Pharmacological Classification of Muscarinic Receptors
Nervous
Involved in the ~~Neural~~ Control of Airway Smooth Muscle
Tone in the Guinea-Pig.

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ABSTRACT

The work in this thesis was carried out to investigate modulation of pulmonary sympathetic neurotransmission by endogenous neurotransmitters and autocoids.

An *in vitro* preparation has been developed which allows selective stimulation of post-ganglionic noradrenergic sympathetic nerves innervating airway smooth muscle of the guinea-pig. When the pressure within the fluid-filled tracheal tube had been raised, stimulation of the sympathetic nerve trunk evoked a relaxation response. Sympathetic nerve-induced relaxations were reduced in the presence of muscarinic agonists in a dose-dependent manner, when compared to those obtained at the same intraluminal pressure in the presence of the stable thromboxane analogue U46619, prostaglandin $F_{2\alpha}$ and histamine. These differences were not due to differences in postjunctional "physiological" antagonism, as noradrenaline attenuated the postjunctional contractile actions of U46619 and acetylcholine to a similar extent.

These results suggest a prejunctional inhibitory action of muscarinic agonists on sympathetic neurotransmission. This suggestion was confirmed with muscarinic antagonists. The selective M_2 antagonists did not alter the postjunctional action of acetylcholine, nor its inhibitory effect on sympathetic nerve-induced relaxations. In contrast, the inhibitory effect of acetylcholine could be blocked with atropine and with M_3 muscarinic antagonists.

Endogenous acetylcholine was also shown to inhibit sympathetic nerve-induced relaxations. Stimulation of the vagal nerve trunk, simultaneously with sympathetic nerve stimulation caused inhibition of sympathetic nerve-induced relaxations. In addition, the anticholinesterase physostigmine attenuated sympathetic nerve-induced relaxations probably via facilitation of spontaneous acetylcholine release.

These results suggest that in the guinea-pig trachea, acetylcholine released from the adjacent parasympathetic nerves inhibits sympathetic neurotransmission via an action on prejunctional muscarinic receptors situated on the noradrenergic nerve terminals. These heteroreceptors appear to be similar to the airway smooth muscle M_3 receptors and differ from the M_2 autoreceptors on parasympathetic nerve terminals. The implication of these results for the rational design of antimuscarinic drugs is discussed.

ACKNOWLEDGEMENTS

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SECTION 1 - INTRODUCTION

1.0 INTRODUCTION

1.1 THE RESPIRATORY SYSTEM

1.1.1 Structure of the Respiratory Tract

After passing through the nose or mouth, pharynx and larynx (the upper airways), air enters the tracheobronchial tree. Starting with the trachea which subdivides into the left and right main bronchi, the air may pass through 23 generations or subdivisions. The first 16 generations of the airways, which include the trachea, bronchi, bronchioles and terminal bronchioles, constitute the conducting or central airways. After passing through the conducting airways, which are anatomically incapable of gas exchange with the venous blood (see below), air passes into the respiratory bronchioles which communicate with ducts leading to the alveolar sacs (respiratory zone) (Tyler 1983). Although airway diameter decreases with each new generation, the total cross section and volume of the airway is enlarged (Ferin, 1984). The entire respiratory tract from the nose to the terminal bronchioles is lined with ciliated cells interspersed with mucus-secreting goblet cells and other secretory cells. In the bronchioles the goblet cells become less frequent and are replaced by secretory Clara cells. The ciliated epithelium, secreted mucus and secretory products of the goblet and Clara cells constitute an important protective mechanism of the lung.

1.1.2 Blood Supply to the Lung

The lungs receive blood via both the bronchial and pulmonary circulation. The bronchial arteries arise from the aorta and supply oxygenated blood to the tracheobronchial tree and supporting tissue of the lung down to the level of the terminal bronchioles (the conducting airways). The peripheral airways, which include the respiratory bronchioles, alveolar ducts, alveolar sacs and alveoli (respiratory zone), receive oxygen directly from the alveolar air and nutrients from the mixed venous blood in the pulmonary circulation.

The pulmonary artery carries non-oxygenated blood from the right ventricle into the lungs. Gas exchange occurs between the alveoli and pulmonary capillaries and oxygenated blood is then returned to the heart via the pulmonary vein. There are approximately 300 million alveoli and nearly 1000 pulmonary capillaries per alveolus resulting in up to $100m^2$ of surface area available for gas exchange by diffusion (Comroe 1966).

The airway vasculature is under hormonal, neural and local mediator control (Deffebach et al 1987).

1.1.3 Airway Smooth Muscle

1.1.3.1 Anatomical Features

The fundamental plan of the musculature of the lungs was first described by Reisseisen (1822) who pointed out that its structure is such as to permit ease of adaptation of the rhythmic length and width changes which are an integral part of the lung inflation and deflation; indeed to take an active part in these movements. It's structure is that of a branched tubular network, neither longitudinal nor circular, extending outwards around the tracheobronchial tree as far as the alveoli (Maklin 1929, Vandam 1952).

The trachea and bronchi are supported by incomplete rings or plates of cartilage which are connected by a band of smooth muscle along the dorsal wall. The bronchioles contain a circular ring of smooth muscle (no cartilage) which becomes thinner towards the alveoli; smooth muscle can even be found in the walls of the alveolar ducts. Throughout the airways, elastic fibres are associated with the muscle layer so that it has been called a myo-elastic layer (Maklin 1929). Smooth muscle taken from different parts of the tracheobronchial tree differs in intrinsic tone and in its reaction to a number of chemical agents (Hahn and Nadel 1981). Reasons for this are not clear but may include mediator release from the surrounding tissue and/or differences in innervation and receptor types (Coburn and Tomita 1973).

The airways are relatively sparsely innervated and therefore, some smooth muscle cells are not directly activated by transmitter released from autonomic varicosities, but are activated indirectly via gap junctions or nexuses. These gap junctions form low resistance pathways which allow electrogenic coupling of activity between cells (Burnstock 1988). Although cell to cell connections exist within individual muscle bundles, electrical communication between adjacent groups of bundles is poor (Dixon and Small 1983, Small and Foster 1986). The proportion of gap junction membrane and degree of cell to cell coupling between smooth muscle cells varies along the length of the airways and is species dependent (Daniel 1988).

1.1.3.2 Electrical Properties

Trachealis muscle taken from several mammalian species has a stable resting membrane potential (-45 to -65 mV) and lacks spontaneous tone (Widdicombe 1963, Small and Foster 1987, Small et al 1988). Spontaneous tone controlled by endogenous mediators has however, been recorded from guinea-pig trachealis muscle (Souhrada and Dickey 1976) and human tracheal and bronchial smooth muscle (Brink et al 1980, Davis et al 1982, Ito et al 1989). This spontaneous tone was decreased by indomethacin, atropine and 5-lipoxygenase inhibitors, suggesting that the observed tone may result from spontaneous release of cyclo-oxygenase products of arachidonic acid, acetylcholine and leukotrienes (Ito et al 1989).

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Furthermore, spontaneous slow oscillations of the membrane potential (slow waves) have been recorded from guinea-pig (McCaig and Souhrada 1980, Small 1982, Allen et al 1986, Small and Foster 1987), human (Honda and Tomita 1987) and bovine trachealis cells (Kirkpatrick 1981) and human bronchial cells (Ito et al 1989).

There is a close correlation between membrane potential and slow wave amplitude (Honda et al 1986). Spasmogens, such as acetylcholine and histamine, that cause depolarization of the membrane, increase the frequency of slow waves (McCaig and Souhrada 1980, Ahmed et al 1984);in contrast, relaxant drugs which produce hyperpolarization of the membrane were shown to suppress slow waves (Honda et al 1986).

The cell membranes of airway smooth muscle exhibit rectifying behaviour and do not pass depolarizing currents as easily as hyperpolarizing ones, as a consequence they cannot initiate action potentials or other propagated active responses (Small and Foster 1987). Rectification involves K+ channels which can be either voltage or Ca^{2+} dependent (Kirkpatrick 1975, Suzuki et al 1976, Ito and Itoh 1984).

1.2   PULMONARY INNERVATION

The airways contain both a motor (efferent) and sensory (afferent) innervation which controls the function of pulmonary smooth muscle, blood vessels and submucosal glands. There are considerable species differences and so care should be taken when extrapolating from one species to another (Richardson 1979,1983).

1.2.1   Afferent Innervation

Several types of afferent nerve fibres have been identified throughout the airways. They carry sensory impulses from irritant, stretch and chemoreceptors in the lungs to the central nervous system, so that appropriate changes in breathing pattern and bronchomotor tone may occur. They arise in the airway epithelium, smooth muscle, submuscosal layer and interalveolar spaces, and terminate in the vagal nuclei (Richardson and Ferguson 1979). Although the vagus nerve is the main afferent pathway there is also evidence for sympathetic afferents (Kostreva et al 1975).

Slowly adapting (stretch) receptors are myelinated nerve terminals found mainly in the smooth muscle of conducting airways. They are mechanoreceptors which respond to changes in tension in the airway walls, but are relatively insensitive to chemical stimuli (Trenchard 1977, Coleridge and Coleridge 1986, Sant'Ambrogio 1987). Via the autonomic nervous system they lead to a reflex bronchodilation by inhibiting vagal tone. They are also responsible for the Hering-Breuer inflation reflex (Widdicombe 1963, Widdicombe and Sterling 1970).

Rapidly adapting (irritant) receptors, unlike slowly adapting receptors, adapt more quickly, fire irregularly and are excited by a wide variety of mechanical and chemical stimuli (Widdicombe 1954a,b). Although the fibres are myelinated, the terminals are non-myelinated. They are situated below the epithelium and between epithelial cells (Sant'Ambrogio 1987, Coleridge and Coleridge 1986). Their most important function may be to signal the onset of pathophysiological changes in the airways. Rapidly adapting receptors in the larynx and trachea are particularly sensitive to mechanical stimuli and particulate irritants, such as cigarette smoke and dust. Intrapulmonary irritant receptors are stimulated by gases, such as ammonia, sulphur dioxide, and ozone, as well as inflammatory mediators such as, PGF_2, histamine and 5-hydroxytryptamine (Coleridge et al 1976). However, autocoids such as bradykinin and PGE_2, which do not contract airway smooth muscle directly, have little effect on these receptors.

C-Fibres are nonmyelinated nerve endings found throughout the airways, usually within the airway epithelium. They respond to many pathological and chemical stimuli such as embolism, congestion, histamine, bradykinin, capsacin, PGF_2, PGE_2, PGI_2 and sulphur dioxide. Stimulation results in rapid shallow breathing (apoea) and pronounced autonomic nervous system changes, including broncho-constriction, mucus secretion, vasodilation, laryngeal closure and often cardiovascular depression (Widdicombe and Stirling 1970, Coleridge et al 1976, Roberts et al 1981,1985, Coleridge and Coleridge 1984, Clozel et al 1985).

Some non-myelinated nerves contain sensory neuropeptides, such as substance P, which may be released as part of an axon reflex (Lundberg et al 1984). They can be activated electrically by antidromic stimulation of the vagus nerve or directly, for example, by capsaicin, the hot extract of pepper, which releases the neuropeptides, substance P and neurokinin A and results in local changes in the vasculature and airway smooth muscle.

Afferent fibres have been identified in both animals and humans which are associated with epithelial cells containing 5-hydroxytryptamine and a variety of regulatory peptides (Lauweryns and Peuskens 1972, Lauweryns et al 1985). The possible sensory functions of these complexes, called neuroepithelial bodies, is unclear but they may sense hypoxia. They are more numerous in foetal and infant airways, possibly suggesting a function related to the developing lung.

1.2.2   Efferent Innervation

1.2.2.1 Overview of the Role of Classical Cholinergic and Adrenergic
           Nerves

The dominant neuronal control of airway smooth muscle is exerted via excitatory parasympathetic (cholinergic) nerves (Olsen et al 1965, Barnes 1986). Cholinergic efferent nerves arising in the vagal nuclei of the brainstem, pass down the vagi and synapse in small ganglia located in an extensive nerve plexus within the airway walls. Short fibres from these ganglia supply target cells, such as, airway smooth muscle and pulmonary submucosal glands (Richardson 1979, Dawson 1984, Nadel and Barnes 1984). Postganglionic parasympathetic nerve endings are

important sites for modulation of acetylcholine release (Maclagan 1987, 1988).

Vagal nerve stimulation results in mucus secretion and bronchoconstriction which are augmented by anticholinesterases and blocked by atropine, indicating release of acetylcholine onto muscarinic receptors on secretory cells and the airway smooth muscle (Basbaum et al 1984, Culp and Marin 1986).

Unlike the parasympathetic innervation, the sympathetic nerve supply to the airways is sparse and not well characterised. Upper thoracic sympathetic preganglionic fibres terminate in the extrapulmonary stellate ganglia. Postganglionic fibres then enter the airways but their exact distribution has not been fully established and is very species dependent (Mann 1971, Richardson 1979).

Pulmonary sympathetic fibres appear to innervate the submucosal glands, bronchial vessels and the airway ganglia of most species. In some species, including the guinea-pig, cat and dog (Mann 1971, O'Donnell and Saar 1973) the airway smooth muscle is also innnervated by sympathetic nerves. In these species stimulation of the cervical sympathetic nerve trunk induces bronchodilation. In other species, for example, man and baboons (Mann 1971, Laitinen et al 1985) the sympathetic innervation to airway smooth muscle is thought to be sparse or absent and postjunctional ß-adrenoceptors which mediate bronchodilation are probably activated by circulating catecholamines. Adrenoceptors which inhibit acetylcholine release have also been demonstrated on the terminals of parasympathetic nerves

innervating airway smooth muscle (Vermeire and Vanhoutte 1979, McCaig 1987). Thus, circulating and neurally released catecholamines can modulate the bronchoconstrictor action of the parasympathetic nervous system, both pre- and postjunctionally.

The effects of both the parasympathetic and the sympathetic nervous systems are not equally distributed throughout the airways. The density of both pulmonary parasympathetic and sympathetic innervation decreases with a decrease in airway diameter (O'Donnell and Saar 1973, O'Donnell et al 1978).

1.2.2.2 Non-Adrenergic, Non-Cholinergic Nerves (NANC)

Both excitatory and inhibitory nonadrenergic noncholinergic (NANC) mechanisms have been reported in airways, but the neurotransmitters involved and their physiological significance will remain uncertain until specific antagonists become available (Said 1987).

The presence of inhibitory NANC nerves has been demonstrated *in vivo* and *in vitro*, in guinea-pig (Coburn and Tomita 1973, Coleman and Levy 1974, Richardson and Bouchard 1975), chicken (Bhatla et al 1980), cat (Diamond and O'Donnell 1980, Altiere et al 1985), toad, baboon (Middendorf and Russell 1980) and human airways (Richardson and Beland 1976).

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In some species, transmural stimulation of isolated airway smooth muscle in the presence of muscarinic, α-adrenoceptor and β-adrenoceptor antagonists produces a bronchodilation which is abolished with tetrodotoxin (Coleman and Levy 1974, Richardson and Bouchard 1975, Richardson and Beland 1976, Diamond and Gillespie 1982). In addition, this bronchodilation is blocked with hexamethonium, indicating the presence of ganglia within the nerves (Chesrown et al 1980, Diamond and O'Donnell 1980, Yip et al 1981).

The identity of the transmitter released from these inhibitory NANC nerves is still unknown. Burnstock (1972) proposed that it might be a purine nucleotide such as adenosine triphosphate (ATP). More recent studies, however, suggest that the transmitter may be a regulatory peptide such as, vasoactive intestinal peptide (VIP) (Matsuzaki et al 1980, Polak and Bloom 1985,1986, Sheppard and Polak 1986).

VIP-immunoreactive nerves have been identified in the lungs of several species (Uddman et al 1978, Uddman and Sundler 1987, Dey et al 1981, Partanen et al 1982). They diminish in the smaller airways, and are virtually absent from bronchioles. NANC relaxation is also almost absent in the smaller airways (Barnes 1988). VIP produces relaxation of airway smooth muscle in vitro which, a) is unaffected by adrenergic or cholinergic antagonists, b) reverses bronchoconstriction induced by 5-hydroxytryptamine (Diamond et al 1983) and c) protects against histamine and prostaglandin $F_{2\alpha}$-induced bronchoconstriction in the dog (Said 1987). VIP is one of the most potent endogenous airway relaxants known and binds to specific receptors on the airway smooth muscle (Palmer et al 1986).

In addition, it mimics the electrophysiological changes produced by NANC nerve stimulation (Ito and Takeda 1981), stimulates cAMP production and is released during relaxation induced by electrical field stimulation of the trachea (Matsuzaki et al 1980).

Autoradiography confirms the presence of VIP receptors on airway glands, epithelium, blood vessels, and bronchi smooth muscle, but not bronchioles (Carstairs and Barnes 1986). VIP also stimulates ion transport in airway epithelium, inhibits secretion from mucous cells stimulated by methacholine and enhances secretion produced by phenylephrine from serous cells (Webber and Widdicombe 1987).

VIP-containing nerve fibres originate in the nodose ganglion, in small ganglia along the vagus nerve, and in ganglia within the trachea wall. VIP-immunoreactive nerves are often associated with cholinergic nerves. VIP may also coexist in the same nerve terminals as acetylcholine, where it may function as a co-transmitter or neuromodulator (Laitinen et al 1985).

All this evidence points towards VIP as at least one of the transmitters released from non-adrenergic inhibitory nerves in the airways. There is, however, evidence which suggests that VIP is not the exclusive transmitter in these nerves. Stimulation of the NANC system causes a further inhibition of airways, which have been maximally relaxed with VIP (Karlsson and Persson 1983) and proteolytic enzymes, which inactivate VIP, do not inhibit NANC relaxations (Altiere and Diamond 1984).

Another possible candidate for the neurotransmitter in NANC inhibitory nerves is peptide histidine methionine (PHM), the human equivalent of peptide histidine isoleucine (PHI). This is structurally similar to VIP and has a similar immunocytochemical distribution in the lung. PHI/PHM immunoactive nerves supply airway smooth muscle (especially the larger airways), bronchial and pulmonary vessels, submucosal glands and airway ganglia (Lundberg et al 1984). PHM is equipotent to VIP as a relaxant of airway smooth muscle, but less potent as a vasodilator, suggesting that it might act on different receptors (Palmer et al 1986).

Electrical stimulation of the guinea-pig bronchi and occasionally trachea, produces a bronchoconstriction which is not inhibited by atropine or adrenoceptor antagonists. These responses are mimicked by substance P(SP) and inhibited by SP-antagonists (Lundberg et al 1983), suggesting that SP may be the transmitter released from NANC excitatory nerves. These antagonists, however, are still relatively non-specific. SP is localised to unmyelinated C-fibres. These SP-containing afferent nerves are found beneath and within the airway epithelium, around blood vessels and to a lesser extent within airway smooth muscle. In addition, SP-containing nerve cell bodies occur in small ganglia along the vagus nerve (Uddman and Sundler 1987). It is possibly released by antidromic stimulation of the vagal afferent nerves or as part of an axon reflex.

In addition to contraction of airway smooth muscle, SP also stimulates mucus secretion, increases airway microvascular permeability, causes vasodilation and increases exudation of plasma into the airway lumen (Barnes 1988).

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Capsaicin, the hot extract of pepper, is a neurotoxin which destroys small sensory afferents and lowers the levels of SP in the respiratory tract. Capsaicin at lower concentrations releases SP from unmyelinated sensory nerve endings and causes bronchoconstriction and microvascular leakage (Lundberg and Saria 1982, Saria et al 1983). Chronic treatment with capsaicin leads to depletion of SP-immunoreactivity and prevents irritants from stimulating airway microvascular leakage (Lundberg and Saria 1983).

SP in sensory neurones is usually found in association with a second tachykinin, neurokinin A (NKA), which has a similar distribution to SP. NKA, however, is more potent than SP in contracting airway smooth muscle (Hall et al 1989) and vasodilating the tracheal vasculature (Salonen et al 1988), whereas SP is more potent at causing microvascular leakage (Rogers et al 1988). These effects are mediated via different neurokinin receptors (Webber 1989).

1.3   PULMONARY PARASYMPATHETIC INNERVATION

1.3.1   Classification of Cholinoceptors

In 1914, Dale suggested that the actions of acetylcholine could be differentiated into two types; those reproduced by the alkaloid muscarine (from the toadstool Amanita muscaria) and those mimicked by nicotine (from tobacco). "Muscarine" actions are abolished by atropine, whereas, "nicotine" actions are blocked by a combination of large doses of nicotine plus tubocurarine. These actions are now referred to as muscarinic and nicotinic actions, and are mediated via muscarinic cholinoceptors and nicotinic cholinoceptors respectively. Muscarinic receptors are located on smooth muscle, gland and cardiac cells. In contrast, nicotinic receptors are found on all autonomic ganglia, the adrenal medulla and at the motor endplates of striated muscles. Although this classification provides the basis for division of acetylcholine receptors into two main classes, each class has now been further subdivided.

1.3.1.1   Subclassification of Nicotinic Cholinoceptors

Nicotinic cholinoceptors are found at ganglia, the neuromuscular junction and in the central nervous system. They do not form a homogenous population and can be subdivided into those selectively blocked by hexamethonium (neuronal location), whereas those at the neuromuscular junction, are blocked by the competitive antagonists, tubocurarine, gallamine, pancuronium and toxins, such as, α-bungarotoxin (see Bowman 1982 for review).

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It has also been suggested that at the neuromuscular junction, nicotinic cholinoceptors are not only located postjunctionally, but also on the motor nerve endings. These prejunctional nicotinic autoreceptors are probably facilitatory and have different pharmacological properties to the post-synaptic receptors (Bowman et al 1984,1987,1988). For example, they lack the abilities to bind various toxins and classical nicotinic antagonists show great variability between receptor subtypes. Hexamethonium, although weak, is relatively selective for prejunctional cholinoceptors; pancuronium acts preferentially on postjunctional nicotinic cholinoceptors and tubocurarine combines with both types of cholinoceptors (Bowman and Webb 1976).

1.3.1.2 Subclassification of Muscarinic Receptors

The first evidence supporting the idea that muscarinic receptors were not homogenous was presented by Riker and Wescoe in 1951. They showed that the neuromuscular blocking drug, gallamine, inhibited the negative chronotropic action of methacholine at concentrations that did not effect the hypotensive action of methacholine. This concept, that muscarinic receptors consist of pharmacologically different subtypes, was supported by the work of Barlow and his group (1972,1982,1985). They showed that the muscarinic antagonist 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) is almost 11 fold more selective for ileal smooth muscle than for atrial muscarinic receptors.

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It was the discovery of the muscarinic antagonist, pirenzepine, which led to general acceptance of different subclasses of muscarinic receptors. Pirenzepine was shown to exhibit heterogenity of binding to different tissues (Hammer et al 1980, Birdsall et al 1984). High affinity sites for pirenzepine are located in the sympathetic ganglia, on vagal nerves innervating the gastric parietal cells and in certain areas of the brain, such as the cerebral cortex, corpus striatum and the hippocampus. In contrast, the muscarinic receptors in the heart and on smooth muscle, show only low affinity for pirenzepine. Tissues, such as the cerebral cortex and sympathetic ganglia, contain both low and high affinity receptors (Birdsall and Hulme 1983, Hirschowitz et al 1984, Eglen and Whiting 1986). The organ distribution of high affinity sites for pirenzepine was confirmed using ^{3}H-pirenzepine directly as the ligand (Watson et al 1983, 1986a,b).

The heterogeneity of muscarinic receptors obtained using receptor binding studies was confirmed in functional experiments, both *in vitro* and *in vivo* (Birdsall and Hulme 1985). Pirenzepine inhibited gastric secretion and blocked the hypertensive action of the ganglionic stimulant McN-A-343 *in vivo* (Hammer and Giachetti 1982). In comparison, it was only a weak antagonist of agonist-induced contraction of the guinea-pig ileum smooth muscle (Brown et al 1980b, Barlow and Kitchen 1982) and of vagally-induced bradycardia (Hammer and Giachetti 1982).

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Muscarinic receptors were therefore classified at the first symposium on muscarinic receptors in Boston in 1983, into two subtypes; *i)* M_1 receptors, which exhibit a high affinity for pirenzepine and are located in neural tissue, and *ii)* M_2 receptors, which exhibit a low affinity for pirenzepine and are located both on neural and visceral tissues (Hirshowitz et al 1984).

Other compounds claimed to possess M_1-selectivity include, adiphenine (Eglen et al 1986), dicyclomine (Giachetti et al 1986a), 4-DAMP (Giachetti et al 1986), scopolamine (Watson et al 1986b) and telenzepine (Eltze et al 1985). Although these compounds have a greater affinity for M_1 receptors in comparison to cardiac M_2 receptors, they do not discriminate between M_1 receptors and glandular or smooth muscle muscarinic receptors (Doods et al 1987). Also, the binding of adipenine, hexahydroadiphenine and dicylomine (Eglen et al 1986) appears to be non-competitive. Thus, at present, pirenzepine is the only compound that can be considered as M_1 selective (Doods 1987).

Although pirenzepine does not distinguish between different subtypes of muscarinic receptors on different visceral organs (Szelenyi 1982, Clague et al 1985) there is increasing evidence for heterogenity of muscarinic receptors which exhibit a low affinity for pirenzepine. The development of more selective agonists and antagonists has demonstrated clear subdivisions in the M_2 receptor subtype (Mihn and Wetzel 1987). M_2 muscarinic receptors on cardiac muscle are distinct from those on glands and smooth muscle of, for example, the airways and ileum, which are at present classified as M_3.

As previously mentioned gallamine selectively blocked the action of acetylcholine on the heart without affecting acetylcholine-induced changes in sweating, salivary secretions and gut motility (Riker and Wescoe 1951). Himbacine (Anwar et al 1986) and the gallamine related neuromuscular blocking drugs, pancuronium and stercuronium (Riker and Wescoe 1951, Clark and Mitchelson 1976, Li and Mitchelson 1980, Ellis and Hoss 1982, Stockton et al 1982, Kharkevich and Shorr 1987) exhibit a similar cardiac selectivity. Although the muscarinic antagonist properties of the latter compounds are complicated by allosteric interactions (Roffel et al 1989) and are non-competitive in nature (Stockton et al 1982), they have proved to be useful tools for distinguishing M_2 receptors from M_1 and M_3 muscarinic receptors.

The synthesis of the pirenzepine analogue AFDX-116 provided additional evidence for the presence of at least 3 different muscarinic receptor subtypes. AFDX-116 has proved to be a competitive M_2 muscarinic antagonist. In receptor binding studies it exhibits approximately 30 fold higher affinity for cardiac than glandular muscarinic receptors (Hammer et al 1986, Doods et al 1987). This cardioselectivity of AFDX-116 also occurs *in vivo* and *in vitro* functional studies. In pithed rats, AFDX-116 is more potent at antagonizing vagally-induced bradycardia than M_1 responses, such as McN-A-343 provoked increases in blood pressure and M_3 responses, such as salivation (Giachetti et al 1986b, De Jonge et al 1986).

Another class of compounds which exhibits selectivity for atria M_2 muscarinic receptors has recently been described (Melchiorre et al 1987b, Melchiorre 1988). The most selective of these polymethylene tetramines is methoctramine, which in functional studies was found to be 54-275 fold more selective for cardiac as opposed to ileal (Melchiorre et al 1987a,b) or tracheal muscarinic receptors (Giraldo et al 1988).

This selectivity for cardiac muscarinic receptors was confirmed in direct binding studies, in which it proved to be 130-158 fold more selective for cardiac M_2 as opposed to exocrine gland muscarinic receptors. When its potency at M_1 and M_2 muscarinic receptors was compared, methoctramine was 16 fold more selective for M_2 cardiac compared to M_1 cerebral cortex muscarinic receptors (Giraldo et al 1988, Michel and Whiting 1988).

The above degree of selectivity was not however confirmed *in vivo*. Although in anaesthetized cats methoctramine was a more potent antagonist of bradycardia responses to bethanechol, than bethanechol induced changes in urinary bladder tone, saliva secretion and blood pressure, it was only approximately 10 fold selective (Giraldo et al 1988). In the anaesthetised guinea-pig methoctramine appears to exhibit only a two-fold greater potency for M_2 muscarinic receptors on the heart and parasympathetic nerve terminals than for M_3 receptors on airway smooth muscle (Watson et al 1989). Methoctramine was also found in the pithed rat to inhibit vagally-induced bradycardia in preference to ganglionic M_1 mediated McN-A-343 induced hypertension (Giraldo et al 1988).

In addition to muscarinic receptor antagonism, methoctramine exhibits non-competitive antagonist potency for nicotinic receptors, weakly antagonises α-adrenoceptors, elicits positive inotropic responses and exhibits allosteric inhibitory effects at cardiac muscarinic receptors (Melchiorre et al 1987b, Eglen et al 1988, Giraldo et al 1988). These properties may complicate its use in muscarinic receptor classification.

Functional and binding studies suggest that muscarinic receptors on the smooth muscle of the airways, ileum, iris and bladder are similar (Barlow et al 1972, Li and Mitchelson 1980, Barlow and Weston-Smith 1985) and therefore belong to the same subtype, that is, M_3 muscarinic receptors. The muscarinic antagonists 4-DAMP, hexahydrosiladifenidol (Mutschler and Lambrecht 1984, Fuder et al 1985) p-floro-hexahydrosiladifenidol (Lambrecht et al 1988) and silabenzhexol (Eglen and Whiting 1987, Waelbroeck et al 1989) have been reported to display higher affinity for these smooth muscle muscarinic receptors than for those on the heart.

At present muscarinic receptors on exocrine glands are also classified as M_3. The question whether smooth muscle and glandular muscarinic receptors are identical is however, very controversial. Recently, on the basis of functional and binding studies, muscarinic receptors were further divided into four subtypes (Batink et al 1987). It has also been shown in both binding studies and functional experiments, that the affinity of AFDX-116 for ileal smooth muscle receptors is approximately 10 fold higher than that for glandular muscarinic receptors (Hammer et al 1986, Giachetti et al 1986b, Batink et al 1987, Doods et al 1987).

It has proved very difficult to classify vascular muscarinic receptors as they have been reported to exhibit high (O'Rourke and Vanhoutte 1987), intermediate (Eglen and Whiting 1985) and low (Hynes et al 1986) affinity for pirenzepine. The confusion may be due to the presence of two different groups of vascular muscarinic receptor, which are situated on the endothelium and the vascular smooth muscle (Hynes et al 1986). Thus, *in vivo* muscarinic agonists cause a decrease in blood pressure, whereas *in vitro* they induce vasodilation or vasoconstriction depending upon whether the endothelium is intact or not. It is therefore possible that vascular muscarinic receptors also consist of a heterogenic population, for example, in rabbit aorta the affinity of ipratropium was more than 10 fold higher for muscarinic receptors mediating relaxation than for those causing contraction (Doods 1987).

Cloning experiments have identified five distinct gene sequences that code for muscarinic receptors with different amino acid sequences (Bonner 1989), confirming the existence of at least three distinct muscarinic receptor subtypes.

1.3.1.3 Neuronal Muscarinic Receptors

Muscarinic receptors which moderate transmitter release have been identified in both the peripheral and central nervous systems. They may be located on the terminal fibres supplying the ganglia ("preganglionic"), on the ganglia cell body ("postganglionic") and on the terminals of

postganglionic fibres innervating target organs, such as, smooth muscle and gland cells in the lung ("prejunctional"). If the neurotransmitter modulates its own release, the receptors involved are called "autoreceptors", to distinguish them from neuronal receptors for other neurotransmitters, autocoids and neuropeptides, which are called "heteroreceptors".

Activation of muscarinic receptors on sympathetic and parasympathetic nerve endings and ganglia cell bodies usually results in inhibition of transmitter release (Hellman 1963, Loffelholz and Muscholl 1970, Fozard and Muscholl 1972, Fosbraey and Johnson 1980). Muscarinic receptors on dopaminergic nerves, however, exert a facilitatory effect on transmitter output (Raiteri et al 1982, Bonanno et al 1985). Pre-junctional muscarinic receptors on noradrenergic, dopaminergic and cholinergic nerve terminals, do not form a homogenous population, although most exhibit a low affinity towards pirenzepine and are therefore not of the M_1 subtype (Mitchelson 1989).

Pre-synaptic muscarinic autoreceptors, which inhibit release from cholinergic nerves, were first discovered in the cat cerebral cortex (Mitchell 1963). Since then, cholinergic neurones in the central nervous system in the cortex, hippocampus and striatum, have all been shown to possess pre-synaptic muscarinic receptors.

Muscarinic inhibitory autoreceptors have also been identified, for example, on postjunctional parasympathetic nerve endings, innervating peripheral effector organs such as, the ileum (Fosbraey and Johnson 1980, Kilbinger and Wessler 1980), heart (Wetzel and Brown 1985), iris (Kilbinger 1984), airways (Fryer and Maclagan 1984, Blaber et al 1985, Maclagan 1987,1988) and on motor neurones of lower vertebrates (Koketsu and Yanada 1982).

1.3.2   Airway Parasympathetic Ganglia

Pre-ganglionic parasympathetic axons, whose cell bodies are located in the medulla oblongata (nucleus ambigius), decend in the vagus and synapse onto ganglion cell bodies in the airway wall (Richardson 1979 Mitchell et al 1985). Ganglia have been observed in chains extending along the length of the trachea (Skoogh 1988). Baker and colleagues (1986) identified ganglia, containing one to four ganglion cell bodies, located in the superficial muscle and gland nerve plexuses. A few ganglia containing 10-40 larger cell bodies were located along a longitudinal nerve trunk, near the trachealis muscle and cartilage junction. In the human airways, ganglia consisting of several ganglion cell bodies (usually less than 20) have also been observed. Occasionally, single ganglion cell bodies are found in the airway wall, for example, in the bronchioles. In the trachea and major bronchi, ganglia are found in the posterior wall, but in the intrapulmonary airways they are more evenly distributed within the wall (Barnes 1986). At least

two different types of ganglia (Baker et al 1986) and nerve endings have been identified (Cameron and Coburn 1984). Electron microscopic sections of the interganglionic nerve trunk show that most fibres are nonmyelinated, though a small proportion are myelinated (Cameron and Coburn 1984).

Acetylcholine, released from preganglionic nerve terminals, induces a fast excitatory postsynaptic potential (fEPSP) which is mediated by nicotinic cholinoceptors in all ganglionic cells (Kuba and Koketsu 1978). In some parasympathetic ganglia the fEPSP is followed by a slow inhibitory postsynaptic potential (sIPSP) and a slow excitatory postsynaptic potential (sEPSP), which are mediated by muscarinic cholinoceptors (Gallagher et al 1982). Intracellular recordings from airway parasympathetic ganglia in ferrets have shown slow after-potentials, suggestive of sIPSP and sEPSP (Cameron and Coburn 1984, Baker et al 1982, 1983).

The existence of facilitatory M_1 muscarinic receptors on pulmonary parasympathetic ganglia has recently been proposed. There however, appears to be considerable species differences and conflicting results have been obtained for some species.

Recently the effect of inhaled pirenzepine and ipratropium bromide on cholinergic muscarinic agonist-induced and reflex bronchoconstriction were compared in allergic human volunteers (Lammers et al 1989). A dose of pirenzepine was found that did not inhibit significantly the muscarinic agonist-induced bronchoconstriction. Whereas, ipratropium bromide blocked this bronchoconstriction. The same dose of

pirenzepine was as effective as ipratropium bromide at antagonizing reflex bronchoconstriction. Pirenzepine was therefore not having a postjunctional effect on airway smooth muscle. The most likely location for its action seems to be pulmonary parasympathetic ganglia, which have been shown to contain a high density of muscarinic receptors in autoradiographic studies of bovine airways (Van Koppen et al 1987). It has also been suggested that M_1 receptors are present in the parasympathetic ganglia of the rabbit bronchi, as pirenzepine was more potent at inhibiting vagally-stimulated than field-stimulated contractions of this tissue (Bloom et al 1988). *In vivo* pirenzepine was shown to antagonize bronchoconstriction induced by preganglionic vagal stimulation in rabbits (Bloom et al 1987) and dogs (Beck et al 1987) and these results were interpreted as evidence for M_1 receptors in the pulmonary parasympathetic ganglia. However, an effect of similar concentrations of pirenzepine on acetylcholine-induced broncho-constriction mediated via postjunctional M_3 receptors on the airway smooth muscle was not excluded. Recently, Maclagan and colleagues concluded that M_1 muscarinic receptors are not present in the pulmonary parasympathetic ganglia of the rabbit (Maclagan and Faulkner 1989) and guinea-pig (Maclagan et al 1989) as pirenzepine was equipotent at antagonizing bronchoconstrictor responses induced by intravenous acetylcholine and vagal stimulation.

Filtering may occur at parasympathetic ganglia, particularly at higher frequencies. Skoogh (1983) demonstrated that postganglionic activation by field stimulation had a greater effect than preganglionic activation by nerve stimulation on contractile responses of the ferret trachea. In addition, although preganglionic nerves may fire at electrical

frequencies as high as 50Hz (Sheppard 1987) and fire at 25Hz during spontaneous inspiration, ganglion cells themselves can only fire at frequencies up to about 15Hz (Mitchell et al 1985).

Evidence for neural inputs other than cholinergic to airway parasympathetic ganglia has been obtained in some species. Both adrenergic and peptidergic (namely VIPergic) nerves have been demonstrated in airway parasympathetic ganglia (Mann 1971, Richardson and Ferguson 1979, Barnes 1986).

There is also morphological evidence for transmitter heterogeneity; some cells within the ganglia contain dense core vesicles, which are believed to contain catecholamines or neuropeptides (Uddman et al 1978).

Although inhibition of transmission in ferret airway parasympathetic ganglia by application of agonists for β_2 and α-adrenoceptors has been reported (Baker et al 1982, 1983, Skoogh 1983, 1988), inhibition of airway parasympathetic ganglionic transmission by noradrenaline following stimulation of the nerve trunk has not yet been demonstrated (Baker et al 1983, Skoogh 1988). Barbiturates have also been shown to depress transmission through parasympathetic ganglia in the airways via an action on the post-synaptic membrane (Skoogh et al 1982). In contrast, neuropeptides may potentiate parasympathetic ganglionic transmission, as removal of peptide-containing fibres by capsaicin pretreatment reduced bronchoconstriction induced by preganglionic vagal nerve stimulation in vivo (Martling et al 1984).

Various inflammatory mediators may also influence ganglionic transmission and mast cells are often found in close association with airway ganglia (Grillo and Nadel 1980, Barnes 1986).

Thus, airway parasympathetic ganglia may be capable of complex modulation and integration of neural inputs.

1.3.3   Post-Ganglionic Parasympathetic Neurones

Relatively short postganglionic nerve fibres innervate target cells, such as the airway smooth muscle and submucosal glands (Richardson 1979). The parasympathetic nervous system has been shown to provide the dominant control of airway smooth muscle calibre, and may be responsible for the small degree of resting bronchomotor tone observed in many animal species. This resting tone may be abolished by section of the vagus and by muscarinic antagonists (Barnes 1986). In the cat, direct recordings from vagal efferent fibres confirm an irregular tonic firing at rest (Widdicombe 1961). In humans, muscarinic antagonists cause bronchodilation (Taylor et al 1989), whereas inhalation of an anticholinesterase caused bronchoconstriction in normal subjects (Quigley et al 1985, Barnes 1986). These results confirm that there is a tonic release of acetylcholine in the airways. At night this tonic vagal activity increases and may be important in the pathophysiology of nocturnal asthma (Morrison et al 1988).

The effects of vagal nerve stimulation are not equally distributed throughout the airways. Using tantalum bronchography to outline the airways, maximum bronchospasm is seen in airways measuring 1-5mm

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in diameter, with relatively little effect in the bronchioles. This is consistent with the distribution of pulmonary cholinergic nerves. As airway diameter narrows, both the density of innervation to and muscarinic receptors on the airway smooth muscle decreases until the terminal bronchioles are reached, where there are very few cholinergic fibres or receptors, and none are seen in the alveoli.

Acetylcholine synthesis by choline acetyltransferase occurs in the neurone body and the varicosities where it is stored in small agranular vesicles (30-60nm in diameter). Inactivation of acetylcholine, following interaction with postganglionic muscarinic receptors, involves hydrolysis by acetylcholinesterase. Acetylcholine is not taken up into parasympathetic prejunctional nerve terminals itself, but choline, produced from hydrolysis of acetylcholine, is actively taken up into the varicosities and is re-used to synthesize acetylcholine (Burnstock 1988).

Post-ganglionic parasympathetic nerve endings are important sites for modulation of acetylcholine release by neurotransmitters, neuropeptides and autocoids (see Maclagan 1987,1988).

In the airways of the guinea-pig (Fryer and Maclagan 1984), cat (Blaber et al 1985), rabbit (Maclagan and Faulkner 1989), dog (Ito and Yoshitomi 1988), rat (Maclagan and Aas 1990 unpublished) and man (Minette and Barnes 1988), the existence of inhibitory muscarinic autoreceptors on parasympathetic nerve terminals have been demonstrated. Muscarinic agonists such as, acetylcholine and pilocarpine, reduce acetylcholine release via activation of these

prejunctional receptors. This inhibitory effect of muscarinic agonists can be blocked with the selective M_2 muscarinic antagonists, gallamine (Fryer and Maclagan 1984) and methoctramine (Watson et al 1989). These M_2 antagonists also potentiated vagally-induced bronchoconstriction *in vivo* and electrical field stimulated and vagally-induced contractions *in vitro*. The involvement of postjunctional muscarinic receptors, the sympathetic nervous system and differences in airway contractility were excluded. These results therefore suggest that inhibitory muscarinic autoreceptors exist on pulmonary parasympathetic nerve endings which are of the M_2 subtype.

In the airways, transmission from parasympathetic nerves is also inhibited by adrenoceptor agonists and simultaneous stimulation of the adjacent sympathetic nerves. Prejunctional adrenergic heteroreceptors have been identified on pulmonary parasympathetic nerves in the guinea-pig (Jones et al 1980a, Grundstrom et al 1981, McCaig 1987), man (Rhoden et al 1988), and the dog (Cabezas et al 1971, Vermeire and Vanhoutte 1979, Danser et al 1987). The involvement of $ß_1$-, $ß_2$- and $α_2$- adrenoceptors has been suggested (see section 1.5).

In some species, the major inhibitory pathway is exerted via nonadrenergic noncholinergic nerves which lie in close proximity to the vagus in the vagal bundle (see section 1.2.2.2). Possible candidates for the neurotransmitter involved include neuropeptides, such as vasoactive intestinal polypeptide (VIP), peptide histidine isoleucine (PHI) and peptide histidine methionine (PHM) (Richardson and Beland 1976, Ito and Takeda 1982, Diamond and Gillespie 1982, Diamond et al 1983) and purines, such as adenosine and ATP (Burnstock 1972).

Adenosine inhibited the potassium evoked release of [^{3}H] acetylcholine from rat bronchi (Aas and Fonnum 1986). In the guinea-pig VIP inhibited vagally-induced contractions *in vivo* (Lundberg et al 1984). *In vitro* VIP had a dual action on contractile responses of the ferret trachea induced by electrical field stimulation (EFS); at concentrations up to 10^{-9}M, it potentiated responses, whereas, at higher concentrations (>10^{-8}M) it inhibited EFS-induced contractions. In the rat bradykinin, angiotensin and neurotensin enhanced cholinergic output in response to electrical field stimulation (Aas and Helle 1982).

Substance P, which may be released from afferent nerves and the related tachykinins, neurokinin A and eledoisin, have been shown to potentiate cholinergic neurotransmission prejunctionally in the rabbit and guinea-pig (Tanaka and Grunstein 1984, Hall et al 1989).

In addition to their direct postjunctional actions on airway smooth muscle autocoids, such as, the prostaglandins, histamine and 5-hydroxytryptamine, have been shown to modulate parasympathetic neurotransmission. For example, prostaglandins E_1, $E_{2\alpha}$, $F_{2\alpha}$ and I_2 inhibit (Jones et al 1980a, Ito and Tajima 1981a, Walters et al 1984, Inoue et al 1984, Inoue and Ito 1985, Inoue and Ito 1986), whereas, 5-hydroxytryptamine (Hahn et al 1978, Sheller et al 1982) and U46619 potentiate (Chung et al 1985) contractions induced by electrical field stimulation. Histamine has been reported to both reduce and increase pulmonary parasympathetic responses (Kikuchi et al 1984, Inoue and Ito 1986, McCaig 1986, Ichinose et al 1989).

Both *in vivo* and *in vitro* experiments indicate that vagal nerve stimulation also increases airway mucus secretion in laboratory animals and humans. Whether there is a functional significant cholinergic supply to airway blood vessels or epithelium is less certain.

1.3.4 Postjunctional Pulmonary Muscarinic Receptors on Airway Smooth Muscle and Glands

Acetylcholine, released from pulmonary postganglionic parasympathetic nerve endings, activates muscarinic receptors present on airway smooth muscle (Murlas et al 1982), submucosal glands (Borson et al 1984), epithelium and possibly mast cells (Richardson 1979). Direct receptor binding studies and autoradiographic mapping indicate a high density of muscarinic receptors on airway smooth muscle of the large airways, this density decreases with the size of the airways (Barnes et al 1983a, Basbaum et al 1984). This distribution of pulmonary muscarinic receptors is consistent with decreasing parasympathetic innervation with a decrease in airway diameter.

These receptors on airway smooth muscle are usually classified as M_3 muscarinic receptors. Muscarinic agonist and vagally-induced bronchoconstriction is not sensitive to M_1 selective concentrations of pirenzepine (Bloom et al 1987, O'Rourke et al 1987, Madison et al 1987), nor M_2 selective concentrations of gallamine, methoctramine (Fryer and Maclagan 1984, Faulkner et al 1986, Watson et al 1989) and AFDX-116, but is antagonized by atropine and the M_3 antagonists 4-DAMP, hexahydrosiladifenidol and telenzepine *in vivo* and *in vitro* (Moore et al 1989).

In fact, vagally-induced, but not muscarinic agonist-induced bronchoconstriction, may be potentiated by M_2 antagonists such as, gallamine and methoctramine, due to inhibition of prejunctional muscarinic autoreceptors on pulmonary parasympathetic nerve endings (see section 1.3.3)(Barnes et al 1988).

Binding studies in guinea-pig lungs indicate a predominance of M_3 receptors whereas human lungs contain both M_1 and M_2/M_3 muscarinic receptors in relatively equal proportions (Casale and Ecklund 1988, Van Koppen et al 1985, Mak and Barnes 1989a). Although in human airways, mucus secretion is mediated via both M_1 and M_3 receptors, in other species, including the guinea-pig, muscarinic receptors on pulmonary submucosal glands are predominantly of the M_3 subtype (Mak and Barnes 1989b).

1.3.5 Secondary Messenger Systems

Activation of muscarinic receptors by acetylcholine released from the parasympathetic nerve endings results in contraction of the airway smooth muscle. The slight delay between activation of the muscarinic receptor and the resultant contractile response is due to the involvement of secondary messenger systems. Contraction is dependent upon a rise in intracellular Ca^{2+} which is itself a secondary messenger (Bolton 1979). There are two possible sources of this Ca^{2+} :- a) extracellular; Ca^{2+} can enter the cell through voltage-operated (VOC) or receptor-operated channels (ROC), and b) intracellular; Ca^{2+}

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can be released from intracellular stores, for example, the sarcoplasmic reticulum. Contractile agonists may utilize one or more methods to raise the concentration of cytosolic free Ca^{2+} (Goodman et al 1987).

Although KCl and TEA-induced contraction of the airway smooth muscle appears to be initiated by the opening of VOC in the cell membrane (Small et al 1988), muscarinic agonists evoke contraction via a different mechanism. Acetylcholine-induced contraction of airway smooth muscle is independent of changes in membrane potential (Coburn 1979), does not involve Ca^{2+} influx (Ahmed et al 1985) and therefore does not appear to be dependent upon uptake of extracellular Ca^{2+} and so it seems to involve the release of Ca^{2+} from intracellular stores (Small and Foster 1986).

Hydrolysis of membrane phosphoinositides is associated with an increase in cytosolic calcium. The major phosphoinositide is phosphatidylinositol (PI) which exists in metabolic equilibrium with the two polyphosphoinositides, phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2). There is increasing evidence to suggest that the products of hydrolysis of PIP_2, diacylglycerol (DAG) and inositol 1,4,5,-triphosphate (IP_3), are the intracellular secondary messengers linking receptor stimulation and potential-independent transmembrane calcium flux and intracellular calcium mobilization (Hall and Chilvers 1989). There is a close

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relationship between occupation of muscarinic receptors and stimulation of PIP_2 turnover (Grandordy et al 1986, Meurs et al 1989). Breakdown of PIP_2 to generate DAG and IP_3 involves a membrane bound phosphoinositide-specific phospholipase, which is thought to be coupled to the muscarinic receptor by a regulatory protein. It has been suggested that this regulatory protein is probably a GTP-binding protein (Cockcroft and Gomperts 1988).

The formation of DAG from PIP_2 parallels that of muscarinic contraction (Baron et al 1984) and the extent of PIP_2 hydrolysis is related to the size of the contractile response (Grandordy and Barnes 1987). In addition, IP_3 causes contraction by mobilizing Ca^{2+} from intracellular stores (Berridge 1987, Taylor 1987). DAG activates protein kinase C, which may sustain contraction of airway smooth muscle (Park et al 1985).

As previously mentioned DAG is an activator of protein kinase C, which may play an important role in altering the sensitivity of the contractile apparatus to Ca^{2+}, resulting in a sustained contraction (Forder et al 1985, Spedding 1987). In addition protein kinase C has the capability to phosphorylate the light chain of myosin via a calmodulin-independent mechanism (Rodger 1986). Protein kinase C activation in airway smooth muscle may also regulate and uncouple ß-receptors (Grandordy et al 1987), thus removing the opposing effect of the sympathetic nervous system to cause relaxation of the airway smooth muscle.

DAG is hydrolysed by diacylglycerol lipase to release arachidonic acid, the precursor of prostaglandins, leukotrienes and thromboxane, or is phosphorylated to phosphatidic acid (Berridge 1985). Arachidonic acid itself or one of its metabolites may then activate guanylate cylase to raise cGMP levels (Gerzer et al 1983).

It has been suggested that phosphatidic acid may act as an ionophore to facilitate the entry of extracellular calcium (Salmon and Honeyman 1980).

When the intracellular Ca^{2+} reaches a threshold (10-100nM) the free Ca^{2+} ions bind to the four binding sites of calmodulin to form an activated Ca^{2+}-calmodulin complex (Cheung 1980). The Ca^{2+}-calmodulin complex can combine with and induce the activity of many different proteins, including, sarcolemmal Ca-Mg ATPase, cyclic nucleotide phosphodiesterases and the myosin light chain kinase. The activated myosin light chain kinase can then phosphorylate the light chain of myosin, this in turn results in activation of myosin ATPase by actin, cross bridge formation and hence contraction (Rodger 1986, Hall and Chilvers 1989).

1.3.6   The Use of Muscarinic Antagonists in the Treatment of Asthma

The parasympathetic nervous system exerts the dominant control of airway calibre and is responsible for the small degree of resting bronchomotor tone. It has been suggested that the bronchoconstriction associated with asthma could be explained by exaggerated pulmonary vagal tone initiated reflexly by activation of irritant receptors (Nadel 1977). The implication of this suggestion was that anticholinergic drugs should be effective in asthma. However, the use of these drugs has often proved to be disappointing in asthma therapy.

Although anticholinergic drugs are effective against challenge by sulphur dioxide, inert dust and irritants, where bronchoconstriction involves a cholinergic reflex, they are not always effective against exercise, hyperventilation and antigen challenge (Gross and Skorodin 1984). Anticholinergic drugs may be more effective in atopic and mild asthmatic subjects than severe asthma, as they do not block the direct bronchoconstrictor actions of inflammatory mediators, such as, histamine, leukotrienes and platelet-activating factor (Hahn 1988).

One of the major strengths of anticholinergic agents are their wide margin of safety. In addition, patients do not appear to develop tolerance to the muscarinic antagonist, ipratropium bromide (Newcombe et al 1985). They may become more useful in the treatment of nocturnal asthma which is associated with an increase in vagal drive to the lungs, implying that vagal mechanisms are

fundamental in its pathophysiology (Morrison et al 1988). The most common side effects associated with the use of muscarinic antagonists are dry mouth and cough.

Investigations into the effects and/or plasma levels of anticholinergic drugs demonstrate slower onset of action, slower increase in blood levels and longer duration of action than for sympathomimetic drugs. In acute asthma, a combination of ipratropium and an adrenergic agonist produces significantly more bronchodilation than either drug given alone (Roester and Reynaert 1987). It may therefore be advantageous in the treatment of asthma to administer an anticholinergic drug in conjunction with a ß-antagonist.

1.4   PULMONARY SYMPATHETIC INNERVATION

1.4.1   Classification of Adrenoceptors

In 1948 Ahlquist examined the effect on different tissues of catecholamines including noradrenaline, adrenaline and isoprenaline. He postulated that the differences in potencies of these sympathomimetics could be explained by the presence of two distinct adrenoceptor subtypes designated α and β. Following the development of more selective adrenoceptor agonists and antagonists β-receptors were further divided into β_1 and β_2 (Lands et al 1967a,b). In general β_1-adrenoceptor predominate in cardiac tissue while β_2-adrenoceptors are present mainly in smooth muscle such as bronchi, uterus and the vasculture, and gland cells. It is unlikely however, that tissues possess only one subtype of β-adrenoceptor. It has been suggested that β_1 and β_2-adrenoceptors may be present in varying proportions in different tissues including the airways, heart and adipose tissue (Carlsson et al 1972, Rugg et al 1978).

Subsequently α-adrenoceptors were found to be heterogenous. α-adrenoceptors on smooth muscle cells and gland cells ("postjunctional") have been shown using a variety of α-adrenoceptor agonists and antagonists to have different properties to those on nerve terminals ("prejunctional"). α-Adrenoceptors were subdivided into α_1 and α_2 respectively (Langer 1974). α_2-Adrenoceptors are however, also present postjunctionally on several tissues including the brain, uterus, parotid gland, and certain areas of vascular smooth muscle.

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The relative potencies of isoprenaline, noradrenaline and adrenaline at the different subtypes of adrenoceptors can be summarized as follows:-

i) At α_1 receptors:- adrenaline $\geq$ noradrenaline > isoprenaline.

ii) At α_2 receptors:- isoprenaline is effective, adrenaline is either more or less potent than noradrenaline depending on the tissue.

iii) At β_1 receptors:- isoprenaline > adrenaline = noradrenaline.

iv) At β_2 receptors:- isoprenaline $\geq$ adrenaline > noradrenaline.

In general the effect of activation of α_1-receptors on smooth muscle is excitatory while activation of β_2-receptors results in an inhibitory response such as, relaxation.

The inhibitory and excitatory effects of noradrenaline and the sympathomimetic drugs can be blocked at the receptor site by specific adrenoceptor antagonists. Propranolol, for example, produces a concentration dependent reduction in responses to adrenergic sympathetic nerve stimulation that are mediated through β-adrenoceptors without altering those mediated via α-adrenoceptors.

Antagonists have also been developed which differentiate between the different subtypes of α- and β-adrenoceptors. β_1-adrenoceptors are, for example, antagonised by practolol and atenolol, while β_2-adrenoceptors are blocked by ICI 118551. Responses mediated via α_1-adrenoceptors are antagonised by prazosin and phenoxybenzamine, whereas, those mediated through α_2-adrenoceptors are antagonised by yohimbine and rauwolscine (Bulbring and Tomita 1987).

1.4.2 Sympathetic Ganglia and Post-Ganglionic Neurones

The sympathetic ganglion cells, which send postganglionic fibres to the lung, are located extrapulmonary in the stellate and middle cervical ganglia (Richardson 1979). One preganglionic synapse may activate 20 or more postganglionic neurones, as well as stimulating secretion of adrenaline from the adrenal medulla (Leff 1988).

Although very little is known about pulmonary sympathetic ganglionic transmission, other sympathetic ganglia have been well studied. In the superior cervical ganglion for example, stimulation of preganglionic nerves produces a fast excitatory post-synaptic potential (fEPSP), followed by a slow inhibitory post-synaptic potential (sIPSP), and a slow excitatory post-synaptic potential (sEPSP) (Eccles and Libet 1961, Gardier et al 1978a, Ashe and Yarosh 1984). Although the fEPSP is mediated by nicotinic cholinoceptors, the sIPSP and sEPSP are mediated by muscarinic receptors. Muscarinic antagonists have been used to differentiate between the subtypes of muscarinic receptors mediating these events. The M_2 antagonists, gallamine, pancuronium and AFDX-116 selectively inhibit the muscarinic receptors mediating

hyperpolarization (sIPSP) (Mochida and Kobayski 1988, Yarosh et al 1988). In contrast, the M_1 selective antagonist, pirenzepine, blocks the muscarinic receptors associated with depolarization (sEPSP) (Gardier et al 1978a, Ashe and Yarosh 1984). The two different subtypes of muscarinic receptors may not occur on the same ganglion cell body (Mitchelson 1989). The IPSP may be due to the release of dopamine from a dopaminergic interneurone (Libet and Tosaka 1970, Gardier et al 1978b), whereas, the sEPSP involves activation of a M_1 muscarinic receptor located on the ganglion cell body, which inhibits a K+ current ("M current") (Brown 1983,1988, Marrion et al 1989).

The only available information concerning pulmonary sympathetic ganglia is that M_1 receptors have been identified in the guinea-pig (Maclagan et al 1989). The existence of muscarinic receptors in the pulmonary sympathetic ganglia of other species has not yet been investigated.

Post-ganglionic neurones enter the lung at the hilum, where they intermingle with the cholinergic nerves to form a dense plexus around the airways and blood vessels (Richardson 1979). In comparison to the pulmonary parasympathetic innervation, the sympathetic innervation is sparse.

Pulmonary sympathetic innervation is very species dependent (Mann 1971, Richardson 1979, Laitinen 1985). In most species adrenergic nerve fibres have been found in close association with submucosal glands (Laitinen 1985), blood vessels and airway ganglia (Richardson and Ferguson 1979), but direct supply to pulmonary smooth muscle is very variable. Sympathetic nerves innervate the pulmonary smooth muscle of dogs, goat, sheep, pig, calf (Mann 1971), cats and guinea-pigs (O'Donnell and Saar 1973, O'Donnell 1984), but few, if any adrenergic fibres have been demonstrated in airway smooth muscle of rats, rabbits (Mann 1971), monkeys or humans (Laitinen et al 1985).

As with parasympathetic nerves, there is a progressive decrease in the density of adrenergic fibres from the trachea, which is densely innervated, to the bronchioles which contain fewer fibres (O'Donnell et al 1978).

Electrical field stimulation in the presence of atropine or selective stimulation of sympathetic nerves via the stellate ganglion, results in relaxation of the guinea-pig isolated trachea. This response is blocked with propanolol, indicating release of noradrenaline onto ß-adrenoceptors. No relaxation of guinea-pig bronchi is observed (Doidge and Satchell 1982).

The ß-adreoceptor antagonists do not alter the bronchomotor tone of airways from normal subjects. Asthmatic subjects, however, develop bronchoconstriction with ß-blockers, suggesting an increase in adrenergic drive to the airways during asthma (Richardson and Sterling 1969).

Enzymes involved in the synthesis and breakdown of noradrenaline (tyrosine hydroxylase, DOPA decarboxylase, dopamine ß-hydroxylase, monoamine oxidase or MAO and some catechol-O-methyltransferase or COMT) are present throughout the adrenergic neurone. Noradrenaline can inhibit its own synthesis by feedback onto tyrosine hydroxylase, which therefore forms the rate-limiting step (Burnstock 1988). Noradrenaline is stored in large opaque vesicles (180-200 mM) present in the varicosities of the terminal nerve fibres (Richardson and Ferguson 1979). Transmitter release from each individual varicosity is quantal (Burnstock and Holman 1962) and occurs intermittently (Blakely and Cunnane 1979, Cunnane 1984, 1987, Brock and Cunnane 1987 a,b). Intermittence is caused by a low probability of release in the invaded varicosity and not by failure of the action potential to invade the varicosity (Brock and Cunnane 1987b). Following activation of ß and α-adrenoceptors on the postjunctional muscle membrane, noradrenaline is removed from the junctional cleft mainly by re-uptake into the nerve. Here it is either taken up into the vesicular stores or degraded by MAO. Some noradrenaline is taken up into the airway smooth muscle cells, where it is inactivated by MAO and COMT (Burnstock 1988).

1.4.3 Pulmonary Adrenoceptors

The presence of both α- and ß-adrenoceptors in the airways has been shown. These adrenoceptors are activated either by noradrenaline released from pulmonary sympathetic nerves (which are sparse or absent in some species, see above) or by circulating catecholamines released from the adrenal medulla predominantly adrenaline.

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ß-adrenoceptors are found on many different cell types within the lung and affect several aspects of lung function (Barnes et al 1982a, Barnes 1984). They have been shown to mediate bronchodilation (Castro de la Mata et al 1962, Guirgis and McNeill 1969), inhibit cholinergic transmission (Vermeire and Vanhoutte 1979, Cabezas et al 1981, Ito and Tajima 1982, Rhoden et al 1988), stimulate mucus secretion, decrease mediator release from mast cells, suppress permeability oedema and stimulate ion and water flux into the bronchial lumen (Knowles et al 1984, Barnes 1984,1986). Autoradiography and functional studies show that mucus secretion is mediated via both β_1 and β_2-adrenoceptors (Phipps et al 1982) whereas, mast cells have only β_2-adrenoceptors (Butchers et al 1980, Hughes et al 1983). Autoradiography shows that more than 90% of all ß-receptors in the lung are localised to the alveolar walls (Carstairs et al 1985).

ß-Adrenoceptors are found in airway smooth muscle from all areas, which is consistent with the observation that ß-agonists are potent relaxants of animal airways, including the guinea-pig and human bronchi, bronchioles and peripheral lung strips in vitro (Davis et al 1982, Goldie et al 1982). Their density increases with decreasing diameter of the airways (Barnes et al 1983a, Carstairs et al 1985).

Although initially ß-adrenoceptors on airway smooth muscle were thought to be purely β_2 (Lands et al 1967a) Furchgott observed that the relative potencies of isoprenaline, noradrenaline and salbutamol on guinea-pig tracheal strips were very variable. He proposed that the observed relaxation involved both β_1 and β_2-adrenoceptors and that the differences in relative potencies in different tracheal preparations was

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due to variations in the ratio of β_2:β_1 adrenoceptors.

The above discrepancies were also observed in experiments using selective β_1 and β_2-agonists and antagonists in which calculated pA_2 values were not reproducible (Furchgott 1978, O'Donnell and Wanstall 1979). The coexistence of β_1 and β_2-receptors were confirmed using direct binding studies (Rugg et al 1978, Barnes et al 1983a). In the guinea-pig airways, binding studies indicated that approximately 85% of β-adrenoceptors are of the β_2 subtype in the trachea, bronchi and parenchyma. However, functional studies demonstrated that relaxation responses of the guinea-pig trachea to exogenous agonists are mediated by both β_1 and β_2 adrenoceptors, whereas relaxations of parenchymal lung strips are mediated solely by β_2-adrenoceptors (Carswell and Nahorski 1983). In the rat lung and mouse lung parenchyma the ratio of β_1:β_2 adrenoceptors is approximately 1:3 (Rugg et al 1978, Henry et al 1990) and in the rabbit the proportion is 3:2 with β_1-adrenoceptors predominating (Rugg et al 1978). In contrast, in the canine trachea β_2-adrenoceptors predominate but approximately 20% of β-adrenoceptors are of the β_1 subtype. In the same tissue β_2-adrenoceptors were shown to mediate relaxation to exogenous β-agonists, whereas β_1-adrenoceptors mediated relaxations to electrical field stimulation of sympathetic nerves (Barnes et al 1983c). Similarly, in the calf trachea, 75% of the β-adrenoceptors are of the β_2 subtype, however, β_1-adrenoceptors seem to mediate relaxations evoked by electrical field stimulation of the sympathetic nerves at low frequencies (0.2-0.8Hz). In contrast, in this tissue low concentrations of exogenously applied noradrenaline relax through β_2-adrenoceptors (Lemoine et al 1989).

In human lung sections the ratio of $\beta_2:\beta_1$ receptors was approximately 3:1 (Carstairs et al 1985). Autoradiography revealed that β-adrenoceptors of airway smooth muscle from both large and small airways were entirely of the β_2-adrenoceptor subtype (Carstairs et al 1985). Thus in humans, where there is no significant pulmonary sympathetic innervation, relaxation of the central airways is mediated only by β_2-adrenoceptors (Davis et al 1980, Carstairs et al 1985).

These findings are consistent with the hypothesis that noradrenaline released from sympathetic nerves activates β_1-adrenoceptors, whereas, circulating adrenaline and exogenously applied β-agonists appear to stimulate predominantly β_2-adrenoceptors with a possible contribution of β_1-adrenoceptors.

In the airways of many species, including the guinea-pig and man, α-adrenoceptor agonists stimulate secretion from serous submucosal glands, increase histamine release, probably via α-adrenoceptors on mast cells and induce vasoconstriction (Barnes 1986). Noradrenaline, adrenaline and/or sympathetic stimulation also cause contraction of the trachealis smooth muscle of the guinea-pig, dog, man, rabbit, cat and old rat, but only after β-receptor blockade (Castro de la Mata et al 1962, Fleisch et al 1970, Mathe et al 1971, McCaig 1986). α-Adrenoceptor mediated contraction of canine and human trachealis muscle appears to depend on basal tone. Pretreatment with spasmogens such as, methocholine, histamine, 5-hydroxytryptamine or excess potassium, markedly potentiated response to α-agonists or sympathetic nerve stimulation (Kneussl and Richardson 1978, Barnes et al 1983d).

In the guinea-pig trachea, sustained stimulation of the sympathetic nerve trunk, in the presence of atropine and propranolol, sometimes evoked a small contractile response which was blocked by the α-adrenoceptor antagonist, phentolamine (Blackman and McCaig 1983).

In the dog, tracheal contractions to noradrenaline were partially antagonized by both prazosin and yohimbine, which block α_1 and α_2-adrenoceptors respectively, suggesting the presence of both α_1 and α_2-adrenoceptors in this preparation (Leff and Munoz 1981). Contraction mediated by α_2-adrenoceptors seems to predominate (Barnes et al 1983d). Radioligand binding involving ^3H prazosin and ^3H yohimbine indicate that in the dog trachea smooth muscle, α_2 outnumber α_1-adrenoceptors by a ratio of 5:1. In contrast, in the peripheral lung the ratio of $\alpha_1:\alpha_2$ receptors is 10:1 (Barnes et al 1982b).

Autoradiography of pulmonary α-receptors has demonstrated few α-adrenoceptors in the large airways, but a higher density in the small bronchioles of the ferret (Barnes et al 1983a). A high density of α_1-adrenoceptors was found in both serous submucosal glands and vascular smooth muscle (Barnes et al 1983b).

1.4.4   Secondary Messenger Systems

Binding of ß-adrenoceptor agonists results in a conformational change in the ß-adrenoceptor and activation of adenylate cylase, via a guanine nucleotide regulatory protein. Adenylate cylase converts ATP to the secondary messenger, 3'5'-cyclic adenosine monophosphate (cAMP). The ß-adrenoceptor agonist concentrations required to evoke cAMP

accumulation and relaxation are comparable. In addition, the time-course of cAMP accumulation and relaxation are similar (Kumar 1978, Small and Foster 1986); cAMP then activates the protein kinases. In airway smooth muscle these cAMP-dependent protein kinases may inhibit contraction via phosphorylation of the myosin light chain kinase stimulates intracellular binding to the plasma membrane and sarcoplasmic reticulum and pumping of Ca^{2+} out of the cell, thereby lowering the intracellular calcium ion concentration (Ito and Itoh 1984, Honda et al 1986, Rodger 1986, Small and Foster 1986, Fujiwara et al 1988).

1.4.5 ß-Adrenoceptor Agonists in the Treatment of Asthma

ß-adrenoceptor agonists are very effective bronchodilator drugs and in many countries are the first line treatment of asthma. They are relatively safe, well tolerated, easy to administer and without problems of significant side effects (Barnes et al 1984).

Adrenaline was the first adrenoceptor agonist used in the treatment of asthma (Bullawa and Kaplan 1903). Adrenaline, however, stimulates both α- and ß-adrenoceptors and the dose which could be given was limited by its α-agonist actions. Isoprenaline, which has only ß-agonist effects, was introduced in 1940 (Konzett 1940). In the 1960's, the mortality rate of asthmatics rose sharply and there was concern that the ß-agonists may have been responsible, although this was unproven. This led to the introduction in the 1970's of selective $ß_2$-adrenoceptor agonists which have predominant effects in the lungs. These include, salbutamol, terbutaline, rimiterol, fenoterol, reproterol and pirbuterol.

The selectivity of the drug is dependent upon : a) the drug characteristics, that is, receptor affinity and efficacy, b) tissue characteristics such as, the distribution of receptors and the nature of receptor-coupling to the biological response, and c) the route of drug administration (Seale 1988).

Unlike the naturally occurring catecholamines, which include dopamine, noradrenaline and adrenaline, these drugs are more resistant to degradation by COMT and MAO, and so have longer duration of action with a half-life of between four and six hours compared to approximately one hour (Tattersfield 1986).

The ß-adrenoceptor agonists have various pharmacological actions which may contribute to their beneficial effects in the treatment of asthma. The major therapeutic effect is probably that they are potent relaxants of human airway smooth muscle. They may also modulate the bronchoconstrictor effect of the parasympathetic nervous system (see section 1.3.3), increase mucocilary clearance and reduce airway microvascular permeability and oedema (Barnes 1984, Seale 1988). In addition, ß-adrenoceptor agonist may inhibit immunologically-induced release of histamine and leukotrienes from pulmonary mast cells (Howarth et al 1985). It is possible that they directly inhibit phospholipase A_2 and therefore inhibit the release of cycloxygenase products, such as thromboxane, which causes contraction of the airway. The relevance of this finding to human asthma is not yet known (Seale 1988).

Unwanted dose-dependent side effects include, tachycardia as a consequence of the β_2 mediated fall in peripheral vascular resistance, tremor resulting from stimulation of skeletal muscle β_2-receptors and restlessness. Metabolic effects are seen after large systemic doses and include increases in plasma-free fatty acids, insulin, glucose and lactate, and reduction in plasma potassium (Taylor et al 1976). Patients may also develop tolerance to β-adrenoceptor agonists.

Unwanted side effects can be kept to a mimimum by choosing the right route of administration. Inhalation is the most logical route, as β-agonists given this way are rapidly effective and virtually free from side effects. Oral administration of β-agonists requires 20-80 times the inhaled dose of the same drug to achieve a similar bronchodilation and is associated with much higher plasma concentrations of the drug and therefore, greater side effects.

The airway bronchodilatory response to a given dose of β-agonist is similar whether given by a metered dose inhaler or nebulizer. The side effects are however greater when a metered dose inhaler is used (Tattersfield 1986). The use of intermittent positive pressure ventilation (IPPV) does not appear to have any advantage over a nebulizer. In fact, in acute severe asthma β-agonists given by a nebulizer seem to be more effective than those given either intraveneously or by IPPV (Lawford et al 1978).

1.5 INTERACTIONS BETWEEN PULMONARY PARA-SYMPATHETIC AND SYMPATHETIC NERVOUS SYSTEMS

In tissues receiving a dual innervation such as the heart and gut, cholinergic and noradrenergic varicosities often lie in close opposition without intervening Schwann cell processes (Loiacono et al 1985). Interactions between the two branches of the autonomic nervous system have been identified in these tissues. Transmitter released from one branch may inhibit release of the other neurotransmitter via an action on a prejunctional heteroreceptor. The presence of inhibitory prejunctional muscarinic receptors on sympathetic nerve terminals have been demonstrated, for example, in the heart of the rabbit (Loffelholz and Muscholl 1970), guinea-pig (Limdmar et al 1968), rat (Boyle and Pollock 1988), cat (Haeusler et al 1968), dog (Levy and Blattberg 1976, Lavellee et al 1978), chicken (Engel and Loffelholz 1976), the guinea-pig ileum (Kilbinger and Wessler 1980) and rabbit jejunum (Manber and Gershon 1979).

The reverse interrelationship has also been shown, that is, inhibition of acetylcholine release via activation of adrenoceptors situated prejunctionally on parasympathetic nerve terminals to the heart (Wetzel and Brown 1985, Wetzel et al 1985) and ileum (Gillespie and Khoyi 1977, Drew 1978, Grundstrom et al 1981). In fact, Boyle and Pollock (1988) were able to demonstrate, in the same preparations, inhibitory α_2-adrenoceptors on the cholinergic nerves and inhibitory muscarinic receptor on noradrenergic nerves to the rat heart.

As with other tissues which receive a dual innervation, parasympathetic and sympathetic nerve fibres innervating airway smooth muscle have been shown to lie in close proximity to one another (Jones et al 1980b, Daniel et al 1986, Daniel 1988). Similarly, inhibition of acetylcholine release via activation of adrenoceptors on pulmonary parasympathetic nerve ending has been reported.

Noradrenaline and isoprenaline were found to inhibit contractions of the canine bronchi following electrical field stimulation (EFS) significantly more than those to exogenously applied acetylcholine. Propranolol abolished the inhibition in both cases and augmented contractile responses to EFS but not those to acetylcholine. These observations suggest that in canine bronchi catecholamines inhibit acetylcholine release via an action on prejunctional adrenoceptors (Vermeire and Vanhoute 1979).

This suggestion was supported by the work of Ito and Tajima (1982) who showed that low concentrations of the catecholamines reduced the amplitude of excitatory junctional potentials (e.j.p.s), but did not alter the membrane potential, membrane resistance or the sensitivity of the muscle membrane to exogenously applied acetylcholine. Propranolol suppressed the inhibitory actions of the catecholamines, in fact, it significantly increased the amplitude of e.j.p.s (Ito and Tajima 1982).

Similarly, in the guinea-pig isoprenaline and noradrenaline reduced contractile responses and depolarization following stimulation of the vagal nerve trunk innervating the trachea. This inhibitory effect was blocked by a combination of propranolol and phentolamine. The size of vagally-induced contractions were also inhibited during concurrent stimulation of the sympathetic nerve trunk. Neither sympathetic stimulation nor applied catecholamines altered mechanical responses to exogenously applied acetylcholine, confirming that their effects on vagally-induced responses are prejunctional (McCaig 1987).

These conclusions were confirmed *in vivo*; in the anaesthesised dog (Cabezas et al 1971) and cat (Baker and Don 1987), sympathetic nerve stimulation also suppressed the bronchoconstriction evoked by vagal stimulation. The involvement of β_1, β_2 and α_2 adrenoceptors present on pulmonary parasympathetic nerve terminals have been implicated. In canine bronchi, tyramine, which stimulates the release of endogenous noradrenaline from sympathetic nerve terminals, inhibited EFS evoked contractions but not contractile responses to exogenously applied acetylcholine. This inhibitory effect of tyramine was blocked by the selective β_1 -adrenoceptor antagonist metoprolol but not the α-adrenergic antagonist rauwolscine (Danser et al 1987). However, the β_2-adrenoceptor agonist, procaterol, has been reported to reduce the amplitude of the e.j.p. and twitch contraction of the dog trachea evoked by EFS. This inhibitory effect was blocked with the β_2-adrenoceptor antagonist ICI 118551 (Ito 1988).

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In isolated human airway smooth muscle, isoprenaline, adrenaline and noradrenaline, in that order of potency, produced a concentration dependent inhibition of comparable contractile responses to EFS to a greater extent than exogenously applied acetylcholine. Inhibition of EFS evoked contractions was prevented by propranolol and the β_2-antagonist ICI 118551 but not by betaxolol, a β_1-antagonist (Rhoden et al 1988).

In contrast, in the guinea-pig trachea, endogenously released noradrenaline decreases acetylcholine release via an action on β and α_2-adrenoceptors situated on the vagal nerve terminals (Jones et al 1980, Grundstrom et al 1981, Widmark and Waldeck 1986, McCaig 1987).

Therefore, in summary, noradrenaline released from sympathetic nerve terminals decreases neurotransmission from parasympathetic nerves innervating airway smooth muscle via activation of prejunctional adrenoceptors.

Very little is known, however, concerning the reverse interrelationship, that is, inhibition of noradrenaline release from pulmonary sympathetic nerves by endogenously released acetylcholine.

It has been shown that exogenously applied muscarinic agonists can inhibit noradrenaline overflow from the perfused rabbit lung (Tong et al 1978), tracheal strips and strips of intrapulmonary airways (Russell and Bartlett 1981). The origin of the ^{3}H noradrenaline was not, however, established and may have been either the airway smooth muscle or pulmonary blood vessels. The former seems particularly unlikely for the rabbit in which the sympathetic innervation to airway smooth muscle is sparse or absent (Mann 1971).

The following experiments were carried out to investigate further the existence of this interrelationship, that is, inhibition of pulmonary sympathetic neurotransmission by acetylcholine released from the adjacent parasympathetic nerves. The guinea-pig was chosen for these studies because there is more information concerning the pulmonary innervation in this species than for other laboratory species. In the guinea-pig, inhibition of pulmonary parasympathetic neurotransmission by the sympathetic nervous system has already been demonstrated. In addition, the airway smooth muscle of the guinea-pig is known to receive a relatively rich sympathetic innervation compared to that of other species.

PARASYMPATHETIC

SYMPATHETIC

M_1

β α_2

M_2 Ach $M?$ NA

M_3

α

$Ca^{2+}\uparrow$ $Ca^{2+}\downarrow$ β_1

CONTRACTION   RELAXATION

AIRWAY SMOOTH MUSCLE CELL β_2
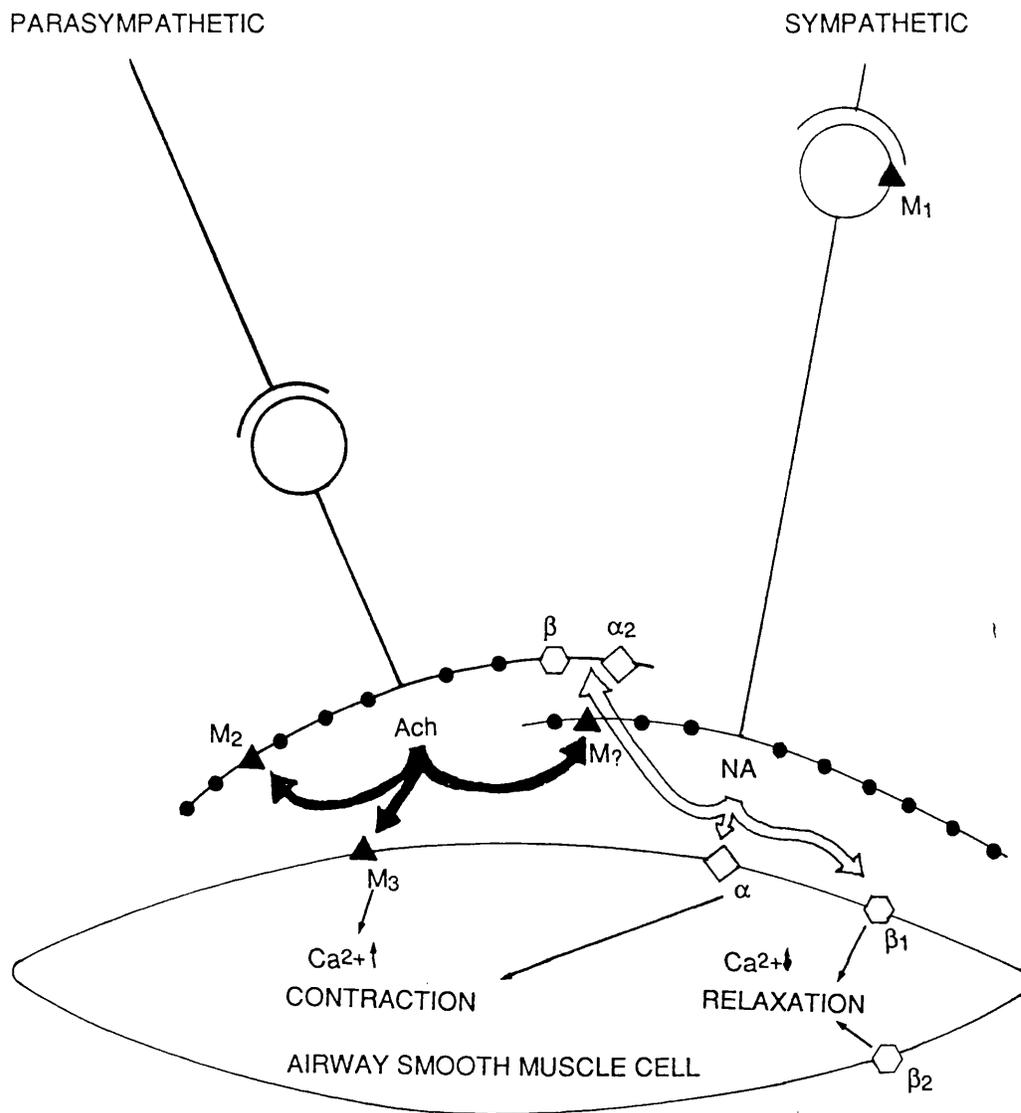
Figure 1.

Schematic representation of the dominant parasympathetic and sympathetic innervation to the airway smooth muscle of the guinea-pig. Acetylcholine (Ach) released from the parasympathetic nerves evokes contraction of the trachealis smooth muscle via activation of postjunctional M_3 muscarinic receptors. In the guinea-pig, stimulation of the sympathetic nerve trunk releases noradrenaline (NA) predominately onto β_1-adrenoceptors on the airway smooth muscle which results in relaxation of the precontracted trachea. Circulating adrenaline and exogenously applied catecholamines induce relaxation via β_2-adrenoceptors. Ach release is inhibited via activation of M_2 autoreceptors and α_2 and β-adrenoceptors on the cholinergic nerve terminals. Whether endogenously released acetylcholine can inhibit pulmonary sympathetic neurotransmission is unknown and is to be investigated($M?$).

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SECTION 2 - METHODS

2.0 METHODS

2.1 INNERVATED TRACHEAL TUBE PREPARATION

Guinea-pigs (200-400g) of the Dunkin-Hartley strain (Graystoke, Hampshire) were anaesthetised with urethane (1.5g Kg^{-1} i.p.). The sympathetic nerve trunk on the right hand side was dissected down to the stellate ganglion, as described by Blackman and McCaig (1983). During the dissection the pulmonary nerves were kept moist with Krebs'-Hensleit solution (composition mM: NaCl 118.4, KCl 4.7, Na HCO$_3$ 25.0, glucose 11.1, KH$_2$PO$_4$ 1.16, Mg SO$_4$.7H$_2$O 1.19 and CaCl$_2$ 2.6) gassed with 95% O$_2$ and 5% CO$_2$. When the dissection was complete, the animal was killed with an anaesthetic overdose and the trachea with the sympathetic nerves attached, was removed. The trachea was cannulated at both ends and mounted horizontally at its *in vivo* length in an organ bath containing gassed Krebs'-Hensleit solution maintained at 37ºC. The lumen of the trachea was filled with Krebs'-Hensleit solution to form a fluid-filled tube which was closed at one end with a clamp, and the other end was attached to a Statham (P23AC) transducer to record intraluminal pressure (ILP) (Figure 2).

The stellate ganglion was placed on bipolar platinum electrodes and stimulated with trains of rectangular pulses (V$_{max}$, 40Hz, 0.2ms for 5s at 90s intervals using a Grass S44 stimulator) to induce relaxation of the trachea. Increases or decreases in intraluminal pressure reflected smooth muscle contraction or relaxation, respectively.

Tissues were left to equilibriate for one hour in the presence of indomethacin (5×10^{-6}M) to remove any prostaglandin-induced tone, after which time a stable baseline was obtained. The trachea could, however, be relaxed further and a minimum ILP in the presence of excess isoprenaline (10^{-3}M) was established at the end of each experiment. All ILP's were expressed as values in mmH_2O above this minimum. After the equilibrium period, drugs which induced airway smooth muscle contraction were then added cumulatively to the organ bath and sympathetic nerve-induced relaxations were measured when the ILP had reached a plateau.
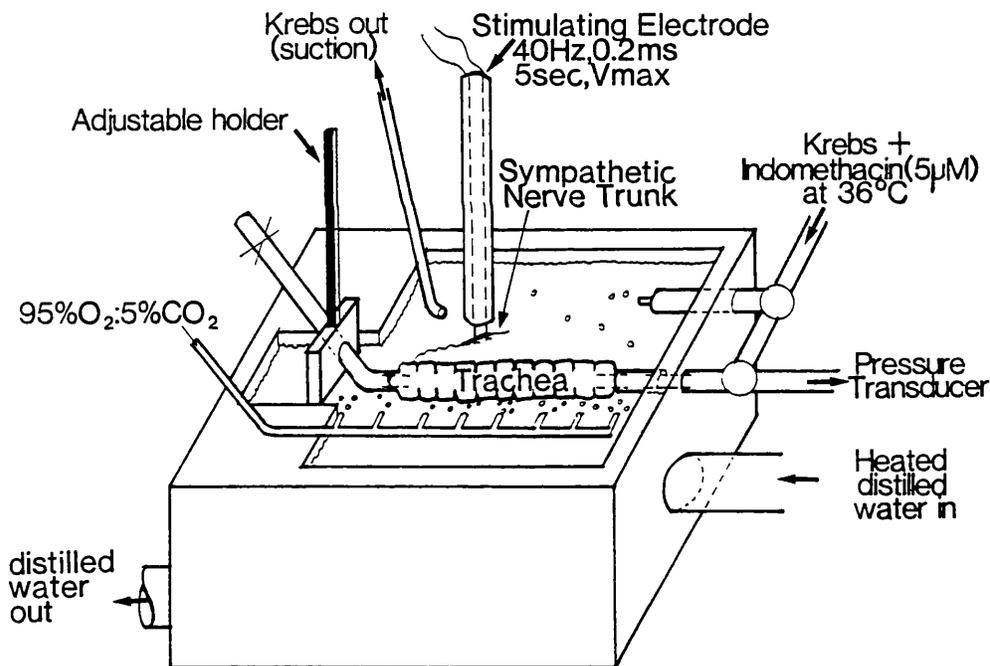
Figure 2.

Diagram of the *in vitro* apparatus showing the orientation of the guinea-pig trachea, sympathetic nerve trunk, stimulating electrodes and gassing equipment.

2.2   EFFECT OF MUSCARINIC AGONISTS

The muscarinic agonists, acetylcholine, pilocarpine and carbachol, which induced contraction of the tracheal smooth muscle, were administered cumulatively to the organ bath at regular intervals (3 minutes between doses for acetylcholine, 6 minutes for carbachol and 7.5 minutes for pilocarpine). Sympathetic nerve-induced relaxation responses were measured in the presence of different concentrations of agonists and compared to those at a comparable ILP when the tone had been raised with the stable thromboxane analogue U46619. The tissue was washed for at least 30 minutes between muscarinic agonist and subsequent U46619 concentration-response curves. This time period was sufficient to wash out the agonist and restore the intraluminal pressure to pre-drug control values.

The effect of the muscarinic agonists on sympathetic nerve-induced relaxations (compared to U46619) was calculated as follows :-

$$\text{percentage change} = \frac{A - B}{A} \times 100\%$$

Where :-

A = The sympathetic nerve-induced relaxation in mmH_2O when the ILP was raised with U46619.

B = The sympathetic nerve-induced relaxation in mmH_2O measured at the same ILP following the administration of a specific concentration of muscarinic agonist.

2.3   SIMULTANEOUS ADDITION OF EXOGENOUS NORADRENALINE AND A SPASMOGEN

When the sympathetic neurotransmitter, noradrenaline, and a spasmogen are administered simultaneously, they have opposing effects on the trachealis smooth muscle; an effect sometimes called either, "physiological" or "functional" antagonism. An attempt was made to quantify this effect using the spasmogens U46619 and acetylcholine.

Cumulative contractile concentration-response curves to U46619 and acetylcholine were constructed in the absence and presence of noradrenaline (10^{-6}, 10^{-5}M); 3 minutes between doses for acetylcholine and 6 or 7.5 minutes between U46619 doses. At the end of the experiment excess acetylcholine was administered to the organ bath to obtain a maximum contraction. The ILP changes produced by the different concentrations of U46619 and acetylcholine were expressed as a percentage of this maximum. The concentrations of U46619 and acetylcholine to produce a 30% maximum contraction in the absence and presence of noradrenaline were calculated (EC_{30}).

The ratio of the EC_{30}s in the presence and absence of noradrenaline was used as a measure of the degree of "physiological" antagonism. This was called the "concentration ratio" and calculated thus :-

$$\frac{EC_{30} \text{ in the } \textbf{presence} \text{ of noradrenaline}}{EC_{30} \text{ in the } \textbf{absence} \text{ of noradrenaline}}$$

2.4   EFFECT OF ENDOGENOUSLY RELEASED ACETYLCHOLINE

2.4.1   Effect of Concurrent Vagal and Sympathetic Stimulation

In this series of experiments, the trachea with the sympathetic nerve trunk on the right hand side and the parasympathetic innervation on both sides attached was removed and mounted horizontally in the organ bath. The vagal nerve trunks and sympathetic stellate ganglion were stimulated through two separate bipolar platinum electrodes with trains of rectangular pulses delivered using two Grass S44 stimulators, via stimulator isolation units to avoid any "leakage" of current.

In the first part of this study, the tone of the guinea-pig trachealis muscle was raised with U46619 in order to observe relaxation responses following stimulation of the sympathetic nerve trunk. When the tone had reached a plateau, sympathetic nerve-induced relaxations (V_{max}, 40Hz, 0.2ms for 5sec at 90sec intervals) were measured in the absence and presence of concurrent submaximal vagal stimulation (20Hz, 0.2ms for 180 or 270sec). Vagal parameters were chosen such that stimulation of the vagus alone caused no significant change in ILP.

In the second part of this study, contractile responses following preganglionic stimulation of the vagal bundles (30Hz, V_{max}, 0.2ms for 5sec at 45sec intervals) were measured. When a stable response had been obtained, the effect of concurrent sympathetic stimulation (40Hz, 0.2ms, 90sec) on these vagally-induced contractions was investigated. Sympathetic parameters were chosen such that stimulation of the sympathetic nerve trunk alone did not alter the ILP.

2.4.2 Effect of an Anticholinesterase Drug

The effect of the anticholinesterase, physostigmine on the internal spontaneous tone, contractile responses following addition of acetylcholine or stimulation of the preganglionic cholinergic nerve fibres (30Hz, V_{max}, 0.2ms, 5sec) of the guinea-pig trachea was measured. Sympathetic nerve-induced relaxations were also measured following at least 15 minutes preincubation with physostigmine (10^{-7}, 10^{-6}M); in the latter experiments the intraluminal pressure was raised with U46619.

2.5 EFFECT OF CHOLINOCEPTOR ANTAGONISTS
2.5.1 Effect of Muscarinic Antagonists

Sympathetic nerve-induced relaxations were measured following administration of U46619 and acetylcholine in the absence and in the presence of the muscarinic antagonists atropine, pirenzepine, hexahydrosiladifenidol, methoctramine and gallamine. The tissue was preincubated with the muscarinic antagonist for at least 15 minutes.

2.5.2 Effect of a Nicotinic Antagonist

Sympathetic nerve-induced relaxations, ILP changes and the inhibitory effect of the muscarinic agonists were measured in the absence and after at least one hour preincubation with the nicotinic cholinoceptor antagonist, hexamethonium (10^{-5}M).

2.6   DRUGS USED

The drugs used in the course of this work are listed below, with their molecular weights (MW) and source.

DRUG	MW	SOURCE
MUSCARINIC AGONISTS		
Acetylcholine bromide	226.1	BDH
Carbachol chloride	182.7	BDH
Pilocarpine nitrate	271.3	BDH
MUSCARINIC ANTAGONISTS		
Atropine sulphate	694.9	BDH
Gallamine triethiodide	891.5	May and Baker
Hexahydrosiladifenidol	331.6	Dr Lambrecht
Methoctramine	728.8	Dr Melchiorre
Pirenzepine dihydrochloride	442.3	Boots
OTHER SPASMOGENS		
Histamine acid phosphate	307.1	BDH
Prostaglandin $F_{2\alpha}$ trimethamine salt	475.6	Sigma
U46619	350.4	Sigma/Semat
ADRENOCEPTOR AGONIST		
Isoprenaline sulphate	556.6	Wellcome
Noradrenaline acid tartrate	337.3	Winthrop
ADRENOCEPTOR ANTAGONIST		
Propranolol hydrochloride	295.8	ICI
GANGLION BLOCKING DRUG		
Hexamethonium bromide	362.2	Koch Light

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DRUG	MW	SOURCE
ANTICHOLINESTERASE		
Physostigmine sulphate	648.8	Sigma
ANAESTHETIC		
Urethane	89.1	Sigma
CYCLO-OXYGENASE INHIBITOR		
Indomethacin	357.8	Sigma

Indomethacin (1mg/ml) was dissolved daily in buffer, (KH_2PO_4 19.76 mM and Na_2HPO_4 118.34 mM; pH adjusted to 7.8 with NaOH) by warming and sonication before addition to the Krebs'-Henseleit solution (final concentration $5 \times 10^{-6}M$). Stock solutions of U46619 and prostaglandin $F_{2\alpha}$ were prepared by dissolving 1mg of the solid in 0.1ml of ethanol and 0.9% saline was added to give a $10^{-3}M$ solution (2.75ml for U46619 and 2ml for prostaglandin $F_{2\alpha}$). 100µl aliquots of these stock solutions were stored at -70°C and were diluted daily in 0.9% saline. All solutions of U46619 and prostaglandin $F_{2\alpha}$ were stored on ice throughout the experiment. All other drugs were dissolved and diluted in 0.9% saline. Stock solutions of isoprenaline were never kept longer than 15 minutes, and so no antioxidant was used. Ascorbic acid was added to prevent oxidation of solutions of noradrenaline and histamine which were kept throughout the experiment.

2.7　EXPRESSION OF RESULTS

2.7.1　Standard Error of the Mean

Groups of data were expressed as the sample mean ($\bar{x}$) $\pm$ standard error of the mean (s.e.m.).

Where,　　　　　s.e.m. $= \dfrac{s}{\sqrt{n}}$

standard deviation　　$s = \sqrt{\dfrac{\Sigma(x - \bar{x})^2}{n - 1}}$

In which,　　　　　n = number of observations

and,　　　　$\Sigma(x - \bar{x})^2$ = the sum of the squares of the

　　　　　　　difference from the mean.

2.7.2　Unpaired Student t-tests

Unpaired student t tests were used to compare the means of two samples. In the first sample there were n_1 observations with mean $\bar{x}_1$ and standard deviation s_1. The corresponding values for the second sample were n_2, $\bar{x}_2$ and s_2. The t value was evaluated as follows:-

1)　The sum of the squares of the differences from the mean for each sample, was calculated separately. For example, for sample 1 :

$\Sigma(x_1 - \bar{x}_1)^2$

2)　The squares of the standard deviations (varience) for both samples were combined to give :-

$$SD^2 = \frac{\Sigma(x_1 - \bar{x}_1)^2 + \Sigma(x_2 - \bar{x}_2)^2}{(n_1 - 1) + (n_2 - 1)}$$

3)   The standard error of the difference between the means was calculated.

$$SE_{diff} = \sqrt{\frac{SD^2}{n_1} + \frac{SD^2}{n_2}}$$

4)   t could then be obtained by dividing the difference between the means by this standard error.

$$Thus, \quad t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\dfrac{SD^2}{n_1} + \dfrac{SD^2}{n_2}}}$$

The probability (p) that there is no significant difference between the means (null hypothesis) was then read from the table of t distribution at, $(n_1-1) + (n_2-1)$, that is, $n_1 + n_2 - 2$ degrees of freedom. If $p < 0.05$, the null hypothesis is unlikely and the means were considered to be significantly different.

SECTION 3 - RESULTS

3.0 RESULTS

3.1 RELATIONSHIP BETWEEN SYMPATHETIC NERVE-INDUCED RELAXATION AND INTRALUMINAL PRESSURE

3.1.1 Sympathetic Relaxations Using U46619 as the Spasmogen

When the tissue was first set up in the organ bath stimulation of the sympathetic nerve trunk resulted in a small or an undetectable relaxation response. In order to observe a relaxation, the intraluminal pressure (ILP) had to be increased with a spasmogen, such as the stable thromboxane analogue U46619, which induced a concentration-dependent increase in the ILP of the guinea-pig trachea (Figure 3a).

As the ILP was raised with U46619, stimulation of the sympathetic nerves caused a rapid fall in the ILP, reflecting relaxation of the trachealis muscle. These sympathetic nerve-induced relaxations were abolished by propranolol (10^{-5}M), but not by hexamethonium (10^{-5}M). This indicates that postganglionic sympathetic nerves had been stimulated to release noradrenaline onto ß-adrenoceptors on the airway smooth muscle. Relaxations increased in a linear manner in the range 100-300 mmH$_2$O, relative to the minimum pressure in the presence of excess isoprenaline (Figure 3b). The maximum contractile response of the tracheal tube to excess acetylcholine was in the range 400-500 mmH$_2$O. In the present studies, however, the majority of experiments were carried out at ILP values below 300 mmH$_2$O. The size of the sympathetic nerve-induced relaxation response at any specific ILP was reproducible during two consecutive cumulative U46619 concentration-response curves (Figure 4). Therefore, sympathetic relaxations in the presence of different drugs were always compared at identical ILP's, to eliminate the effect of changes in muscle tone on the response.
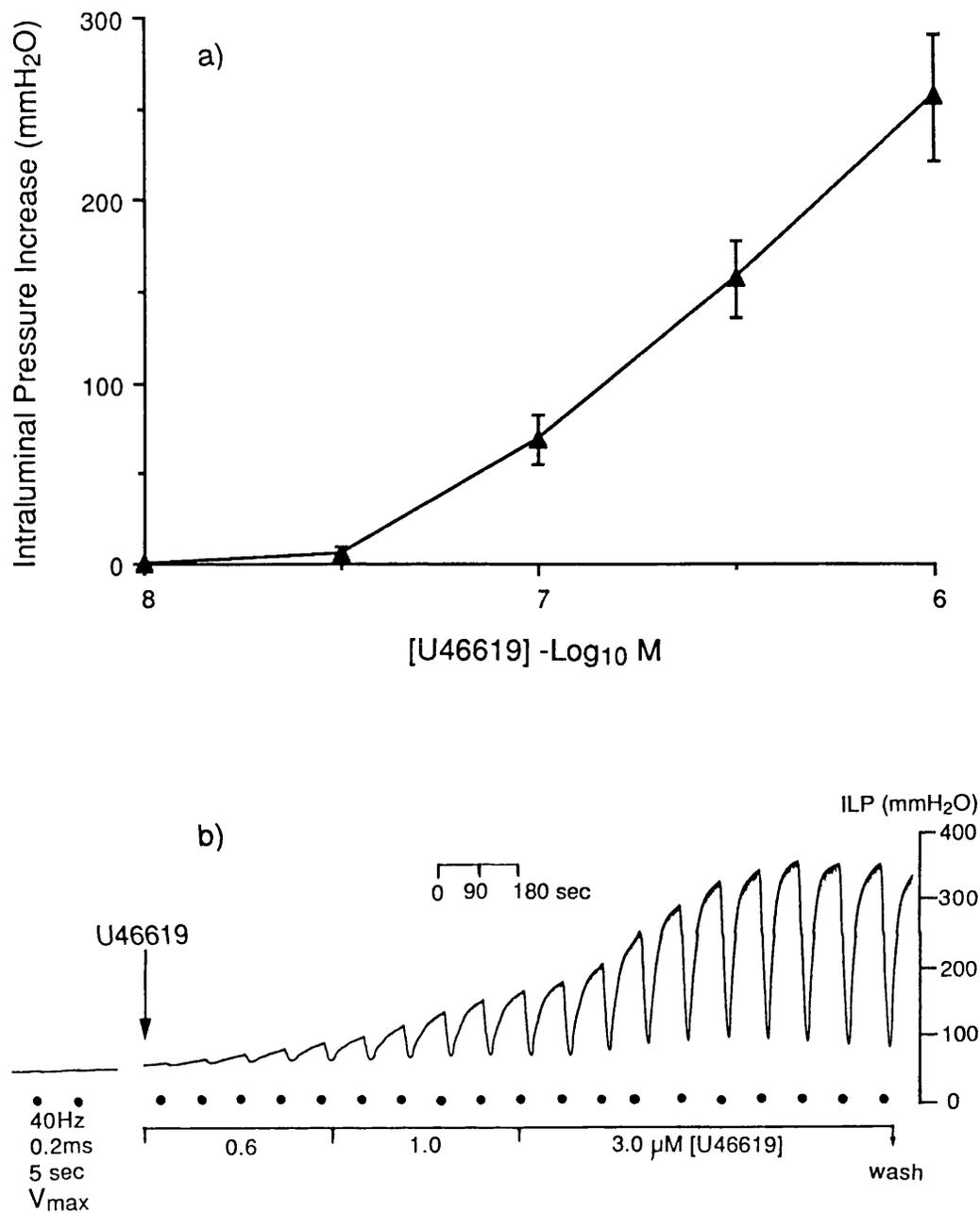
Figure 3.

a) Effect of cumulative addition of U46619 to contract the fluid-filled guinea-pig tracheal tube preparation. Increases in intraluminal pressure (ILP) reflect contraction of the trachealis smooth muscle. Each point is the mean ± s.e.m. for 8 different tissues. b) Experimental record showing relaxation responses of the guinea-pig trachea, recorded as rapid falls in the ILP, following stimulation of the sympathetic nerve trunk (indicated by the dots; 40Hz, V_{max}, 0.2ms, 5sec). The size of the relaxation responses increased as the pressure was raised by cumulative addition of U46619. Indomethacin (5×10^{-6}M) was present throughout.

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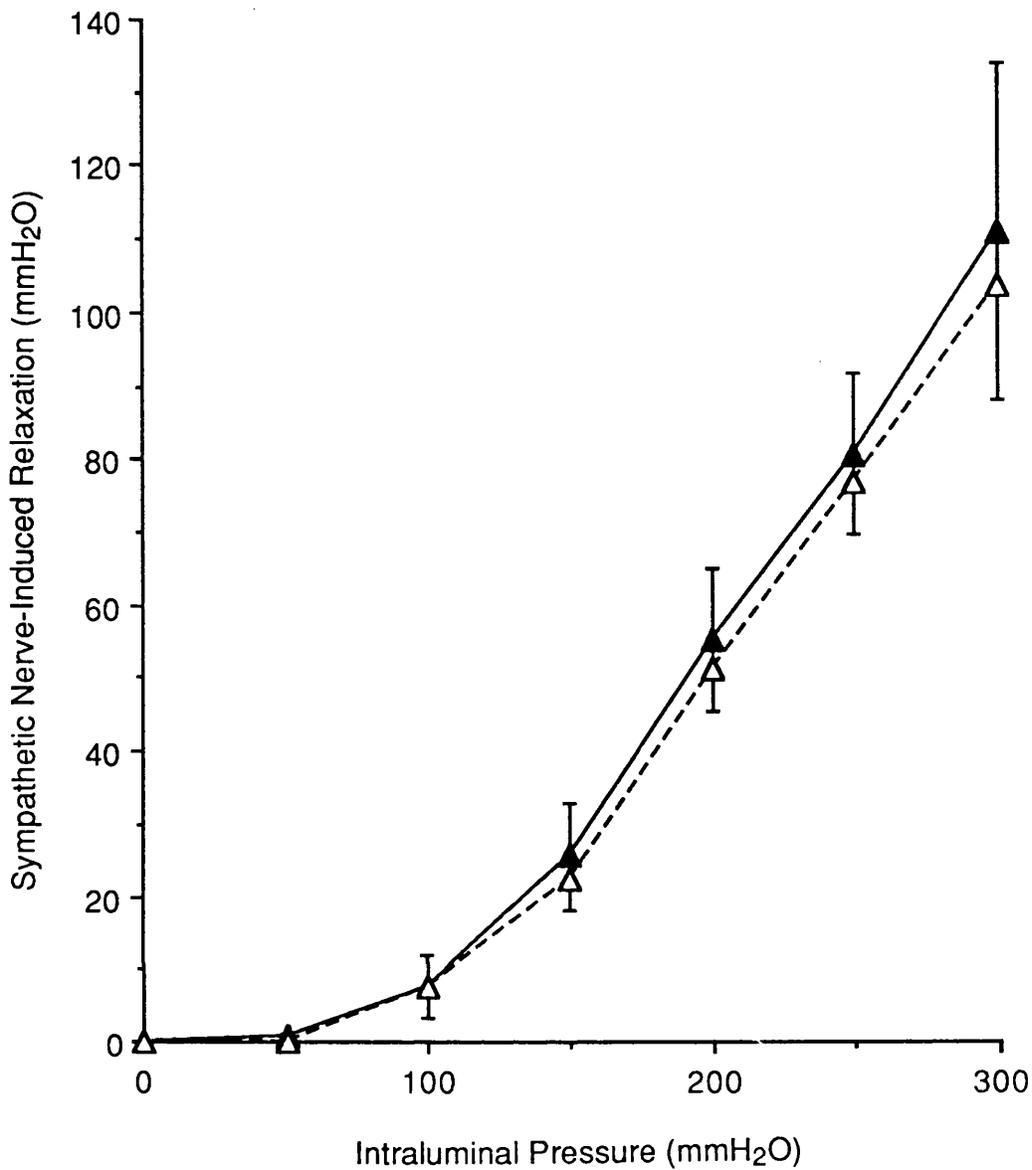
Figure 4.

Relationship between sympathetic nerve-induced relaxations following stimulation of the stellate ganglion (40Hz, V_{max} 0.2ms, 5sec) and the intraluminal pressure of the guinea-pig trachea in the presence of two consecutive administrations of U46619. $-\triangle-$ 1st dose response curve; $-\blacktriangle-$ 2nd dose response curve (mean ± s.e.m; n >7).

Tissues did not respond immediately to stimulation of the sympathetic nerve trunk. Approximately 4 seconds elapsed between the start of the stimulus train and the beginning of the relaxation response, and the duration of the relaxation was often greater than 1 minute. Sympathetic nerve-induced relaxations increased with stimulation frequency, a maximum being observed with 40Hz.

When the ILP had been raised following the administration of a spasmogen, stimulation of the sympathetic nerve trunk via the stellate ganglia evoked a pure relaxation in 90% of the preparations. In the remaining tissues, an initial contraction occurred before the relaxation response (<5% relaxation response); this contractile response could be blocked with atropine and could not be avoided by changing the position of the stimulating electrodes or by taking more care when separating the sympathetic and parasympathetic nerves apart. This suggests that in these tissues there were interconnections between the two branches of the autonomic nervous system.

3.1.2   Effect of Histamine

Histamine induced a concentration-dependent increase in the ILP of the guinea-pig trachealis muscle which reached a peak after 6 minutes (Figure 5b). Histamine was approximately equipotent to acetylcholine at contracting the trachealis muscle. Sympathetic nerve-induced relaxations in the presence of U46619 and histamine were not significantly different in the ILP range 0-300 mmH_2O above the minimum in the presence of excess isoprenaline (Figure 5).
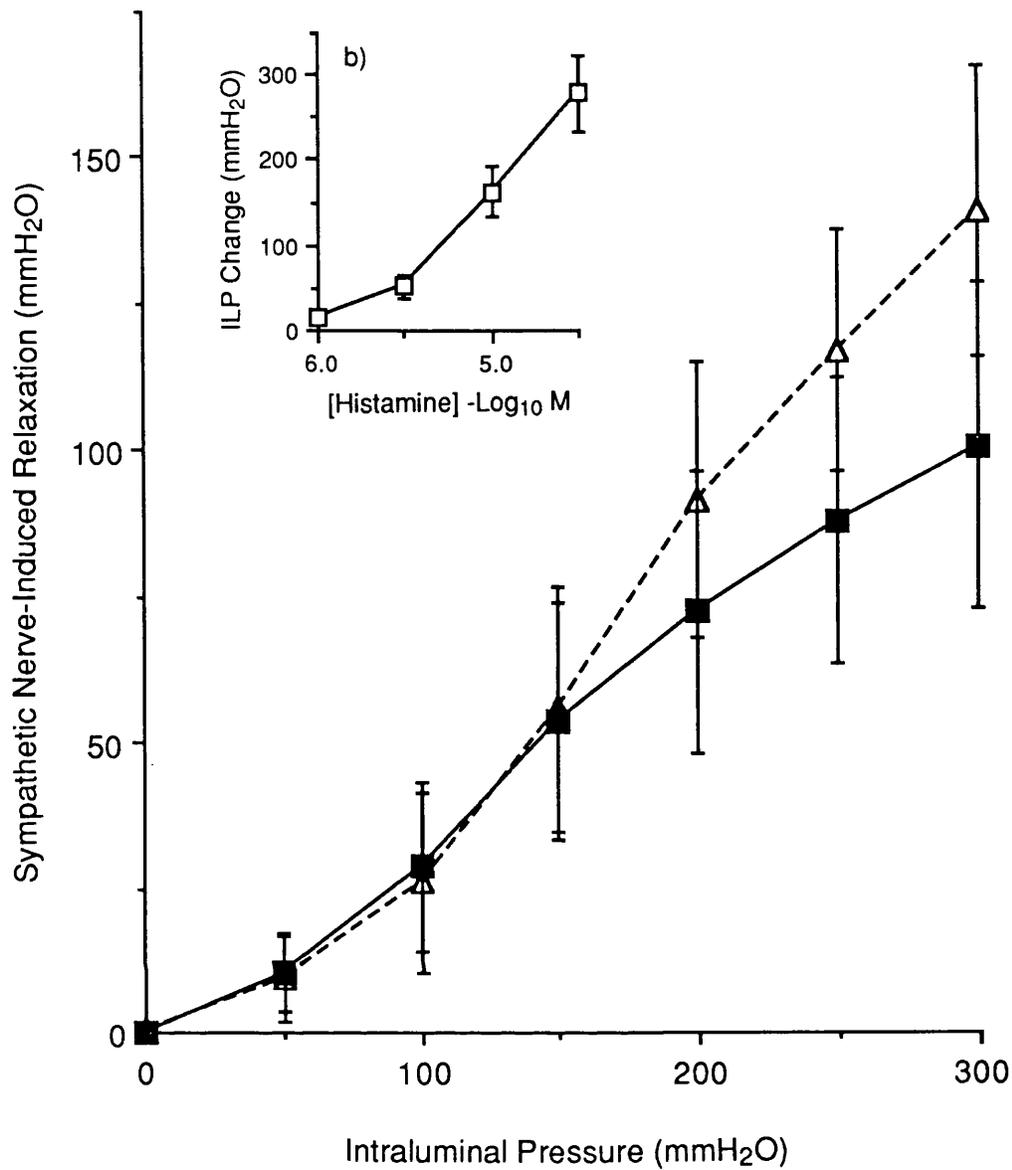
Figure 5.

Relaxation responses of the guinea-pig trachea induced by stimulation of the sympathetic nerve trunk (40Hz, V_{max}, 0.2ms, 5sec) when the intraluminal pressure (ILP) was raised with U46619 (-△-) and histamine (-■-). The insert b) shows the postjunctional contractile action of histamine to increase the ILP of the tracheal tube. Sympathetic nerve-induced relaxations in the presence of U46619 and histamine at any ILP were not significantly different (mean ± s.e.m.; n>5).

3.1.3 Effect of Prostaglandin $F_{2\alpha}$

Prostaglandin $F_{2\alpha}$ induced a concentration-dependent increase in the ILP of the guinea-pig trachealis muscle which reached a peak after 7.5 minutes (Figure 6b). Sympathetic nerve-induced relaxations in the presence of prostaglandin $F_{2\alpha}$ increased linearly with this increase in ILP. At any given ILP (0-250 mmH_2O), sympathetic relaxations were comparable in the presence of prostaglandin $F_{2\alpha}$ and U46619 (Figure 6).

3.2 EFFECT OF MUSCARINIC AGONISTS

3.2.1 Postjunctional Effect on Airway Smooth Muscle

The muscarinic agonists, acetylcholine, pilocarpine and carbachol caused a concentration-dependent increase in the ILP of the guinea-pig trachea. The order of potency on the postjunctional muscarinic receptor on the trachealis muscle was carbachol > pilocarpine > acetylcholine (Figure 7).

3.2.2 Effect on Sympathetic Nerve-Induced Relaxations

Figures 8 and 9 illustrate two separate experiments in which the guinea-pig trachea was initially contracted with U46619 followed after washout by either acetylcholine (Figure 8) or pilocarpine (Figure 9). In both experiments, sympathetic nerve-induced relaxations were smaller in the presence of the muscarinic agonist, when compared to those at a comparable ILP in the presence of U46619.
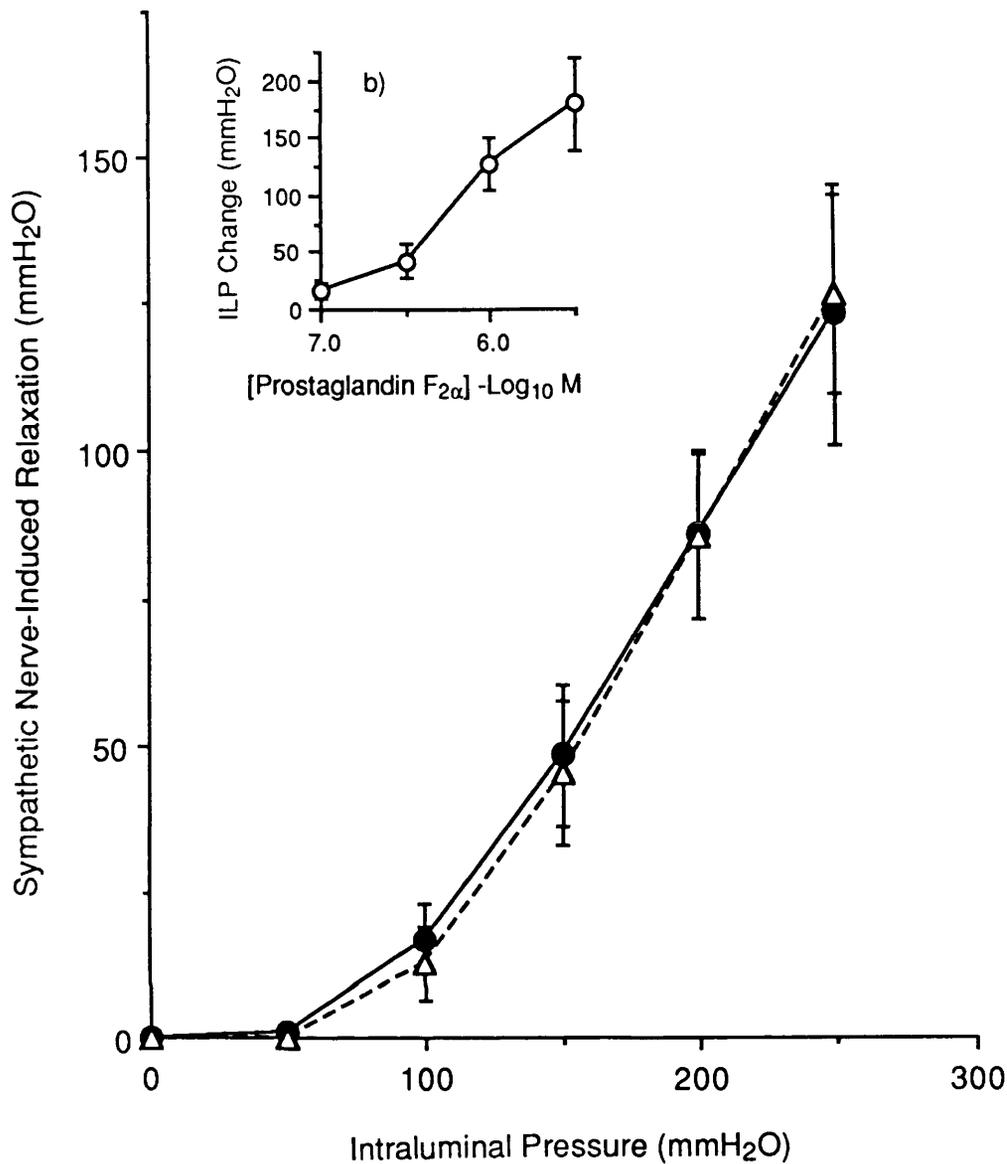
Figure 6.

Relaxation responses of the guinea-pig trachea induced by stimulation of the sympathetic nerve trunk (40Hz, V_{max}, 0.2ms, 5sec) when the intraluminal pressure (ILP) was raised with U46619 (- △ -) and prostaglandin $F_{2\alpha}$ (—●—). The insert b) shows the postjunctional contractile action of prostaglandin $F_{2\alpha}$ to increase the ILP of the tracheal tube. Sympathetic nerve-induced relaxations in the presence of U46619 and prostaglandin $F_{2\alpha}$ at any ILP were not significantly different (mean ± s.e.m.; n>5).
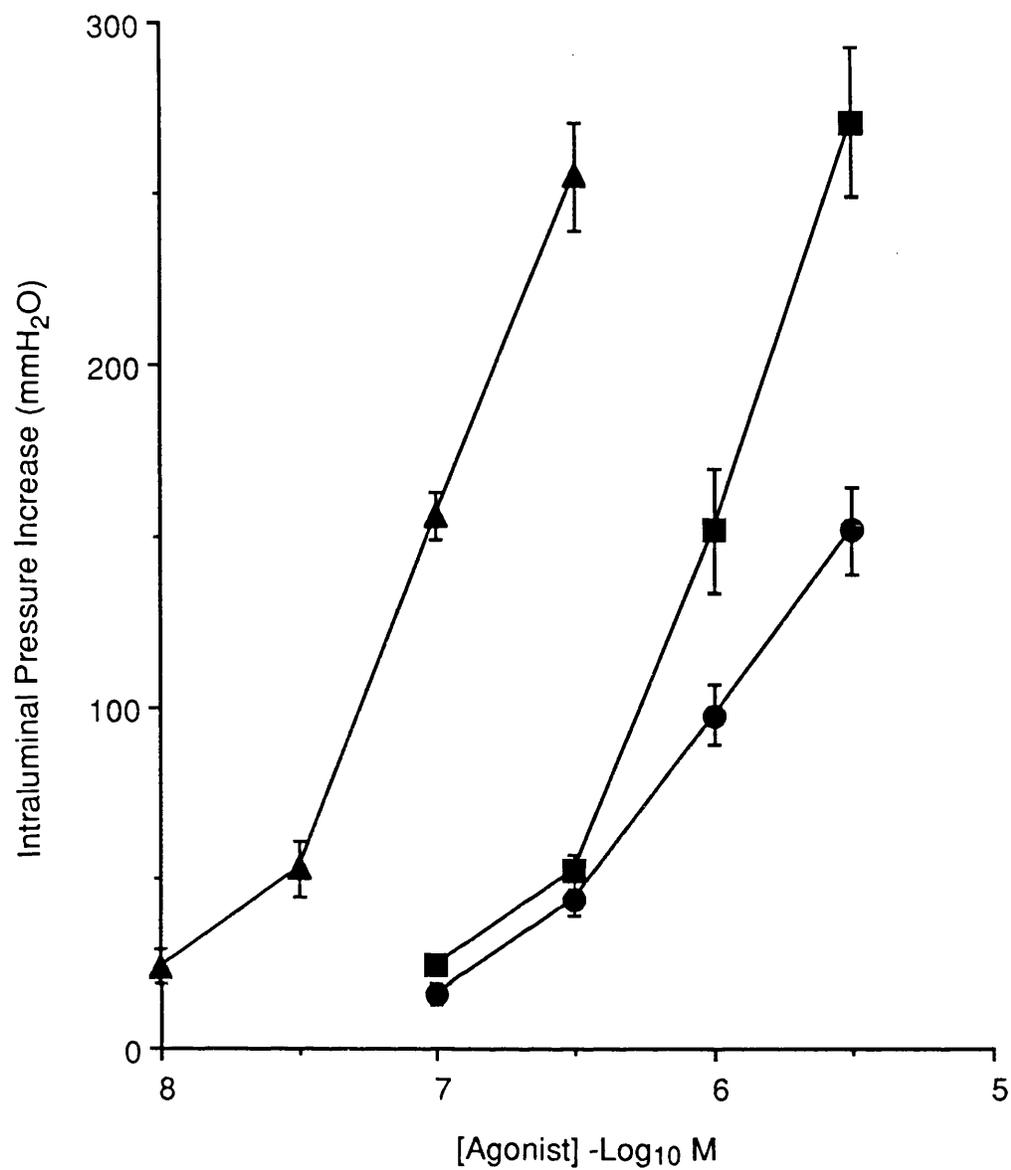
Figure 7.

Comparison of the effect of the muscarinic agonists carbachol (▲),
pilocarpine (■) and acetylcholine (●) on the intraluminal pressure of the
guinea-pig tracheal tube preparation (mean ± s.e.m; n = 10).
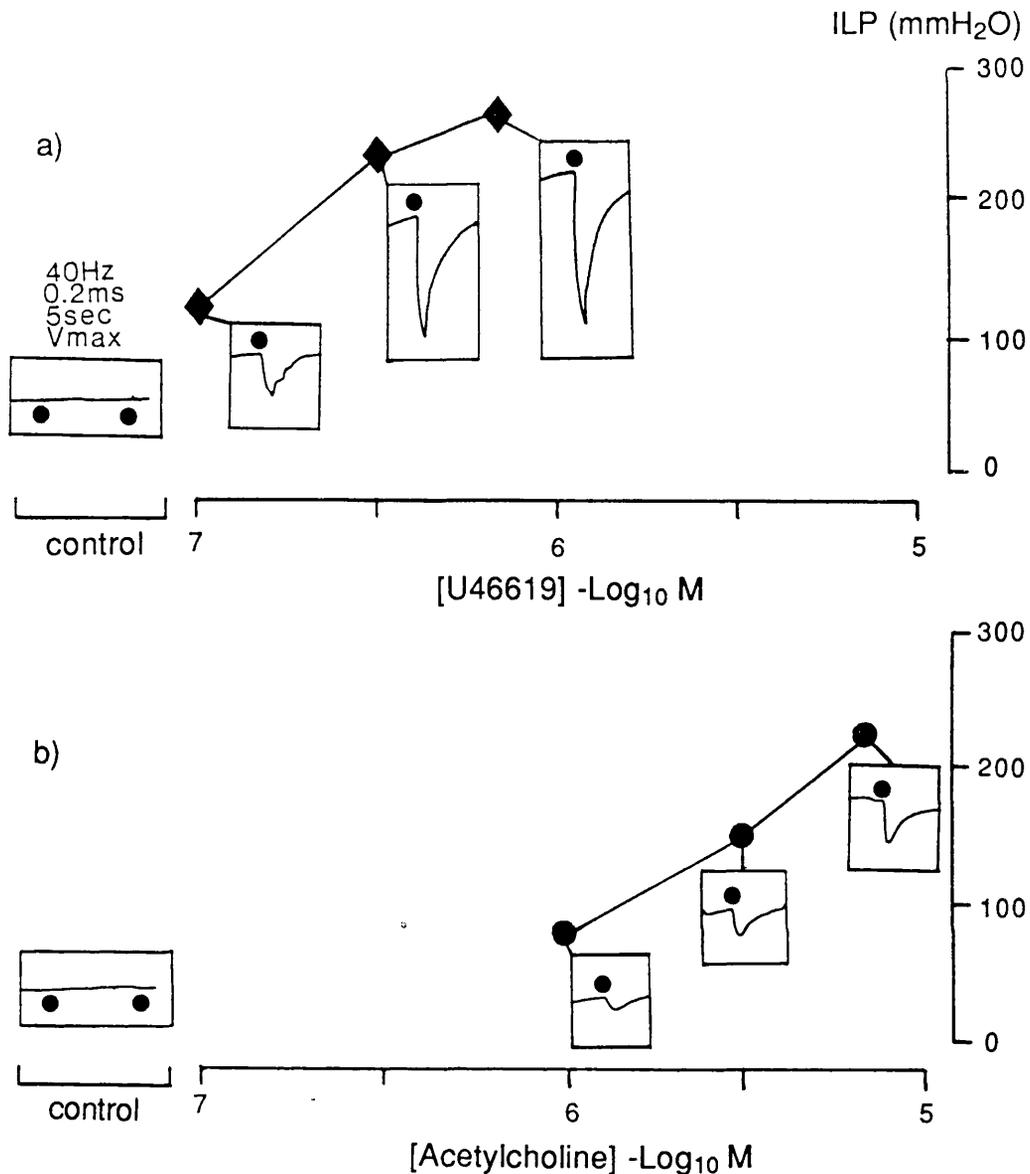
Figure 8.

Experimental record showing relaxation responses of the guinea-pig
trachea following stimulation of the sympathetic nerve trunk via the stellate
ganglion (indicated by the dots; 40Hz, V_{max}, 0.2ms, 5sec) in the presence
of a) U46619; and b) acetylcholine added cumulatively to the organ bath.
Bath concentrations of the spasmogens were selected to produce
comparable increases in the intraluminal pressure (ILP) of the fluid-filled
trachea.
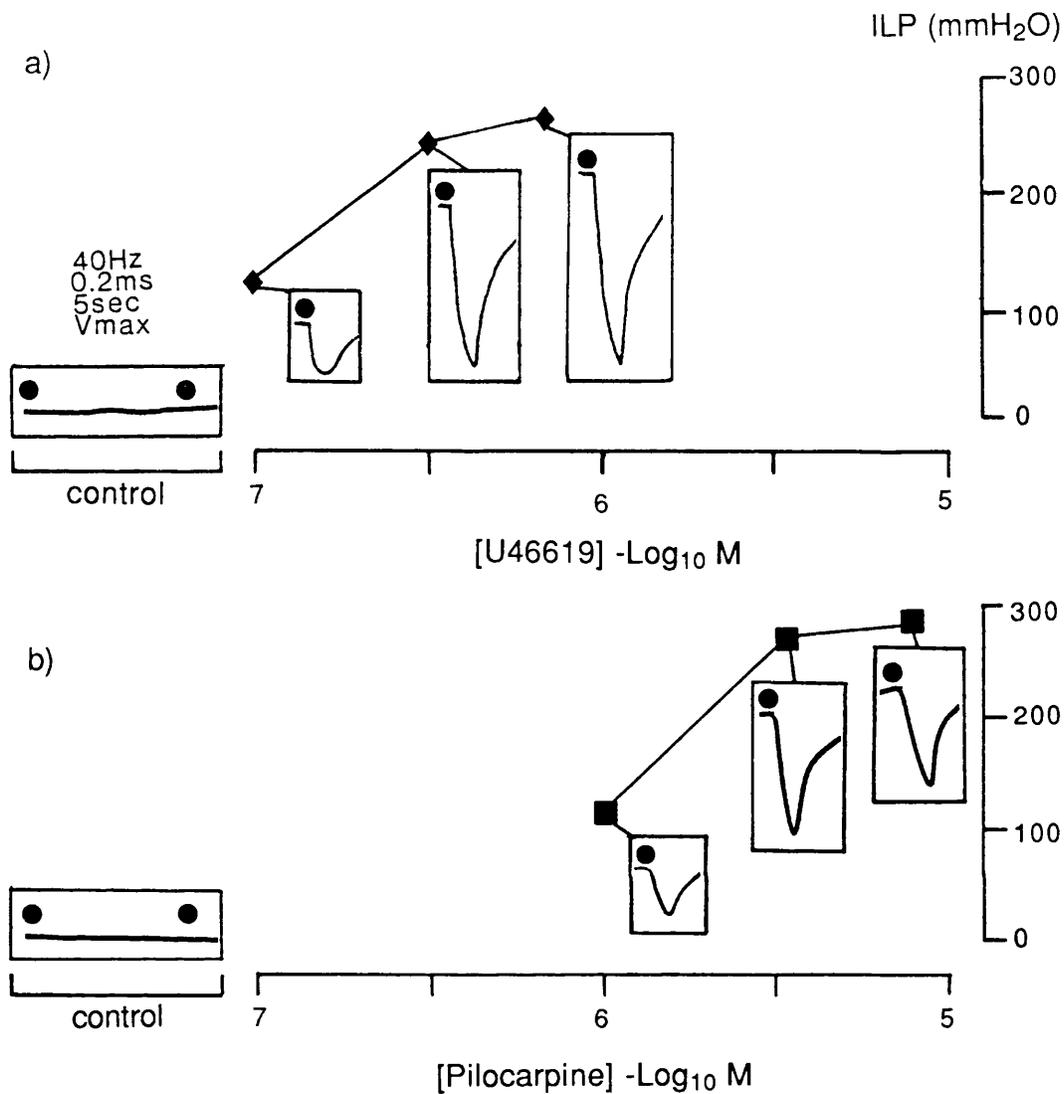
Figure 9.

Experimental record showing relaxation responses of the guinea-pig trachea following stimulation of the sympathetic nerve trunk via the stellate ganglion (indicated by the dots; 40Hz, V_{max}, 0.2ms, 5sec) in the presence of a) U46619; and b) pilocarpine added cumulatively to the organ bath. Bath concentrations of the spasmogens were selected to produce comparable increases in the intraluminal pressure (ILP) of the fluid-filled trachea.

Figure 10 summarises experiments in which sympathetic nerve-induced relaxation responses in the presence of the three muscarinic agonists, acetylcholine, carbachol, and pilocarpine were compared to those recorded in the same tissues in the presence of the stable thromboxane analogue U46619. At any given ILP (for example 300 mmH$_2$O above the minimum ILP in the presence of isoprenaline) sympathetic nerve-induced relaxations in the presence of acetylcholine or pilocarpine were smaller than those obtained when the tissue had been contracted to the same tone with carbachol or U46619. This suggests that acetylcholine and pilocarpine were inhibiting sympathetic nerve-induced relaxations. From Figure 10, it appears that acetylcholine is more potent as an inhibitor of sympathetic relaxations than pilocarpine. Such a comparison is however, an oversimplification, as interpretation is complicated by the different postjunctional potencies of the three muscarinic agonists (carbachol > pilocarpine > acetylcholine).

When the inhibitory effect of the muscarinic agonists was related to the concentration of agonist in the bath, acetylcholine and pilocarpine were found to inhibit sympathetic nerve-induced relaxations to a similar degree, in a concentration-dependent manner (Figures 11 and 12 respectively).
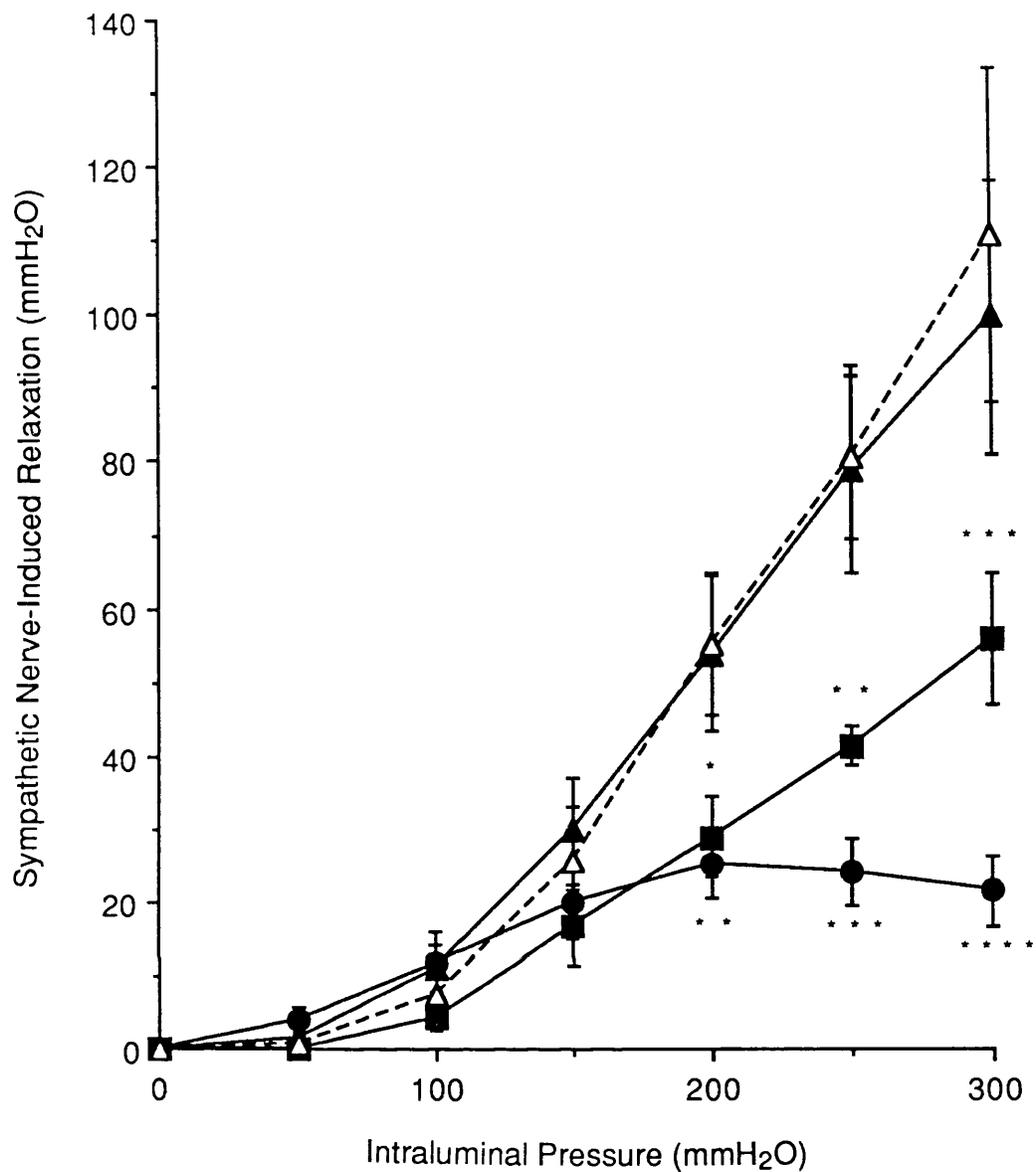
Figure 10.

Relaxation responses of the guinea-pig trachea following stimulation of the sympathetic nerve trunk via the stellate ganglion (40Hz, V_{max}, 0.2ms, 5sec). The intraluminal pressure (ILP) was raised with U46619 (-△-), carbachol (▲), pilocarpine (■) and acetylcholine (●). Sympathetic responses were reduced in the presence of acetylcholine and pilocarpine when compared to those at a comparable ILP in the presence of U46619 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$, mean ± s.e.m.; n>5).
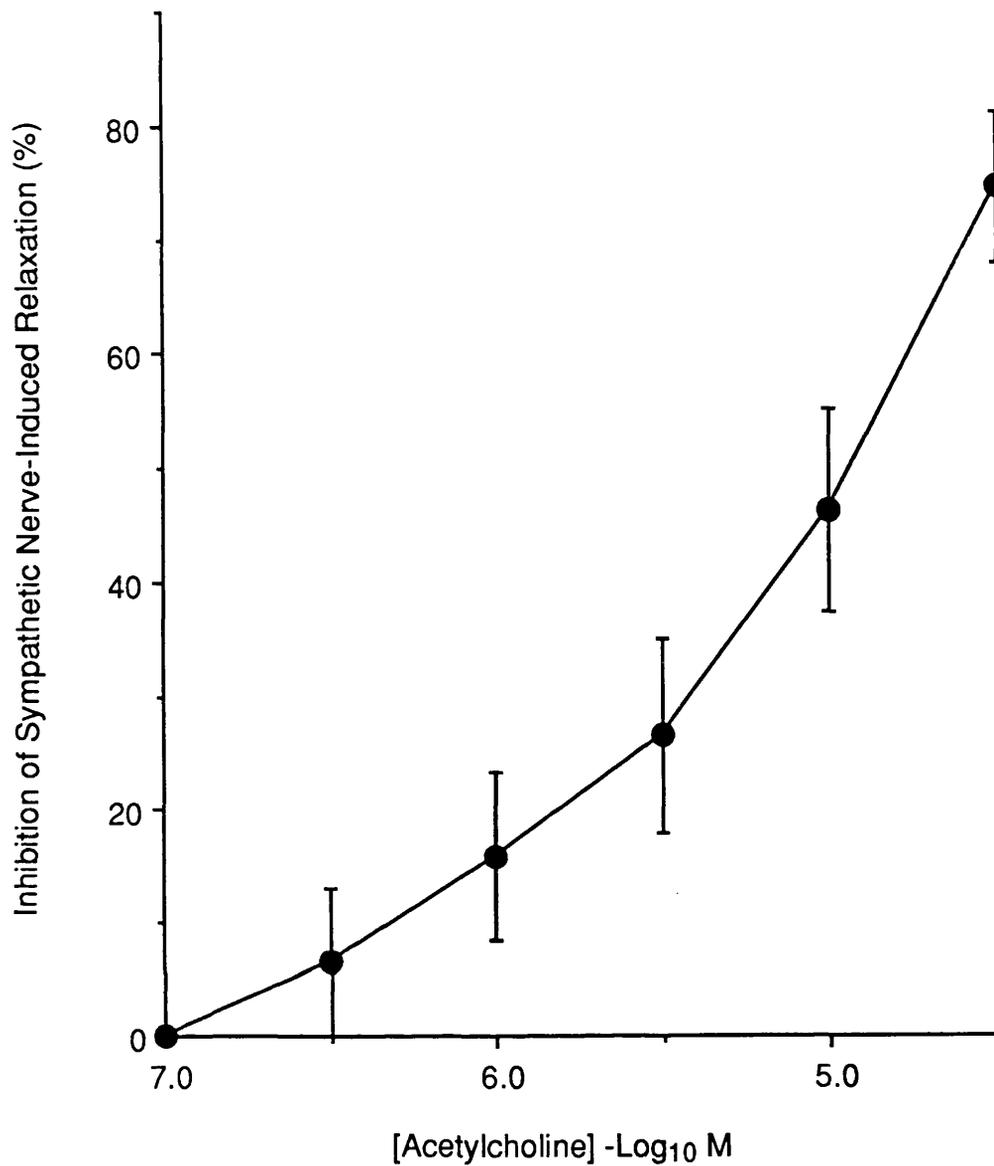
Figure 11.

Relationship between the concentration of acetylcholine and its inhibitory effect on relaxation responses of the guinea-pig trachea induced by stimulation of the sympathetic nerve trunk (40Hz, V_{max}, 0.2ms, 5sec).

$$\% \text{ inhibition} = \frac{A\text{-}B}{A} \times 100\%$$

where A is the sympathetic nerve-induced relaxation in the presence of U46619 and B is the relaxation response at an identical intraluminal pressure(ILP) when the ILP has been raised with acetylcholine (mean ± s.e.m.; n > 5).
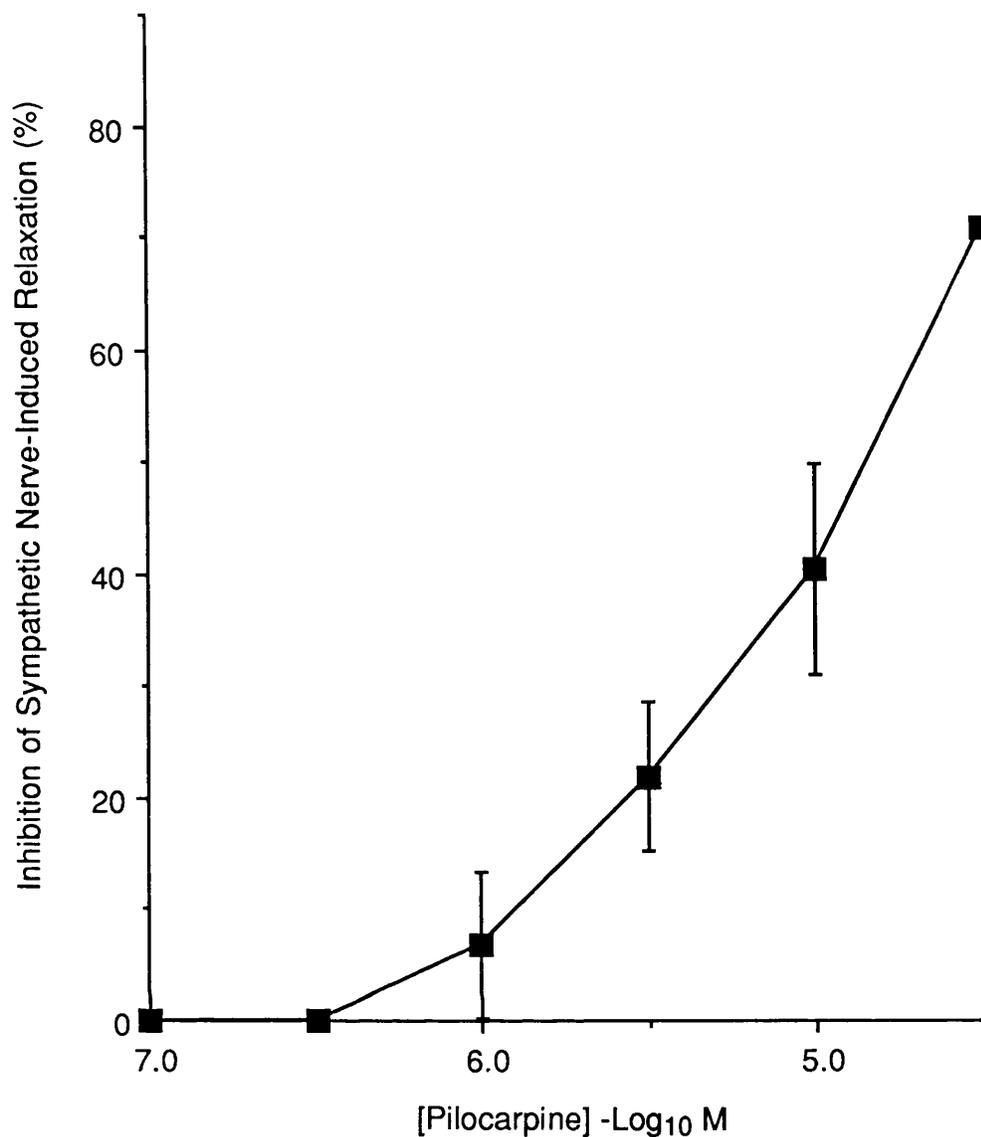
Figure 12.

Relationship between the concentration of pilocarpine and its inhibitory effect on relaxation responses of the guinea-pig trachea induced by stimulation of the sympathetic nerve trunk (40Hz, V_{max}, 0.2ms, 5sec).

$$\% \text{ inhibition} = \frac{A-B}{A} \times 100\%$$

where A is the sympathetic nerve-induced relaxation in the presence of U46619 and B is the sympathetic relaxation at an identical intraluminal pressure in the presence of pilocarpine (mean ± s.e.m.; n > 8, except in the presence of 3×10^{-5}M pilocarpine, which is the mean of two experiments).

In contrast to the other two muscarinic agonists, carbachol did not appear to inhibit sympathetic nerve-induced relaxations (Figure 10). Carbachol was, however, at least ten times more potent for the postjunctional muscarinic receptor than the other two muscarinic agonists; for example, the concentration of carbachol to which produced a tone rise of 300 mmH_2O was approximately $3x10^{-7}M$ compared to $3x10^{-6}M$ for pilocarpine and $> 10^{-5}M$ for acetylcholine. At this lower concentration $(3x10^{-7}M)$ neither acetylcholine (Figure 11) nor pilocarpine (Figure 12) significantly inhibited sympathetic nerve-induced relaxations (% inhibition 0 ± 0 and 6.5 ± 6.5 respectively).

When carbachol was added in concentrations greater than $3x10^{-7}M$, the ILP increased above 300 mmH_2O (Figure 13). As the tone was raised, sympathetic nerve-induced relaxations no longer increased, but were reduced. These inhibitory concentrations of carbachol $(10^{-6}M - 10^{-5}M)$ were in the same range as the inhibitory concentrations for the other muscarinic agonists. Thus, when the comparison was made in this way, carbachol, like the other two muscarinic agonists, also caused inhibition of sympathetic nerve-induced relaxations.
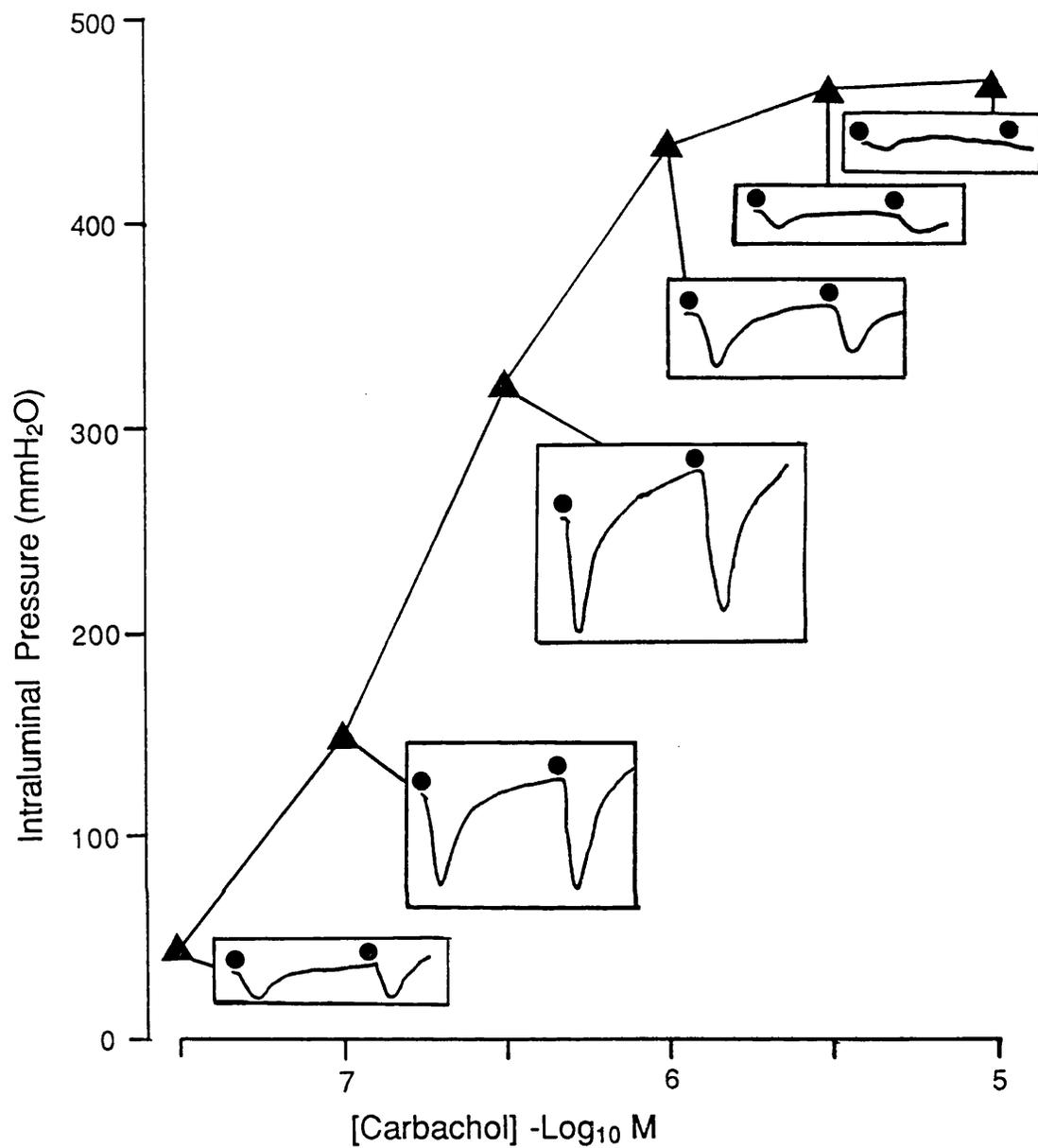
Figure 13.

Experimental record showing relaxation responses following stimulation of the sympathetic nerve trunk (indicated by the dots; 40Hz, V_{max}, 0.2ms, 5sec) at increasing levels of intraluminal pressure of the fluid-filled guinea-pig trachea in the presence of carbachol added cumulatively to the organ bath.

Thus, all three muscarinic agonists appear to be inhibiting sympathetic nerve-induced relaxations. This inhibitory effect of muscarinic agonists could be explained by either :-

1) differences in the degree of postjunctional "physiological"antagonism existing between the sympathetic neurotransmitter, noradrenaline, which induces relaxation of the airway smooth muscle and either U46619 or the muscarinic agonists, which contract the trachea, or,

2) a prejunctional action of the muscarinic agonists on pulmonary noradrenergic nerve endings.

The following experiments were designed to investigate these possibilities.

3.3 SIMULTANEOUS ADDITION OF EXOGENOUS NORADRENALINE AND A SPASMOGEN

Both U46619 and acetylcholine caused a concentration-dependent contraction of the guinea-pig trachealis muscle (Figures 3a and 7) via activation, respectively, of prostanoid receptors and muscarinic cholinoceptors situated postjunctionally on the airway smooth muscle. In contrast, ß-adrenoceptor agonists such as isoprenaline and the sympathetic neurotransmitter, noradrenaline, cause relaxation of the trachea.

The concentrations of either U46619 or acetylcholine to produce a 30% maximum contraction in the absence and presence of noradrenaline were calculated (EC_{30}). EC_{30}s in the absence and presence of noradrenaline were compared to give a "concentration ratio".

Table 1 shows the "concentration ratios" obtained when the guinea-pig trachea was contracted with either spasmogen in the presence of noradrenaline (10^{-6} and 10^{-5}M), compared to the spasmogen alone. At a concentration of 10^{-6}M, noradrenaline did not significantly inhibit the postjunctional contractile action of either spasmogen and as a result the calculated "concentration ratios" were close to unity. Noradrenaline at a higher concentration (10^{-5}M), however, inhibited the postjunctional action of U46619 and acetylcholine to a similar degree and caused approximately a 20 fold shift of both U46619 and acetylcholine contractile concentration-response curves.

Thus, the degree of postjunctional "functional" or "physiological" antagonism existing between the neurotransmitter and either spasmogens appears to be comparable. Therefore, differences in the degree of "physiological" antagonism existing between the sympathetic neurotransmitter and the different spasmogens, does not appear to explain the inhibitory effect of acetylcholine and other muscarinic agonists on sympathetic nerve-induced relaxations observed in the previous section (Section 3.2). Thus, the inhibitory effect of the muscarinic agonists appears to be due to an action on prejunctional muscarinic receptors on the sympathetic nerve endings.

Noradrenaline Concentration (μM)	Concentration Ratio (mean $\pm$ s.e.m. n > 5)	
	Acetylcholine	U46619
0	1	1
1	2.2 ± 0.5	1.7 ± 0.4
10	22.5 ± 5.4	21.8 ± 3.7

Table 1

The "physiological" antagonism existing between noradrenaline and either acetylcholine or U46619, expressed as concentration ratios.

$$\text{concentration ratio} = \frac{EC_{30} \text{ in the } \textbf{presence} \text{ of noradrenaline}}{EC_{30} \text{ in the } \textbf{absence} \text{ of noradrenaline}}$$

Noradrenaline inhibited the postjunctional contractile actions of U46619 and acetylcholine to a similar degree.

3.4 EFFECT OF ENDOGENOUSLY RELEASED ACETYLCHOLINE

3.4.1 Effect of Concurrent Vagal and Sympathetic Stimulation

Figure 14 shows an experiment in which the tone of the guinea-pig trachea was raised with U46619 (10^{-7}M). As previously described, sympathetic nerve-induced relaxations increased as the ILP was increased. When the tone had reached a plateau, repetitive stimulation of the sympathetic nerve trunk resulted in reproducible relaxations. When the vagus was stimulated simultaneously with the sympathetic nerve trunk, it caused inhibition of succeeding sympathetic relaxation responses.

This inhibitory effect of the parasympathetic nervous system on the sympathetic nervous system occurred in approximately 50% of the preparations and was not dependent upon the reverse relationship (that is, inhibition of vagal responses by the sympathetic nervous system) nor was it related to stimulation of either the right or the left vagal nerve trunk. The inhibitory effect of the parasympathetic nervous system on the sympathetic neurotransmission was; a) very variable between tissues, b) not reproducible in the same tissue, and, c) declined with successive periods of stimulation. It was therefore not possible to investigate the effect of muscarinic antagonists on this inhibitory effect. When vagal stimulation was ceased, sympathetic nerve-induced relaxations returned immediately to control values (Figure 14). The neuropeptides, VIP (10^{-9} - 10^{-8}M), neurokinin A (10^{-9} - 10^{-6}M), substance P (10^{-8} - 10^{-6}M) and endothelin (10^{-9} - 10^{-8}M) did not inhibit sympathetic nerve-induced relaxations excluding the involvement of NANC nerves.

ILP(mmH$_2$O)

STIMULATE VAGUS

20Hz 0.2ms

40Hz
0.2ms
5sec
Vmax

U46619 (100nM)

wash

0   90 180sec
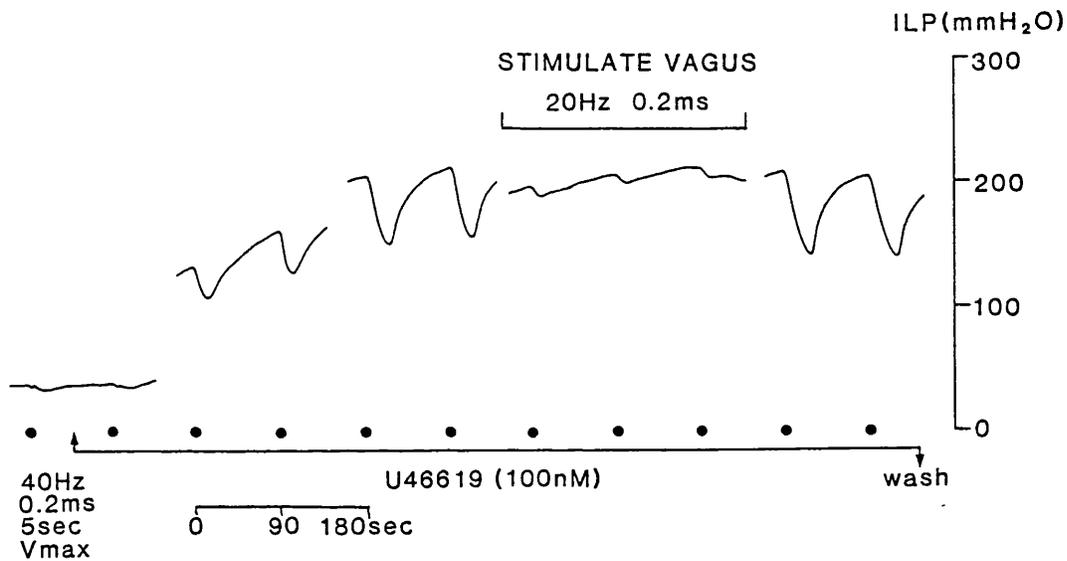
Figure 14.

Effect of concurrent stimulation of the vagal nerve trunk (shown by the
brackets; 20Hz, 0.2ms) on relaxation responses of the guinea-pig trachea
induced by stimulation of the sympathetic nerve trunk (indicated by the
dots; 40Hz, V$_{max}$, 0.2ms, 5sec). Concurrent stimulation of the vagal nerve
trunk did not alter the intraluminal pressure (ILP) but inhibited sympathetic
nerve-induced relaxations.

The reverse relationship is shown in Figure 15. Stimulation of the preganglionic vagal nerve fibres resulted in reproducible contractile responses, which were abolished with atropine. These contractile responses increased with increasing frequency, a maximum being observed at 30Hz and were rapid in onset (0.5 sec). In almost 80% of the preparations concurrent stimulations of the sympathetic nerve trunk resulted in a reduction in the size of the vagal contractile response. The degree of inhibition was reproducible in any one tissue (Figure 15a,b). This inhibitory effect of the sympathetic nervous system on the parasympathetic nervous system was almost abolished with propranolol $(10^{-5}M)$ (Figure 15c).

3.4.2 Effect of an Anticholinesterase Drug

In the absence of nerve stimulation, the anticholinesterase drug, physostigmine $(10^{-8}$ - $10^{-6}M)$ caused a concentration-dependent contraction of the fluid-filled trachea which was slow to develop and reached a maximum after about 15 minutes exposure, after which time the contraction was maintained or slowly declined (Figure 17b). In the same experiment, physostigmine was also found to potentiate the postjunctional action of exogenously applied acetylcholine in a concentration-dependent manner (Figure 16b).
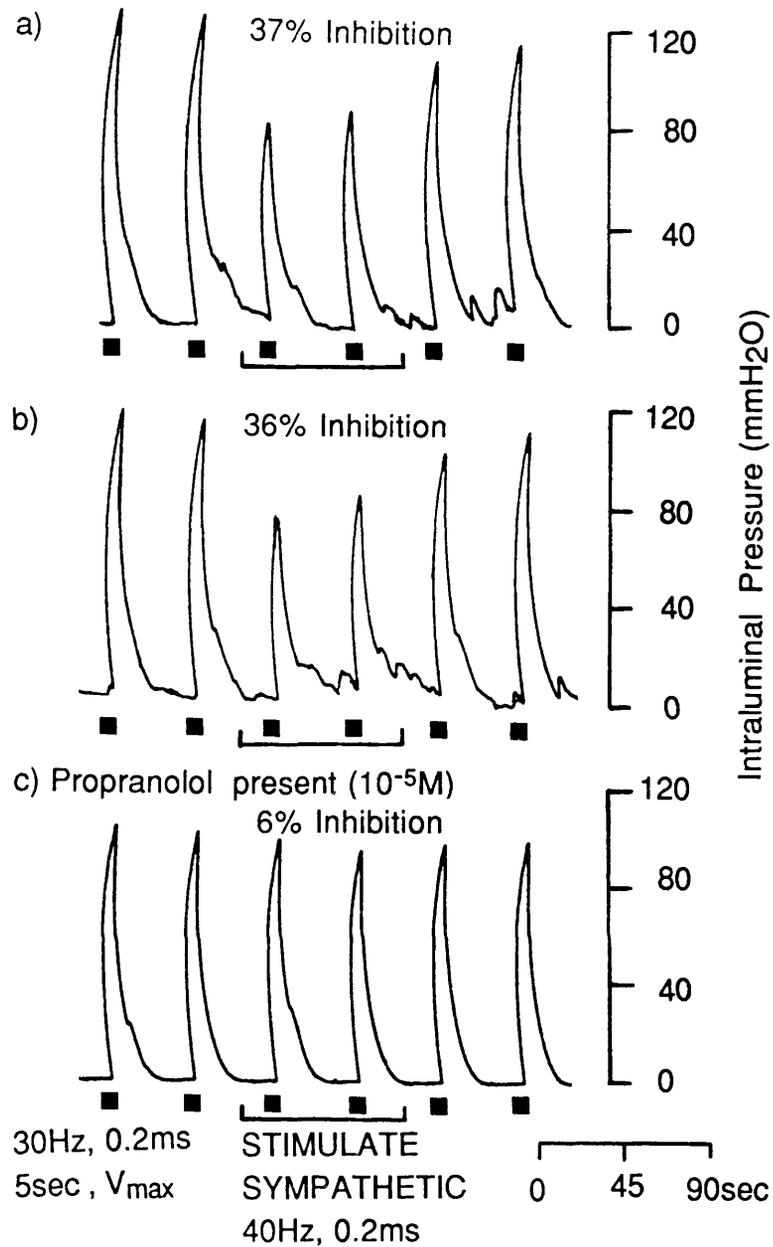
Figure 15.

Effect of concurrent stimulation of the sympathetic nerve trunk (shown by the brackets; 40Hz, 0.2ms), on contractile responses of the guinea-pig trachealis muscle induced by preganglionic stimulation of the vagal nerve trunk (shown by the dots; 30Hz, V_{max}, 0.2ms, 5sec). a) and b) show two separate periods of concurrent sympathetic stimulation in the same tissue; c) shows a period of concurrent sympathetic stimulation after the tissue had been preincubated with propranolol (10^{-5}M). Propranolol (10^{-5}M) antagonised the inhibitory effect of the sympathetic nervous system on contractions induced by vagal stimulation.

Physostigmine at the lowest concentrations used (10^{-8}, 3×10^{-8}M) had very little effect on vagally-induced contractions; at the intermediate concentrations (10^{-7}, 3×10^{-7}M) it caused an overall potentiation of vagally-induced contractions, whereas, in the presence of the highest concentration (10^{-6}M) of physostigmine, vagally-induced contractions were reduced (Figure 16). Physostigmine, particularly at the higher concentrations, increased the duration of vagally-induced contractions. The large error bars on Figure 16 are, however, an indication of the variability between tissues.

When sympathetic nerve-induced relaxations were studied in the presence of physostigmine, they were either unaffected (10^{-7}M), or inhibited (10^{-6}M) (Figure 17).

Atropine (10^{-7}M) blocked both the inhibitory effect of physostigmine (10^{-6}M) on sympathetic nerve-induced relaxations and its postjunctional action to contract the guinea-pig trachealis smooth muscle indicating the involvement of muscarinic receptors.
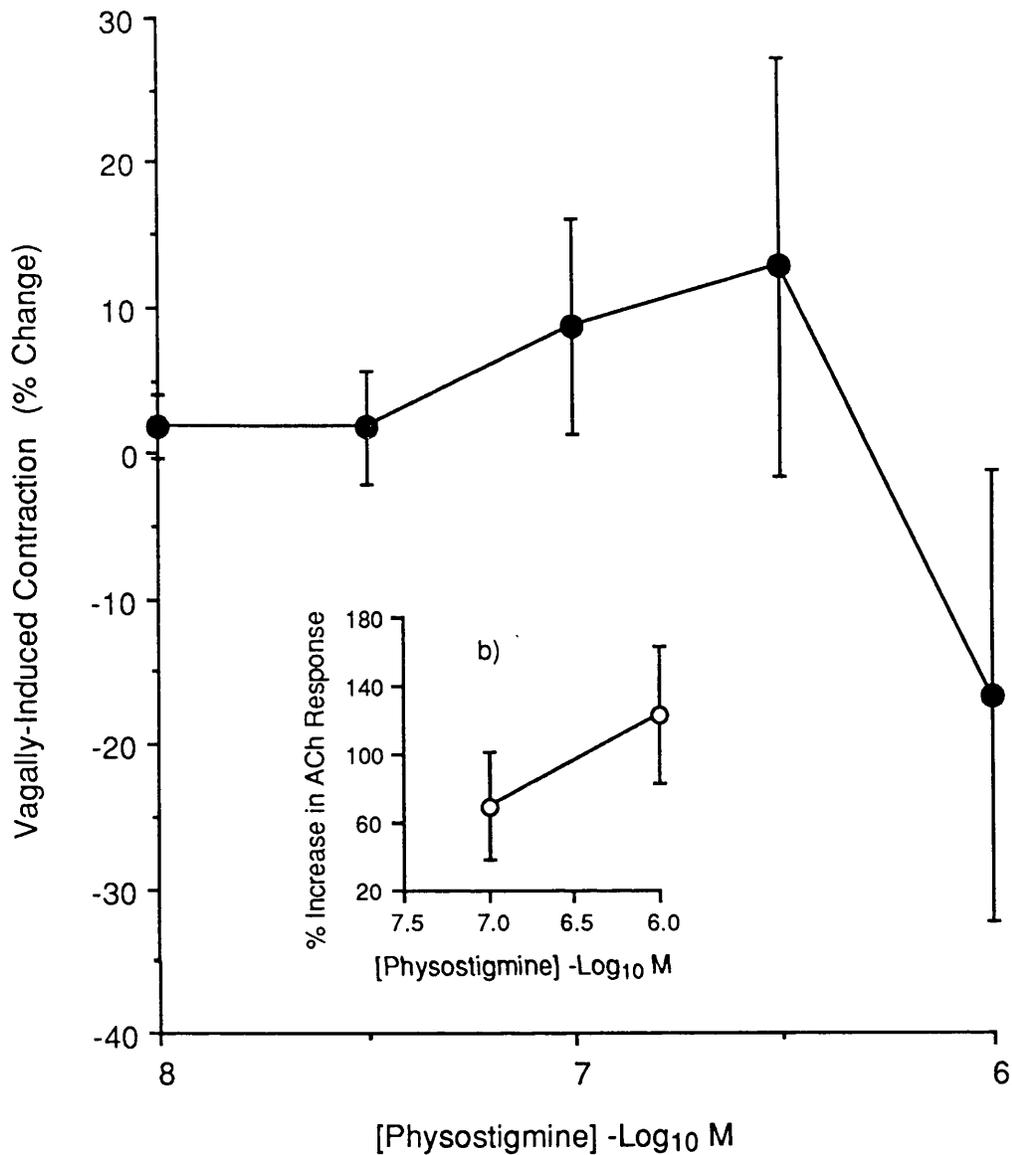
Figure 16.

Effect of physostigmine on contractions of the guinea-pig trachea induced by stimulation of the vagal nerve trunk (30Hz, V_{max}, 0.2ms, 5sec). The insert b) shows the effect of physostigmine (10^{-7}, 10^{-6}M) on the postjunctional contractile action of acetylcholine (10^{-5}M) to increase the tone of the trachealis smooth muscle. Physostigmine at a concentration of 10^{-6}M caused an overall inhibition of vagally-induced contractions whereas the postjunctional action of acetylcholine was potentiated (mean ± s.e.m; n>5).
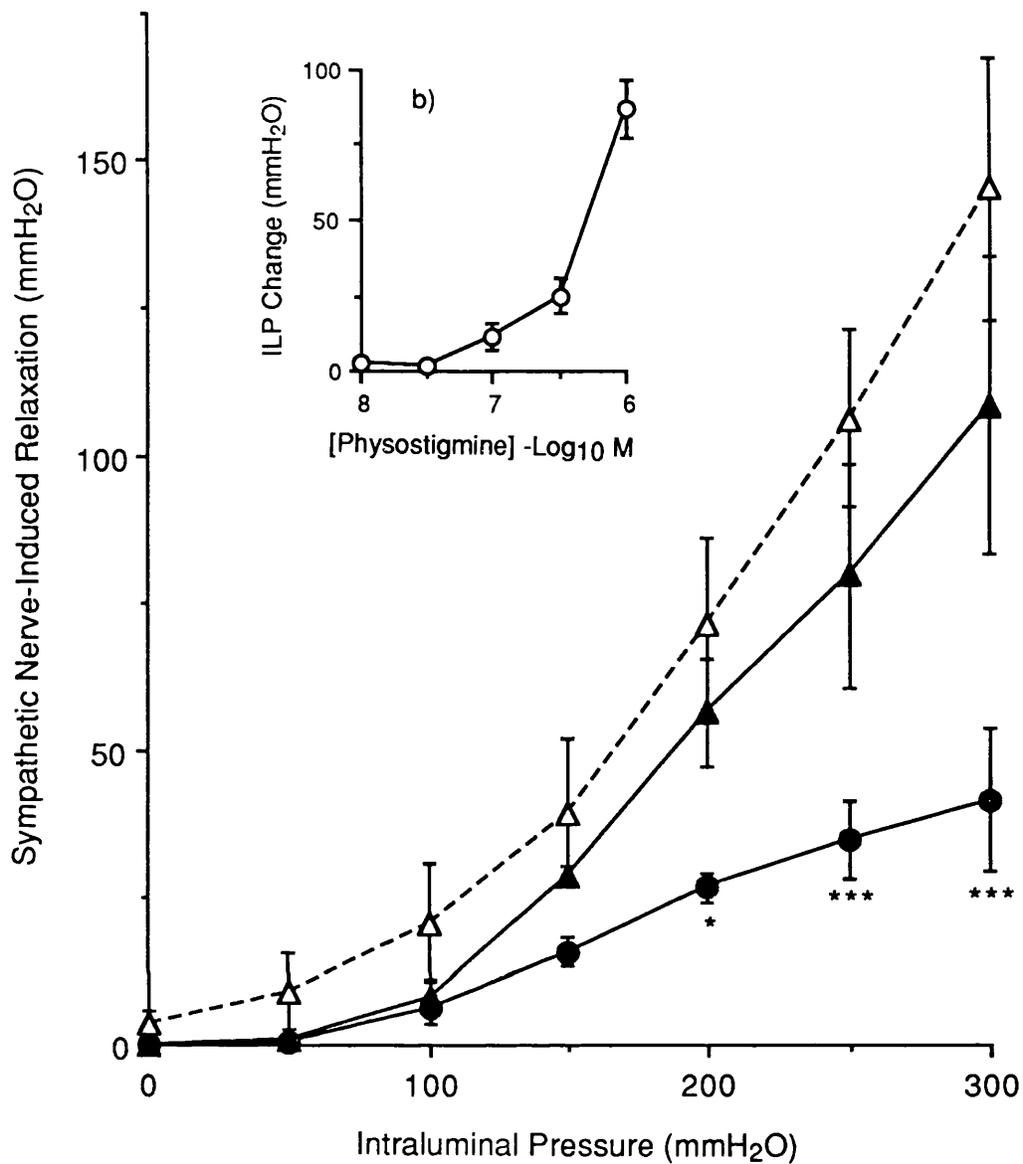
Figure 17.

Sympathetic nerve-induced relaxation responses of the guinea-pig trachea (40Hz, V_{max}, 0.2ms, 5sec), in the absence (- △ -) and presence of physostigmine 10^{-7}M (—▲—) and 10^{-6}M (—●—).The intraluminal pressure was raised with U46619. The insert b) shows the postjunctional contractile action of physostigmine. Although physostigmine (10^{-6}M) increased the intraluminal pressure, sympathetic nerve-induced relaxations were inhibited in the presence of this concentration of physostigmine (*p<0.05, ***p<0.005, mean ± s.e.m.; n<5).

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3.5   EFFECT OF CHOLINOCEPTOR ANTAGONISTS

3.5.1   Effect of Muscarinic Antagonists

If the inhibitory effect of the muscarinic agonists is due to an action on muscarinic receptors situated on the sympathetic nerve endings, the effect should be abolished with muscarinic antagonists. Figures 18 and 19 show two separate experiments in which the effects of the selective M_2 antagonists, gallamine and methoctramine were investigated on the effect of acetylcholine to inhibit sympathetic nerve-induced relaxations. Sections a) and b) show sympathetic nerve-induced relaxations measured in the presence of U46619 and acetylcholine respectively. It can be seen that sympathetic relaxations at any given ILP were smaller when the tone had been raised with acetylcholine than those in the presence of U46619 as previously shown in section 3.2.2. Section c) shows sympathetic nerve-induced relaxations in the presence of acetylcholine following at least 15 minutes preincubation with the M_2 muscarinic antagonists, gallamine (Figure 18) and methoctramine (Figure 19). Sections b) and c) of both figures are identical, indicating that the presence of the M_2 antagonists altered neither the postjunctional action of acetylcholine to contract the airway smooth muscle, nor its inhibitory effect on sympathetic nerve-induced relaxations. In addition, M_1 selective concentrations of pirenzepine ($<10^{-6}M$) did not antagonise the inhibitory effect of acetylcholine.
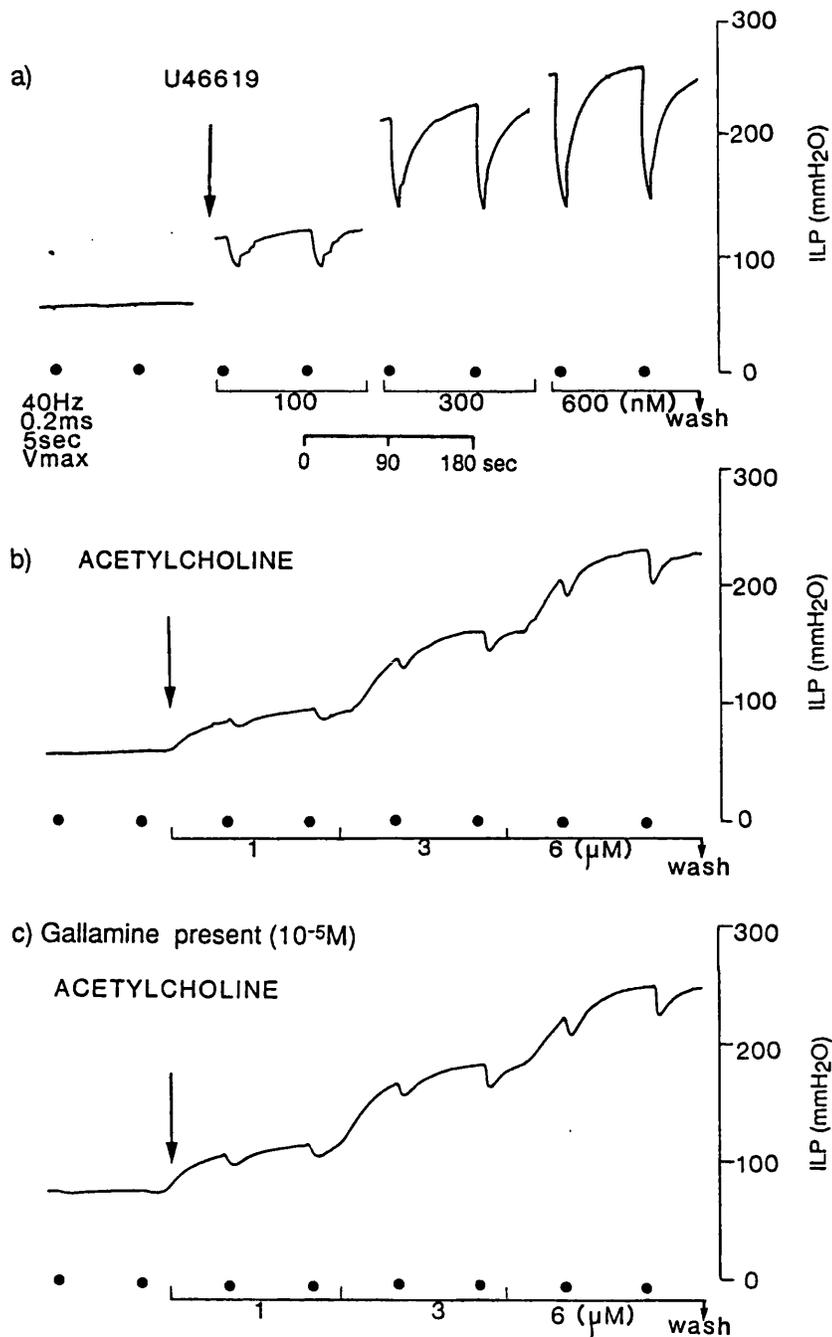
Figure 18.

Experimental record showing the effect of the muscarinic M_2 antagonist, gallamine, on intraluminal pressure changes (ILP) and relaxation responses of the guinea-pig trachea induced by stimulation of the sympathetic nerve trunk (shown by the dots; 40Hz, V_{max}, 0.2ms, 5sec). The ILP was raised with a) U46619; b) acetylcholine; and c) acetylcholine in the presence of gallamine (10^{-5}M). Both the postjunctional contractile action and inhibitory effect of acetylcholine on sympathetic nerve-induced relaxations were unaltered by gallamine (10^{-5}M).
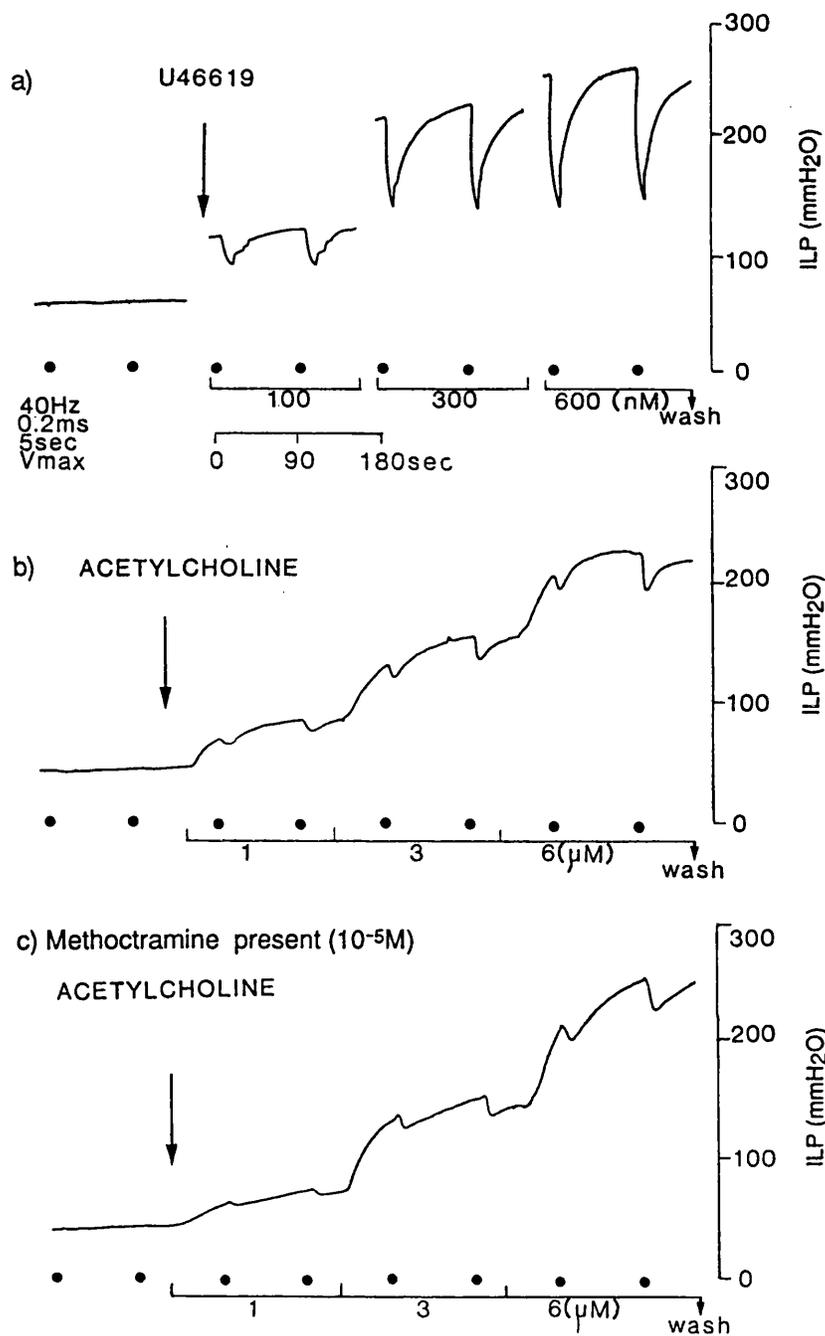
Figure 19.

Experimental record showing the effect of the muscarinic M_2 antagonist, methoctramine, on intraluminal pressure changes (ILP) and relaxation responses of the guinea-pig trachea induced by stimulation of the sympathetic nerve trunk (shown by the dots; 40Hz, V_{max}, 0.2ms, 5sec). The ILP was raised with a) U46619; b) acetylcholine; and c) acetylcholine in the presence of methoctramine (10^{-5}M). Both the postjunctional contractile action and inhibitory effect of acetylcholine on sympathetic nerve-induced relaxations were unaltered by methoctramine (10^{-5}M).

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The postjunctional contractile action of acetylcholine was antagonised by several of the muscarinic antagonists, atropine, hexahydrosiladifenidol and pirenzepine (higher concentrations), due to blockade of M_3 muscarinic receptors on the airway smooth muscle. Therefore, in order to investigate the inhibitory effect of acetylcholine on sympathetic nerve-induced relaxations in the presence of M_3 blocking concentrations of the muscarinic antagonists, the trachea had to be precontracted with a spasmogen which does not act through muscarinic receptors, such as U46619. When the tone had reached a plateau, acetylcholine was administered at concentrations which had been shown previously in the same tissue to inhibit sympathetic nerve-induced relaxations. From Figures 20, 21 and 22, it can be seen that in the presence of M_3 blocking concentrations of the muscarinic antagonists, atropine, hexahydrosiladifenidol and pirenzepine (higher concentrations), acetylcholine no longer had an inhibitory effect on sympathetic neurotransmission.

a) U46619

-300

ILP(mmH₂O)

-200

-100

-0

40Hz
0.2ms
5sec
Vmax

10   30   60   100   200nM   wash

0 90 180 sec

b)

ACETYLCHOLINE

-200

ILP(mmH₂O)

-100

-0

0.3   0.6   1   3   6µM   wash

c)Atropine present (10⁻⁹M)   ACETYLCHOLINE

-200

ILP(mmH₂O)

-100

-0

1   3   6   10   20µM   wash

d)Atropine present (10⁻⁷M)   ACETYLCHOLINE

-100

ILP(mmH₂O)

-0

3   6   10   30µM   wash

e)Atropine present (10⁻⁷M)   ACETYLCHOLINE   3   6   10   30µM   wash

U46619

-200

ILP(mmH₂O)

-100

-0

10   30   60   100nM   wash
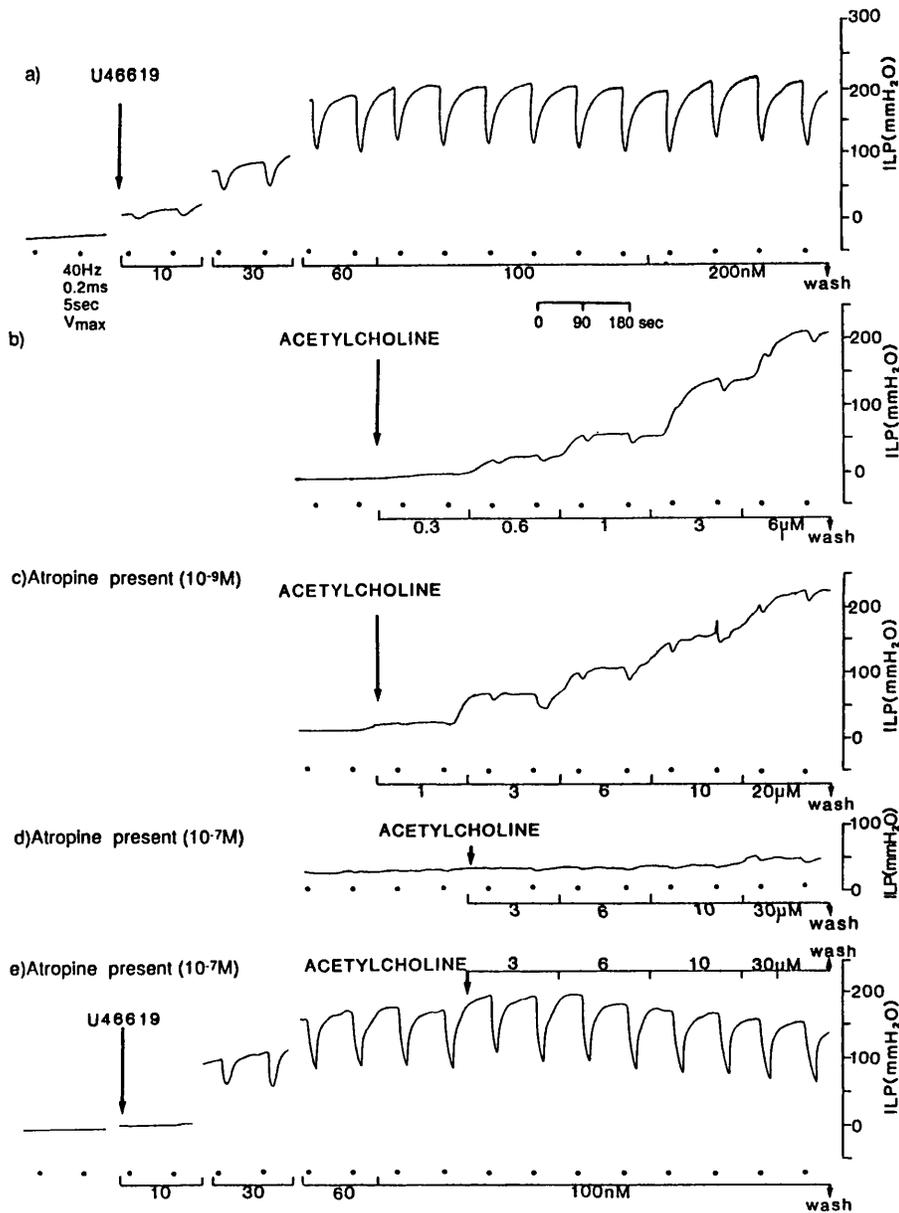
Figure 20.

Experimental record showing the effect of the muscarinic antagonist,
atropine, on the action of acetylcholine to inhibit relaxation responses of
the guinea-pig trachea induced by stimulation of the sympathetic nerve
trunk (indicated by the dots; 40Hz, V_{max}, 0.2ms, 5sec). Sympathetic nerve-
induced relaxations and intraluminal pressure (ILP) changes are shown
when the ILP was raised with a) U46619; b) acetylcholine; c) acetylcholine
in the presence of atropine ($10^{-9}M$); d) acetylcholine in the presence of
atropine ($10^{-7}M$); and e) U46619 and acetylcholine in the presence of
atropine ($10^{-7}M$). Atropine ($10^{-7}M$) abolished both the postjunctional
contractile action of acetylcholine and its inhibitory action on sympathetic
nerve-induced relaxations.

100

ILP (mmH2O)

a) U46619

40Hz
0.2ms
5sec
Vmax

100   150   200 (nM)   wash

0   90 180 sec

b) ACETYLCHOLINE

1   3   6 (µM)   wash

c) Hexahydrosiladifenidol present (10^{-5}M)   ACETYLCHOLINE

1   3   6 (µM)   wash

d) Hexahydrosiladifenidol present (10^{-5}M)

ACETYLCHOLINE
3   6   10 (µM)   wash

U46619

100   150   200 (nM)   wash
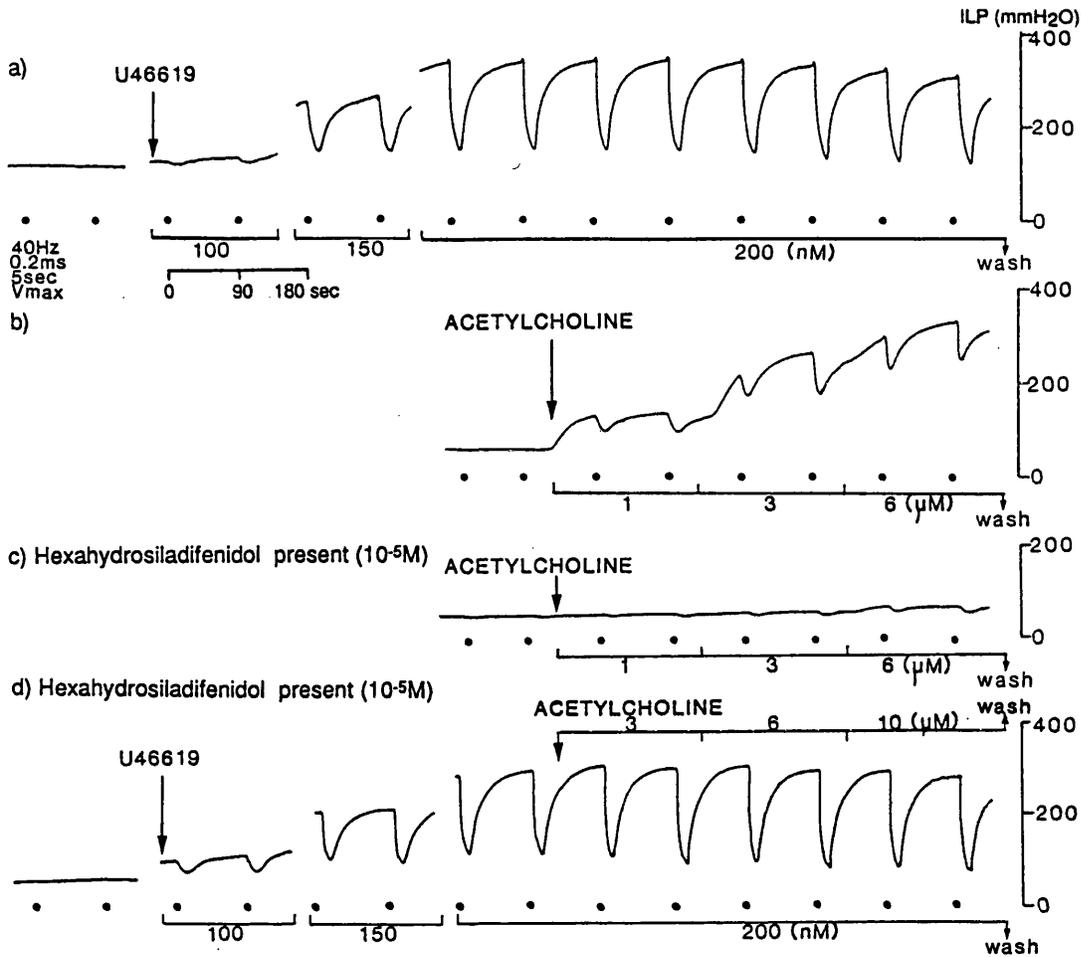
Figure 21.

Experimental record showing the effect of the muscarinic M_3 antagonist, hexahydrosiladifenidol, on the action of acetylcholine to inhibit relaxation responses of the guinea-pig trachea induced by stimulation of the sympathetic nerve trunk (indicated by the dots; 40Hz, V_{max}, 0.2ms, 5sec). Sympathetic nerve-induced relaxations and intraluminal pressure (ILP) changes are shown when the ILP was raised with a) U46619; b) acetylcholine; c) acetylcholine in the presence of hexahydrosiladifenidol (10^{-5}M); and d) U46619 and acetylcholine in the presence of hexahydrosiladifenidol (10^{-5}M). Hexahydrosiladifenidol (10^{-5}M) abolished both the postjunctional contractile action of acetylcholine and its inhibitory action on sympathetic nerve-induced relaxations.

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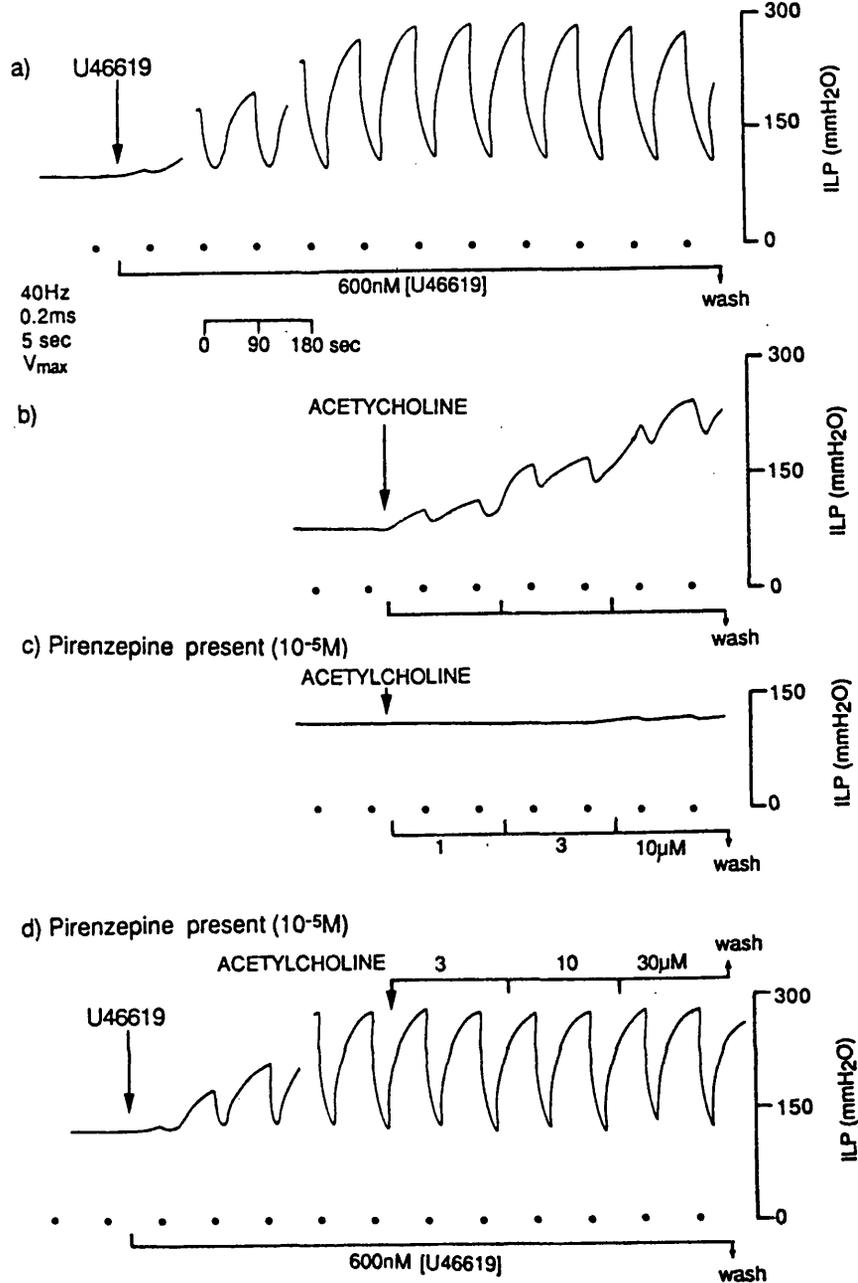
Figure 22.

Experimental record showing the effect of the muscarinic antagonist, pirenzepine, on intraluminal pressure changes (ILP) and relaxation responses of the guinea-pig trachea following stimulation of the sympathetic nerve trunk (shown by the dots; 40Hz, V_{max}, 0.2ms, 5sec). The ILP was raised with a) U46619; b) acetylcholine; and c) acetylcholine in the presence of pirenzepine (10^{-5}M). At higher concentrations pirenzepine (10^{-5}M) abolished both the postjunctional contractile action of acetylcholine and its inhibitory effect on sympathetic nerve -induced relaxations. The inhibitory effect of acetylcholine on sympathetic responses was unaltered by M_1 selective concentrations of pirenzepine.

3.5.2 Effect of a Nicotinic Antagonist

To exclude the involvement of prejunctional facilitatory nicotinic cholinoceptors, sympathetic nerve-induced relaxations, ILP changes and the inhibitory effect of the muscarinic agonists on sympathetic neurotransmission, were measured in the absence and presence of hexamethonium $(10^{-5}M)$. Preincubation with hexamethonium $(10^{-5}M)$ for at least one hour resulted in no observable change in tone or significant change in these three parameters.

Figure 23, for example, illustrates sympathetic nerve-induced relaxations in the absence and presence of hexamethonium $(10^{-5}M)$ when the tone of the guinea-pig trachea has been raised with a) U46619, b) carbachol and c) pilocarpine. Hexamethonium caused no significant change in sympathetic nerve-induced relaxation responses. A similar insignificant effect was observed on ILP changes induced by U46619, carbachol and pilocarpine or the inhibitory effect of pilocarpine and carbachol on sympathetic nerve-induced relaxations (not shown here).

Similarly, hexamethonium $(10^{-5}M)$ did not alter significantly the postjunctional contractile action of acetylcholine on the airway smooth muscle, nor sympathetic nerve-induced relaxations in the presence of acetylcholine nor the inhibitory effect of acetylcholine on sympathetic neurotransmission (Figure 24).
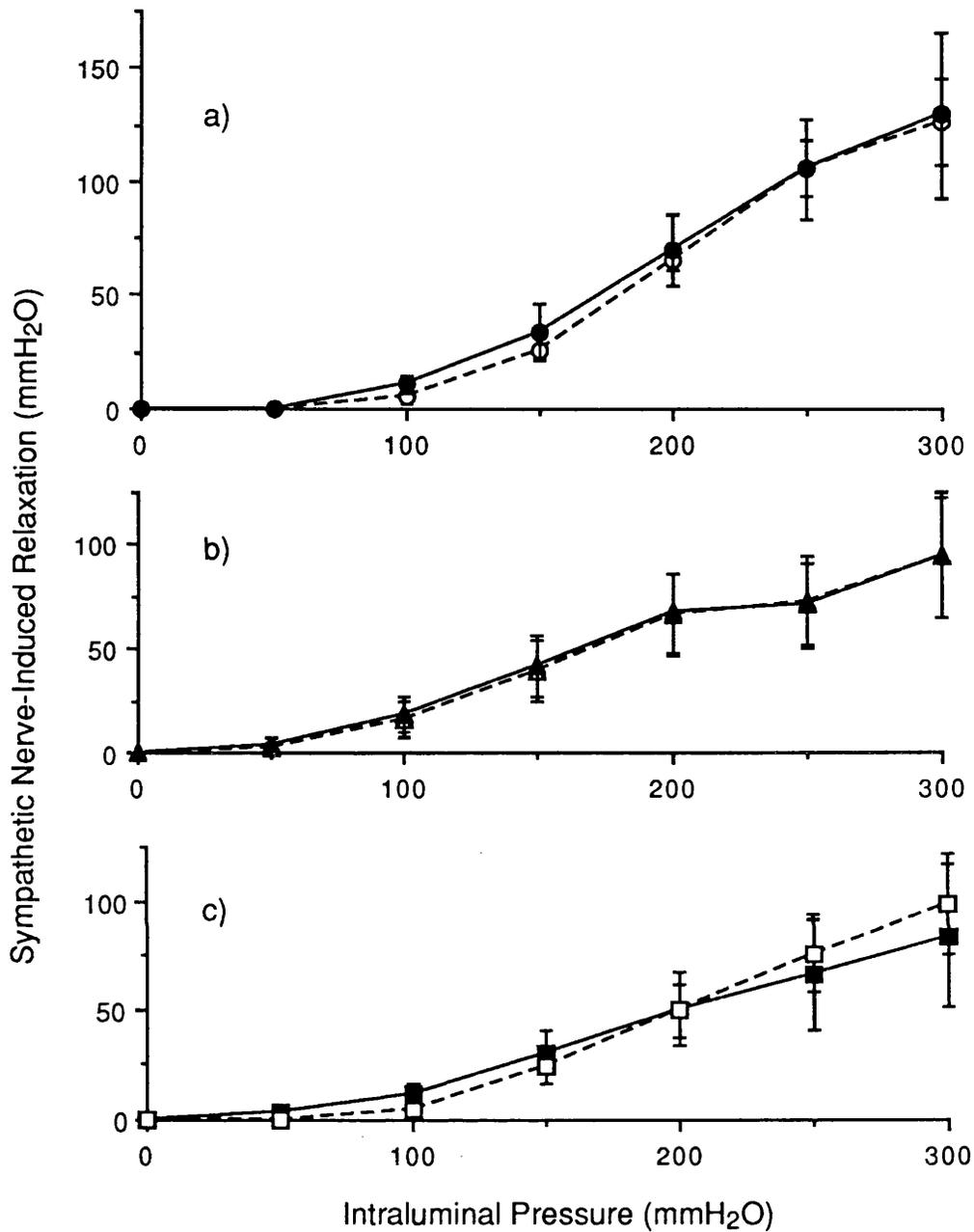
Figure 23.

Sympathetic nerve-induced relaxation of the guinea-pig trachealis muscle in the absence (open symbols) and presence (closed symbols) of hexamethonium ($10^{-5}M$) when the intraluminal pressure (ILP) was raised with a) U46619; b) carbachol; and c) pilocarpine. Sympathetic relaxations in the presence of all three spasmogens at any given ILP were not significantly different in the presence and absence of hexamethonium ($10^{-5}M$) thus, at some ILPs the open symbols are concealed due to the close opposition of the two points (mean ± s.e.m; n>5).
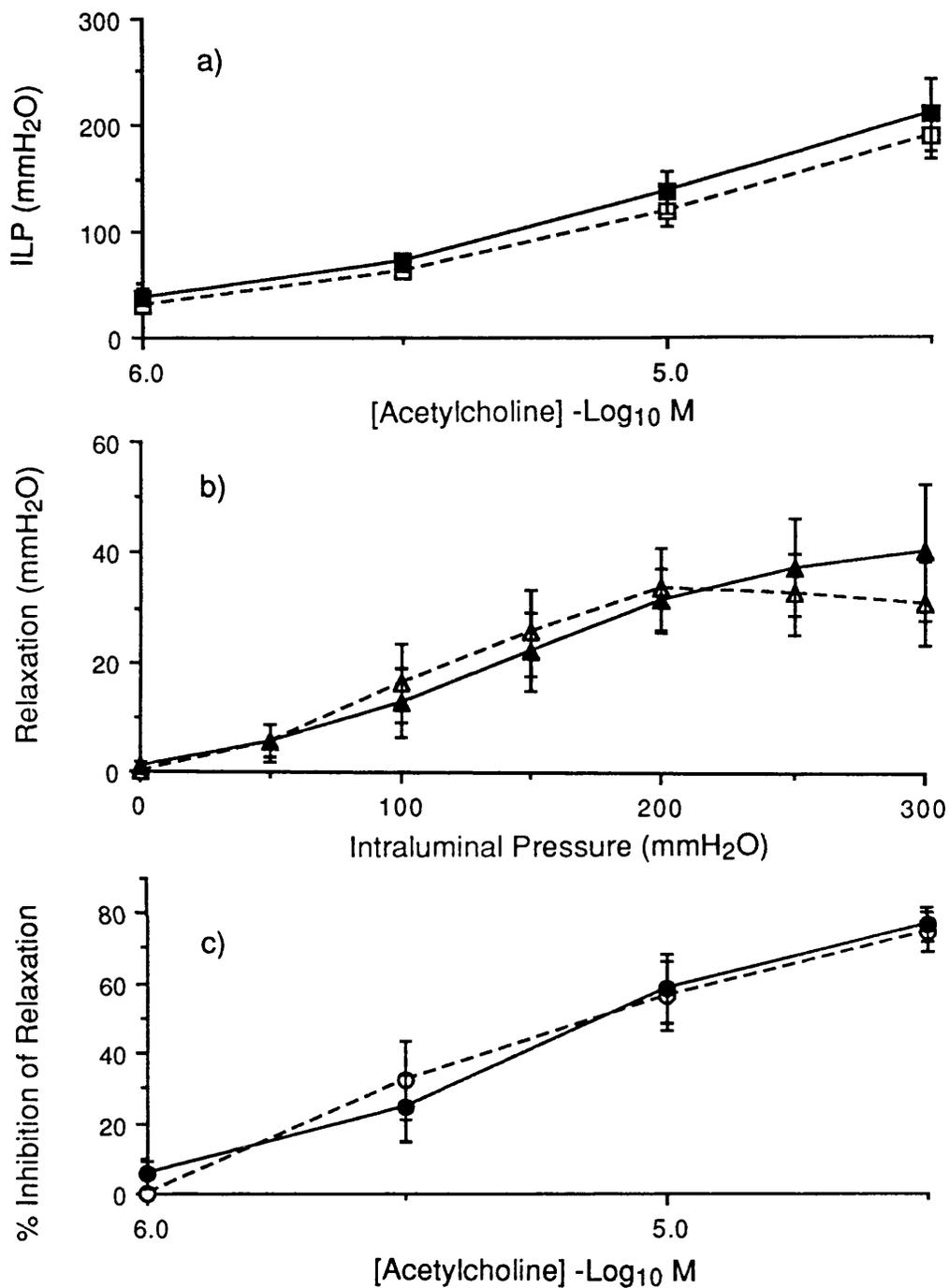
Figure 24.

Effect of hexamethonium (10^{-5}M closed symbols) on a) the postjunctional contractile action of acetylcholine to raise the intraluminal pressure of the guinea-pig trachea; b) sympathetic nerve-induced relaxations when the intraluminal pressure (ILP) has been increased with acetylcholine; and c) the inhibitory effect of acetylcholine on sympathetic nerve-induced relaxations of the guinea-pig trachea.The open symbols represent control data in the absence of hexamethonium (mean ± s.e.m.; n>5).

SECTION 4 - DISCUSSION

4.0   DISCUSSION

4.1   SYMPATHETIC NERVE-INDUCED RELAXATION RESPONSES OF THE INNERVATED TRACHEAL TUBE PREPARATION

Autonomic regulation of airway smooth muscle tone is very complex. In addition to the classical cholinergic and noradrenergic pathways there is evidence for both inhibitory and excitatory nonadrenergic noncholinergic nerves (NANC) whose transmitters have not yet been identified.

Investigations into modulation of the different branches of the pulmonary autonomic nervous system usually involves transmural stimulation of the airway smooth muscle. A disadvantage of this type of preparation is that all nerve endings within the tissue are stimulated simultaneously. Although this problem has been partially circumvented by the use of cholinoceptor and adrenoceptor antagonists, selective antagonists for the NANC neurotransmitters, however, are not available. In addition, the presence of antagonists does not allow the effects of drugs on different branches of the autonomic nervous system to be investigated in the same tissue. Their presence also prevents investigations into the interactions existing between the different branches, in particular the effect of the parasympathetic nervous system, which is dominant in the lung, on the sympathetic nervous system.

The preparation used in the present experiments allows selective discrete stimulation of the sympathetic and parasympathetic nerves to the airway smooth muscle either alone or simultaneously. This preparation is a modification of that previously described by Blackman and McCaig (1983).

Indomethacin ($5x10^{-6}$M) was present throughout our experiments to remove any prostaglandin-induced tone (Vane 1971, Farmer et al 1974, Ito and Tajima 1981a,b). Thromboxane and arachadonic acid metabolites, such as prostaglandin $E_{2\alpha}$ and prostaglandin $F_{2\alpha}$ have been shown to modulate both sympathetic and parasympathetic neurotransmission in many tissues (Body and Kadowitz 1974, Hedqvist 1977, Malik 1978).

Lung tissues from all species so far investigated can synthesize and release prostaglandins in response to immunological, chemical and mechanical stimuli (Piper and Vane 1971, Robinson and Holgate 1986, Burka 1988).

In the airways prostaglandins inhibit pulmonary parasympathetic neurotransmission (Ito and Tajima 1981a,b, Inoue et al 1984, Walters et al 1984, Inoue and Ito 1986) and evoke spontaneous activity of the trachealis smooth muscle (Boyle et al 1988). Contractile responses and excitatory junction potentials (e.j.p.s) of the canine trachealis muscle to electrical field stimulation (EFS) diminished in a time dependent manner

(Walters 1984, Inoue et al 1984, Inoue and Ito 1986). Addition of indomethacin or a prostaglandin antagonist to the bathing medium prevented this inhibition and markedly increased contractile responses to EFS suggesting that prostaglandins were responsible for the time dependent inhibition (Inoue et al 1984, Walters et al 1984).

In the same studies, $PGE_{2\alpha}$ and $PGF_{2\alpha}$ inhibited EFS-induced contractions in a concentration-dependent manner. Acetylcholine-induced contractions were unaffected by incubation alone, addition of indomethacin and $PGE_{2\alpha}$ (Walters et al 1984).

$PGE_{2\alpha}$ release from the smooth muscle was also shown to increase with time. After two hours the bath concentration was equivalent to a level of $PGE_{2\alpha}$ which inhibited the generation of e.j.p.s and contractions evoked by EFS when added exogenously (Inoue et al 1984, Walter et al 1984). Thus, endogenously released prostaglandins inhibit acetylcholine release from pulmonary parasympathetic nerve endings at a prejunctional level.

In addition, prostaglandins of the F-series cause contraction, whereas, those of the E-series cause relaxation of airway smooth muscle though a direct action on postjunctional prostanoid receptors. (Mathe and Hedqvist 1975, Coleman and Kennedy 1980, Gardiner and Collier 1980).

Unlike the preparations of Blackman and McCaig (1983), our preparations therefore did not have any tone induced by release of endogenous prostaglandins. Thus in order to observe relaxation responses following selective stimulation of the sympathetic nerve trunk via the stellate ganglion the tone had to be raised with a spasmogen. Sympathetic nerve-induced relaxations were completely abolished by propranolol (10^{-5}M) but unaffected by hexamethonium (5×10^{-5}M) indicating stimulation of sympathetic postganglionic fibres which release noradrenaline onto ß-adrenoceptors on the airway smooth muscle. Sympathetic nerve-induced relaxations were found to increase linearly with increase in tone. Relaxations up to a value of approximately 300mmH$_2$O were observed. In previous experiments by other workers (Blackman and McCaig 1983, McCaig 1986) in which endogenous tone was not controlled, relaxations evoked by stimulation of the stellate ganglion were much smaller (<50 mmH$_2$O). Thus, the presence of indomethacin in our preparations allows greater control of the airway smooth muscle tone, which is essential.

A maximum relaxation was obtained at a frequency of 40Hz which is in good agreement with Blackman and McCaig (1983) who found that the size of the depressor responses increased with frequency to a maximum at between 20 and 80Hz. In succeeding experiments involving measurement of sympathetic relaxations, tissues were stimulated at 40Hz as this produced reproducible relaxations at all ILP's. Cunnane and colleagues (Cunnane and Stjarne 1984a, Brock and Cunnane 1987a, 1988) have shown that transmitter release from sympathetic nerves increases with an increase in frequency. This frequency-dependent facilitation can not be explained by a frequency

dependent increase in the amplitude of the action potential of the nerve, nor by an increase in the number of quanta released per secreting site. They suggested that facilitation involves the recruitment of previously silent varicosities (Brock and Cunnane 1988).

In our preparations a slight delay occurred between stimulation of the sympathetic nerve trunk and the evoked relaxation. Although the tracheal smooth muscle contains a relatively high proportion of ß-adrenoceptors, pulmonary innervation is relatively sparse. Therefore, not all airway smooth muscle cells are innervated but they are electrically coupled to each other via gap junctions (Richardson and Ferguson 1979). In addition, transmitter release from sympathetic nerve endings is quantal and occurs intermittently (Burnstock and Holman 1962, Blakely and Cunnane 1979, Cunnane and Stjarne 1982, 1984a,b, Cunnane 1984, 1987, Brock and Cunnane 1987a,b, 1988). Thus, the lapse between sympathetic stimulation and the evoked relaxation may be related to the lack of pulmonary sympathetic innervation and intermittency of transmission, which lead to delays in the build up of noradrenaline concentrations in the synapse. The time lapse may also be sufficient to allow noradrenaline to overflow from sympathetic nerves innervating pulmonary blood vessels and secretory cells.

In contrast, airway smooth muscle is densely innervated by cholinergic nerves and there is no detectable delay between stimulation of the cervical end of the vagi and the resultant contraction. These vagally-induced contractile responses were abolished by both hexamethonium (5×10^{-5}M) and atropine (10^{-7}M) indicating stimulation of preganglionic cholinergic fibres.

The thromboxane analogue U46619, $PGF_{2\alpha}$ and histamine induce contraction of the guinea-pig trachealis muscle via different types of receptors present postjunctionally on the airway smooth muscle. Never-the-less sympathetic nerve-induced relaxations, of the guinea-pig trachea, at any given ILP were comparable when the tone had been raised with any of these three spasmogens.

A postjunctional prostaglandin "contractant" receptor is involved in U46619-induced contraction of the guinea-pig trachea (Gardiner and Collier 1980, Coleman et al 1981). U46619 has also been shown to have a prejunctional action in the airways. It potentiates pulmonary parasympathetic neurotransmission (Chung et al 1985). Although U46619 facilitates noradrenaline release from sympathetic nerves to the rat anococcygeus muscle (Timimi et al 1978) and the rabbit vas deferens (Trachte 1986), its effect on pulmonary sympathetic neurotransmission is unknown.

$PGF_{2\alpha}$ also causes contraction of airway smooth muscle via a "contractant" prostanoid receptor. It has been proposed that distinct receptors exist for each of the five naturally occurring prostaglandins (Coleman et al 1985). Thus, $PGF_{2\alpha}$ and the thromboxane analogue, U46619, may contract the trachea via different specific receptors. $PGF_{2\alpha}$ facilitates sympathetic neurotransmission in vascular smooth muscle and facilitates catecholamine release from the adrenal medulla.

Noradrenergic transmission to effectors, such as, the nictitating membrane, gut and heart were however not potentiated (Brody and Kadowitz 1974). In the airways, $PGF_{2\alpha}$ is known to inhibit pulmonary parasympathetic neurotransmission (Inoue et al 1984, Inoue and Ito 1986), but its effect on noradrenaline release from pulmonary sympathetic nerves has not yet been studied.

Histamine has a dual action on airway smooth muscle, it causes bronchoconstriction through stimulation of H_1 receptors and bronchodilation via H_2 receptors (Chand and DeRoth 1979). In the present experiments the dominant action of histamine was to contract the trachealis muscle. This contraction was not well maintained, an effect which may be due to the opposing bronchodilatory effect of histamine.

In addition, in the airways it has been demonstrated that histamine can both inhibit and potentiate acetylcholine release preganglionically and prejunctionally (postganglionically) from cholinergic nerve endings via different subtypes of histamine receptors (Kikuchi et al 1984, McCaig 1986, Inoue and Ito 1986, Ichinose et al 1989).

Although histamine stimulates transmission in sympathetic ganglia (Trendelburg 1956), it inhibits sympathetic neurotransmission in the saphenous vein, tibial artery and perivascular nerves (McGrath and Shepherd 1976, Ishikawa and Sperelakis 1987).

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The effect of histamine on pulmonary sympathetic neurotransmission is unclear. In the guinea-pig trachea, relaxations induced by sympathetic nerve stimulation were unaltered in the presence of $10^{-6}M$ histamine (McCaig 1986). However, in the same experiments a higher concentration of histamine ($2 \times 10^{-5}M$) was reported to have a dual effect on sympathetic nerve-induced relaxations. At lower frequencies of stimulation it inhibited responses, and at higher frequencies it potentiated sympathetic inhibitory responses (McCaig 1986). The change in ILP of these preparations due to the postjunctional contractile action of histamine was not however taken into consideration. In McCaig's experiments, histamine ($2 \times 10^{-5}M$) was reported to increase the ILP by 13.5 ± 2.7 cmH$_2$O (n=6). It is therefore likely that the observed potentiation was simply due to the increase in tone as we have shown, in the experiments reported in this thesis, that sympathetic nerve-induced relaxations increase linearly as the tone rises.

4.2 EVIDENCE FOR INHIBITORY MUSCARINIC RECEPTORS ON PULMONARY SYMPATHETIC NERVE ENDINGS

4.2.1 Inhibitory Effect of Muscarinic Agonists

Exogenous applied muscarinic agonists have been shown to inhibit release of noradrenaline from postganglionic sympathetic nerve terminals in many different tissues including, the heart of the rabbit (Loffelholz and Muscholl 1970, Fozard and Muscholl 1972), rat (Loiacono et al 1985, Boyle and Pollock 1988), cat (Haeusler et al 1968), chicken (Engel and Loffelholz 1976), dog (Lavellee et al 1970, Levy and Blattberg 1976), and guinea-pig (Muscholl 1980), the dog saphenous vein (Vanhoutte et al 1973), the guinea-pig ileum (Kilbinger and Wessler 1980), the rabbit ear artery (Rand and Varma 1970, Allen et al 1975), rat portal vein (Remie et al 1989, 1990) and the guinea-pig vas deferens (Alberts et al 1981).

Most of the studies involving pulmonary tissue performed so far have involved the measurement of noradrenaline overflow. Inhibition of noradrenaline overflow from the rabbit pulmonary artery by the muscarinic agonist, methacholine, has been observed (Tong et al 1978). Acetylcholine has also been shown to inhibit ^{3}H-noradrenaline overflow following electrical field stimulation from canine tracheal and bronchial strips (Russell and Bartlett 1981). These studies do not exclude the possibility of "contamination" of the airway smooth muscle preparations by vascular smooth muscle, which is more densely innervated by noradrenergic nerves.

In our experiments selective stimulation of pulmonary sympathetic nerves allowed the effect of muscarinic agonists on sympathetic neurotransmission to be examined by investigating their effect on the postjunctional relaxation response of the trachealis smooth muscle.

Sympathetic nerve-induced relaxation responses were reduced in the presence of the muscarinic agonists, acetylcholine and pilocarpine, in a concentration-dependent manner when compared to those obtained at the same ILP in the presence of U46619. As previously mentioned, sympathetic nerve-induced relaxations were comparable in the presence of $PGF_{2\alpha}$, U46619 and histamine, which contract the trachea via different receptors. It therefore seems unlikely that U46619 was potentiating sympathetic nerve-induced relaxations, as that would suggest that these three spasmogens were facilitating sympathetic nerve-induced relaxations to the same extent. Thus, the muscarinic agonists appear to be inhibiting sympathetic relaxations. Sympathetic responses were also inhibited by higher concentrations of carbachol $(3 \times 10^{-7}M)$.

Our next aim was to determine whether this inhibitory effect was due to a postjunctional action on airway smooth muscle or a prejunctional action via activation of neuronal muscarinic receptors to inhibit noradrenaline release from pulmonary sympathetic nerve terminals.

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4.2.2 "Physiological Antagonism"

When two substances interact with different receptor systems present postjunctionally on the effector tissue, to oppose each other, there exists a "physiological" or "functional" antagonism between them. For example, contractile substances such as, U46619 or muscarinic agonists, physiologically oppose the relaxant action of the sympathetic neurotransmitter, noradrenaline, on the trachealis smooth muscle to produce the opposite "physiological" response.

U46619 and the muscarinic agonists acetylcholine, carbachol and pilocarpine evoke contraction of the guinea-pig trachealis muscle via different mechanism involving prostanoid and M_3 muscarinic receptors respectively. One possible explanation for the fact that the muscarinic agonists have an inhibitory effect on sympathetic relaxations, whereas U46619 lacks this effect, is that U46619 and the muscarinic agonists might antagonise the postjunctional relaxant action of noradrenaline by different amounts, that is, U46619 and the muscarinic agonists may exert different degrees of "physiological" antagonism to noradrenaline. This possibility was excluded, in our experiments, as noradrenaline suppressed the postjunctional contractile actions of acetylcholine and U46619 to the same extent.

Therefore in the guinea-pig trachea, muscarinic agonists inhibit sympathetic nerve-induced relaxations via activation of neuronal muscarinic receptors located prejunctionally on sympathetic nerve endings.

4.2.3 Effect of a Nicotinic Antagonist

It was difficult to demonstrate that cabachol was an agonist for neuronal inhibitory muscarinic heteroreceptors. As carbachol has both nicotinic and muscarinic actions, it was possible that it could have been activating both a facilitatory nicotinic and an inhibitory muscarinic cholinoceptor simultaneously. Therefore, all experiments were repeated in the presence of the nicotinic antagonist, hexamethonium.

Hexamethonium selectively blocks nicotinic receptors on ganglion cell bodies compared to those situated postjunctionally on the motor end plate of the neuromuscular junction (Paton and Zaimis 1949). It is therefore often used to exclude ganglionic nicotinic actions of cholinomimetics when investigating the involvement of muscarinic receptors. In addition, hexamethonium has been shown to have a prejunctional action at the neuromuscular junctions via an action on a facilitatory nicotinic autoreceptor (Bowman et al 1984). These receptors appear to be different from those present postjunctionally, as hexamethonium, although weak, was 250 fold more potent at antagonising pre- than postjunctional nicotinic receptors at the neuromuscular junction (Wessler 1989).

Preincubation with hexamethonium did not antagonise sympathetic nerve-induced relaxations indicating stimulation of postganglionic nerve fibres. Hexamethonium had no significant effect on the postjunctional contractile actions or sympathetic responses in the presence of several spasmogens, including the muscarinic agonist carbachol. The inhibitory

effect of the muscarinic agonists on sympathetic neurotransmission was also unaltered by hexamethonium.

Therefore, the involvement of a facilitatory hexamethonium sensitive nicotinic receptor was excluded.

Hexamethonium was not used in succeeding experiments as it has been shown to have both anticholinesterase and weak muscarinic antagonist properties, both allosteric and competitive, which vary according to the agonist, antagonist and tissue used (Barlow et al 1972, Leung and Mitchelson 1982, Clague et al 1985, Eglen et al 1989). In functional and radioligand binding studies for example, hexamethonium appears to exhibit at least a 10 fold selectivity for cardiac M_2 as opposed to smooth muscle/glandular M_3 receptors, but can not distinguish between peripheral M_1 and M_2 receptors (Eglen et al 1989).

4.3 EVIDENCE FOR ACTIVATION OF INHIBITORY MUSCARINIC HETERORECEPTORS BY ENDOGENOUSLY RELEASED ACETYLCHOLINE

4.3.1 Inhibitory Effect of Concurrent Vagal Stimulation

It was important to establish whether the inhibitory muscarinic receptors on pulmonary sympathetic nerve endings could be activated by acetylcholine released under physiological conditions from the adjacent cholinergic nerves.

Using discrete stimulation of the pulmonary parasympathetic and sympathetic nerve trunk, we were able to confirm this interaction. Concurrent stimulation of the vagal nerve trunk inhibited sympathetic nerve-induced relaxations. The involvement of postjunctional antagonism was excluded by stimulating the vagal nerve trunk at parameters which caused no increase in ILP. Stimulation of the vagal nerve trunk, not only stimulates the vagi, but also NANC nerves. The possibility that the inhibitory effect of concurrent vagal stimulation was due to release of neuropeptides from NANC nerves is however unlikely, as when VIP, neurokinin A, substance P and endothelin were administered exogenously, they did not inhibit sympathetic nerve-induced relaxations. Thus, in the airways it appears that endogenously released acetylcholine can activate inhibitory muscarinic receptors present on the adjacent sympathetic nerve endings.

We also confirmed the reverse relationship, that is, prejunctional inhibition of vagally-induced contractile responses via activation of predominately ß-adrenoceptors by endogenously noradrenaline released following concurrent stimulation of the sympathetic nerve

trunk. These findings are consistent with the observations that in the airways, the two branches of the autonomic nervous system lie in close proximity and may interconnect (Jones et al 1980b, Daniel et al 1986, Daniel 1988).

4.3.2   Effect of Anticholinesterase Drugs

As we had shown that endogenous acetylcholine could inhibit sympathetic neurotransmission, it was interesting to see the effect of an anticholinesterase on this interaction. Anticholinesterases such as, physostigmine and neostigmine, cause contraction of the trachealis muscle. It has been suggested that this contractile effect may not depend totally on their ability to prevent breakdown of acetylcholine. Physostigmine evoked contraction of the guinea-pig tracheal muscle may also be due to potentiation of acetylcholine release from cholinergic nerve endings (Douglas 1951, Carlyle 1963, Kirkpatrick and Rooney 1979).

Carlyle (1963) observed that neostigmine and physostigmine still contracted the guinea-pig trachea after pretreatment with high concentrations of the anticholinesterase diisopropylphosphodiamidic fluoride which did not itself cause any further contraction. These contractile responses were inhibited by factors known to inhibit release of acetylcholine from nerve endings, such as, anaesthetic, cooling, ionic changes, and hemicholinium.

Although, potentiation of vagally-induced contractions of the guinea-pig trachea by the anticholinesterases, neostigmine and physostigmine has

been reported (McCaig 1986, Widmark and Waldeck 1986), other workers have reported inhibition of potassium evoked release of ^{3}H-acetylcholine from the rat bronchi by the anticholinesterase soman (Aas et al 1986,1987).

In our experiments, the effect of the anticholinesterase, physostigmine, on vagally-induced contractions of the guinea-pig trachea was not clear cut. Contractile responses elicited by stimulation of the vagal nerve trunk were potentiated and inhibited in the presence of 10^{-7} and 10^{-6}M physostigmine, respectively. At a concentration of 3×10^{-7}M, physostigmine in some preparations produced an increase, and in others a decrease in vagally-induced contractions. Thus mean results showed a large scatter. Aas (1988) showed that long term exposure to the anticholinesterase, soman, reduces the acetylcholine-induced contraction of the rat bronchi, probably via a reduction in the number of muscarinic receptors. However, the inhibition of vagally-induced contractions by 10^{-6}M physostigmine, in our experiments, does not appear to be due to desensitization of postjunctional muscarinic receptors, as 10^{-6}M physostigmine caused a greater increase in the postjunctional contractile action of 10^{-5}M acetylcholine than 10^{-7}M physostigmine. The inhibitory effect is therefore, probably a result of acetylcholine accumulating in the synapse and feeding back onto muscarinic autoreceptors on the cholinergic nerve endings to inhibit its own release. This complication has previously been observed in gut experiments involving measurement of acetylcholine release in the presence of anticholinesterases to prevent its breakdown (Kilbinger 1984).

Widmark and Waldeck (1986) reported a facilitatory effect of physostigmine on vagally-induced contractions of the guinea-pig trachea. The disagreement between the effects of physostigmine (10^{-6}M) on vagally-induced contractions observed in the present experiments and those performed by Widmark and Waldeck (1986) may be attributed to the length of time the tissue was left in contact with the anticholinesterase. In the present experiments, physostigmine was administered cumulatively and was allowed to stay in contact with the tissue for much longer, during which time the concentration of acetylcholine may have built up to such an extent that it inhibited its own release via prejunctional muscarinic receptors.

In the presence of the larger concentration of physostigmine (10^{-6}M), sympathetic nerve-induced relaxations were also attenuated. This inhibitory effect of physostigmine was blocked by atropine (10^{-7}M). Similarly, an inhibitory effect of the anticholinesterase neostigmine was observed on sympathetic relaxations which was shown to be reduced by atropine (M[c]Caig 1986). The inhibitory effect of the anticholinesterases on sympathetic responses is therefore likely to be due to accumulation of acetylcholine released spontaneously from pulmonary parasympathetic nerves which activates an inhibitory muscarinic heteroreceptor on the nearby sympathetic nerve terminals.

These results reinforce the suggestion that endogenously released acetylcholine can inhibit sympathetic neurotransmission in the guinea-pig trachea. The anticholinesterase potentiated this inhibitory effect.

4.4 CLASSIFICATION OF NEURONAL MUSCARINIC HETERORECEPTORS

Muscarinic cholinoceptors were initially classified as M_1 or M_2 receptors depending on the receptor binding and pharmacological profile of pirenzepine. Receptors with a high affinity for pirenzepine were designated M_1 and those with a low affinity as M_2 receptors. With the discovery of more selective antagonists M_2 receptors were further subdivided into M_2 and M_3 receptors. M_2 receptors are predominately found in cardiac tissue and are selectively blocked by gallamine, AFDX116, himbacine and methoctramine (Brown and Crout 1970, Giachetti et al 1986, Melchiorre et al 1987), whereas M_3 receptors are found on smooth muscle and glands, and are selectively antagonised by 4-DAMP and hexahydrosiladifenidol (Barlow and Kitchen 1982, Mutschler and Lambrecht 1984).

The presence of more than one subtype of muscarinic receptors have now been recognised in many tissues including the guinea-pig trachea (Eglen and Whiting 1986, Mitchelson 1989, Barnes et al 1988). The muscarinic receptors present postjunctionally on guinea-pig airway smooth muscle which mediate contraction, are classified as M_3 because they are blocked by 4-DAMP and hexahydrosiladifenidol. In contrast, neural muscarinic autoreceptors which inhibit acetylcholine release from pulmonary parasympathetic nerve endings of the cat, guinea-pig and rat are sensitive to gallamine and methoctramine and are therefore classified as M_2 (Fryer and Maclagan 1984,1987, Faulkner et al 1986, Watson et al 1989).

In our experiments atropine which universally blocks all muscarinic receptors, antagonised the inhibitory effect of the muscarinic agonists on sympathetic nerve-induced relaxations confirming the involvement of a muscarinic receptor. In contrast, the M_2 antagonists gallamine and methoctramine depressed neither the postjunctional contractile action of acetylcholine nor its inhibitory effect on sympathetic relaxations. Thus, M_2 receptors do not appear to be involved in the inhibition of sympathetic neurotransmission in the airways and the prejunctional heteroreceptors on pulmonary sympathetic nerve ending differ from the muscarinic autoreceptors present on pulmonary parasymathetic nerve terminals, which are of the M_2 subtype. It also seems unlikely that the inhibitory action of a muscarinic agonist such as, acetylcholine, is mediated via M_1 receptors, as M_1 receptor blocking concentrations of pirenzepine did not alter the inhibitory effect of acetylcholine on sympathetic neurotransmission.

The inhibitory effect of acetylcholine on sympathetic nerve-induced relaxations was, however, sensitive to the M_3 receptor antagonist hexahydrosiladifenidol and higher concentrations of pirenzepine which also blocked the postjunctional contractile action of acetylcholine mediated via M_3 muscarinic receptors.

We were unable to study the effect of muscarinic antagonists on the inhibitory effect of concurrent vagal stimulation on sympathetic neurotransmission, because this inhibitory effect was not reproducible in any one tissue, decreased with successive periods of stimulation and was very variable between tissues.

4.5   CONCLUSIONS

These results suggest that in the guinea-pig trachea, muscarinic agonists inhibit sympathetic neurotransmission via prejunctional M_3 muscarinic receptors on sympathetic nerve terminals.

These inhibitory muscarinic receptors thus appear to resemble those previously described on the cholinergic nerves innervating the guinea-pig ileum. In this tissue both the prejunctional autoreceptors and the postjunctional muscarinic receptors are sensitive to hexahydro-siladifenidol and are therefore of the M_3 subtype (Fuder et al 1985).

In the airways, if the inhibitory M_3 muscarinic receptors on the sympathetic nerves are activated *in vivo* by endogenous acetylcholine released from the adjacent cholinergic nerves, a selective M_3 antagonist would, decrease the bronchoconstrictor action of the parasympathetic nervous system on airway smooth muscle via blockade of postjunctional M_3 muscarinic receptors and remove the prejunctional inhibitory effect of the parasympathetic nervous on sympathetic neurotransmission, that is, have a sympathoexcitatory effect. Both of these effects would contribute to bronchodilation and would be clinically benificial.

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