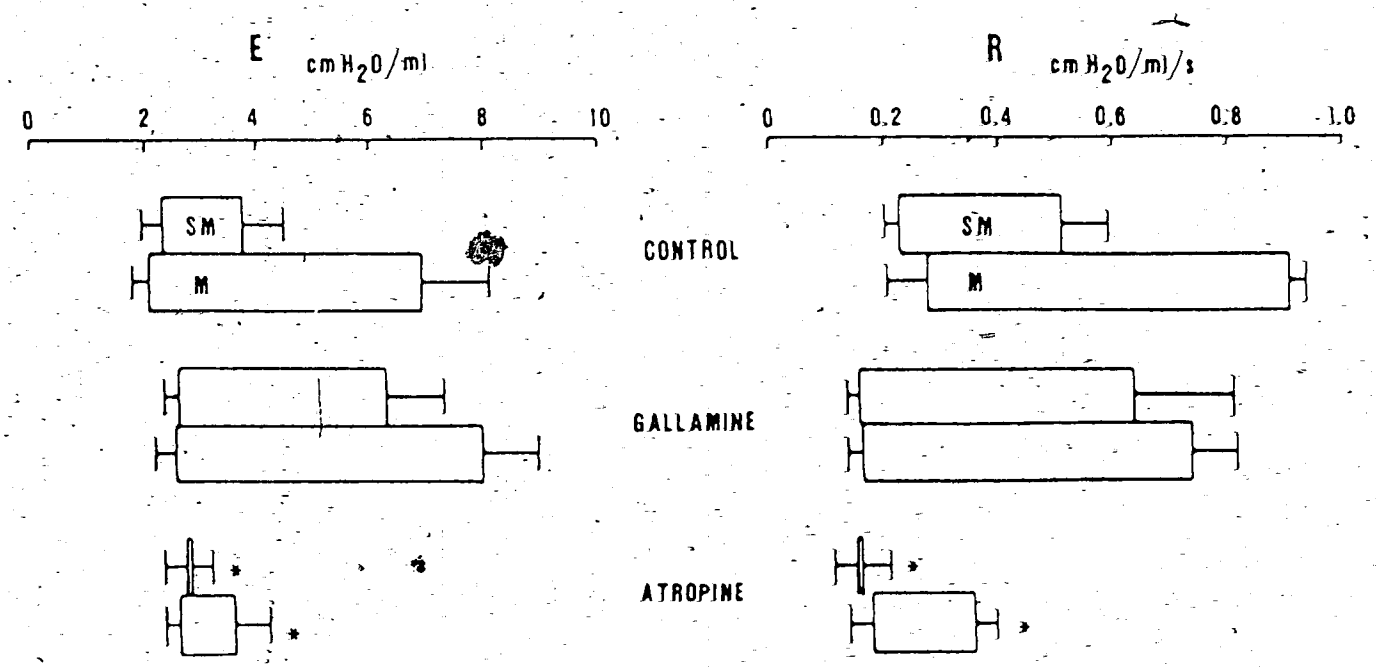


Figure 4.16 Effects of an injection of gallamine (5.0 mg/kg) and a subsequent injection of atropine (0.5 mg/kg) on increases in R and E induced by submaximal (SM) and maximal (M) stimulation in vehicle-treated animals. (*$p<0.05$; $n=4$).

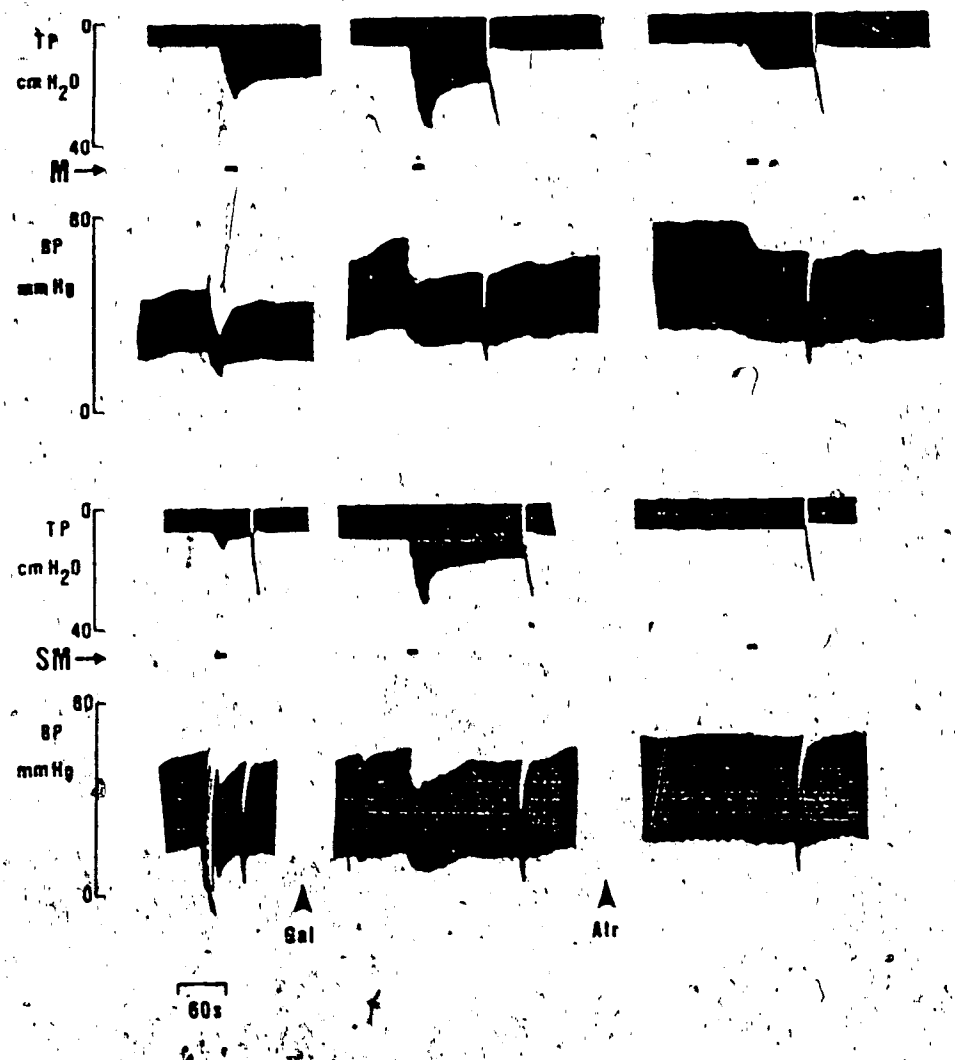


Figure 4.17 Tracheal pressure (TP) and blood pressure (BP) tracings of a vehicle-treated animal during submaximal (SM) and maximal (M) stimulation before and after gallamine (5.0 mg/kg) and atropine (0.5 mg/kg).

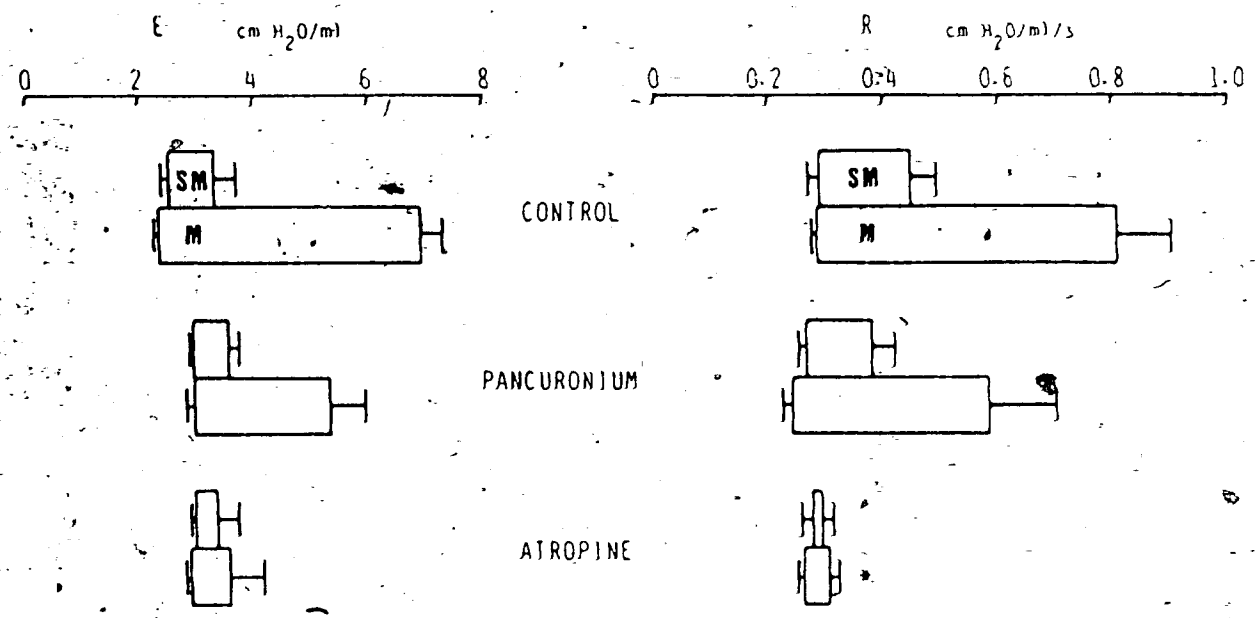


Figure 4.18 Effects of an injection of pancuronium (0.1 mg/kg) and a subsequent injection of atropine (0.5 mg/kg) on increases in R and E to submaximal (SM) and maximal (M) vagal stimulation in vehicle-treated animals. (*$p<0.05$; $n=4$).

4.3.1.2 Effects of exogenous MCh

MCh, 2 and 4 μg/kg, increased R and E and induced bradycardia and hypotension in these control animals. These effects had rapid onsets and peaked within 30 s after injection before returning to baseline values, usually within 3-5 min. Figure 4.19 shows the pulmonary and cardiovascular effects of MCh 4 μg/kg in a control and nonylvanylamide-pretreated animal. These results are summarized in Figure 4.27 and compared to those of the nonylvanylamide-pretreated animals.

4.3.2 Effects of nonylvanylamide-pretreatment

Submaximal stimulation did not alter baseline values of R and E in these animals. Maximal stimulation induced small increases (< 50% of baseline values) in R and E. Increases in R were consistently larger than increases in E. When the voltage was increased from 15 to 50 V, increases were slightly larger. These were termed "supramaximal" responses although they were not significantly larger than the responses evoked by maximal stimulation. Stimulation did not induce bronchoconstriction at all in some animals. The responses in animals treated with nonylvanylamide were different from responses of the controls because they were significantly smaller and lasted for a shorter time. The shape of the response also differed from that of the control animals (see Figure 4.20). The differences can also be seen and compared from the tracheal pressure tracings (Figure 4.21). In control animals, the responses to maximal stimulation consisted of a fast and a slow component; nonylvanylamide-pretreatment abolished the slow component of the response. Because of the absence of the slow phase in the responses of nonylvanylamide-treated animals, the overall duration of the responses in these animals was shorter. Although vagal stimulation had minimal effects on the airway smooth muscle, its cardiovascular effects were evident by the fall in MABP and the decrease in HR observed even during submaximal stimulation when no pulmonary effects were apparent (see Figure 4.22).

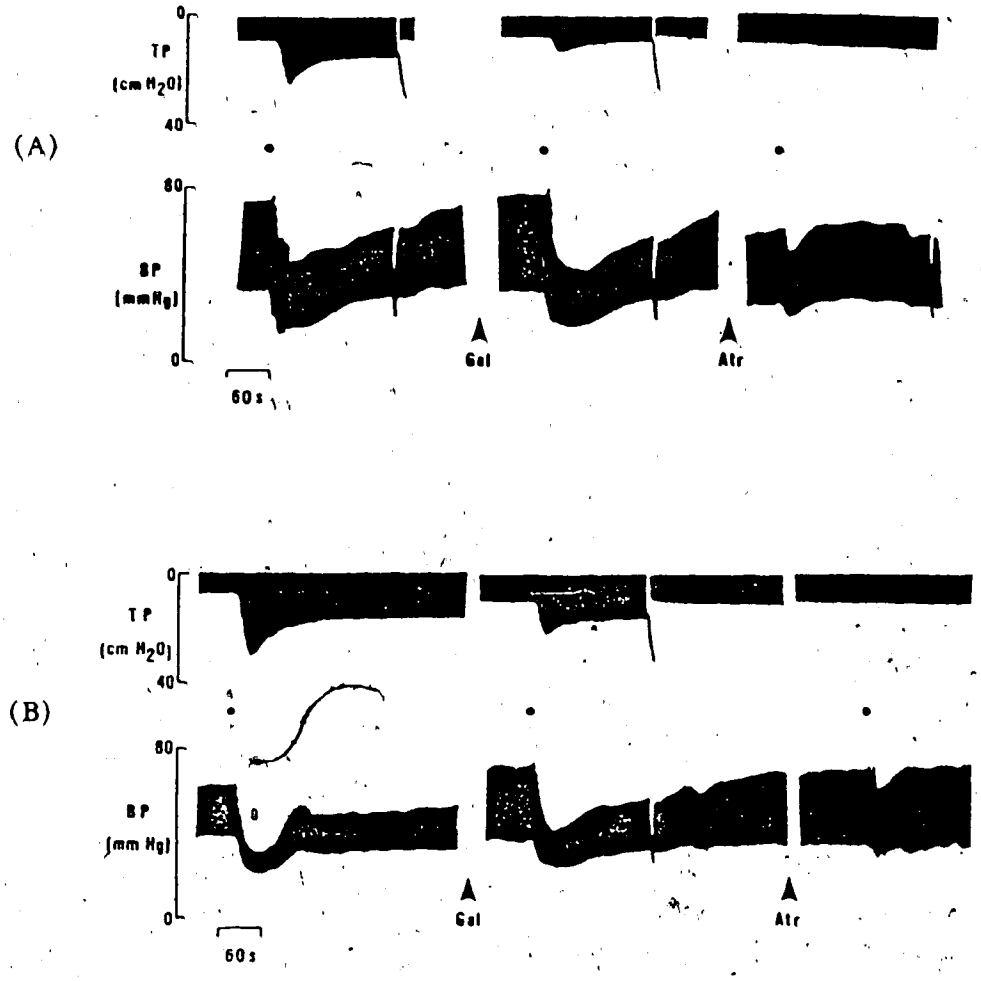


Figure 4.19 Changes in tracheal pressure (TP) and blood pressure (BP) induced by MCh 4 μg/kg in (A). a vehicle-treated and (B). a nonylvanylamide-treated animal, before and after gallamine (5.0 mg/kg) and atropine (0.5 mg/kg). • indicates the time of injection.

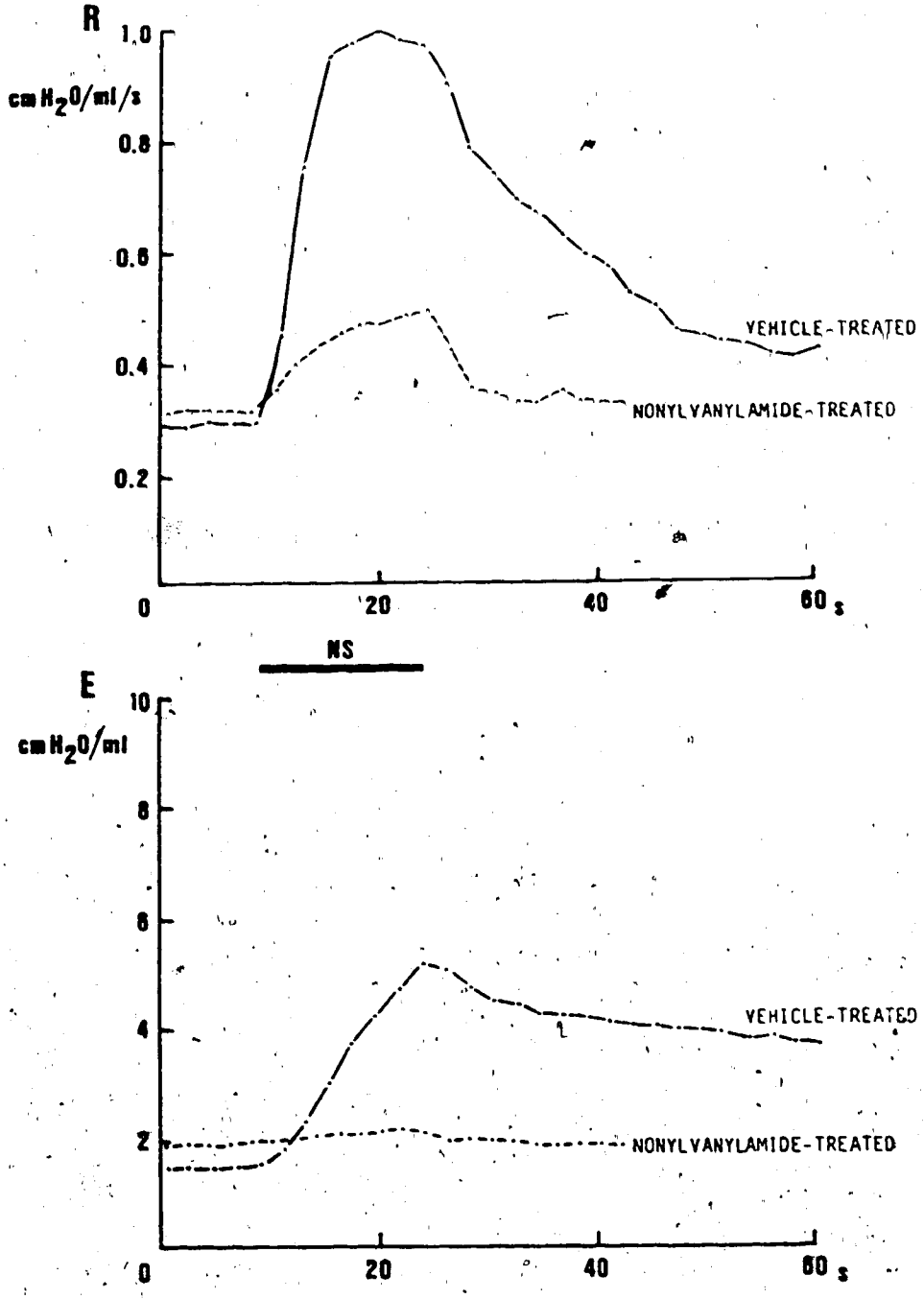


Figure 4.20 Effects of maximal vagal stimulation in vehicle- and nonylvanylamide-treated animals.

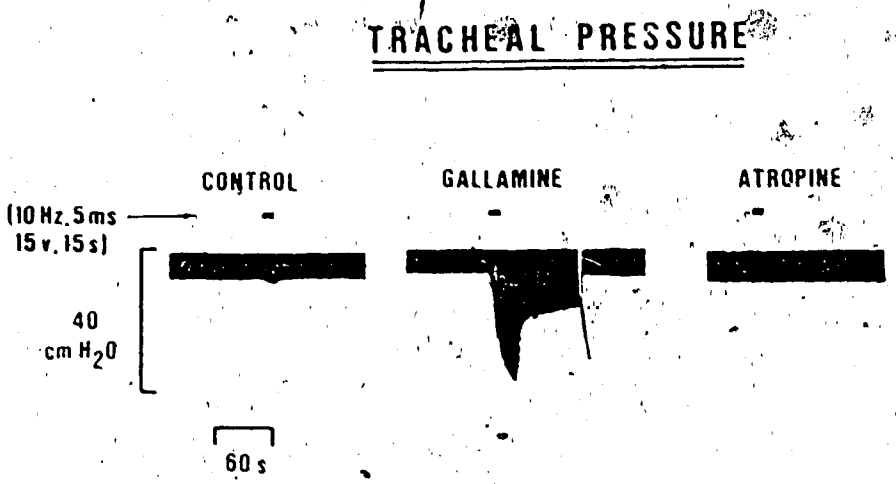


Figure 4.21 Increases in tracheal pressure (TP) in response to maximal vagal nerve stimulation before and after gallamine (5.0 mg/kg) and atropine (0.5 mg/kg) in a nonylvanylamide-treated animal.

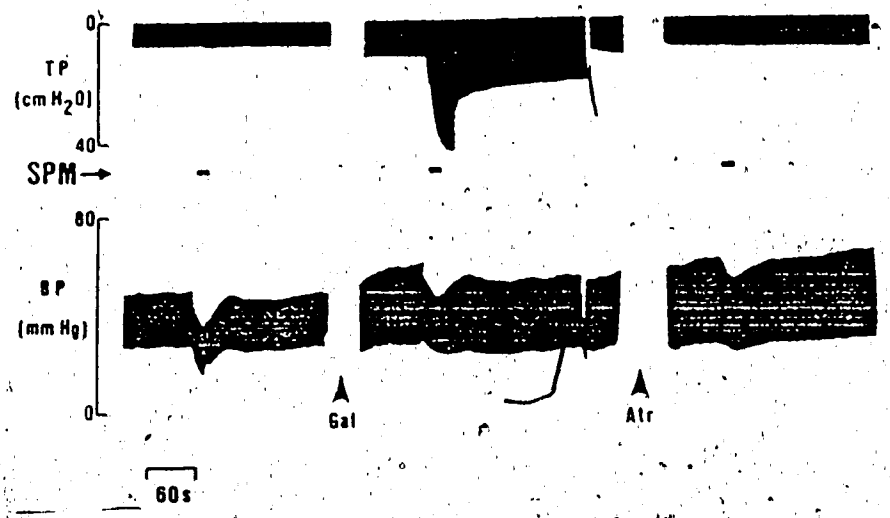


Figure 4.22 Pulmonary and cardiovascular effects of supramaximal stimulation in a nonylvanylamide-treated animal before and after gallamine (5.0 mg/kg) and atropine (0.5 mg/kg).

Nonylvanylamide (2 μg/kg i.v.) did not increase baseline values of R and E in nonylvanylamide-pretreated animals. At higher doses (>16 μg/kg), only small, or no, increases in R and E were observed.

4.3.2.1 Effects of gallamine and pancuronium

After injection of gallamine (5 mg/kg), stimulation-induced increases in R and E were potentiated and reached levels similar to those of vehicle-treated controls. Figures 4.23 and 4.24 show the typical increases in R and E in response to maximal and supramaximal stimulation respectively, before and after gallamine and atropine in a nonylvanylamide-pretreated animal. Potentiation of the responses to submaximal stimulation also occurred in nonylvanylamide-treated animals, but these were not quantified. As in the control animals, gallamine abolished the bradycardia that accompanied stimulation. Figure 4.25 summarizes the effects of gallamine and atropine on increases in R and E induced by maximal stimulation in 4 nonylvanylamide-pretreated animals.

Experiments with pancuronium yielded results similar to those with gallamine (see Figure 4.26). However, the potentiation of the responses was smaller in magnitude.

Atropine (0.5 mg/kg) abolished the vagally-induced increases in R and E in nonylvanylamide-treated animals, unlike in the controls in which responses were only reduced.

4.3.2.2 Effects of exogenous MCh

Exogenous MCh 2 and 4 μg/kg, induced similar effects in nonylvanylamide-pretreated animals as in the controls: R and E were increased and bradycardia and hypotension were induced. Figure 4.19 shows the tracheal pressure and blood pressure effects of MCh 4 μg/kg in a vehicle-pretreated animal and a nonylvanylamide-pretreated animal. The effects of MCh in control and nonylvanylamide-pretreated animals are summarized in Figure 4.27. The changes in R

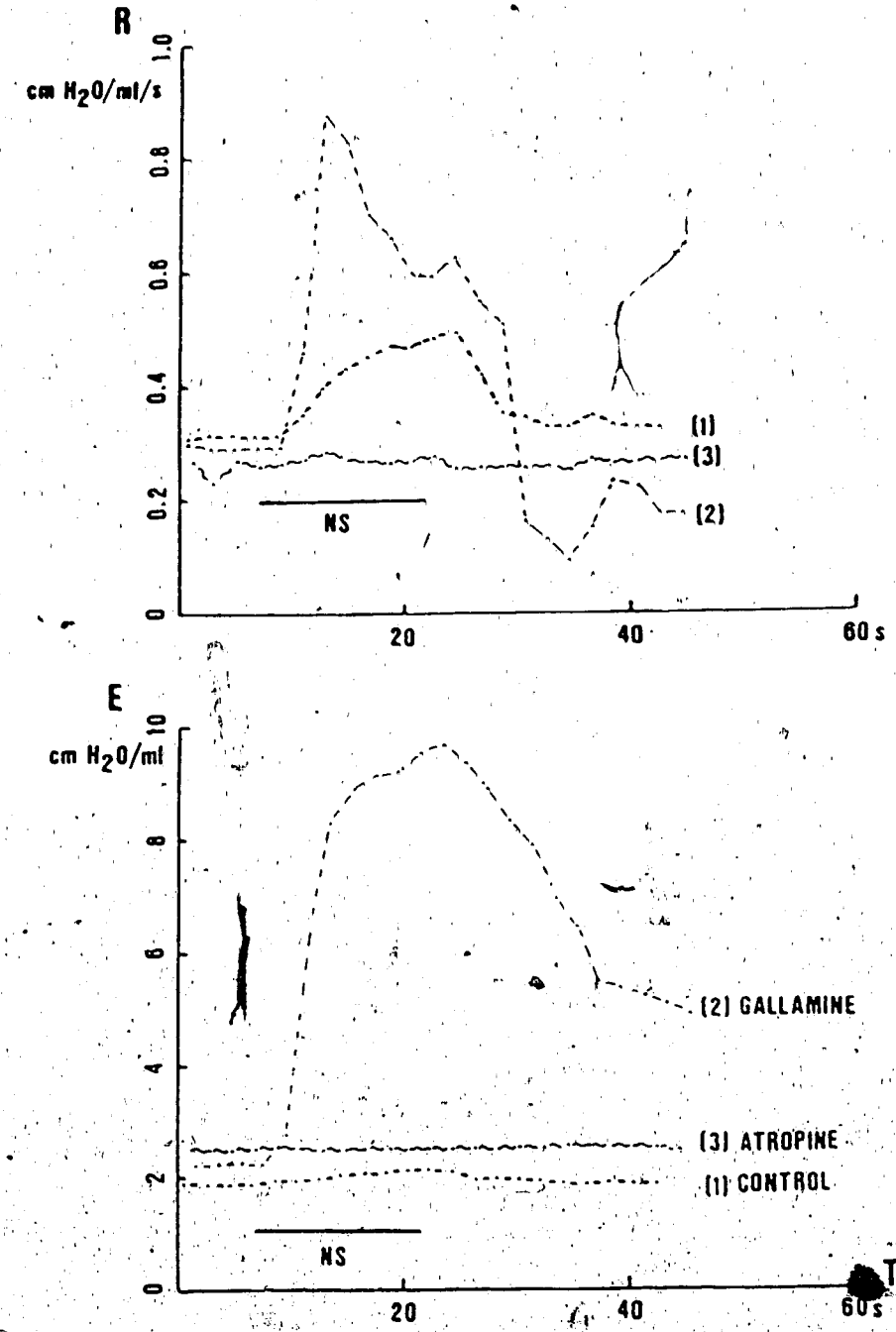


Figure 4.23 Increases in R and E in response to maximal stimulation before and after gallamine (5.0 mg/kg) and atropine (0.5 mg/kg) in a nonylvanylamide-treated animal.

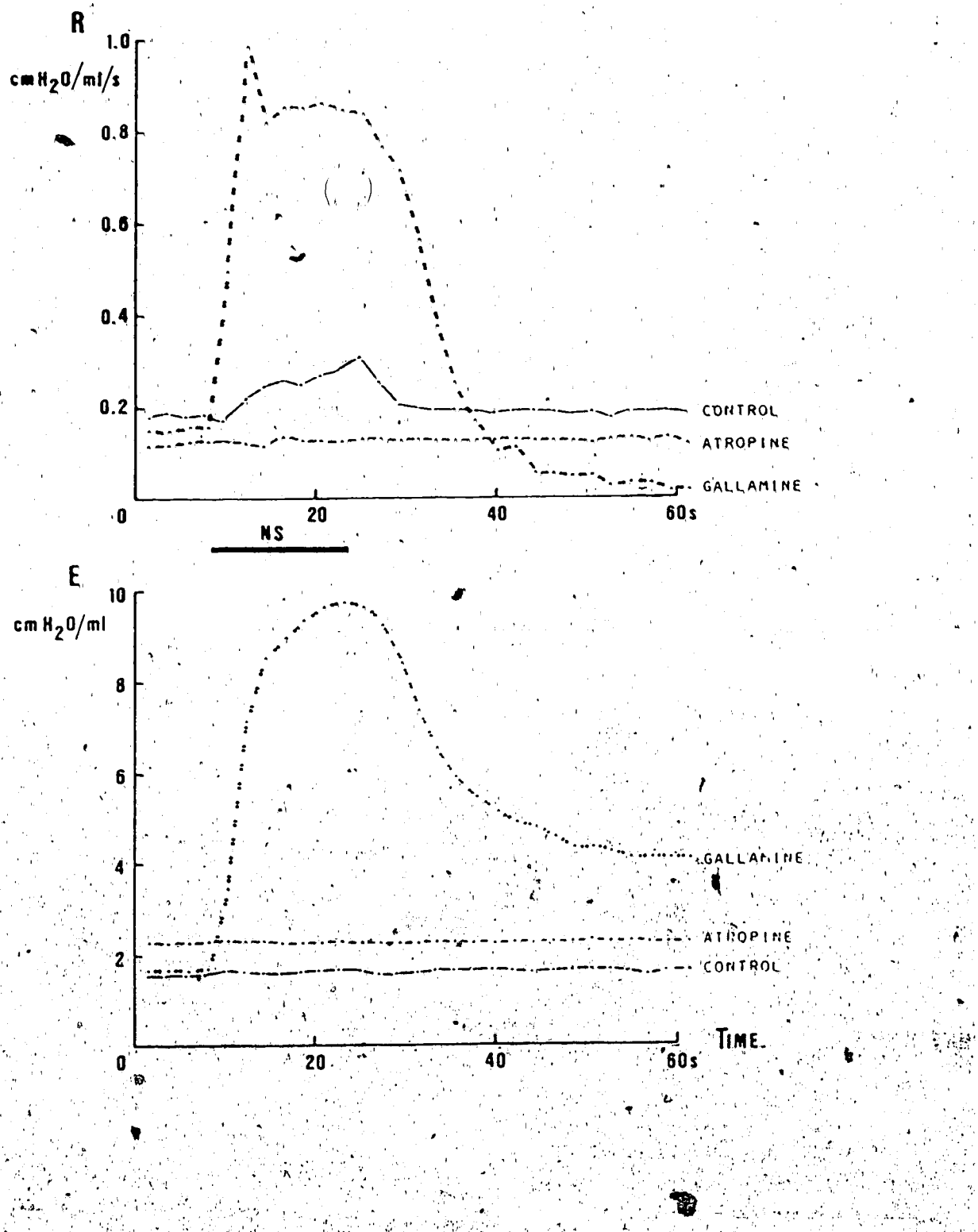


Figure 4.24 Increases in R and E in response to supramaximal stimulation before and after gallamine (5.0 mg/kg) and atropine (0.5 mg/kg) in a nonylvanylamide-treated animal.

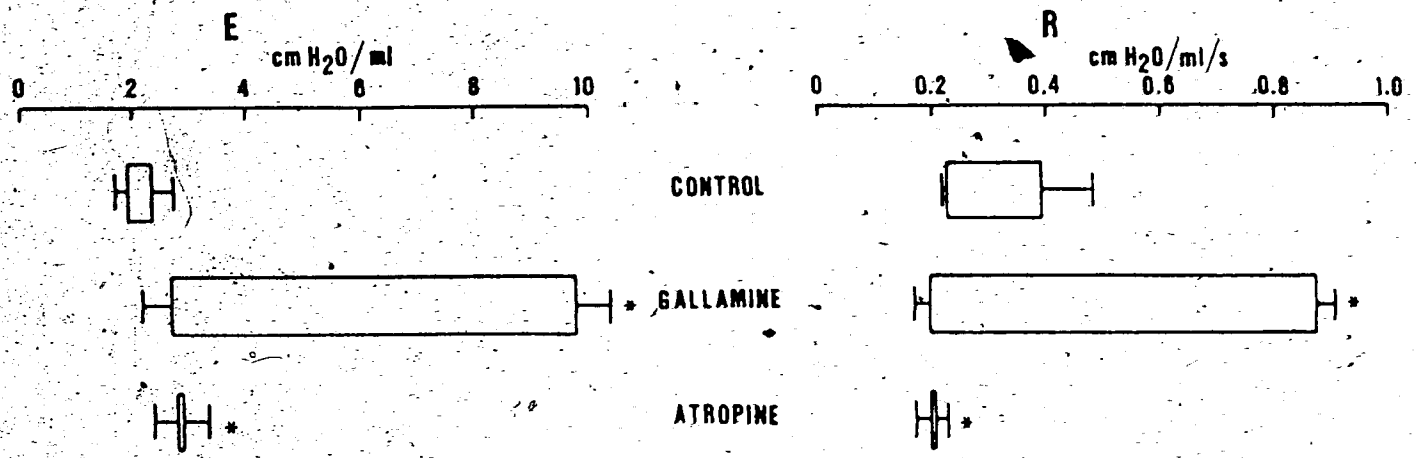


Figure 4.25 Effects of gallamine (5.0 mg/kg) and atropine (0.5 mg/kg) on increases in R and E to maximal vagal stimulation in nonylvanylamide-treated animals. ($^*p<0.05$; $n=4$).

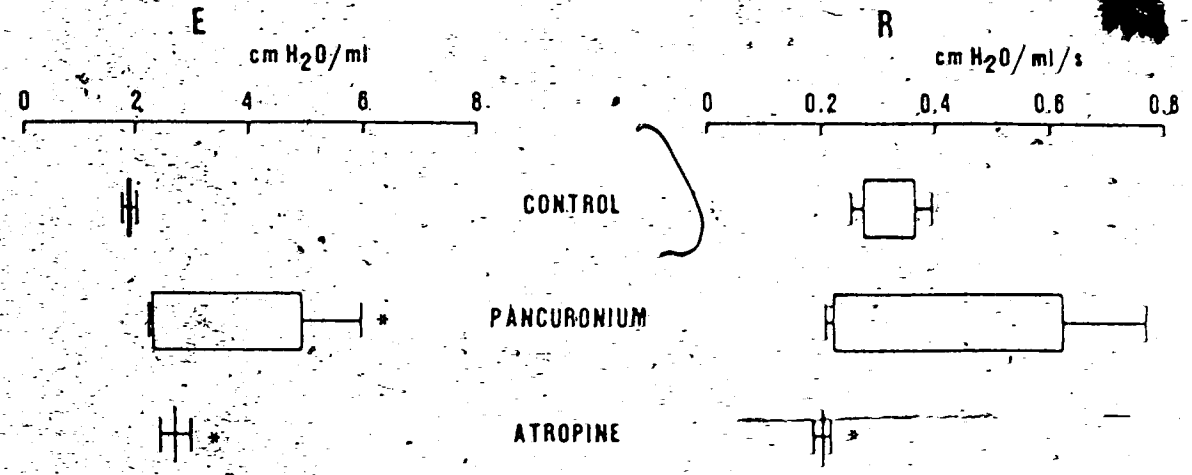


Figure 4.26 Effects of an injection of pancuronium (0.1 mg/kg) and a subsequent injection of atropine (0.5 mg/kg) on increases in R and E to maximal vagal stimulation in nonylvanylamide-treated animals. (*$p<0.05$; $n=4$).

and E induced by MCh are not significantly different between these 2 groups of animals.

4.3.2.3 Effects of hexamethonium

Hexamethonium (2.5 mg/kg) abolished bronchoconstriction induced by vagal stimulation in animals pretreated with nonylvanylamide (2 experiments). Figure 4.28 is a tracheal pressure tracing showing the effect of maximal vagal nerve stimulation before and after hexamethonium.

4.3.3 Experiments on the inhibitory NANC system

These experiments were conducted on nonylvanylamide-pretreated animals to abolish any interference from the excitatory NANC system. Animals underwent vagotomy and glossopharyngealotomy. These procedures had minimal effects on baseline values of R and E. Stimulation of the vagi induced increases in R and E that were abolished by giving atropine (0.5 mg/kg). Propranolol (2.0 mg/kg) was administered to these atropinized animals to block any effects of circulating catecholamines. This agent did not potentiate responses to stimulation significantly. Serotonin was infused to increase values of R and E to about 200% above baseline. Maximal and supramaximal stimulation then resulted in a small initial increase in R and E, followed by little or no decrease below baseline values, i.e. no significant bronchodilation occurred in the airways. The maximal decrease in R observed in one animal was about 10%. The other 3 animals failed to demonstrate any significant decrease in the values of R and E in response to nerve stimulation. An additional dose of atropine (0.5 mg/kg) abolished the initial increases in R and E. The infusion of serotonin induced an initial transient fall in MABP followed by an increase to above baseline values. Hexamethonium (2.0 mg/kg) abolished the small decrease noted in response to vagal stimulation in animals given atropine and propranolol. VIP was infused as a test relaxant, and its effects on bronchoconstriction induced by vagal stimulation were quantified. This agent did not block vagally-induced bronchoconstriction in these animals.

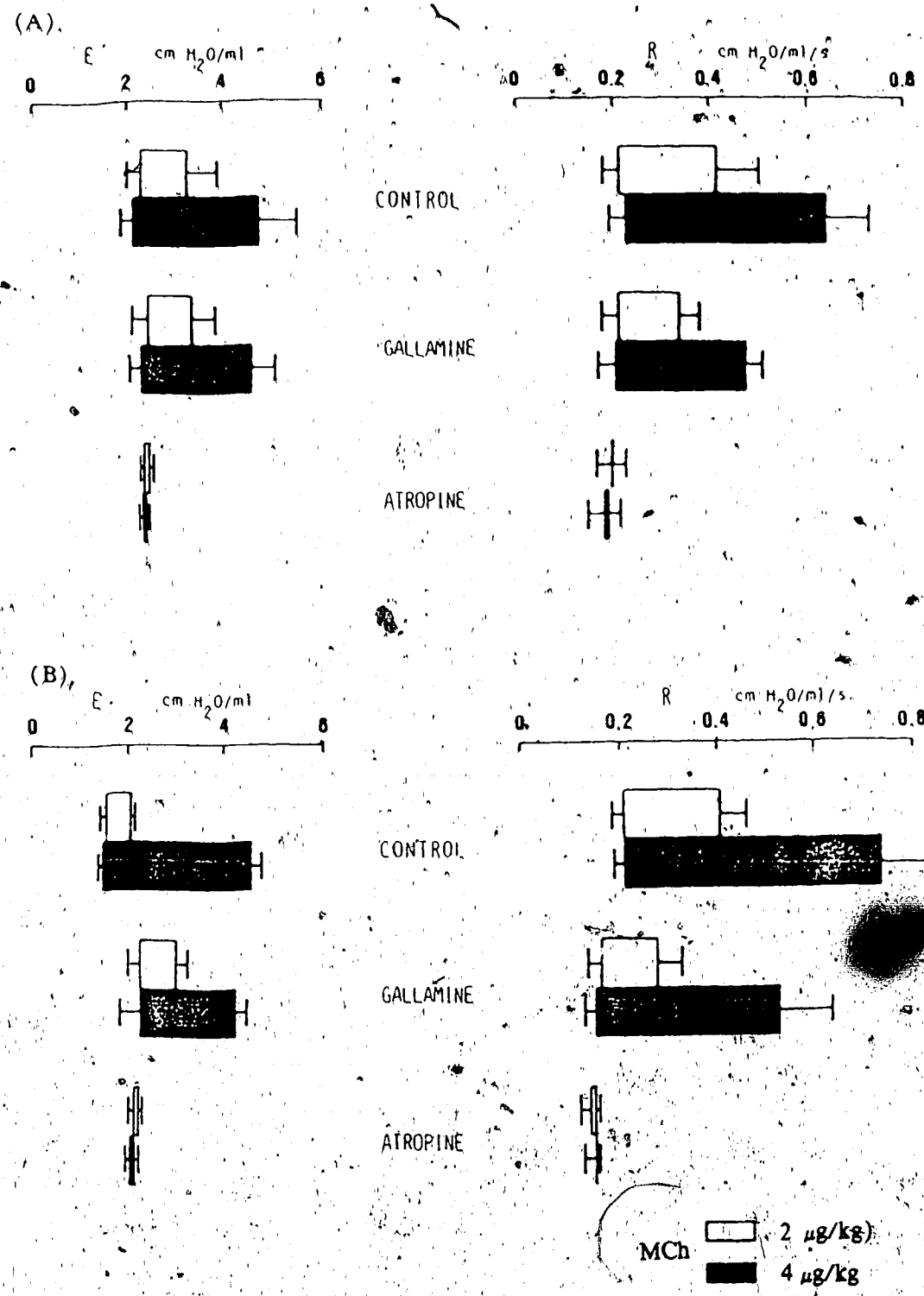


Figure 4.27 Increases in R and E induced by MCh (2 and 4 μg/kg) in (A) vehicle- and (B) nonylvanylamide-treated animals. (*$p<0.05$; $n=4$).

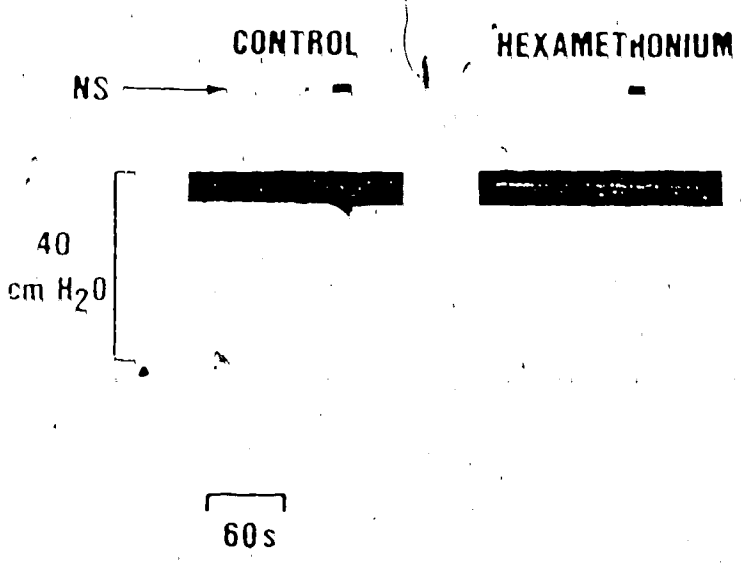


Figure 4.28 Changes in tracheal pressure (TP) induced by maximal stimulation before and after hexamethonium (2.5 mg/kg) in a nonylvanylamide-treated animal.

4.4 Time course of effects of nonylvanylamide-pretreatment

The time course of the effects of nonylvanylamide-pretreatment on pulmonary responses to vagal stimulation and to bronchospastic drugs were studied in animals 2, 8, 16, 32, and 64 days after treatment, and compared to the pulmonary responses of vehicle-pretreated controls to similar stimuli. The bronchospastic drugs injected were MCh (2 and 4 μg/kg), Hist (2 and 4 μg/kg), and SP (4 and 8 μg/kg).

4.4.1 Vehicle-pretreated animals

4.4.1.1 Responses to nerve stimulation

These responses were similar in all the vehicle-treated groups of animals. However, the potentiation of increases in E in response to submaximal stimulation by gallamine was significantly greater in the group of animals used 2 days after treatment. The increases in R and E in response to submaximal and maximal stimulation were not enhanced significantly by gallamine in most of the control groups. Increases in R and E seen in all groups were significantly reduced by atropine and responses to submaximal stimulation were virtually abolished. Figures 4.29 and 4.30 summarize the results obtained with submaximal and maximal stimulation respectively. R and E were increased by approximately 100% and 200% during submaximal and maximal stimulation respectively. After atropine (0.5 mg/kg), peak response times were significantly delayed.

4.4.1.2 Responses to bronchospastic drugs

MCh, Hist and SP (i.v.) all increased R and E. The increases were prompt in onset and reached peak values within 30 s after injection, before slowly decreasing to baseline. All 3 agents also induced hypotension. The hypotension associated with MCh and Hist injections recovered within 2 min, but that caused by SP lasted for 15-20 min. Increases in R and E were unaltered after giving gallamine in all groups of control animals. Atropine, as expected, blocked the effects of MCh but failed to alter the

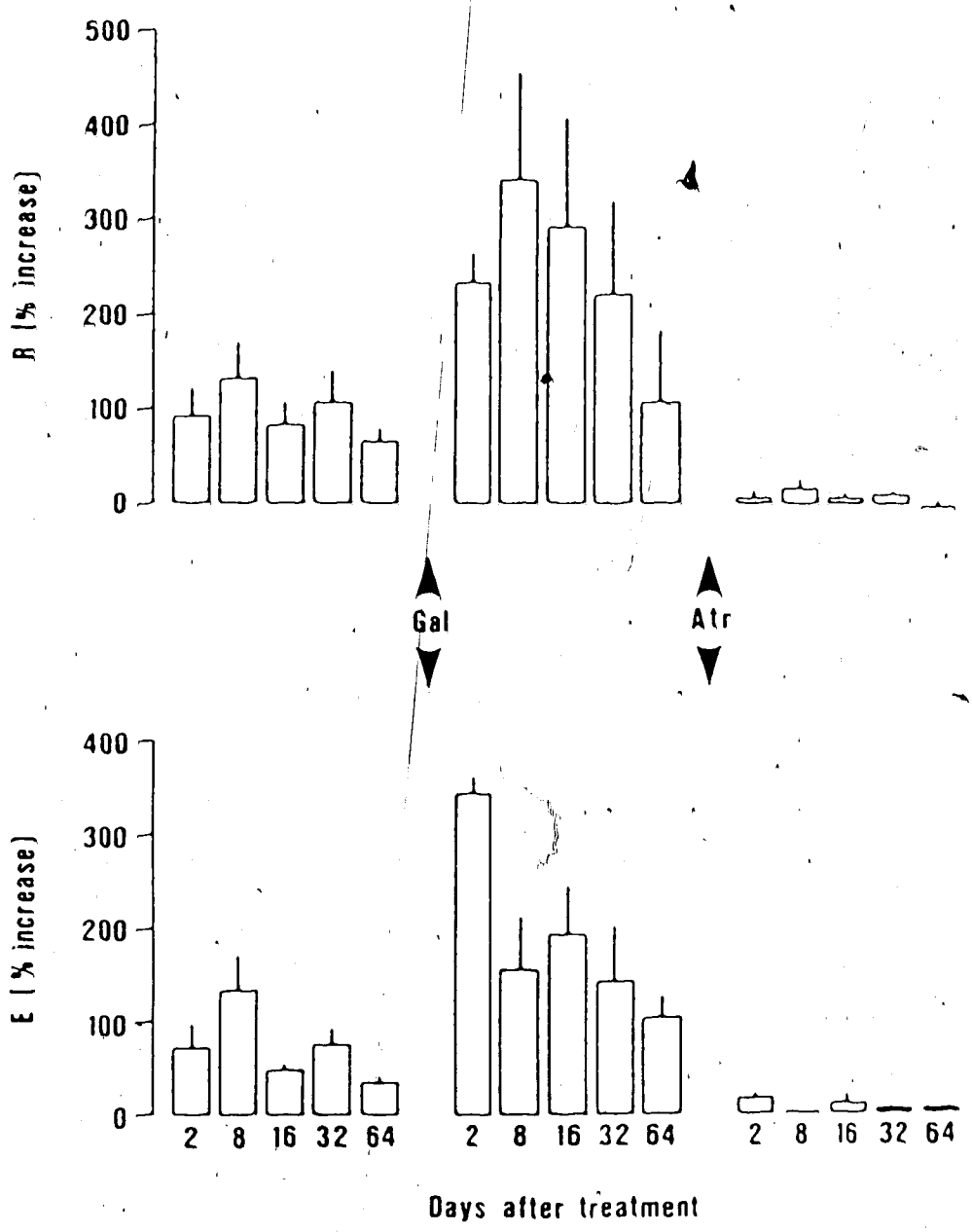


Figure 4.29 Effects of an injection of gallamine (5.0 mg/kg) and a subsequent injection of atropine (0.5 mg/kg) on increases in R and E to submaximal vagal stimulation in vehicle-treated groups. (n=4 for each group).

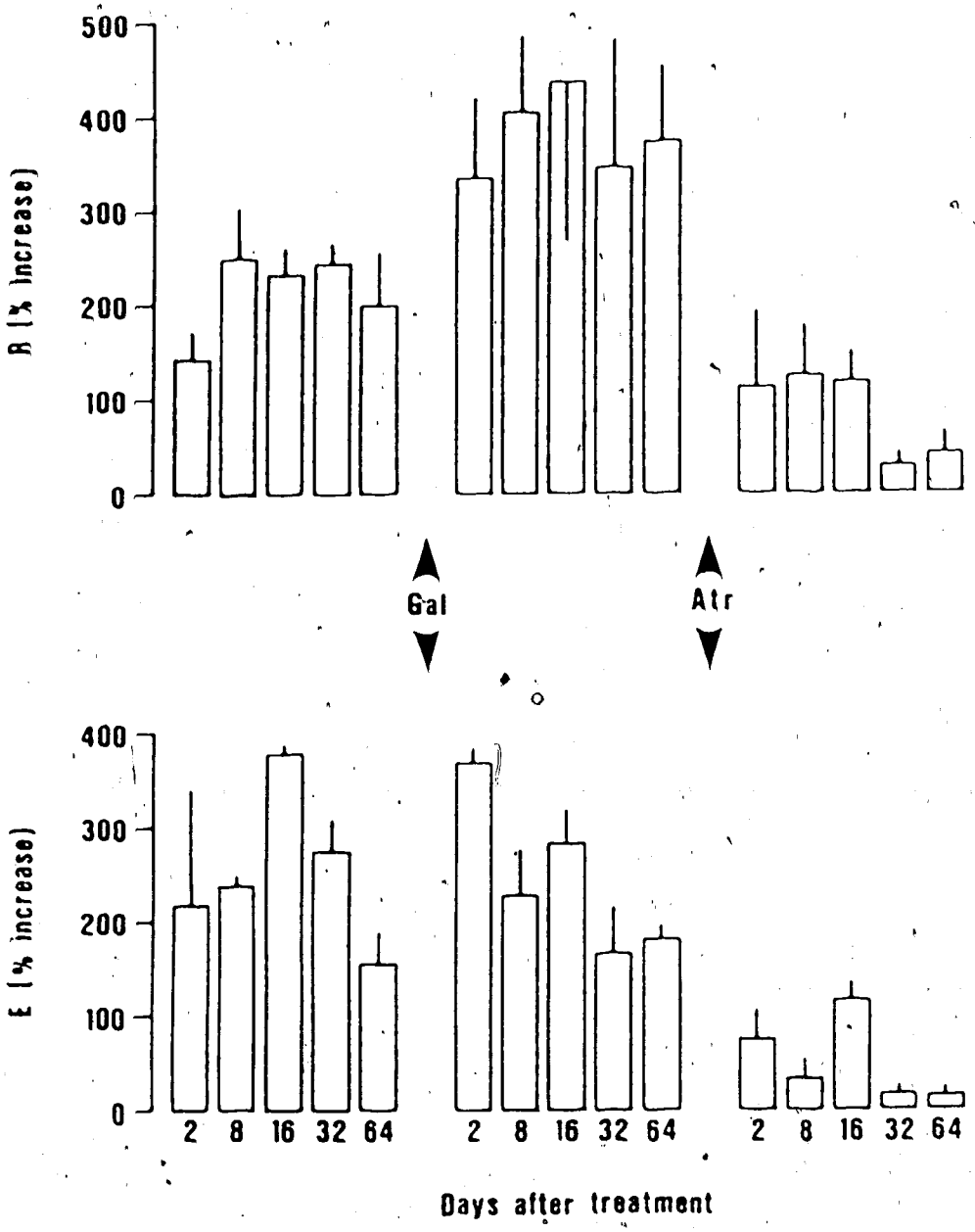


Figure 4.30 Effects of an injection of gallamine (5.0 mg/kg) and a subsequent injection of atropine (0.5 mg/kg) on increases in R and E to maximal vagal stimulation in vehicle-treated groups. (n=4 for each group).

responses to Hist and SP. Figures 4.31, 4.32, and 4.33 summarize the results obtained with these agents in all the vehicle-pretreated groups of control animals.

4.4.2 Nonylvanylamide-pretreated animals

4.4.2.1 Responses to nerve stimulation

There were no differences in pulmonary responses elicited by vagal stimulation among all the groups of animals treated with nonylvanylamide for different durations. In all groups, stimulation produced greater changes in the R than E values.

After gallamine, stimulation-induced increases in R and E were greatly potentiated. However, animals used 2 days after nonylvanylamide-treatment did not show the same degree of potentiation of increases in R as the other nonylvanylamide-treated groups. After gallamine, increases in R and E reached peak values similar to those of control animals after the same drug. Vagally-induced increases in R and E were abolished by atropine in these nonylvanylamide-pretreated animals, unlike in the controls in which there was only a partial reduction. Responses to maximal and supramaximal stimulation did not differ significantly. Figures 4.34 and 4.35 summarize the results obtained with maximal and supramaximal vagal nerve stimulation in these animals.

4.4.2.2 Responses to bronchospastic drugs

Results obtained with the bronchospastic agents were generally similar to those obtained from vehicle-treated controls; responses were neither potentiated nor decreased by gallamine at all times post-treatment, and atropine abolished only the responses to MCh. No evidence of supersensitivity to any of these agents were observed. Figure 4.36, 4.37, and 4.38 summarize the results obtained with these agents.

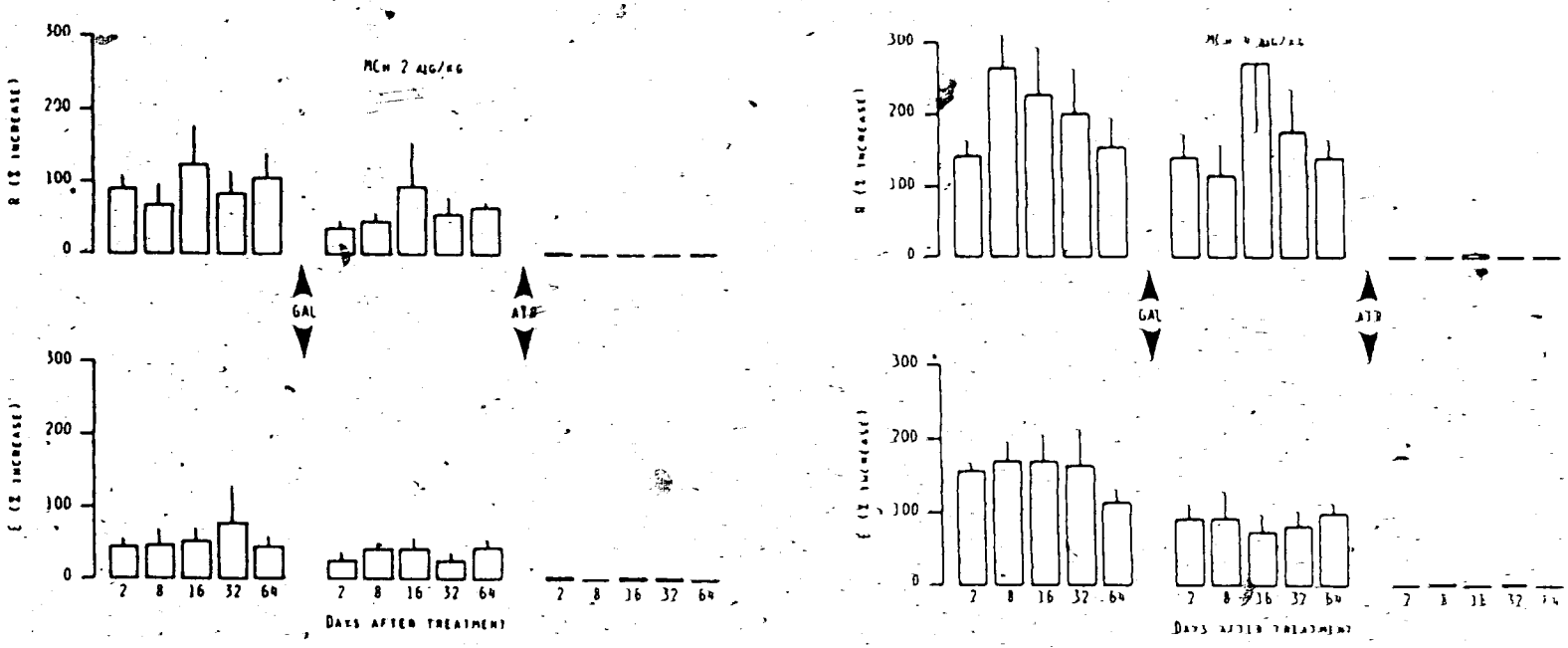


Figure 4.31 Effects of MCh (2 and 4 μg/kg) in vehicle-treated groups. (n=4 for each group).

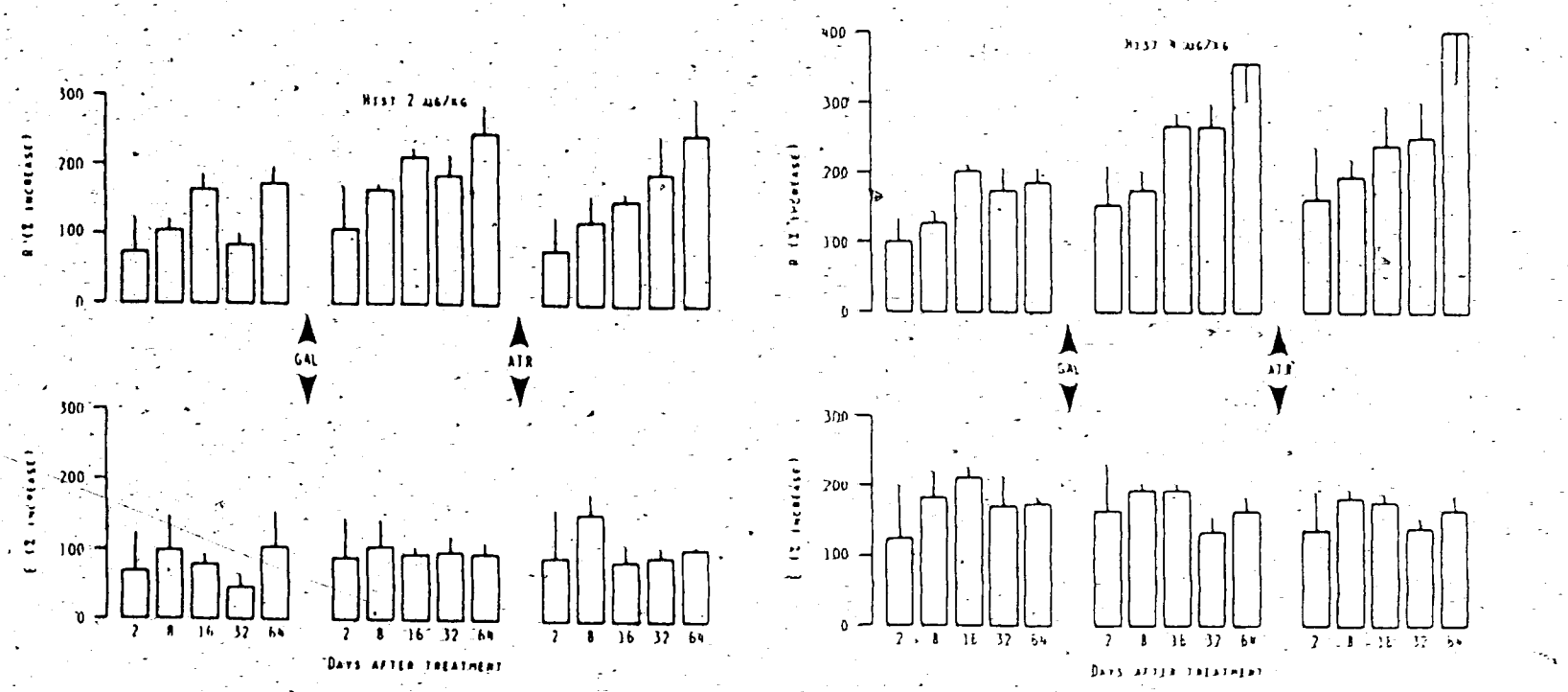


Figure 4.32 Effects of Hist (2 and 4 μg/kg) in vehicle-treated groups. (n=4 for each group).

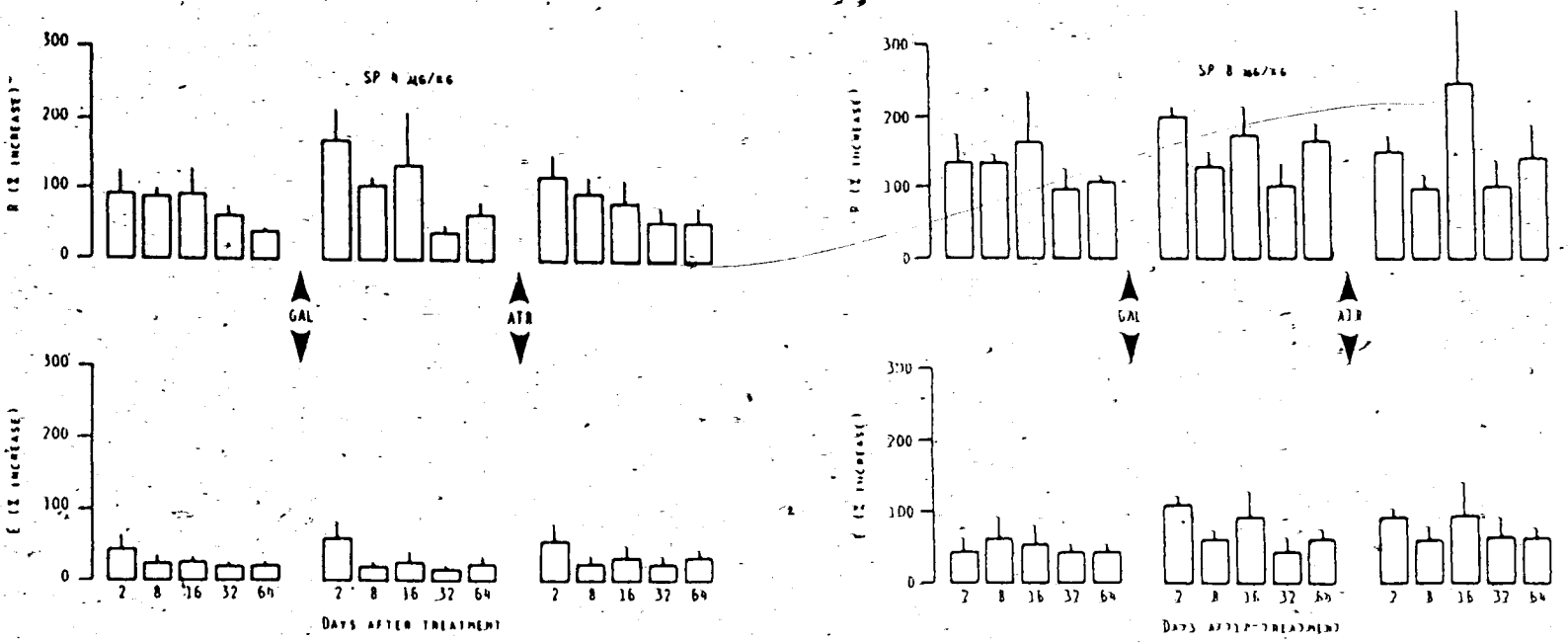


Figure 4.33 Effects of SP (4 and 8 μg/kg) in vehicle-treated groups. (n=4 for each group).

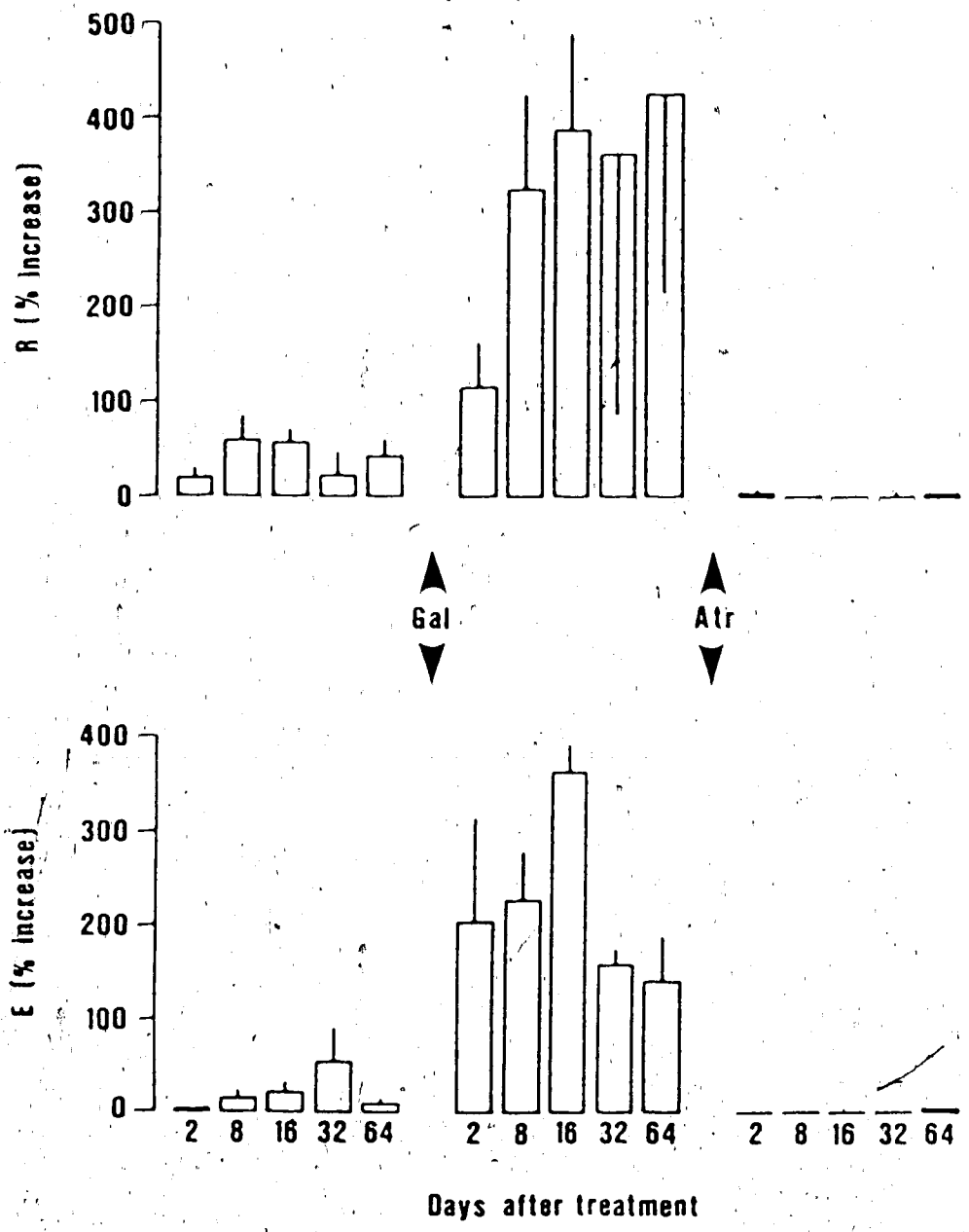


Figure 4.34 Effects of an injection of gallamine (5.0 mg/kg) and a subsequent injection of atropine (0.5 mg/kg) on increases in R and E to maximal vagal stimulation in nonylvanylamide-treated groups. (n = 4 for each group).

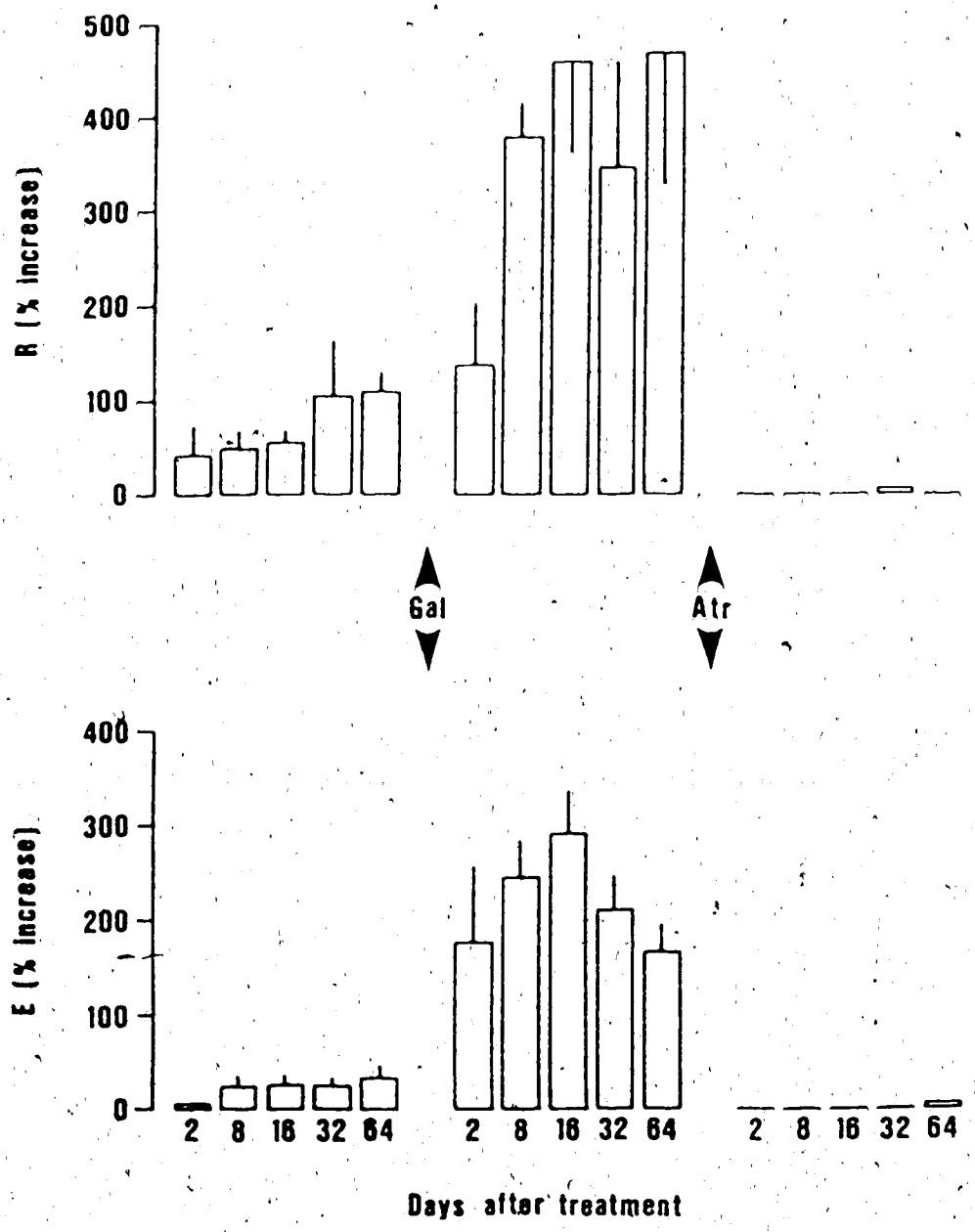


Figure 4.35 Effects of an injection of gallamine (5.0 mg/kg) and a subsequent injection of atropine (0.5 mg/kg) on increases in R and E to supramaximal vagal stimulation in nonylvanylamide-treated groups. (n=4 for each group).

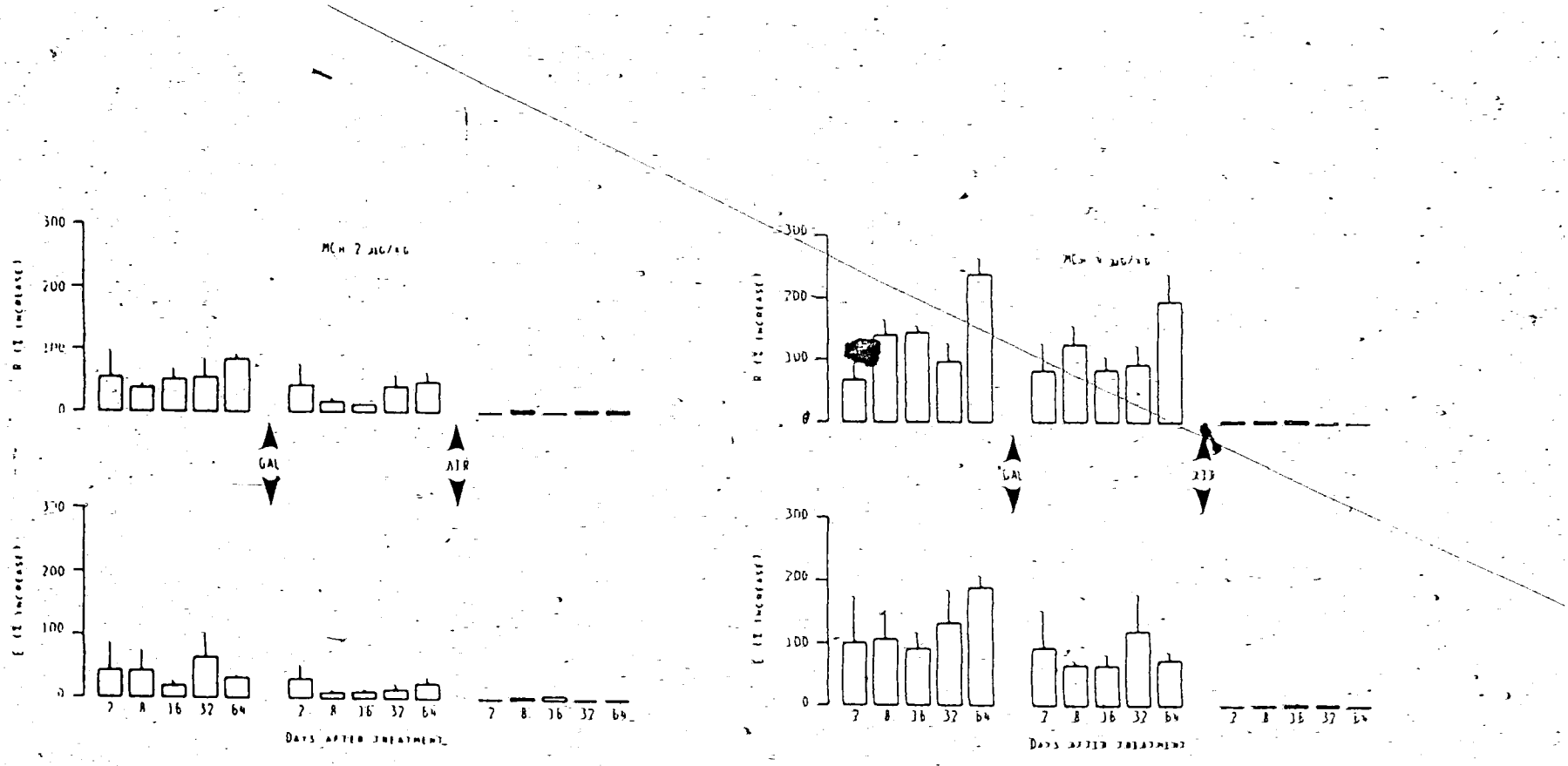


Figure 4.36 Effects of MCh (2 and 4 μg/kg) in nonylvanylamide-treated groups. (n=4 for each group).

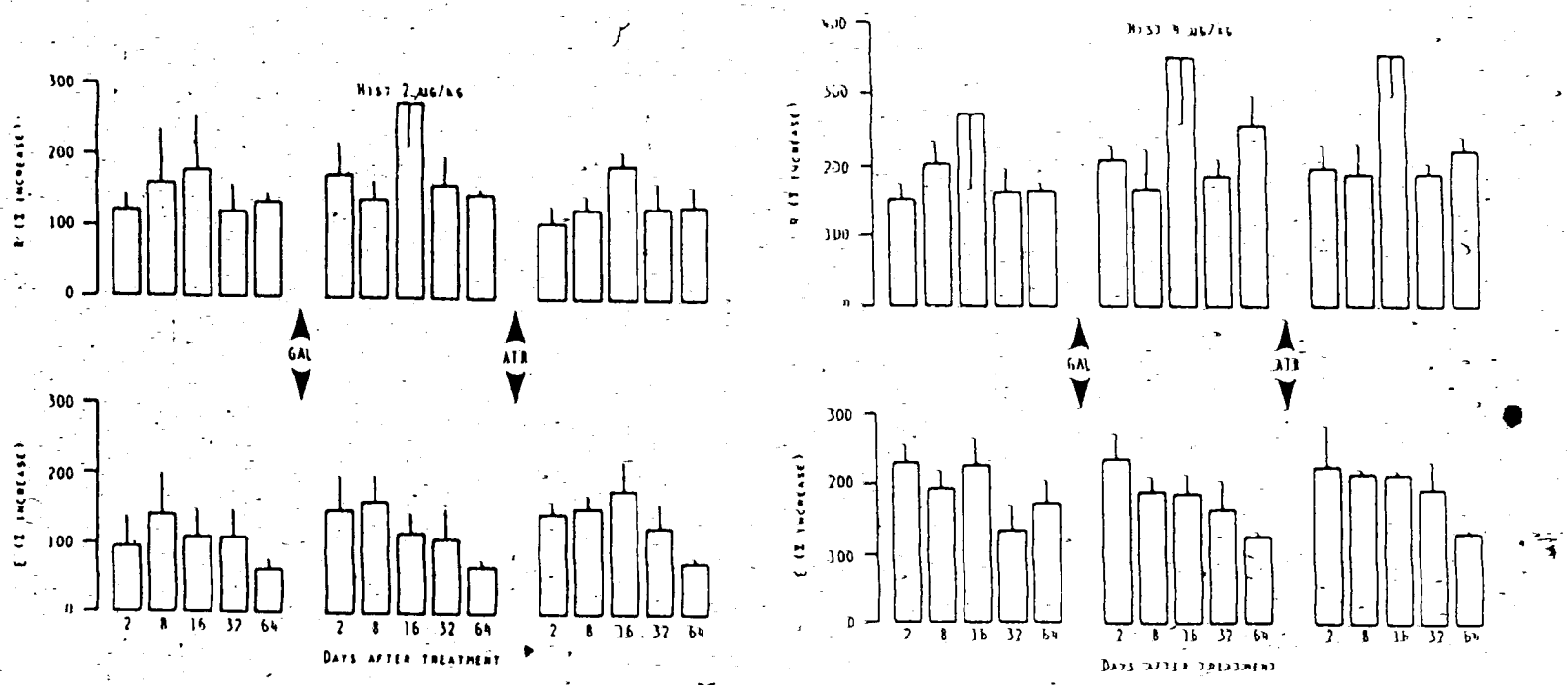


Figure 4.37 Effects of Hist (2 and 4 μg/kg) in nonylvanylamide-treated groups. (n=4 for each group).

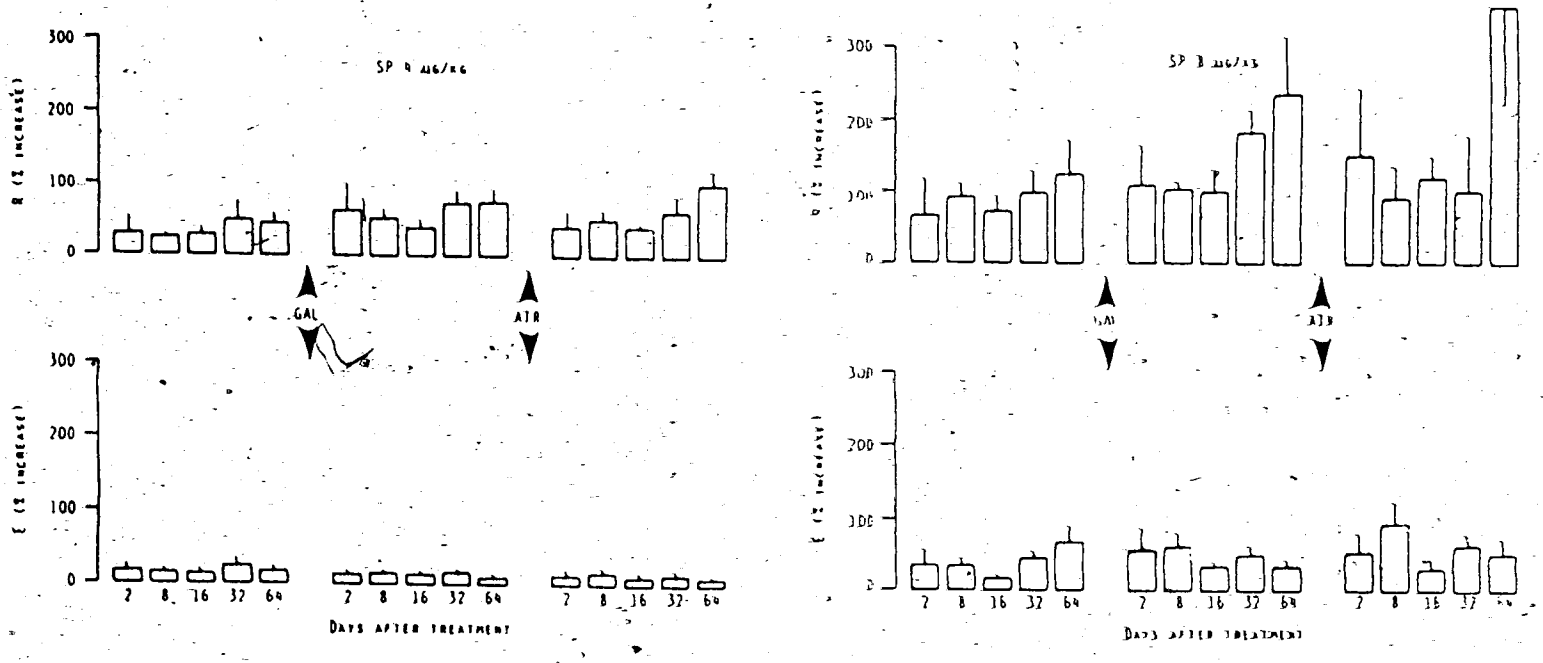


Figure 4.38 Effects of SP (4 and 8 μg/kg) in nonylvanylamide-treated groups. ($n=4$ for each group).

5. DISCUSSION

The objective of the first part of this research was to determine whether the efferent parasympathetic innervation to the lungs contained presynaptic muscarinic receptors that modulated the release of neurotransmitter. Presynaptic muscarinic receptors modulating ACh release have been described in the myenteric plexus of guinea pigs (Kilbinger, 1984a,b; Fosbraey & Johnson, 1980,1982), and I postulated that similar receptors might exist in guinea-pig lungs.

I began my work by characterizing the responses to electrical stimulation of the distal ends of the cut cervical vagus nerves. These experiments were conducted in normal (untreated) guinea pigs, and in animals treated with nonylvanylamide or the vehicle used to dissolve the nonylvanylamide (controls). In normal guinea pigs, vagal stimulation induced bronchoconstriction evidenced as marked increases in values of R and E from baseline. The increases were rapid in onset and peaked during or just after nerve stimulation. Nerve stimulation also induced bradycardia and hypotension. The size of the increases in R and E observed depended upon several factors. Unilateral stimulation induced much smaller increases in R and E than bilateral stimulation. Bilateral stimulation was used for the rest of the experiments because airway responses were larger, and any "buffering" action due to *pendelluft* would be absent. Also, any variation that might arise from regional and lateral differences in the distribution of the vagal innervation to the lungs would be eliminated. Evidence of vagal crossover is lacking in guinea pigs (see Goel, 1986), however, Olsen *et al.* (1965) detected a small amount of crossover in dogs, but only after animals were treated with a cholinesterase inhibitor.

Increases in R and E were dependent upon the strength of the electrical stimulus and it was possible to induce submaximal or maximal responses that had different characteristics. Thus, submaximal stimulation elicited responses that were blocked by hexamethonium (5.0 and 10.0 mg/kg), mecamylamine (1.0 and 2.0 mg/kg), and by atropine (0.5 mg/kg). The β-blocker propranolol (2.0 mg/kg) increased responses but the increases were not statistically

significant. These findings show that submaximal stimulation induces "classical" efferent parasympathetic effects that involve a ganglionic relay and muscarinic receptors. Endogenous or exogenous β-tone is not an important factor in determining the size of the responses. Interestingly, when the vagi were stimulated submaximally the percentage increase in R was always greater than the percentage increase in E. This implies that greater changes occurred in the "large" rather than the "small" airways (Nadel *et al.*, 1971), and this matches the distribution of the parasympathetic innervation of the airways (Barnes *et al.*, 1983a,b). However, it is important to point out that the technique used to measure R and E does not differentiate flow rate, volume, and pressure signals into the two parameters absolutely; considerable overlap exists between these measurements.

Maximal vagal stimulation induced prompt increases in R and E that peaked during stimulation and were of longer duration than those seen after submaximal nerve stimulation. The increases were reduced, but not abolished, by atropine, and were unchanged after propranolol. The ganglionic blockers hexamethonium and mecamylamine, only reduced the size of the responses. Interestingly, both hexamethonium and mecamylamine altered baseline values of R and E. Mecamylamine increased baseline values of both R and E, while hexamethonium only increased that of E. Examination of the time course of the responses after atropine revealed: (a) that responses now reached a maximum after nerve stimulation ceased, and (b) that most of the bronchoconstriction that had occurred during nerve stimulation was eliminated by the drug. Thus, the responses to nerve stimulation appear to comprise a fast phase that is blocked by giving atropine, and a slow phase that peaks after stimulation ceases and is resistant to block by atropine. Ganglionic blockers produced similar effects, abolishing the fast phase and leaving the slow phase intact. After ganglionic blockers, atropine induced an additional small decrease in the response to nerve stimulation. Goel (1986) and others (Lundberg & Saria, 1982; Lundberg *et al.*, 1983b) have suggested that the slow response results from the release of peptide(s) from vagal afferent endings via antidromic electrical stimulation. Our findings support this suggestion. Antagonists to SP

reduced the responses to nerve stimulation in animals pretreated with atropine and mepyramine. Thus, [D-Pro^2,D-Trp^{7,9}]-SP eliminated the increases in R but not those in E, [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]-SP reduced increases in both R and E. [D-Arg^1,D-Pro^2, D-Trp^{7,9},Leu^{11}]-SP is specific for SP-P receptors and [D-Pro^2,D-Trp^{7,9}]-SP is specific for SP-E receptors (Lee *et al.*, 1982; Brown & Hill, 1983). These findings suggest that both types of receptors are involved in the pulmonary responses to vagal stimulation.

The inability of these antagonists to SP to induce complete block of the responses induced by stimulation could have several explanations. One possible explanation is the antagonists are far from ideal or "pure" (see Karlson *et al.*, 1984; Post *et al.*, 1985; Folkers *et al.*, 1985). They possess some agonist activity and also release histamine from mast cells. Theodorsson-Norheim *et al.* (1985) and Hua *et al.* (1985) have shown that there are other peptides, in addition to SP, present in capsaicin-sensitive neurons. The effects of these peptides (if released) may not have been blocked by the SP antagonists used in our studies.

It is possible that more than one peptide is released from vagal afferents during stimulation. Immunohistochemical studies have shown that SP-immunoreactive material coexists with calcitonin gene-related peptide-immunoreactive (CGRP-IR) material in capsaicin-sensitive afferents in the lung (Lundberg *et al.*, 1985). CGRP is a more potent constrictor of human airway smooth muscle than SP (Palmer *et al.*, 1985).

It is clear that responses induced by submaximal stimulation differ from those induced by maximal nerve stimulation in that they lack the delayed phase. This finding implies that a certain threshold has to be reached before the release of peptides can be evoked. The submaximal stimulation parameters used were presumably subthreshold for peptide release because the responses elicited were of cholinergic origin as they were abolished by atropine. However, it is possible for SP released during stimulation to act both directly on the smooth muscle and indirectly via cholinergic neurons. In this case, atropine would block both the neuromodulatory effects of SP and the effects of ACh. However, this appears to be unlikely because increases in R and E induced by SP i.v. before and after atropine were not

significantly different. Thus, the effects of SP on airway smooth muscle appear to involve only direct actions of SP on SP-P and SP-E receptors — indirect effects could not be detected in this preparation.

The vagus nerve should contain (at least) 3 separate groups of nerve fibers: parasympathetic efferent, NANC efferent, and afferent. The first two groups contain a ganglionic relay. My findings support the existence of all 3 groups. Thus, results from experiments with submaximal and maximal stimulation, atropine, and ganglionic blockers clearly demonstrate the existence of the classical parasympathetic efferent pathway. Effects mediated via NANC inhibitory efferent fibers are difficult to demonstrate (see Results and later in this discussion), however, our findings with ganglionic blockers offer indirect evidence of their existence. These findings imply that the increases in R and E observed in response to vagal stimulation represent the arithmetic sum of contractile and relaxant effects on airway smooth muscle. However, NANC-tone cannot be overly important or easy to detect in this preparation because atropine induces only small statistically insignificant decreases in baseline values of R and E. However, as my preparations have low tone, it would be difficult to demonstrate additional relaxation and thus distinguish NANC inhibitory effects from the loss of cholinergic excitatory effects.

As stated earlier, both submaximal and maximal nerve stimulation induced bradycardia and hypotension. The former was abolished by atropine and ganglionic blockers, however, some hypotension persisted to nerve stimulation. This atropine-resistant hypotension was quite marked in most animals. It probably represents the systemic depressor response to SP and/or related peptides released via antidromic stimulation of afferents. Doses of atropine and ganglionic blockers required to eliminate the bradycardia were much lower than those necessary to eliminate the airway responses to vagal stimulation. This implies possible differences in the accessibility of the antagonists to their site of action, or alternatively, that pulmonary ganglia and muscarinic receptors on airway smooth muscle are resistant to the blockers used.

In animals given vehicle 7-8 days before experiment, pulmonary and circulatory responses to vagal stimulation were essentially the same as those seen in untreated control animals. These responses were not characterized in the detail set out above. Responses in nonylvanylamide-treated animals were characterized in detail. Submaximal vagal stimulation failed to elicit any pulmonary responses but still induced bradycardia and hypotension. Maximal or supramaximal stimulation induced small pulmonary responses that peaked during stimulation, and were abolished by atropine and ganglionic blockers. Responses appeared to consist entirely of the fast phase, no slow peptidergic phase was apparent. Pretreatment with capsaicin results in the long term loss of SP-like immunoreactivity from primary sensory nerves in guinea pigs (Papka *et al.*, 1984). Thus, capsaicin-treatment appears to have eliminated the peptidergic slow phase of the pulmonary response to vagal stimulation.

The reduction in the size of the pulmonary responses we observed has been reported by others (Lundberg & Saria, 1982; Martling *et al.*, 1984). It does not appear to arise via alteration of post-synaptic cholinergic mechanisms as responses to exogenous MCh were unchanged. Postsynaptic SP-receptors are probably not involved since pulmonary responses to exogenous SP were unchanged and similar to those of the control animals. This also implies that SP has no "normal" transmitter role involving airway smooth muscle for, if this were the case, one would have expected to observe supersensitivity to SP.

In nonylvanylamide-pretreated animals, administration of ganglion blockers did not increase baseline values of E, by contrast with normals. This implies that nonylvanylamide treatment eliminated the NANC inhibitory transmitter(s) as well as SP and SP-related peptides. If this is so, then the nonylvanylamide-treated guinea pig may well represent an ideal model for studying the efferent parasympathetic nervous system to the lungs. The peptidergic excitatory slow-phase and any NANC inhibitory effects are apparently eliminated by this treatment, leaving only the atropine-sensitive, parasympathetic efferent system intact.

Unfortunately, the action of capsaicin is not restricted to the peptidergic efferents and afferents in the vagal nerves supplying the airways, for capsaicin pretreatment significantly

reduced the cholinergic parasympathetic component — pulmonary responses were 10-20% of those seen in controls. This effect has been reported by Martling and coworkers (1984). Papka and coworkers (1981) have also reported that capsaicin-pretreatment induced a loss of acetylcholinesterase-containing nerve fibers in the heart. This does not necessarily mean that efferent cholinergic nerves are altered in some way by capsaicin because sensory nerves may also contain acetylcholinesterases. The responses to exogenous MCh were similar to those of control animals, in agreement with the findings of Martling and coworkers (1984). Thus, capsaicin-pretreatment does not alter the integrity of the airway smooth muscle or its muscarinic receptors, and any change brought about is probably of neural origin.

After characterizing the bronchoconstrictor responses in guinea pigs to vagal nerve stimulation, I determined the effects of some selective antimuscarinic agents, namely gallamine and pancuronium, on vagally-mediated bronchoconstriction. These agents are curare-like neuromuscular blockers, routinely used in surgical procedures to relax skeletal muscles. Their cardioselective antimuscarinic effects are well known (Riker & Wescoe, 1951). Fryer and Maclagan (1984) and Blaber *et al.* (1985) have reported that gallamine potentiated bronchoconstriction induced by vagal stimulation. I examined the effects of gallamine, pancuronium, and decamethonium first in normal (untreated) animals to verify the reported results. Decamethonium, a depolarizing neuromuscular blocking agent, was used as a control drug.

My findings on vagal stimulation and the effects of gallamine concur with those of Fryer and Maclagan (1984). However, these workers recorded bronchoconstriction as an increase in the tracheal pressure over the basal insufflation pressure. This method is not as sensitive as the one used in our laboratories and it does not differentiate between constriction in the large and the small airways. Furthermore, they used only submaximal stimulation parameters (responses were abolished by atropine), and they did not examine effects of gallamine on responses to maximal stimulation. The release of noncholinergic excitatory mediators via antidromic stimulation of vagal afferents would seem unlikely in their case.

The ability of gallamine and pancuronium to increase pulmonary responses to nerve stimulation in nonylvanylamide-treated animals is striking. In these animals, either drug restored the responses to maximal nerve stimulation to levels that approximated those seen in vehicle-treated controls after gallamine or pancuronium. Although the ability of capsaicin to reduce pulmonary responses to nerve stimulation is well-known, this is the first time that a means of (apparently) increasing this effect has been described. Gallamine and pancuronium also increase responses to nerve stimulation in normal and vehicle-treated control animals, however the effect is less marked than in nonylvanylamide-treated animals and difficult to interpret because of the complex nature of the response to nerve stimulation in control animals.

Gallamine's effects could arise via several possible mechanisms, alone or in combination. These will be discussed in a sequence that ends with a consideration of the effects of the drug at presynaptic, release-modulating muscarinic receptors.

Gallamine may have significant anticholinesterase activity (see Bowman & Rand, 1980). Thus, gallamine's ablility to enhance responses to nerve stimulation could arise by actions that prolong the half-life of acetylcholine released by nerve endings. If this were so, then exogenously applied cholinergic muscarinic agonists such as MCh should also induce increased effects after gallamine; however, this did not occur. Responses to MCh were similar before and after gallamine in normal and vehicle-treated control, and in nonylvanylamide-treated animals. Therefore, it is unlikely that gallamine acted via this mechanism.

Gallamine's effects could arise via a sympatholytic effect that eliminated β-tone in the airways. There is some evidence that suggests that gallamine has sympatholytic effects on the cardiovascular system (Bowman & Rand, 1980), though the drug's effects were small. In my experiments, β-tone in the airways is minimal as evidenced by the inability of propranolol to enhance airway responses to vagal stimulation and drugs, in control and nonylvanylamide-treated animals. Also, pretreatment of animals with either guanethidine or propranolol failed to prevent gallamine's ability to enhance airway responses to nerve

stimulation. Thus, it is very unlikely that gallamine has any significant blocking activity on the sympathetic nervous system or on catecholamines released from extrapulmonary sites. There is a remote possibility that gallamine may affect α-receptors. Richardson (1979, 1983) have described a sympathetic innervation to the peribronchial plexuses in several species. However, there are no reports of gallamine possessing α-adrenoceptor blocking activity in the literature, so I elected not to pursue this aspect further.

Gallamine could also be selectively blocking the NANC inhibitory system in the lungs. If gallamine had this action, then administering this drug should increase baseline values of R and E — similar to findings after giving hexamethonium and mecamylamine. However, gallamine had no significant effect on baseline values of R and E and did not enhance responses to injected agonists. It is possible that gallamine enhances NANC excitatory effects in the airways. To test this hypothesis, I treated animals with atropine and then determined the effects of gallamine on the slow phase of the airways' responses to vagal stimulation. Responses were unaltered by gallamine. If gallamine's effects on the excitatory NANC system are mediated via muscarinic receptors, this result would have been expected. To test this possibility, I treated animals with mecamylamine to eliminate the cholinergic fast phase of responses, and to abolish any inhibitory NANC effects. Gallamine had no potentiating effect on the slow phase in these experiments, and I concluded that this drug could not be acting to enhance the effects of the excitatory NANC system in the airways.

This leaves consideration of the actions of gallamine on nicotinic and muscarinic receptors in the cholinergic efferent excitatory and the inhibitory NANC pathways. Could gallamine induce potentiation via selective enhancement or blockade of ganglionic nicotinic receptors? This seems highly unlikely. Gallamine's potentiating effects are confined to the fast "cholinergic" phase of the response to vagal stimulation, and, as discussed above, there is no evidence of selective block of the inhibitory NANC system via its ganglia. Though the possibility has not been totally excluded, it does appear remote. Thus, gallamine must act at receptors at synapses in the parasympathetic efferent ganglia, or at post-ganglionic

neuron/smooth muscle junctions. Because I stimulated only preganglionic fibers, I am unable to distinguish between these two possibilities. I have shown that *muscarinic* receptors must be involved — atropine blocked gallamine's effects — though this could represent simply postsynaptic blockade. However, the likely site of action of gallamine is the muscarinic receptor. The bradycardia induced by both vagal stimulation and ACh or MCh was abolished by gallamine — gallamine potentiated only the pulmonary responses to vagal stimulation and not those to exogenous cholinergic agonists. This indicates that the pulmonary effects of gallamine are separate from its cardiovascular effects, and that different populations of muscarinic receptors are involved. It also suggests that the potentiation is mediated neurally and that the site of action is not the postsynaptic muscarinic receptors on the airway smooth muscle. However, gallamine could still act on postsynaptic receptors via an allosteric mechanism. It is known to bind to a second binding site distinct from the ligand binding site. By doing so, it may perhaps modulate the effects of ACh. However, this is an unlikely mechanism since gallamine did not potentiate the effects of exogenous ACh or MCh. The postsynaptic receptors mediate constriction, and their blockade would only result in a reduction and not increase in bronchoconstriction. Thus, the muscarinic receptors involved are probably located on nerve terminals.

Gallamine could produce potentiation by blocking either excitatory muscarinic receptors in the inhibitory sympathetic neurons supplying the lungs, or by blocking inhibitory muscarinic receptors in parasympathetic neurons to airway smooth muscle. The former possibility can be excluded by the lack of effect of guanethidine and propranolol on the pulmonary effects of gallamine (see above), the latter possibility is more likely. Release-modulating muscarinic receptors are present in the parasympathetic ganglia of cat bladder (Gallagher *et al.*, 1982) and in parasympathetic nerve terminals in guinea-pig ileum (Fosbraey & Johnson, 1980,1982; Kilbinger, 1983,1984; Kilbinger & Wessler, 1980). However, the actions of gallamine on these receptors have not been tested.

Gallamine and pancuronium have been shown to be at least 10 times more active on presynaptic muscarinic receptors than on postsynaptic receptors in the saphenous veins of dogs (Vercruysse *et al.*, 1979). ACh has 2 actions on this preparation: a direct action on smooth muscle cells to cause contraction — an effect mediated by postjunctional muscarinic receptors (Vanhoutte, 1977; Vanhoutte & Shepherd, 1973), and an inhibitory action on the output of noradrenaline released from adrenergic nerve endings — an effect mediated by presynaptic release-inhibiting muscarinic receptors (Vanhoutte, 1977). In guinea-pig airways, presynaptic muscarinic receptors may decrease the evoked release of ACh during stimulation through a negative feedback mechanism. A nonselective muscarinic antagonist such as atropine would be expected to block all muscarinic receptors regardless of their locations. This would account for the lack of effect of gallamine in atropinized animals. Gallamine selectively blocks the presynaptic muscarinic receptors and impairs the negative feedback mechanism thus causing an exaggeration of bronchospastic responses during stimulation. The fact that gallamine slightly decreased the increases in R and E to exogenous MCh suggests that some postsynaptic receptors were blocked by this agent. However, at the dose of gallamine used, the postsynaptic effects were minimal. The exact location of these receptors on parasympathetic nerves has not been determined. They could be on the terminals of either pre- or postganglionic nerve fibers, or both (see Fig. 5.1). Maclagan *et al.* (1985) and Faulkner *et al.* (1986) have shown that inhibitory muscarinic receptors are present in the postganglionic parasympathetic nerves innervating both the cervical trachea and smaller conducting airways of the guinea pig. However, they could not rule out their presence on preganglionic nerve fibers.

Additional evidence supporting the hypothesis that in guinea pigs presynaptic muscarinic receptors modulate ACh release in response to nerve stimulation is supplied by considering the differential effects of gallamine, pancuronium, and decamethonium. Gallamine and pancuronium increased responses to nerve stimulation in control and nonylvanylamide-treated animals; decamethonium had no potentiating activity. Thus, this

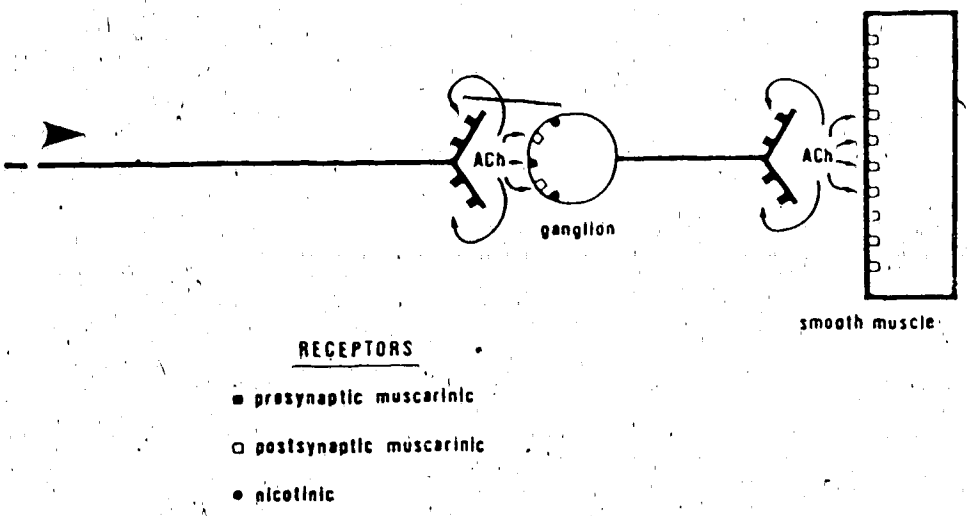


Figure 5.1 Possible locations of presynaptic muscarinic receptors.

potentiating action appears unrelated to the neuromuscular blocking activity of the 3 drugs. In addition, the relative activities of gallamine and pancuronium in my system mirror those seen in other systems with better defined muscarinic receptors such as the guinea-pig ileum.

The enhancement of increases in E by gallamine was greater than that of increases in R. This could mean that small airways are affected to a greater degree than the large airways. As mentioned earlier, the values of R and E computed are not the "true R" and "true E" since there is some overlap between these 2 parameters.

How capsaicin reduces the cholinergic component of bronchoconstriction is not known. However, it does not appear to involve the degeneration of cholinergic nerves, the alteration of choline-acetyltransferase activity, or changes in the affinity or numbers of muscarinic receptors (Martling *et al.*, 1984). Barthó and Vizi (1985) reported that capsaicin releases ACh from the myenteric plexus of guinea pigs. Their results suggest the existence of capsaicin-sensitive nerve endings capable of activating intrinsic cholinergic neurons.

Perhaps a more subtle change was effected, and this was not reflected in any of the variables measured. Since I have been able to enhance the pulmonary cholinergic responses to vagal stimulation to levels similar to those of the vehicle-treated controls, the effect of nonylvanylamide on this pathway is potentially reversible. It is possible that nonylvanylamide increased the affinity or the numbers of presynaptic muscarinic receptors on the nerve endings, thus reducing the pulmonary responses to stimulation. A similar effect would result from the reduction in affinity or numbers of postsynaptic muscarinic receptors on airway smooth muscle, but this is not likely because exogenous MCh elicited responses of similar magnitude to the controls. Perhaps nonylvanylamide induced an allosteric change in the receptor binding site that locked the conformation of the receptor into a "permanently" activated state. Gallamine, by binding to a second binding site, could alter the conformation and reverse this activated state, but still prevent the binding of ACh. Thus, the cholinergic response to vagal-nerve stimulation would be enhanced and the effects of nonylvanylamide on the cholinergic pathway reversed. The existence of the second binding site on the muscarinic

receptor has prompted Birdsall and coworkers (1984) to suggest that it might be a means of selectively 'tuning' a functioning receptor by modulation of the action of neuronally released ACh.

The objective of the second part of this research was to determine the duration of the effects of nonylvanylamide pretreatment on airway responses of guinea pigs. The previous experiments were conducted on animals that had been pretreated 7-8 days before the experiments. I wanted to find out whether there is time-related optimal effect of nonylvanylamide. For these experiments, animals were pretreated with either nonylvanylamide or vehicle before experiments, and used 2, 8, 16, 32, and 64 days after treatment. Each group of vehicle- or nonylvanylamide-pretreated animals consisted of at least 4 animals. The inhibitory effects of nonylvanylamide on responses to vagal stimulation were examined in treated animals, and comparisons were made among the groups treated for the various durations. The degree of restoration of responses by gallamine, and their sensitivity to atropine were also studied. The bronchoconstriction produced by MCh, Hist, and SP was also quantified in these animals before and after the administration of gallamine and atropine.

The results show that the effects of nonylvanylamide on the cholinergic and peptidergic components of vagally-induced bronchoconstriction are rapid in onset and long term, lasting for at least 64 days. The effect on the cholinergic pathway could be reversed by gallamine throughout this period. There was no major difference in the responses to nerve stimulation among all except one of the groups of nonylvanylamide-pretreated animals. The group used after 2 days contained 2 animals (out of 4) that did not demonstrate the same degree of inhibition of responses as the rest of the nonylvanylamide-treated animals. This can mean either that the long term effects of nonylvanylamide are not always evident after 2 days of treatment, or that these 2 animals were not properly desensitized. In all these experiments, the success rate of desensitization is not always 100%, and for this reason a large dose of nonylvanylamide (16 μg/kg) was injected before all experiments to confirm that desensitization had occurred. The effects of nonylvanylamide after 2 days of treatment could

be confirmed by doing more experiments. The receptors for MCh, Hist and SP were functional and unaffected by nonylvanylamide at all times post-treatment.

My results provide inferential evidence for the existence of presynaptic muscarinic receptors that modulate ACh release on pulmonary vagal nerves. My hypothesis could be strengthened by demonstrating modulation of ACh release in synaptosomes derived from pulmonary vagal nerve endings and by electrophysiological studies, with intracellular recording from isolated pulmonary ganglia. To the best of my knowledge, no work has been published on this topic thus far. Many of my explanations are speculative, but if these receptors really do exist, they could form a basis for the development of therapeutically useful drugs for the treatment of bronchial hyperreactive conditions. The antimuscarinic drugs currently used in the treatment of asthma (atropine sulfate and ipratropium bromide) are potent bronchodilators, but are of limited efficacy and associated with systemic side effects (Gross & Skorodin, 1984). The latter could be attributed to their nonselective action on all muscarinic receptors. Muscarinic agonists with a selective affinity for presynaptic receptors in airway smooth muscle would exert a specific action and could be more efficaceous and reduce the incidence of side effects seen with the nonselective antagonists. Our nonylvanylamide-treated guinea pig model could be used to screen potentially selective muscarinic agonists for pulmonary vagal presynaptic receptors, and also antagonists for postsynaptic receptors, since it appears to be comprised exclusively of the classical parasympathetic pathway.

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