The effect of phorbol ester on airway smooth muscle reactivity.

A thesis presented for the degree of
Master of Science

by

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Declaration

The experimental work and other research contained within this thesis was undertaken wholly by myself, with the technical assistance of Mrs Louise Young. Some of the results have been published during this period of study, details of which are given below.

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Summary

In asthma the airways demonstrate hyperreactivity to a variety of stimuli including acetylcholine and histamine (Hargreave et al., 1981). The mechanism accounting for this heightened airway responsiveness is unknown but could be explained by an abnormality in airway smooth muscle function (Barnes et al., 1988).

The histamine (H1) and muscarinic cholinergic receptors, are coupled to contraction by the phosphatidylinositol pathway. Agonist-receptor binding results in the formation of the intracellular second messengers, diacylglycerol (DAG) and inositol triphosphate. DAG activates phospholipid-dependent, calcium-activated protein kinase (protein kinase C; PKC) which is thought to be involved in the sustained phase of smooth muscle contraction (Rasmussen & Barratt, 1984; Forder et al., 1985; Chattergee & Tejada, 1986).

It has been suggested that the inappropriate activation of smooth muscle PKC may be involved in the pathogenesis of the late phase in asthma (Obianime et al., 1989) in which the airways remain hyperreactive to a number of endogenous agonists for a period of 6-12 hours after the triggering of an asthmatic attack (Cartier et al., 1982). This study was undertaken to examine the consequences of sustained activation of PKC on the reactivity of isolated rabbit and human airways.

Phorbol myristate acetate (PMA), a potent phorbol ester which can substitute for DAG (Castagna et al., 1982; Yamanishi et al.,1983), was used to directly activate PKC. The effect of incubation with PMA was examined on the contractile response to electrical field stimulation and to the endogenous agonists acetylcholine and histamine. Bronchial rings were suspended in baths containing oxygenated Krebs-Henseleit solution and changes in tension were measured isometrically.

PMA (1-100μM) potentiated cholinergic neurotransmission. The contractile response to electrical field stimulation (EFS) at 16Hz was increased in a concentration- and time-dependent manner in both human and rabbit bronchial rings, reaching a plateau at approximately 45-60 minutes. At this time point responses in the presence of PMA (10μM) were significantly greater than in the time control tissues. In the rabbit preparation both control and potentiated responses were abolished by atropine (0.1μM). Potentiation was absent after incubation with the calcium channel blocker verapamil (10μM) and reduced after the putative PKC inhibitors H7 (10μM) and staurosporine (0.5μM).

At 45 minutes into incubation, PMA (10μM but not 1μM) significantly enhanced the contractile response of rabbit bronchi to individual concentrations of ACh (0.1-30μM) within a cumulative ACh concentration response curve (0.01-300μM). There was a small leftward shift in the curve but no increase in the maximum response to ACh. The enhancement is thus of the type that would be expected of a synergism taking place beyond the receptor, rather than an increase in sensitivity of receptors. Repeating the experiment in a low calcium environment produced a rightward shift in control responses which was not seen in PMA-incubated tissues, indicating that PMA may produce an increase in the sensitivity of the preparation.

Against sequential responses (at 15 minute intervals starting 45 minutes into the incubation period) to a single sub-maximal concentration of Ach (10μM), PMA (10μM but not 1μM) significantly enhanced the first response in rabbit and human bronchi but the second and subsequent responses were not significantly greater than time controls. In the rabbit preparation the increase in response to ACh was inhibited by the calcium channel blockers nifedipine (0.3μM) and verapamil (10μM), implicating calcium influx via voltage-dependent channels in the response, and attenuated by amiloride (100μM), an inhibitor of Na^+/H^+ exchange. In contrast to their effects versus EFS, the protein kinase-C antagonists H7 (10μM) and

staurosporine (0.5μM) did not prevent the PMA-induced potentiation of response to ACh. The cyclo-oxygenase inhibitor indomethacin (10μM) had no effect on the PMA-induced potentiation of response but itself produced a small but significant increase in response to ACh indicating an ongoing inhibitory influence of a cyclo-oxygenase product versus neurotransmission and/or contraction.

The response of rabbit bronchial smooth muscle to a submaximal concentration of histamine (10μM) was also significantly increased during incubation with PMA, indicating that PMA may induce a change in intracellular coupling rather than an effect on (two sets of) receptors. Following washout in fresh Krebs second and third responses remained elevated.

These results demonstrate that PMA can cause increased reactivity of rabbit and human airway smooth muscle to cholinergic nerve activation and to stimulation by ACh and histamine. They suggest that phorbol ester-induced activation of PKC is responsible, and that the potentiation of response is mediated, at least in part, by increased fluxes of calcium and sodium ions and is independent of prostaglandin production. The findings, therefore, support the hypothesis that sustained activation of PKC *in vivo* could contribute to airway hyperreactivity to contractile stimuli.

Sections 1-4

INTRODUCTION

1.1 Asthma

Asthma is a reversible, obstructive disease characterised by increased responsiveness of the airways. It is one of the most common chronic diseases in industrialised countries and there is evidence that the prevalence and severity are increasing (Ayres, 1986; Fleming, 1987). Despite advances in therapy, asthma deaths in the 5- to 34-year-old age group have risen over the last decade in the UK by an average of 4.7% per annum, at a time when mortality from most other treatable diseases is declining (Burney, 1986, 1987).

1.2 Bronchial hyperreactivity

A cardinal feature of asthma is the hyperreactivity of the airways to a wide variety of stimuli that do not produce this response in non-asthmatic subjects. Bronchial hyperreactivity is virtually universal in asthma and the extent of this hyperreactivity seems to correlate well with the clinical severity of the condition (Cockcroft et al., 1977). In the absence of a "gold standard" test for asthma (the disease shares several characteristics with chronic bronchitis and emphysema), tests of bronchial hyperreactivity have often been employed (ATS committee report, 1962). In their review of allergic asthma, Jacobs and Kaliner (1989) conclude that the presence of airway hyperreactivity is necessary for asthma to develop but that it is not itself sufficient to produce asthma. Although the precise cause of the hyperreactivity is not known it is likely that there is no single factor and that several interact to produce the effect. Possible mechanisms postulated to produce an increase in reactivity include an alteration in the "normal" neural pathways or in the epithelium or smooth muscle of the airways.

The degree of hyperreactivity is not constant and may increase after upper respiratory tract viral infections (Empey et al., 1976) or after exposure to air pollutants

(Holtzman et al., 1979) or allergens (Boulet et al., 1983). Recent studies have suggested that inflammation may play an important role in the development of bronchial hyperreactivity via the interaction of inflammatory cells, mediators and nerves with the components of the airway wall (Barnes, 1989). Histopathologic examination of the airways of asthmatic subjects show a marked airway inflammation, with an infiltration of inflammatory cells, particularly eosinophils. These cells, along with neutrophils and platelets, have been implicated in the modulation of bronchial hyperreactivity (Nadel, 1984; Chung, 1986). Neutrophil and eosinophil products have recently been shown to increase airway responsiveness in isolated human bronchial tissue (Hallahan et al., 1990).

A wide range of inflammatory mediators have been implicated in the pathogenesis of bronchial hyperreactivity, and of asthma, including histamine, prostanoids, leukotrienes, PAF, adenosine, bradykinin, and sensory neuropeptides. These mediators produce their effects by activating specific cell surface receptors which are localised to a variety of cells. Receptor activation may result in contraction of airway smooth muscle, mucus and fluid secretion, microvascular leakiness, chemotaxis of inflammatory cells and neuronal activation (Barnes, 1987). The mucus hypersecretion and increase in vascular permeability results in oedema and exudation of plasma. The resultant mucosal thickening leads to a narrowing of the airways, altering the airway geometry such that the same degree of constriction produces a greater narrowing of the airway lumen (Moreno et al., 1986).

Epithelial damage is a prominent feature of asthma and is implicated in the development of bronchial hyperreactivity. Both the common cold and the influenza virus damage the airway epithelium and can produce hyperreactivity to histamine or methacholine (Empey et al., 1976). Epithelial damage can also be caused by mediators released from the inflammatory cells commonly found in asthmatic airways. Eosinophils secrete a variety of toxic substances including major basic

protein (MBP) which causes death and shedding of epithelial cells (Gleich et al., 1988). Oxygen-derived free radicals produced by neutrophils are similarly toxic to the epithelium.

Epithelial damage is important as this cell layer performs a variety of essential functions. As well as its role in mucociliary clearance and the provision of a physical barrier against noxious agents, the epithelium modulates the environment of the underlying smooth muscle via the metabolism and regulation of mediators and the production of relaxant, constrictor or chemotactic factors. Damage to the epithelium may also expose the underlying sensory nerves which may result in exaggerated vagal reflex reactions. It is also possible that functional biochemical abnormalities may be present in the epithelial cells of asthmatics resulting in the characteristic bronchial hyperreactivity (Cuss & Barnes, 1987).

An imbalance in the neural control of smooth muscle, an increase in excitatory input (cholinergic or a-adrenergic) or conversly a decrease in inhibitory (b-adrenergic or non-adrenergic non-cholinergic (NANC)) input to airway smooth muscle, could result in an increased reactivity of the airways. Inflammatory mediators may influence neurotransmitter release from airway nerves and conversely neurotransmitters may be pro-inflammatory (Barnes, 1989a). Similarly, neuropeptides in capsaicin-sensitive sensory nerves, such as substance P, have potent pro-inflammatory effects. If these are released by an axon reflex, they may amplify the inflammatory response in asthma (termed neurogenic inflammation; Barnes et al., 1990).

Although any one of these mechanisms may dominate in a particular situation it seems unlikely that a single mechanism will explain the cause of airway narrowing in every case. Rather, it is more likely that the hyperreactivity results from an interplay of several of the above mentioned mechanisms and the underlying smooth muscle.

Smooth muscle hypertrophy or hyperplasia is common in asthmatic airway (Dunnil et al., 1969; Heard & Hossain, 1973) and while the increased amount of muscle can exert a greater degree of force and therefore constriction, a more significant change may be in the cell-to-cell signalling or excitation-contraction coupling mechanisms at the intracellular level.

Possible defects in smooth muscle that could result in hyperreactivity include a change in the number of gap junctions, altered nervous control or a change in the number of receptors, altered excitation-contraction coupling or an increased sensitivity of the contractile proteins themselves. Similarly, that asthmatic airways show an exaggerated response to a variety of different spasmogens utilizing many different receptors suggests that the possible abnormality lies beyond the receptors, in the smooth muscle itself. It is possible that there is a change in the regulation of contraction/relaxation in the smooth muscle. Alternatively there could be an enhanced coupling of many receptors, perhaps by an alteration in phospholinositol hydrolysis, a system which is utilised in excitation-contraction coupling of many of the receptor systems in smooth muscle (Barnes, 1987). A defect in this, final, pathway of contraction provides a possible mechanism for the non-specific bronchial hyperreactivity of asthma.

This project was undertaken with the funding of the National Asthma Council to study the role of protein kinase C, an integral component of the phosphotidyl inositol pathway, in the development of bronchial hyperreactivity, in animal and in human *in vitro* preparations.

Section 2

CHARACTERISTICS OF THE BRONCHI

2.1 Nerve supply to the bronchi

All the nerves leading to the trachea and bronchi are made up of a mixture of afferent and efferent fibres, a parasympathetic supply from the vagus and a sympathetic supply from the neighbouring sympathetic chain. On entering the lung the fibres become entwined in a plexus behind the hilum of each lung, from which nerve fibres distribute to the airways. Neural control is especially important in the 4-7th (medium-sized) bronchi which have a denser innervation than either the trachea or the periphery (Daniel et al., 1986). In a systematic electron micrograph study of bovine tracheal smooth muscle Cameron & Kirkpatrick (1977) reported an axon/muscle cell ratio of 1/28. None of the nerves came within 200nm of the muscle and discounting those further than 1000nm away they obtained a ratio of 1/90. In medium-sized bronchi the density of nerve supply is approximately twenty times that of the trachea, i.e. 18 axons per 100 muscle cells (Daniel et al., 1986). Nerve bundles are found in trachea and in the bronchiolar regions but the alveoli are also innervated. The bundles run mainly within the intramuscular septa rather than in the muscle itself, and are arranged parallel to the muscle (Gabella, 1989).

Ganglia lie outside the smooth muscle layer in the bronchial wall. Their complex structure includes several types of neuronal bodies including cholinergic, adrenergic and non-adrenergic non-cholinergic (NANC) elements, which interact to integrate and coordinate the neural input to smooth muscle (Coburn, 1987; Dey et al., 1981).

Cholinergic innervation

The principal input, and main excitatory input, to the lungs is cholinergic (Widdicome, 1963; Foster, 1964). The non-myelinated post-ganglionic cholinergic fibres may be activated reflexly by sensory receptor stimulation or pre-junctionally by inflammatory mediators. Muscarinic receptors are found principally in central airways

and rapidly decrease in number towards the periphery (Barnes et al., 1983). They have been subdivided pharmacologically into M_1 (neuronal), M_2 (cardiac) and M_3 (glandular & smooth muscle) subtypes. M_1 receptors are located on airway ganglia where they may have a facilitatory effect on ganglion neurotransmission (Lammers et al., 1989). Pre-synaptic M_2 autoreceptors on nerve terminals inhibit release of ACh (Minette & Barnes, 1988) and the smooth muscle M_3 receptors mediate contraction (Barnes, 1989b).

Adrenergic innervation

A small alpha-adrenergic excitatory effect can be seen in the rabbit *in vivo* (Mustafa et al., 1982) and in human tracheal smooth muscle *in vitro* (Kneussel & Richardson, 1978). Adrenergic fibres are scarce in the bronchi of rabbit (Mann, 1971) and human (Pack & Richardson, 1984; Laitinen, 1985) but are abundant in bronchial muscle of cat and dog (Silva & Ross, 1974; Knight et al., 1980). Some adrenergic fibres are closely associated with cholinergic nerves or terminate on parasympathetic ganglia and may modulate the transmission via α2-adrenoceptor effects (Jacobowitz et al., 1973; Knight et al., 1980; Daniel et al., 1986). Mammalian lung has a high density of β-receptors and these increase in density from the trachea to the terminal bronchioles (Barnes et al., 1983). Autoradiographic techniques indicate that only β2-receptors are present on human airway smooth muscle and this subspecies mediates all relaxation of central and peripheral airways in vitro (Carstairs et al., 1985; Goldie et al., 1984). However, adrenergic blockade has no effect on the inhibitory component of the response of human bronchial to EFS *in vitro*, indicating that there is no functional sympathetic innervation of smooth muscle (Doige & Satchell, 1982; Richardson & Beland, 1976; Davis et al., 1982). Subsequent immunohistochemical studies confirmed this supposition, showing that no adrenergic nerve terminals terminated in smooth muscle (Partanen et al., 1982; Sheppard et al., 1983). In

guinea-pig airways adrenoceptor-mediated relaxation is seen in the trachea but not in the bronchus or bronchi (Doige & Satchell, 1982; Grunstrom et al., 1981).

NANC innervation

A second inhibitory component reaches the airways via the vagus nerve and is present mainly in proximal airways. This NANC response has been demonstrated in guinea pig (Coburn & Tomita, 1973; Coleman & Levy, 1974) and in human (Richardson & Beland, 1976) and is the principal inhibitory pathway in human airways (Barnes, 1986a).

The principal candidate for mediation of the NANC inhibitory response is VIP, a potent relaxant of human smooth muscle (Palmer et al., 1985a). VIP receptors have been localised to airway epithelium, submucosal glands and smooth muscle (Barnes & Carstairs, 1986). The density of VIP-containing nerves diminishes in the small airways (Dey et al., 1981) and ultrastructural studies suggest that VIP may co-exist in the same nerve terminals as ACh (Schultzberg et al., 1982). A second peptide, peptide histidine isoleucine (PHI), has also been implicated in the NANC response, shares an identical distribution in the airways with VIP (Lundberg et al., 1984) and is possibly released with VIP. A related peptide, peptide histidine methionine (PHM), has been found in human airways and may mediate the NANC response in man.

NANC excitatory nerves containing substance P, calcitonin gene related peptide and other tachykinins have been identified in human and animal airways (Barnes, 1986b) and receptors for substance P have been localised to smooth muscle and submucosal glands (Carstairs & Barnes, 1986). The release of tachykinins upon sensory nerve stimulation suggests a possible involvement in local axon reflexes.

Afferent nerves

Afferent fibres are carried in the vagus from sensory receptors in the surface epithelium and submucosa including rapidly adapting cough and irritant receptors, slowly adapting stretch receptors and juxtacapillary receptors deep in the lung. These fibres may mediate an axon reflex, which can be induced in human bronchi *in vitro* (Lundberg et al., 1983), resulting in smooth muscle contraction and an increase in vascular permeability.

2.2 *Neural control of the bronchi*

The degree of neural control of the bronchi varies considerably between species. The smooth muscle may be organised as a multi-unit structure, with a very dense innervation and many nerve fibres per muscle cell, or as a single-unit structure which has good electrical coupling via many gap junctions, allowing simultaneous contraction of blocks of muscle fibres, and has spontaneous oscillations of membrane potential. Gap junction connections are seen in human airways (2.7 junctions per 100 muscle cells; Daniel et al., 1986). However the smooth muscle is only sparsely innervated and has no spontaneous activity (Stephens et al., 1986; Cameron & Kirkpatrick, 1977) and may therefore be considered an intermediate structural type, neither multi- nor single-unit.

The number and size of gap junctions can change with stimulation and with time, within minutes in human tracheal smooth muscle *in vitro* (Daniel et al., 1986; Kroeger & Stephens, 1975; Kannan & Daniel, 1978). Such a structural change *in vivo* could be important in the development of bronchial hyperreactivity as movement from a multi- to a single-unit structure would increase the excitability of airway smooth muscle.

2.3 Musculature

The muscular system of the lung is entirely composed of smooth muscle (Fig. 2.1), which can be divided into three morphological groups: (tracheo)bronchial, pulmonary and interstitial.

The course and location of tracheal muscle differs according to the species and the region of the trachea examined. In man muscle is only seen in the membranous part for most of the length of the trachea, where strong muscle bundles of approximately 300-400 cells each (Stephens, 1988) connect the dorsal ends of the tracheal cartilages, but near its bifurcation annular (ring-like) muscles surround the entire circumference. This differs from the arrangement in dog where the trachea is surrounded by strong annular muscles over its entire length. The insertion of muscle on the cartilage is internal in man but external in the rabbit. External to the layer of muscle of the membranous part of the trachea is a small quantity of longitudinal muscle.

The bronchi are defined as those airways that have cartilage in their walls. Bronchial muscle is situated in the airway wall from the main bronchi to the terminal bronchioles. In man the muscles of the main bronchi are somewhat similar to those of the inferior portion of the trachea; thus the lumen becomes smaller mainly due to contraction of the muscle of the membranous part. As the bronchi get smaller the muscle changes its course from annular to spiral and this tendency is increased in the more peripheral regions. The smooth muscle cells are spindle shaped, approximately 1000μm long and 5-10μm at the widest part. They have a single, central, cigar-shaped nucleus and are unbranched, unlike those of the membranous part of the trachea which may have branches and anastomoses. The smooth muscle is arranged in spiral strands around the airway and completely encircles the bronchi internal to the cartilagenous plates. The larger bronchi have two sets of smooth muscles, opposing

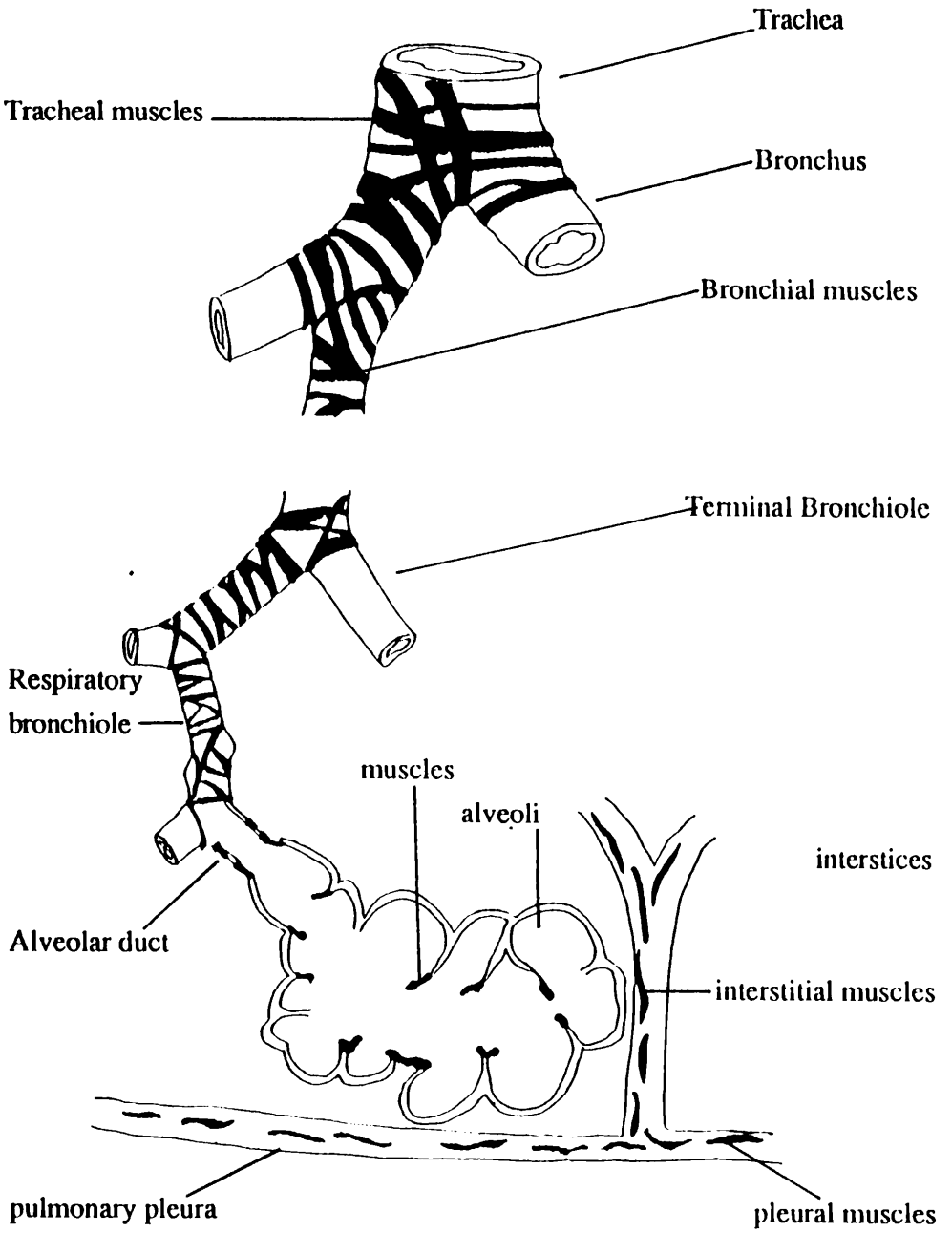


Figure 2.1 The Musculature of the Airways.

(from Nagaishi et al., 1972)

right and left spirals, surrounded by elastin and collagen and with nerve fibres and blood vessels running in the connective tissue between the strands. Contraction of these muscles results in the narrowing and shortening of the airway. In the peripheral bronchi the mucus membrane may produce shelf-like folds during muscle contraction. When the contraction of the muscle becomes stronger, the formation of folds becomes even more evident, the surfaces of the membrane folds touch each other and the circulation of air may be suspended temporarily.

Pulmonary muscle refers to muscles which are distributed in alveolar regions. From the respiratory bronchiole to the alveolar duct the muscle spirals become elongated, and in the more peripheral regions near the alveoli the muscle is net-like and surrounds the alveolar rings in a sphincterlike formation. This arrangement of muscles has been termed a "geodesic network" (Nagaishi et al., 1972; shortest distance between two points on a spherical surface).

2.4 Electrical Properties of smooth muscle

Airway smooth muscle cells respond to neurotransmitter substances, bronchoactive autocoids or depolarisation by KCl with a graded increase in membrane potential rather than by producing spike depolarisations, indeed many mediators produce a contraction without significantly changing the membrane potential (Bolton, 1979; Middleton, 1984). Under normal conditions no spontaneous electrical activity is observed. This electrical quiescence is due to voltage-sensitive potassium channels which act to stabilize the membrane potential. As the muscle depolarises, these channels open thereby increasing membrane potassium conductance and limiting further depolarisation. There is evidence for the presence of at least two types of voltage-sensitive delayed rectifier potassium channels in the canine airway smooth muscle cell (Kotlikoff, 1987, 1989). Voltage-activated calcium channels will also open when the cell is depolarised from its resting potential but the depolarisation that

attends this inward current is blunted by the almost simultaneous activation of the outward currents. Unless the rise is tightly compartmentalised, it is likely that calcium-activated potassium channels are also activated whenever the cytosolic calcium concentration rises (Kotlikoff, 1989). Electrical activity is increased when rectification is reduced by potassium channel inhibitors such as tetra-ethylammonium (TEA; Tomita, 1989).

Depolarisation is conducted through the muscle bundle with relatively little decrement, thus changes in membrane potential are conducted down the preparation by cable-like spread (Kirkpatrick, 1981). The small amount of depolarisation may be sufficient to intitiate the contractile process as the membrane need only be depolarised by 5mV before contraction (Suzuki et al., 1976).

Although tracheal smooth muscle generally has a relatively low level of excitability, the degree of spontaneous activity varies between species and even within species with location in the bronchial tree. Canine trachea, a commonly used preparation in electrophysiological studies, has a stable membrane potential of approximately -54 to -60mV (Farley & Miles, 1977; Kannan et al., 1983; Kroeger & Stephens, 1975) while the membrane potential of the bronchi is reported to be 3mV higher (Souhrada et al., 1983). In contrast, human tracheal smooth muscle has a higher muscle tone and degree of rhythmic electric activity, consisting of oscillatory fluctuations of membrane potential (slow waves). Tracheal smooth muscle strips obtained from autopsy had a resting potential of -45mV and exhibited slow waves with a frequency of 19.7 waves per minute (approximately 0.3Hz) and an amplitude of 7.7mV (Honda & Tomita, 1987). Slow waves are converted to spikelike action potentials when rectification is reduced by TEA (Kroeger & Stephens, 1975; Kannan et al., 1983). Tone and activity seem to be related and both are likely to be produced by endogenous arachidonate derivatives: prostaglandins in the guinea pig and leukotrienes in Man (Tomita, 1989).

Section 3

CONTRACTION OF AIRWAY SMOOTH MUSCLE

3.1 Contraction of bronchial smooth muscle

Smooth muscle cells contain the contractile proteins actin, myosin and tropomyosin. The contractile elements are the same as those of striated muscle (although lacking in troponin), but the amount of myosin present in airway smooth muscle is only a fifth of that in striated muscle and the relative proportion of the two proteins is different (approximately 13 actin to 1 myosin instead of 6 to 1; Dillon & Murphy, 1982).

The actin filaments form a helix of 2 thin polymer chains. Myosin is a thicker filament made up of two heavy chains (200,000 Da each) and two pairs of light chains (one pair each of 17,000 and 20,000 Da) forming the head of the molecule (Rodger, 1988). Although the contractile elements of airway smooth muscle are not arranged in regular sarcomeres as in skeletal smooth muscle, individual myofibrils are striated and electron micrographs of airway smooth muscle show the presence of dark bands in the sarcolemma. Actin is attached to the cell membrane at these points and also to similar structures in the cytoplasm known as dense bodies (Stephens, 1987). Somylo *et al.* (1984) have suggested that these structures are not haphazardly arranged but that they show periodicity and can be regarded as analogous to the Z-bands of striated muscle. The contractile elements lie in parallel and crossbridges may be seen connecting the strands of actin and myosin. It is possible that the basic mechanism of contraction is similar in both striated and smooth muscle and that the sliding filament model of Huxley and Neidergerke (1954) may be applicable to smooth muscle. According to this theory, actin is actively transported along the myosin molecule by attachment, flexion and detachment of the myosin head.

In striated muscle this process is regulated by calcium. The interaction of actin and myosin is inhibited by troponin via its effect on the location of tropomyosin on the thin filament. The binding of calcium to troponin results in a change in the structure

of the thin filament (Squire, 1981), the interaction of actin and myosin, hydrolysis of ATP and the movement of actin past myosin.

Similarly, contraction in non-striated muscle is regulated by calcium. While smooth muscle does not possess troponin, tropomyosin and caldesmon may play similar role in the regulation of actin (de Lanerolle, 1989). The important reaction in the regulation of smooth muscle contraction appears to be the phosphorylation of the 20kDa subunits of myosin light chain (MLC) which stimulates the actin activated Mg^{2+}ATP-activity of the myosin (Sobieszek, 1977) (*see below*) unlike striated muscle which is regulated by the actin-linked troponin-tropomyosin system.

3.2 Control of smooth muscle contraction.

The state of activation of the airway smooth muscle cell is controlled by two calcium-dependent mechanisms which are activated by receptor occupation.

Agonist binding at membrane receptors results in the cleavage of inositol phospholipids into two second messengers, diacylglycerol (DAG) and inositol triphosphate (I-1,4,5-P_3), by membrane-bound phospholipase C (*section 4.1*). I-1,4,5-P_3 releases calcium from intracellular stores while DAG activates protein kinase C (PKC) (Castagna et al., 1982; Nishizuka, 1986). The I-1,4,5-P_3-induced release of calcium is important in the immediate, transient response to agonist-receptor binding whereas it is thought that activation of PKC is involved in the sustained phase of the subsequent contraction (Rasmussen & Barratt, 1984; Forder et al., 1985; Chattergee & Tejada, 1986). Neither second messenger alone is sufficient for complete signal transduction and it has been shown in several tissues that calcium mobilization and activation of PKC act synergistically to produce the full physiological response (Yamanishi et al., 1983; Nishizuka, 1984 & 1986; Baraban et al., 1985; Park & Rasmussen, 1985).

The signal initiating contraction in smooth muscle appears to be the I-1,4,5-P_3-induced rise in the intracellular calcium concentration ($[Ca^{2+}]_i$). When the $[Ca^{2+}]_i$ rises from resting levels of approximately 0.1μM into the 1μM range, the free Ca^{2+} is bound by calmodulin (CaM) which is fully activated by the binding of 4 molecules of Ca^{2+}. MLC inhibits the actin-activated ATPase activity and this inhibition is released by its phosphorylation to MLC-P by myosin light chain kinase (MLCK) which requires Ca^{2+}_4-CaM for activity (Adelstein & Klee, 1981; Dabrowska et al., 1978). MLC-P can interact with actin resulting in the activation of the ATPase in the myosin headgroup. Hydrolysis of ATP to ADP provides energy for the flexion of the myosin head and the subsequent filament movements result in contraction of the smooth muscle. Relaxation is brought about by cessation of stimulation, resequestration of calcium and dephosphorylation of the MLC by a specific phosphatase system.

The dependence of contraction on MLC phosphorylation was shown in *in vitro* studies on canine tracheal smooth muscle (de Lanerolle & Stull, 1980). The peak in MLC phosphorylation was later demonstrated to precede the generation of maximum force and the force generated by a smooth muscle was shown to be related to the level of phosphorylation (de Lanerolle et al., 1982; Silver & Stull, 1982; Kamm & Stull, 1985).

Use of skinned muscle preparations allowed the investigators to manipulate the ionic and protein environment of the contractile and regulatory proteins while measuring the tension and protein phosphorylation. Such experiments provided evidence strongly suggesting that MLCK-stimulated phosphorylation of MLC is an essential prerequisite for smooth muscle contraction (Cassidy et al., 1979; Hoar et al., 1979; Kerrick et al., 1980).

3.3 Mechanism of force maintenance in smooth muscle

While the above mechanism of initiation of force generation is widely accepted there is much debate on the mechanism of force maintenance. This was initiated by the observation, in arterial smooth muscle, that force can be maintained despite the dephosphorylation of myosin to almost resting levels (Dillon et al., 1981). This finding was substantiated by Aksoy *et al.* (1982 & 1983), Silver & Stull (1982) and Kamm & Stull (1985) who demonstrated that, following initiation of contraction, MLC phosphorylation frequently decreases over prolonged stimulation despite the fact that steady-state tension is maintained. However, this dephosphorylation was not observed by several other groups (Kong et al., 1984; de Lanerolle & Stull, 1980; de Lanerolle et al., 1982; Gerhoffer, 1986) who found a good correlation between MLC phosphorylation and sustained tension. This apparent anomaly lead to the formation of several hypotheses to explain the biochemical basis of steady state force maintenance

On the basis of their observations in vascular smooth muscle, Murphy and co-workers suggested that a special actomyosin crossbridge (latch bridge) is functional in the maintained phase of contraction (Dillon et al., 1981). According to the latch bridge hypothesis, the crossbridges are initially of the normal rapidly-cycling type but 2—5 seconds into the contraction (Dillon et al., 1981; Stephens et al., 1986) these are replaced by very slowly-cycling latchbridges which are much more efficient, utilizing only a quarter of the energy expended by the rapidly-cycling crossbridges (Seigman et al., 1980). Oxygen uptake falls to 3-4 times less than in the initial phase (Stephens et al., 1986; Seigman et al., 1980). While the ability to shorten has fallen to almost zero, the ability to develop force is maximal in this state (Dillon et al., 1981). It is not clear whether these latch bridges represent a discrete population or are simply converted from normal cross bridges.

There are, therefore, two discrete bodies of data, the first suggesting a close correlation between myosin phosphorylation and force, and the second demonstrating force maintenance in the face of myosin dephosphorylation. The evidence for each of these hypotheses was examined by de Lanerolle (1989) who reviewed the data supporting each mechanism. However, as this author pointed out, there is no reason to assume that the same regulatory system is operating in all smooth muscles and the most convincing evidence for the existence of latch bridges has come from work in vascular smooth muscle.

An alternative regulatory mechanism was suggested by Rasmussen and coworkers (1987) in which the sustained response is largely due to the phosphorylation by PKC of structural and regulatory components of a fibrillar domain. This hypothesis is based on ultrastructural studies which showed two spatially different fibrillar domains in mammalian smooth muscle cells (Small et al., 1986; Furst et al., 1986). The first of these consists of actin and myosin filaments and their associated proteins. The second contains filamin (and other actin-binding proteins), actin, and desmin (and other intermediate filament proteins e.g. synemin) and is referred to as the F-A-D domain. According to their hypothesis the latch mechanism is caused by a "reorganisation and stablisation of the supramolecular organisation of the components of the F-A-D domain". They suggest that phosphorylation by activated PKC of late phase proteins (desmin, synemin, caldesmon and several unspecified cytosolic proteins) leads to the stabilisation of the F-A-D protein domain in a new configuration such that it is maintained even when MLC-P is dephosphorylated.

3.4 Role of PKC in sustained responses

While activation of PKC is central to the fibrillar domain hypothesis (Rasmussen et al., 1987), it may also be important in the other models of force maintenance as both MLC and MLCK have been shown to be substrates for PKC (Nishikawa et al., 1983). PKC phosphorylates MLC at a site distant from that of MLCK. Each enzyme incorporates two moles of PO_4 per mole myosin; sequential phosphorylation of MLC by MLCK and PKC can occur resulting in the incorporation of four moles PO_4 per mole myosin (Nishikawa et al., 1983). These sequential reactions are known to decrease the activity of the actomyosin ATPase. Thus activated PKC may modulate the activity of smooth muscle myosin.

PKC has previously been proposed to be involved in the maintenance of sustained contraction and has been found to phosphorylate (*in vitro*) the actin-binding proteins vinculin and filamin as well as smooth muscle MLC and MLCK (reviews: Nishizuka, 1986; Abdel-latif, 1986). It has also been suggested that PKC increases the affinity of the contractile proteins for calcium (Rodger, 1986) and that it might therefore be directly involved in the maintained tension phase of airway smooth muscle contraction via its action on the contractile apparatus.

Chattergee and Tejada (1986) showed that phorbol esters, substances which activate PKC (*section 4.5*), induced stress in vascular smooth muscle that was not proportional to MLC phosphorylation and was not preceeded by high levels of MLC phosphorylation. The responses were obtained at concentrations that were too low alone to support stress of MLC phosphorylation and this high calcium sensitivity, coupled with the dissociation between maintained tension and MLC phosphorylation, make PKC a potential candidate for involvement in the latch state.

An alteration in the distribution, quantity or activity of PKC in airway smooth muscle could, therefore, have a significant effect on the ability of the muscle to maintain tension. Inappropriate or excessive activation of PKC might play a significant role in the degree and duration of bronchoconstriction. With the integral role of the enzyme in cell signalling (section 4) it is clear that many signals focus on PKC and it is possible that deviation from its normal pattern of activation could be a contributing factor to hyperreactivity in bronchial smooth muscle.

Section 4

SECOND MESSENGERS AND PROTEIN KINASE C

4.1 Calcium-mobilising Receptors

The muscarinic cholinergic receptor and the histamine receptor are among a group of receptors, collectively termed calcium-mobilising receptors, which are linked to phosphoinositide turnover. This group has over 25 members (Michell, 1982) and includes receptors for neurotransmitters, hormones and growth factors. In airway smooth muscle contractions induced by acetylcholine, histamine and serotonin are related to phosphatidylinositol breakdown (Barnes et al., 1986). The involvement of inositol phospholipids in receptor mechanisms was first described in two papers by Mabel and Lowell Hokin appearing in 1953 and 1954 which showed a marked turnover of phosphatidylinositol (PI) on activation of muscarinic cholinergic receptors (reviewed: Hokin, 1987).

Binding at a calcium-mobilizing receptor stimulates the hydrolysis of inositol phospholipids by phospholipase C (Michell, 1975). This has been shown in membrane preparations to be dependent on GTP (Litosh et al., 1985), suggesting that a G protein(s) is involved in regulating enzyme activity. Conversion from the inactive (GDP) state to the active (GTP) state is dependent on receptor occupancy. Phosphatidyl inositol 4,5-bisphosphate (PI-4,5-P_2) is hydrolysed by phospholipase C to inositol 1,4,5-trisphosphate (I-1,4,5-P_3) and *sn*-1,2-diacylglycerol (DAG; Fig. 4.1). These molecules act as second messengers and function as two arms of a bifurcating signal pathway (Fig. 4.2). I-1,4,5-P_3 is released into the cytosol where it functions to release calcium from the endoplasmic reticulum (reviewed: Berridge, 1984; Berridge & Irvine, 1984). DAG remains in the membrane and activates protein kinase C. Neither of the second messengers is sufficient for complete signal transduction and it has been shown in several tissues that calcium mobilization and activation of PKC act synergistically to produce the full physiological response (Yamanishi et al., 1983; Nishizuka, 1984 & 1986; Baraban et al., 1985; Park & Rasmussen, 1985).

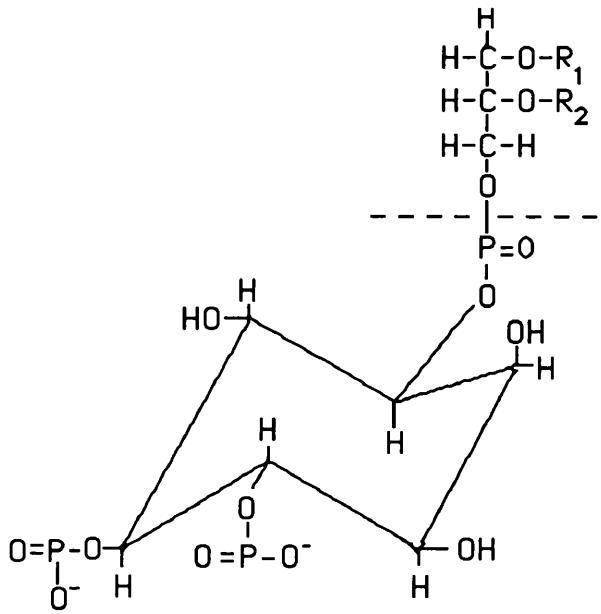


Figure 4.1

Schematic diagram of the molecular structure of phosphatidylinositol 4,5-bisphosphate showing line of cleavage to form inositol 1,4,5-trisphosphate and diacylglycerol.

(from Baron, 1989)

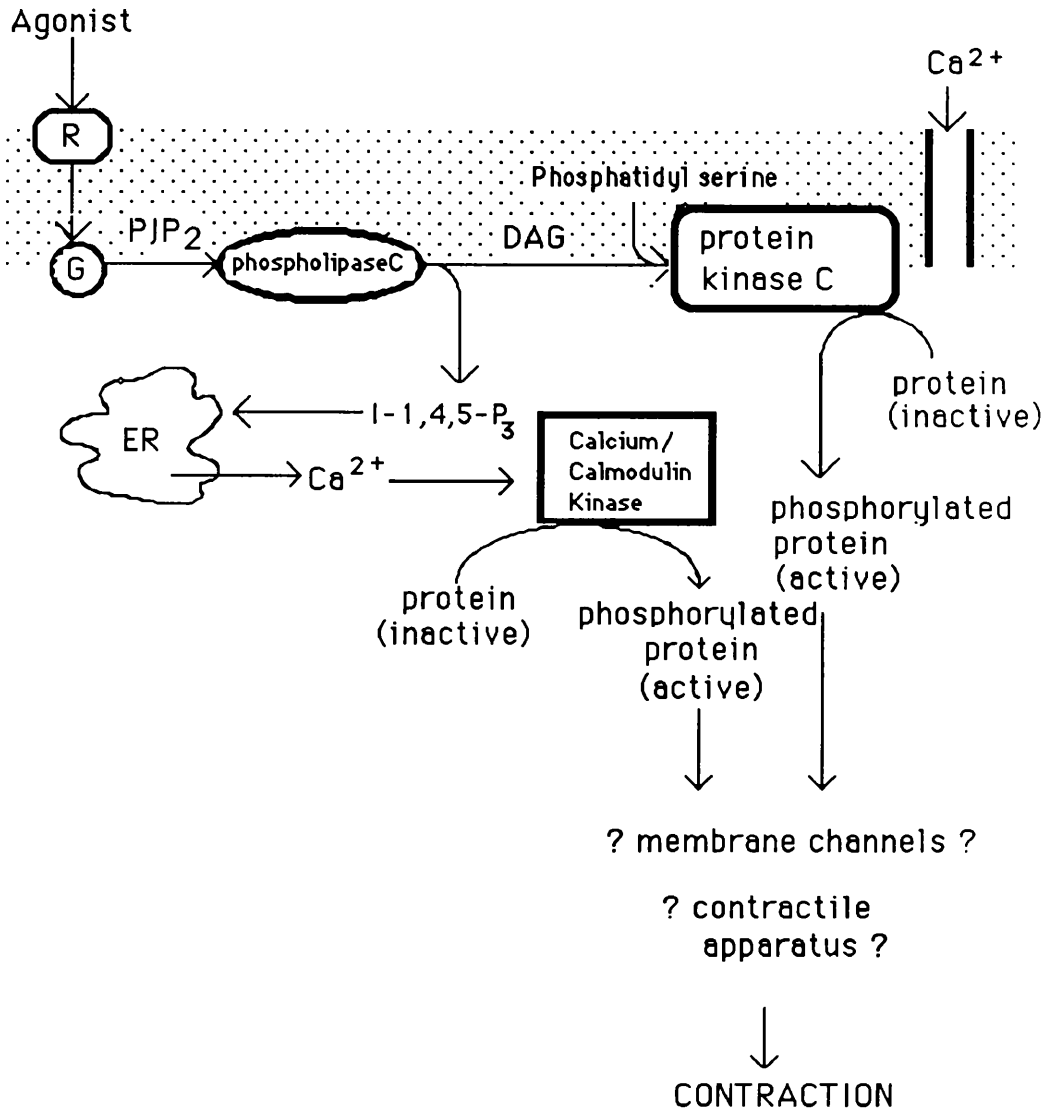


Figure 4.2

Schematic diagram of the intracellular coupling of calcium mobiliising receptors.

4.2 Inositol trisphosphate

Cholinergic stimulation of isolated membranes causes hydrolysis of PI, PI-4-P and PI-4,5-P_2 (Dunlop & Malaisse, 1986). Hydrolysis of PI-4,5-P_2 has been reported in airway smooth muscle (Takuwa et al., 1986; Baron & Coburn, 1987) and production of I-1,4,5-P_3 and other phosphates has been observed in smooth muscle from bovine and canine trachealis smooth muscle (Grandordy et al., 1986; Takawa et al., 1986; Baron & Coburn, 1987; Baron et al., 1989).

I-1,4,5-P_3 mediated release of Ca^{2+} from internal stores has been demonstrated in canine tracheal smooth muscle (Hashimoto et al., 1985). The common method employed to study calcium mobilization involves the addition of I-1,4,5-P_3 to permeabilized cells followed by measurement of the increases in fluorescence of the Ca-sensitive drugs quin-2 or fura-2, or efflux of Ca^{2+} (Suematsu et al., 1984; Somylo et al., 1985; Goldman et al., 1986).

Ca^{2+} is constantly cycling across the endoplasmic reticulum membrane; the passive efflux is balanced by ATP-dependent pumping of Ca^{2+}. The electrogenic potential created by the active uptake of Ca^{2+} is balanced by passive efflux of potassium, which re-enters via K^+/Cl^- co-transport (Prentki et al., 1984).

Ca^{2+} release is preceded by the binding of I-1,4,5-P_3 to a specific receptor present on some but not all of the endoplasmic reticulum (Spat et al., 1986). There appear to be distinct I-1,4,5-P_3-sensitive and insensitive compartments within the endoplasmic reticulum and it is likely that the I-1,4,5-P_3 -sensitive pool is localized to a region close to the cell surface (Biden et al., 1986). Only about 50% of the calcium stored in the endoplasmic reticulum is able to be released by I-1,4,5-P_3 (Muallem et al., 1985) and this appears to be accomplished via an increase in the Ca^{2+} efflux.

The transient increase in I-1,4,5-P_3 therefore seems to be the signal for the equally transient increase in Ca^{2+}. Both return to basal or near-basal levels while the stimulus, and the contraction, are sustained. Rapid metabolism of I-1,4,5-P_3 is accompanied by Ca^{2+} reuptake into the endoplasmic reticulum. Cytoplasmic I-1,4,5-P_3 is reduced by a phosphatase, to produce inositol bisphosphate (I-1,4-P_2), or by phosphorylation to the tetraphosphate I-1,3,4,5-P_4 (Batty, 1985; Heslop et al., 1985; Irvine et al., 1986), which recent reports have indicated may have a role in regulating entry of Ca^{2+} across the plasma membrane (Irvine & Moor, 1986), followed by dephosphorylation to an inactive triphosphate, I-1,3,4-P_3, which is unable to release Ca^{2+} from stores (Downes et al., 1986, Rossier et al., 1986).

The mechanism by which Ca^{2+} entry is subsequently stimulated is still unknown. It has been suggested that I-1,4,5-P_3 acts on the plasma membrane to increase calcium permeability much as it does on the endoplasmic reticulum membrane, but evidence is conflicting on the subject (Slack et al., 1986; Kuno & Gardner, 1987; Streb et al., 1984). An attractive hypothesis is that of the capacitive model of Ca^{2+} entry (Putney, 1986). Briefly, it is suggested that emptying of the intracellular calcium pool by I-1,4,5-P_3 may secondarily open a pathway from the extracellular space into the pool and subsequently into the cytosol. Irvine and Moor (1986) further suggested that I-1,3,4,5-P_4 is also involved in this process, that the release of Ca^{2+} by I-1,4,5-P_3 is a necessary prerequisite, and that the two compounds are together necessary for Ca^{2+} entry.

4.3 Diacylglycerol

The second messenger DAG is lipid soluble, remains in the membrane and activates PKC. Only 1,2-*sn*-DAGs are effective activators of PKC and the DAG is mainly of the 1-stearoyl-2-arachidonyl-sn-glycerol variety (Nomura et al., 1986). The increase in DAG may be as transient as that of I-1,4,5-P_3 as it is rapidly degraded to

arachidonate and subsequent eicosanoid products or rejoins the phosphatidyl inositol cycle via conversion to phosphatidic acid by an ATP-dependent phosphorylation. Phosphatidic acid reacts with cytidine triphosphate (CTP) to yield CTP-DAG which interacts with myo-inositol to form phosphatidylinositol (Esko & Raetz, 1983) which is then transferred from the endoplasmic reticulum to the plasma, and other, membranes.

Sn-1,2-DAGs stereospecifically activate PKC (Takai et al., 1979; Kishimoto et al., 1980; Rando & Young, 1984), the 3-OH group and 1,2-carbonyls are required for activation (Cabot & Jaken, 1984; Ganong et al., 1986). The long chain fatty acid is required for at least one (either) of the 1,2 positions (Mori et al., 1982) with activity increasing from 3-11 carbons (Hannun et al., 1986a). Abdel-Latif (1986) concluded that the acyl ester must satisfy certain physical properties (i.e. water and lipid solubility) such that it partitions correctly into the membrane bilayer and obtains proper access to PKC.

The complex lipid mixture alters the enzyme conformation and DAG increases the affinity of PKC for phospholipid 2-3 fold while the calcum sensitivity is increased 1000-fold to micromolar levels. (Kishimoto et al., 1980; Wise et al., 1982; Hannun et al., 1986a; Rasmussen & Barratt, 1984; Castagna et al., 1982). In the absence of DAG, 100μM Ca^{2+} is needed for enzyme activation (Kikkawa et al., 1986). Conversely both phospholipid and Ca^{2+} increase the affinity of the enzyme for DAG (Hannun et al., 1986a). Kinetic studies indicate that one molecule of DAG activates one molecule of PKC in the presence of 4-10 phosphatidyl serine (Abdel-Latif, 1986; Nishizuka, 1984) and a model has been proposed in which a "primed" $(PKC)_1$ $(phospholipid)_4(Ca^{2+})_1$ complex is formed to which one molecule of DAG binds to activate the enzyme (Bell, 1986).

DAG can also be generated from phosphatidic acid. Hydrolysis of phosphatidylcholine by phospholipase D generates phosphatidic acid as its lipid product, which is then converted to DAG by the action of phosphatidate phosphohydrolase (Peleck & Vance, 1989). Phospholipase D activity does not appear to be desensitised in the same rapid manner as phospholipase C and may therefore result in sustained production of DAG (Cook & Wakelam, 1989; Cook et al., 1990).

The duration of an increase in the DAG content of the plasma membrane, and thus of PKC activation, may therefore vary widely. Mellgren (1987) demonstrated that sustained association of PKC with the plasma membrane results in its proteolytic cleavage by calcium-dependent proteases (termed calpains by Murachi et al., 1981), to a 50kDa subunit, PKM, which has been implicated in mitogenesis (Murray et al., 1987). This process in speeded up in the presence of DAG and Ca^{2+}.

PKC can also be activated by arachidonic acid (Nishizuka, 1986). Thus, stimulation of phospholipase A_2 can result in an increase in PKC activity and involvement of the inositol phospholipid signalling system is not essential for DAG generation or for PKC activation. Phospholipase A_2 has a lower affinity for Ca^{2+} than does phospholipase C (Billah et al., 1980) suggesting that arachidonic acid production by this pathway may be stimulated by agonist-evoked increases in intracellular calcium. PKC is also known to regulate phospholipase A_2 activity, thus these interactions allow for the subtle variation in the effects of different mediators according to the enzymes to which their receptors couple.

4.4 Protein kinase C

PKC is a calcium and phospholipid dependent protein kinase of molecular weight 77,000 Da. First identified in 1977 as an undefined protein kinase present in many tissues, it was found to be activated by limited hydrolysis with calpain (Takai et al., 1977; Inoue et al., 1977). Cleavage results in the release of a catalytically fully active fragment which is rapidly removed from the cell. Later it was shown that in the absence of proteolysis it has activity that is dependent on Ca^{2+} and phospholipid, and enhanced by DAG (Takai et al., 1979; Kishimoto et al., 1980; Kuo et al., 1980). PKC is widely distributed in nature. In mammals the highest levels are found in platelets, spleen, vas deferens and brain, and the lowest levels in heart, adipose tissue and skeletal muscle (Kuo et al., 1980; Girard et al., 1986; Kikkawa et al., 1982). The subcellular location of PKC is hormonally regulated (see below) and varies with the tissue (Kuo et al., 1980; Girard et al., 1986; Kikkawa et al., 1982).

Structure

The enzyme is a single polypeptide chain composed of two domains. The hydrophobic domain interacts with calcium and phospholipid, binds to the membrane, and plays a regulatory role keeping the catalytic part inactive. The catalytic centre is located in the hydrophilic domain and is activated when DAG (or phorbol ester) binds to the regulatory domain (Ruegg & Burgess, 1989; Nishizuka, 1984; Abdel-latif, 1986). The ATP binding site and much of the rest of the catalytic portion shows a large degree of homology with other protein kinases (Ruegg & Burgess, 1989).

The PKC family is comprised of several subspecies of PKC which respond differently to phospholipid, DAG and Ca^{2+} but which share a common basic structure. In brain tissue at least seven subspecies can be distinguished, one of which

(the γ-subspecies) is expressed only in central nervous tissue (reviewed: Kikkawa et al., 1989). Subspecies may be differently located in different cell types and many cell types contain several subspecies in variable ratios. The subspecies show subtle differences in their mode of activation, sensitivity to Ca^{2+}, and catalytic activity (Rana & Hokin, 1990). Following cell stimulation, the subspecies may be down-regulated at different rates and may therefore have distinct roles in the regulation of a variety of physiological and pathological cellular responses. It is possible that different subspecies of PKC may be activated by different phospholipid metabolites (e.g. DAG, arachidonic acid or lipoxin A) that are produced in successive phases of the cellular response to surface receptor occupation (Nishizuka, 1988).

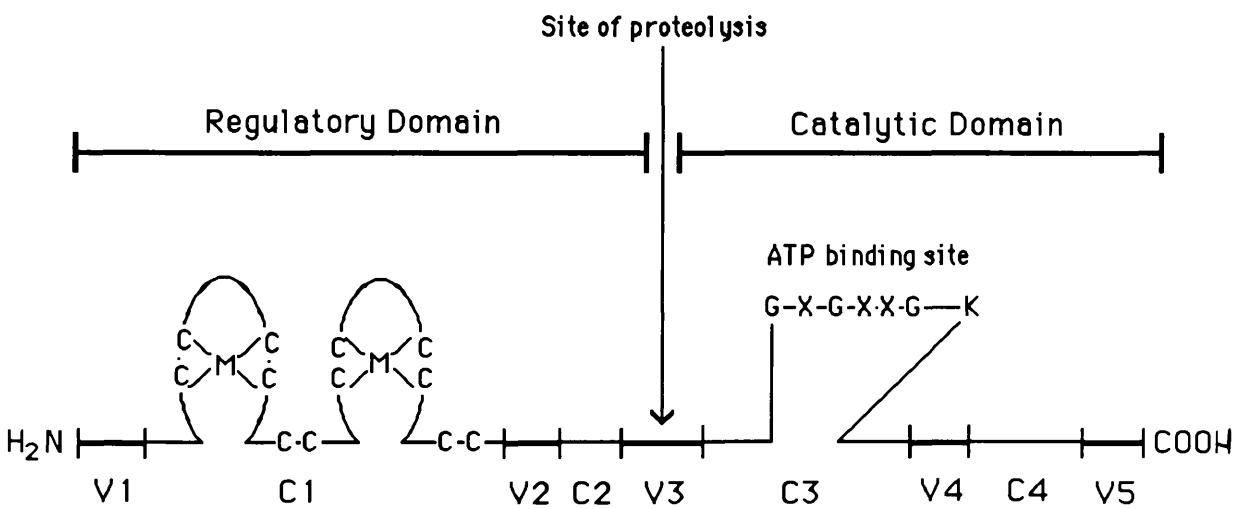


Fig. 4.3

Schematic structure of protein kinase C peptide chain. (from Kikkawa et al., 1989)

Genes for the α, β1, β11 and γ subspecies have been identified on different chromosomes (Coussens et al., 1986) and δ, ε and ζ subspecies have also now been found (Ono et al., 1987 & 1988). The subspecies thus far cloned have the same primary structure, shown schematically above, of a single polypeptide chain consisting of four conserved (C1-C4) and five variable (V1-V5) regions, however the δ, ε and ζ subspecies lack the second conserved region (C2). In the diagram C, G, K,

X and M represent cysteine, glyceine, lysine, any amino acid and metal respectively. The carboxyl terminal appears to include the catalytic domain. It contains the ATP binding site and shows significant sequence homology with other protein kinases (Ono et al., 1986; Parker et al., 1986).

The conserved region of the regulatory domain of PKC contains an amino acid sequence, between residues 19 and 36, that resembles a substrate phosphorylation site in its distribution of basic residues necessary for substrate recognition (House & Kemp, 1987). This area, termed a pseudosubstrate structure, may be responsible for maintaining the enzyme in an inactive form in the absence of activators such as phospholipids. The corresponding synthetic peptide acts as a potent and specific substrate antagonist, inhibiting both autophosphorylation and substrate phosphorylation in a competitive manner (House & Kemp, 1987). The peptide was uncompetitive at varying ATP concentrations, however, indicating that inhibition was not at the ATP binding site. Substitution of one amino acid, serine for alanine, transformed the synthetic peptide into a potent agonist.

The results suggest that the pseudosubstrate region binds to the active site of PKC and inhibits its activity. Activation of the enzyme by phospholipids could cause a conformational change that removes the pseudosubstrate sequence from the active site and therefore allows access to protein substrates.

Activation

In the non-activated cell PKC exists in the cytosol as an inactive soluble enzyme and has a low sensitivity to calcium (Kikkawa et al., 1982). When the DAG concentration of the plasma membrane increases following PI hydrolysis, the enzyme is translocated to the plasma membrane where it binds to the inner surface and associates with the proper mix of phospholipids to become active (Nishizuka, 1986).

While PKC can be activated by the synergistic action of increased intracellular calcium concentrations and increased formation of DAG (detailed above), it can also be activated by limited proteolysis with calpain and is most suceptible to this in its membrane-bound state (Nishizuka, 1986).

Although PKC generally remains active for only a short time, the consequences of PKC activation may persist considerably longer depending on the stability (resistance to proteases) of the phosphate covalently attached to the substrate molecule (Nishizuka, 1986). Numerous substrate proteins for PKC have been discovered *in vitro* (see below).

There is a large body of evidence suggesting that PKC has a dual action, exerting positive as well as negative control over various steps of the cell signalling process (Reviewed: Nishizuka, 1986 & 1988; Kikkawa & Nishizuka, 1986). However, most of the published data to date suggests that activation of PKC does not result in exaggerated responses to receptor-mediated events. In the early stages of cell responses PKC is thought to act synergistically with Ca^{2+} as part of a positive forward response. Subsequently it frequently acts as a negative feedback control, for example PKC is implicated in the down regulation of some receptors which occurs after agonist stimulation (Nishizuka, 1986). Receptors that are phosphorylated by PKC include those for muscarinic and β-adrenoceptor agonists and endothelial growth factor (Safran et al, 1987; Sibley et al, 1986; Davis & Czech, 1986; Friedman et al, 1984; Iwashita & Fox, 1984). Phosphorylation is thought to reduce the affinity of the receptor for the agonist, or to interfere with agonist binding by initiating internalisation of the receptor. However, in aortic smooth muscle cells, activation of PKC inhibited phosphatidylinositol responses to angiotensin II but did not alter receptor affinity or numbers (Katada et al, 1985), suggesting that intracellular coupling may be altered, perhaps due to phosphorylation of the receptor linked G protein(s). Activation of PKC by PMA can inhibit PIP_2 hydrolysis in response to

muscarinic receptor occupation without altering the binding properties of the receptor (Orellana et al., 1985; Itoh et al., 1988).

PKC is implicated in control of secretion & exocytosis, smooth muscle contraction, DNA synthesis, gene expression and cell proliferation (Nishizuka, 1986 & 1988; Kaibuchi et al., 1986; Kikkawa & Nishizuka, 1986). In all of these processes activated PKC acts in synergism with the increased intracellular calcium concentration. However, in some processes an additional pathway is involved in eliciting full activation: for example for rapid cell proliferation, some growth factor, even in low concentration, is needed.

Other lines of evidence suggest that PKC modulates ion conductance by phosphorylation of membrane proteins such as channels, pumps, and ion exchange mechanisms. In various cell types PKC stimulates Ca^{2+} extrusion by activating the Ca^{2+}-transport ATPase and the Na^{+}/Ca^{2+} exchange protein (reviewed: Nishizuka, 1986 & 1988).

Clearly many signals focus on PKC and this enzyme, in turn, plays a central role in the regulation of a wide variety of cellular processes, from calcium signalling to the contraction of the smooth muscle itself. An alteration in the pattern or extent of PKC activation could, therefore, be a factor in the hyperreactive airways of the type found in asthmatics.

4.5 Chemicals influencing PKC directly.

PKC can be artificially activated by several substances. Activators of PKC include the phorbol esters (Castagna et al., 1982; Fig. 4.4), the membrane soluble analogues of DAG: oleoylacetyl glycerol (OAG) and dioctanoyl glycerol (DOG), and several other

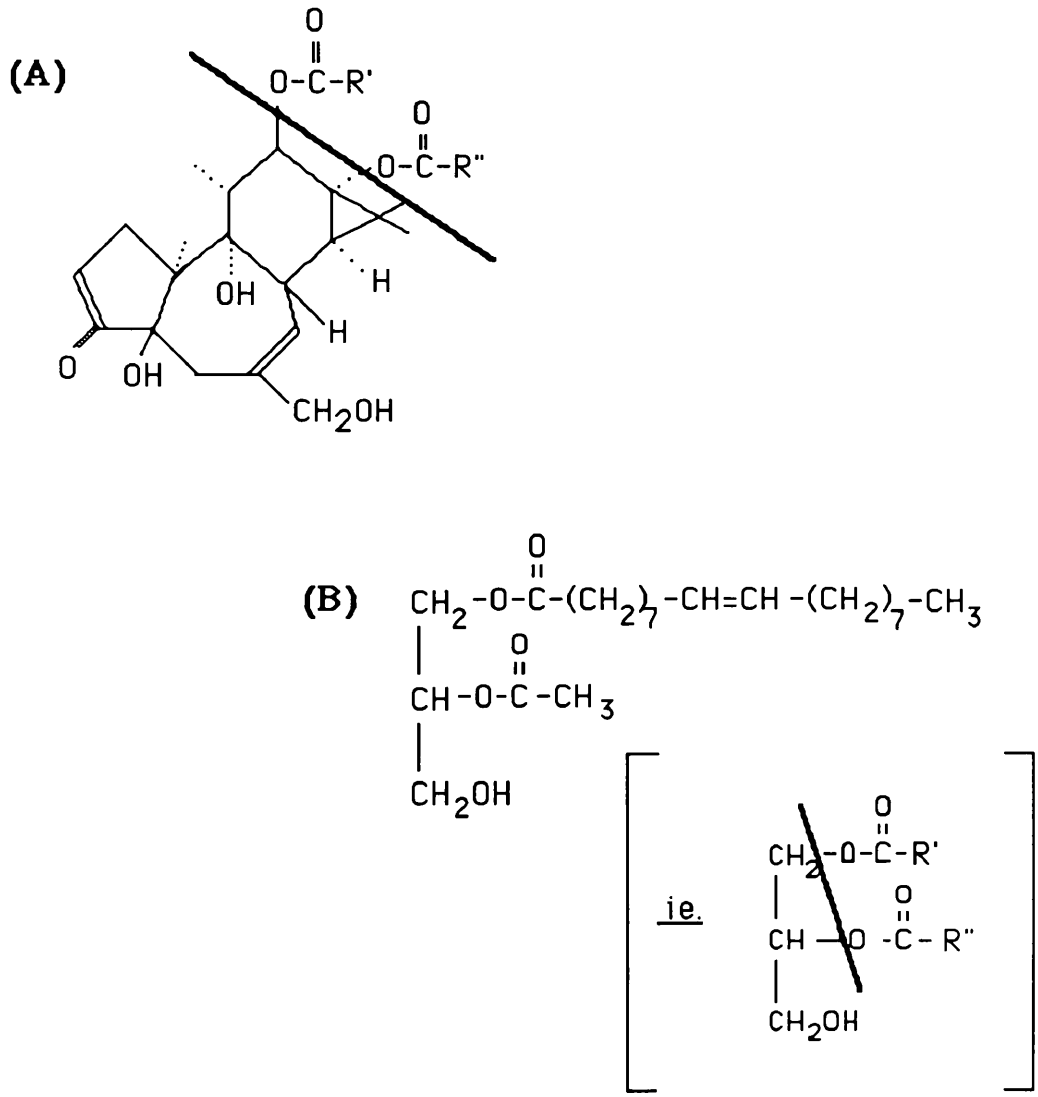


Figure 4.4

Schematic diagram of the general molecular structure A) of phorbol esters (Anwyl, 1989) and B) of diacylglycerol (Nishizuka 198x). R' and R" are ester side chains of varying length.

classes of compound. The phorbol esters are the most potent of the PKC activating drugs and consequently the most useful experimentally (Epand & Lester, 1990). Several phorbol esters have been used in the investigation of the properties of PKC, including the weak promotor 12-deoxyphorbol 13-isobutyrate (DPB), phorbol 12,13-didecanoate (PDD) and the photoaffinity probe phorbol 12-*p*-azidobenzoate 13-benzoate (PaBB; Declos & Blumberg, 1983). However, the esters most frequently employed are phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-dibutyrate (PDBu) which each have an active, 4β, and an inactive, 4α, isomer. Nomenclature confusion arises due to the use, by some authors, of the name 12-O-tetradecanoylphorbol 13-acetate (TPA) which is the same compound as PMA.

PDBu has been used in studies to demonstrate specific, saturatable binding in cells (*section 4.6*) as the use of the more lipophillic PMA results in a high degree of non-specific binding. However, PMA is the most potent of the phorbol esters and has a higher binding affinity than PDBu in all the systems studied. Thus, although PDBu is used as the ligand of convenience for measuring binding, the receptor being measured is more properly considered as a PMA receptor (Blumberg et al., 1983).

Phorbol esters.

Phorbol esters are a class of plant toxins produced by the *Euphorbias* and are widely known as tumour promotors, agents which are not themselves carcinogenic but which induce tumours in animals exposed to a subthreshold dose of carcinogen. Rohrscheider & Boutwell (1973) suggested they might act by binding to some physiological receptor, but as recently as 1981 the site of their action was still unknown. With the discovery of PKC by Nishizuka and coworkers (Inoue et al., 1977; Takai et al., 1977), and the interest this protein kinase aroused, it became apparent that the distribution of the phorbol ester binding activity and of the enzyme were generally very similar. Castagna and coworkers (1982) demonstrated that the

dose response curve for PMA activiation of PKC was very similar to the saturation curve for PMA binding to its receptor and subsequent investigations by the same group confirmed this to be the case (Sano et al., 1983; Yamanishi et al., 1983).

The use of a radioactively labelled phorbol ester [^{3}H]PDBu revealed high affinity binding sites in a wide range of species and tissue preparations. Phorbol ester receptors were present not only in the various vertebrate systems examined but also in nematodes, sea-urchins and fruit flies (Blumberg et al., 1983). Highest levels of binding were seen in brain tissue where the absolute value, 30pmol/mg protein, was much greater than that for neurotransmitter receptors (<2pmol/mg; Blumberg et al., 1983).

Phorbol esters bind to the particulate fraction of cells in a specific, saturatable and reversible manner (Dreidger & Blumberg, 1980) which can be competitively inhibited by diolein, a derivative of DAG (Blumberg et al., 1984). The highly lipophilic phorbol esters intercalate with the membrane and mimic the actions of DAG by acting at the same site on the enzyme to activate protein kinase C both *in vitro* and *in vivo*. (Castagna et al., 1982; Yamanishi et al., 1983). Interaction with phorbol esters is regulated by calcium and phospholipid and occurs at the same site as DAG (Kikkawa et al., 1983; Hannun & Bell, 1986). Indeed DAG competitively inhibits phorbol ester binding to a brain cytosolic phorbol ester aporeceptor (Sharkey et al., 1984). Phorbol esters substitute for DAG at very low concentrations (1000-fold lower than DAG) both *in vitro* and *in vivo* (Nishizuka, 1984). However PMA is hardly degraded and may therefore distort the normal sequence of phosphorylation events.

Although a degree of heterogeneity in phorbol ester binding was seen in certain tissues (Blumberg et al., 1984), and despite the fact that phorbol esters are known to have effects that are not mediated by protein kinase C (*section 4.6*), this enzyme is generally accepted as the principal site of action of the phorbol esters (Yamanishi et

al., 1983; Baraban et al., 1985; Forder et al., 1985). Copurification of kinase activity and phorbol ester binding (Ashendel et al., 1983; Kikkawa et al., 1983; Neidel et al., 1983) confirmed that PKC is a major phorbol ester receptor in cells. The basis for the phorbol receptor heterogeneity could be multiple genes, arising from gene duplication and divergence, that code for receptor variants (*section 4.1*).

Frezard *et al.* (1989) monitored the interaction of PMA with phosphatidylserine-containing unilamellar vesicles using circular dichroism (CD) and observed changes in the CD spectra of PMA upon binding. They assigned these to the embedding of the phorbol ester in the membrane layers and calculated that only 2-5% of the amount of phorbol ester added was embedded after 1 minute incubation. This results suggests that either phorbol esters entering the membrane is not necessary for the activation of PKC or that the amount of phorbol ester needed is very small. The latter is likely as a 1:1 ratio of PMA (or DAG) to PKC is reported *(section 4.3)*.

Several reports (Ballester & Rosen, 1985; Blackshear, 1985; Hepler et al., 1988) indicate that PMA elicits the translocation of the enzyme from the soluble fraction to the membrane and its subsequent depletion, termed down-regulation. Indeed Neidel and Blackshear (1986) cite papers covering 11 cell types in which phorbol esters have been shown to cause translocation. Kuo (1986) reports that after 30 minutes incubation with 200nM PMA all measurable PKC was bound to the plasma membrane while after 2 hours no PKC staining was found, presumably because the enzyme had been degraded or freshly secreted by the cell. Such a situation could result in desensitization to agonist stimulation.

The effects of PKC activation by phorbol esters have been studied in a variety of tissue preparations. However most of the studies undertaken on the action of phorbol esters in smooth muscle have utilised mammalian vascular tissue, in which they are reported to induce a progressive contraction that is slow in onset (Danthuluri & Deth,

1984; Forder et al., 1985; Spedding, 1987). The effect of phorbol ester-induced PKC activation on the response to calcium mobilizing agonists is less clear-cut. They have been shown to inhibit α_1-adrenoceptor-mediated responses and the associated release of I-1,4,5-P_3 (Danthuluri & Deth, 1984; Carmelo et al., 1988), to increase or decrease the amplitude of contraction to ACh (Itoh et al., 1988) and to inhibit or enhance calcium-induced contractions (Baraban et al., 1985).

The limited amount of work carried out on airway smooth muscle is similarly inconclusive. Despite the central role played by PKC in smooth muscle excitation-contraction coupling, relatively little work has been done on the effects of phorbol esters in airways and the reported responses have been variable. Park and Rasmussen (1985) found that PMA itself had no effect on bovine trachea, but when added with the calcium ionophore A23187 the resulting response was similar to that produced by the muscarinic agonist carbachol and could be potentiated by BAY-K8644. Souhrada & Souhrada (1989a) reported a triphasic contractile response to PMA in guinea pig trachea while Schramm & Grunstein (1989) saw a concentration dependent effect in rabbit trachea, high concentrations of PMA producing a relaxation of methacholine-induced tone and lower concentrations producing a contraction.

4.6 PKC-independent effects of phorbol esters

Phorbol esters have been shown to exert effects that are independent of PKC. For example both PDBu and its 4α-isomer depressed whole cell barium current through calcium channels in hippocampal neurons (Doerner et al., 1990). PDBu exerted its effect via the activation of PKC, which could be blocked by H7. However the 4α-isomer is ineffective at activating PKC yet still exerted a (100-fold weaker) effect on the barium current. 4α-PDBu was only effective on the outside of the cell, suggesting it had its effects via an extracellular binding site or that its orientation in the membrane

was crucial to its effects on calcium channels. PKC-independent effects began to appear in the range of 5-10μM.

Maleci and coworkers (1990) showed that PMA pre-treatment of a glioma cell line (SNB-19) made them less susceptible to lymphokine-activated killer cell-mediated lysis. This effect was produced by low concentrations of PMA, was reversible, and was not blocked by PKC-inhibitors. PMA may have exerted its effects by a PKC-independent mechanism, or these glioma cells may possess an isozyme of PKC which is insensitive to the inhibitors employed.

At high concentrations (50μM) phorbol esters and analogues of DAG are thought to have non-specific effects. At this concentration PMA and OAG have been shown to produce a significant release of serotonin from platelets in the absence of a significant increase in $[Ca^{2+}]$. These release reactions are possibly due to the compounds acting as membrane perturbers or fusigens or stimulating superoxide production (Kaibuchi et al., 1983; Yamanishi et al., 1983; Witz et al., 1980).

Section 5

METHODS

5.1 Introduction to methods section: In vitro study of airway smooth muscle.

Overview

Although most modern *in vitro* studies of airway smooth muscle function make use of organ bath techniques, early qualitative studies were performed on the isolated trachea or whole bronchial tree. Thin slices of lung from the gelatine-filled bronchial tree were examined with a microscope during the addition of drugs (Sollman & Gilbert, 1937) while isolated tracheal tube preparations were used in pharmacological and physiological studies (Carlyle, 1964; Hakansson & Toremalm, 1967).

Quantitative studies have provided more detailed information. The direct measurement of airway smooth muscle contraction or relaxation in response to an agonist is possible with the use of organ baths coupled with force transducers and recorders. Castillo and DeBeer, Akcasu and Patterson did much of the early work on tracheal smooth muscle. Castillo and DeBeer (1947) sectioned guinea-pig trachea into 12 rings which were tied together with the smooth muscle in alignment, kept under tension by the natural tendency of the cartilage to spring open. This method was subsequently adapted by Akcasu, first in 1952, when to obtain greater sensitivity, he opened up the rings by cutting through the cartilage and later in 1959, when he cut away most of the cartilage and tied the remaining cartilagenous ends together. The technique was further modified by Foster (1960) who, in order to minimise the interanimal variation, connected the odd numbered (open) rings from one animal with the even numbered ones from a second.

Rosa & MacDowell (1951) and Patterson (1958) developed a bronchial preparation that involved less manipulation of the tissue. They found that bronchi cut into a single spiral was more sensitive than the chain of connected rings, provided a spiral of

sufficient length could be obtained. This method was rediscovered by Constantine (1965) and is still used. (Fleisch et al, 1970; Brink et al, 1980) .

The subsequent development of techniques utilizing single tracheal (Danko et al., 1968) and bronchial (Stephens et al., 1968) rings allowed the preparation of several tissue rings from a single piece of tracheal or bronchial tissue. Tension is usually recorded isometrically and the ring preparations used for physiological and pharmacological studies; the effects of several drugs or conditions could be tested in paired rings taken from the same animal. Second or third generation airways (about 1mm diameter) can be studied this way, by the simple modification of using two 30-gauge disposable needles bent into shape to mount the tissue rings (Hooker et al., 1977).

In order to investigate the properties of smooth muscle of very small airways, use has been made of tissue strips. Strips of lung parenchyma have been used to record tension changes or mediator release from the most peripheral parts of the lung (Kapanci et al., 1974; Lulich et al., 1976; Drazen & Schneider, 1978). This method has been criticised however, as the preparation may be heavily contaminated with other types of cells, eg. vascular smooth muscle cells, which could contribute to any response produced (Finney et al., 1984, 1985). Other groups have developed techniques using tracheal or bronchial muscle strips, dissected free of connective tissue and cartilage, indeed as long ago as 1912 Trendelenburg experimented on strips of muscle and mucosa taken from the bronchi of calves and pigs and cleaned of cartilage. Trachealis muscle is considered to be the purest muscle preparation of the bronchial tree (approximately 75% muscle; Stephens et al., 1969). This technique has been considerably refined and has been used in a variety of electrophysiological and pharmacological experiments (Offermeier & Ariens, 1966; Coburn & Tomita, 1973, Richardson & Bouchard, 1975).

Choice of preparation

In asthma both the central and the peripheral airways are thought to be involved in the bronchoconstrictor response. However, most work has been undertaken on the smooth muscle of the trachea and large bronchi. With the objectives raised against the use of the parenchymal strip preparation, the single bronchial ring provided the best available means of measuring small airway responses and was therefore utilized in this study†. Several of the groups working in airway smooth muscle physiology/pharmacology at present utilize closed or opened bronchial rings (Minette & Barnes, 1988; Roberts et al., 1984/1985) with the result that a bank of literature is available for consultation.

†Recent developments in myography, utilizing devices designed to monitor tone in small resistance blood vessels of down to 80μM diameter (Mulvany et al., 1990), may permit even more accurate measurement of small airway responses. This technique has not yet been applied to bronchioles but should allow direct measurement of the responses of airways in the periphery of the lung to be made.

5.3 Methods - The rabbit preparation

Adult New Zealand White rabbits of either sex, weighing 2.5-3.5 kg were killed by a blow to the cervical spine. Secondary bronchi were isolated from the lungs, cleaned of loose connective tissue and placed in well oxygenated Krebs Henseleit (Krebs) solution.

The Dissection

An incision was made at the base of the rib cage and the sternum cut up the midline for its full length. Lateral cuts were made at the top and bottom of the incision on either side, allowing the rib cage to be opened up to reveal the thoracic viscera.

The conical thoracic cavity contains the thymus gland, heart and lungs (Fig. 5.1). The thymus resembles a large mass of fatty tissue and, especially in younger rabbits, may almost cover the heart. Beneath the heart and thymus lie the right and left lungs, the right having four lobes while the left has only two.

The main thoracic blood vessels, the aorta and vena cava, are closely associated with the lungs. From the heart the aorta curves leftwards then dorsally to run under the lungs on the dorsal wall of the thoracic cavity. The posterior vena cava approaches the heart from its opening in the diaphragm (over and between the lungs), and is accommodated in a deep groove in the ventral surface of the right lung. Figure 5.2 shows the relative positions of the different vessels in a transverse section of the thoracic cavity passing through the lower portion of the heart.

While it is possible, with care, to remove the lungs without damaging the heart and vasculature, the simplest and fastest technique, and that used here, is to cut through the cervical portion of the trachea, oesophagus and blood vessels. The trachea can

Figure 5.1 (from Whitehouse & Grove, 1967)

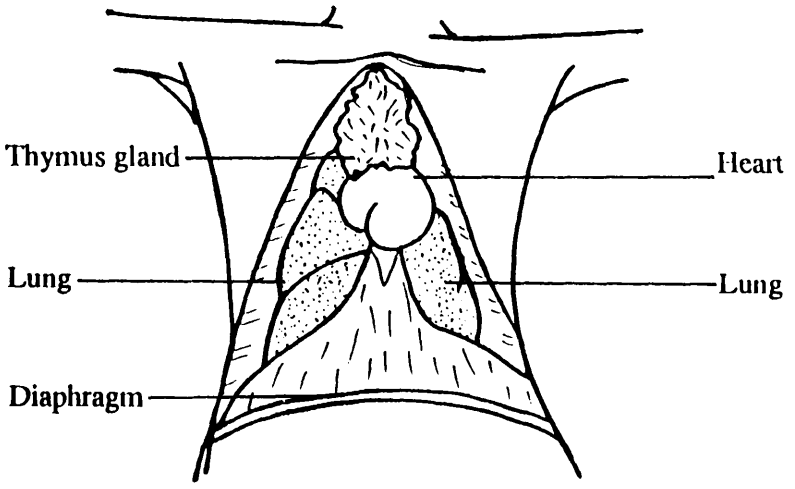
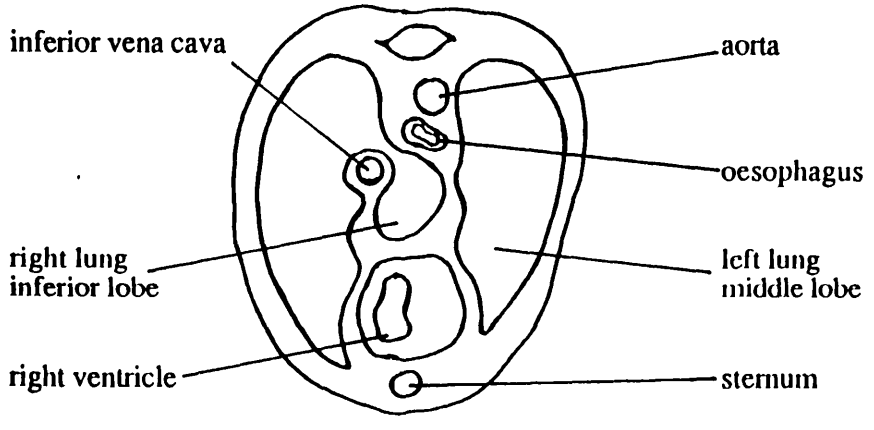


Figure 5.2 (from Craigie, 1951)



then be raised up with forceps and the connective tissue carefully cut away enabling the entire heart and lungs to be lifted free and cleaned up outside the thoracic cavity.

The walls of the rabbit airway contain a substantial amount of cartilage in the trachea, primary and secondary bronchi whereas those of the third order bronchi contain only fragments of cartilage (Fujiwara, 1988). In these studies rings of second order bronchi were prepared from airway immediately above the bifurcation to third order bronchi, where there was relatively little cartilage. When the airways were sufficiently large rings of third order bronchi were utilized.

Bronchi were isolated by blunt dissection and the loose connective tissue gently removed with fine forceps. Care was taken to avoid handling the epithelial surface of the airway.

5.4 Methods - Human tissue

Human bronchial smooth muscle samples were obtained from the thoracic surgical units at the Western Infirmary, Glasgow and Hairmyres Hospital, East Kilbride. Samples of second to sixth order bronchi were obtained from patients undergoing surgery for bronchial carcinoma. Immediately after surgical resection specimens of macroscopically normal tissue were provided be the pathologist and transported to the lab in Krebs fully saturated with 5% CO_2 in O_2. Bronchi were dissected free of loose connective tissue and washed in fresh Krebs to remove any blood. Bronchial rings were cut into 3-4mm sections and used immediately or stored overnight at 4°C in well-oxygenated Krebs. This procedure has been shown to have no significant effect on the response of the tissue to contractile or relaxant agonists (Brink, 1980; De Jongst et al., 1985).

Results in human tissue are obviously the most interesting and clinically relevant for the study of processes related to asthma. Unfortunately the tissue is in short supply and varies widely in quantity and condition. As the tissue is obtained at thoracotomy the supply is irregular and suitable specimens are therefore precious. Thus, all initial experiments were done in the rabbit airway preparation and this tissue was subsequently used to tease out the mechanism once the important positive result was obtained in human airway.

Notes on the human tissue:

Although the lung tissue was macroscopically normal most of the donors had pulmonary carcinoma and most would have been cigarette smokers. In vivo responsiveness to histamine is increased in patients who are heavy smokers (Roberts et al., 1985). Although differences in responsiveness might therefore be expected between lung tissue from diseased and healthy patients, several studies have been unable to find a relationship between in vivo hyperresponsiveness and in vitro airway reactivity (Vincenc et al., 1983; Roberts et al., 1984, 1985). It is unlikely that the responses obtained from these specimens would differ significantly from those from pulmonary disease-free tissue.

Recent work suggests that neutrophil products may increase the reactivity of bronchial smooth muscle (Hallahan et al., 1990). Phorbol esters are known to activate a variety of inflammatory cells and to stimulate release of pre-formed mediators which could produce modulation of bronchial smooth muscle contractility. In an attempt to remove all inflammatory cells from the preparation tissues were washed frequently in fresh Krebs. Nevertheless, it is likely that some cells remained in the specimen. While mediator release may play a role in the response of the smooth muscle to PMA and cannot be discounted, previous studies in airway tissue found no significant effect of activated inflammatory cells on acetylcholine responsiveness in vitro (McAlpine et al., 1989).

5.5 *Setting up*

Bronchi (3-5mm diameter) were cut into rings of approximately 3mm length and suspended between two wire hooks in 5ml water baths. The lower support was attached to a modified tissue holder and the upper hook attached by a cotton thread to an isometric transducer (Fig.5.3). The Krebs was aerated with a carbogen mixture (5%CO2 in O2) and maintained at 37ºC. Tissue rings were placed under 1g tension and allowed to equilibrate in the bath for 1hr, during which time they were washed with fresh Krebs every 15 minutes. By 60 minutes all tissues had reached a stable tension that was unaltered by further washing. If necessary (if a tissue had relaxed to less than 0.5g) tissues were retensioned and allowed to recover till they remained at a steady tension of between 0.5 and 1.0 grams. Changes in tension were measured on Grass isometric (FT03) force displacement transducers and recorded on a Linseis (L2005 series) strip chart recorder.

5.6 *Electrical field stimulation studies*

The modified tissue holders included two platinum wire electrodes which were connected to a Square One electrical stimulator. Tissue rings underwent electrical field stimulation with various parameters. A 10 second train of 0.1msec pulses at a supramaximal voltage of 50V was applied at frequencies from 0.5-100Hz in order to construct a frequency response curve. In subsequent experiments rings were stimulated at 4, 16 or 30Hz which represent approximately 25, 65 & 90% of the maximal response to EFS. Stimulation was repeated at 5 minute intervals until 3 successive contractile responses were within 10%. The average of these 3 responses was designated 100% and all subsequent responses expressed as a percentage.

Following addition of PMA, stimulation continued every 5 minutes for an hour, with further stimulation at 75 and 90 minutes. In order to assess the cholinergic nature of

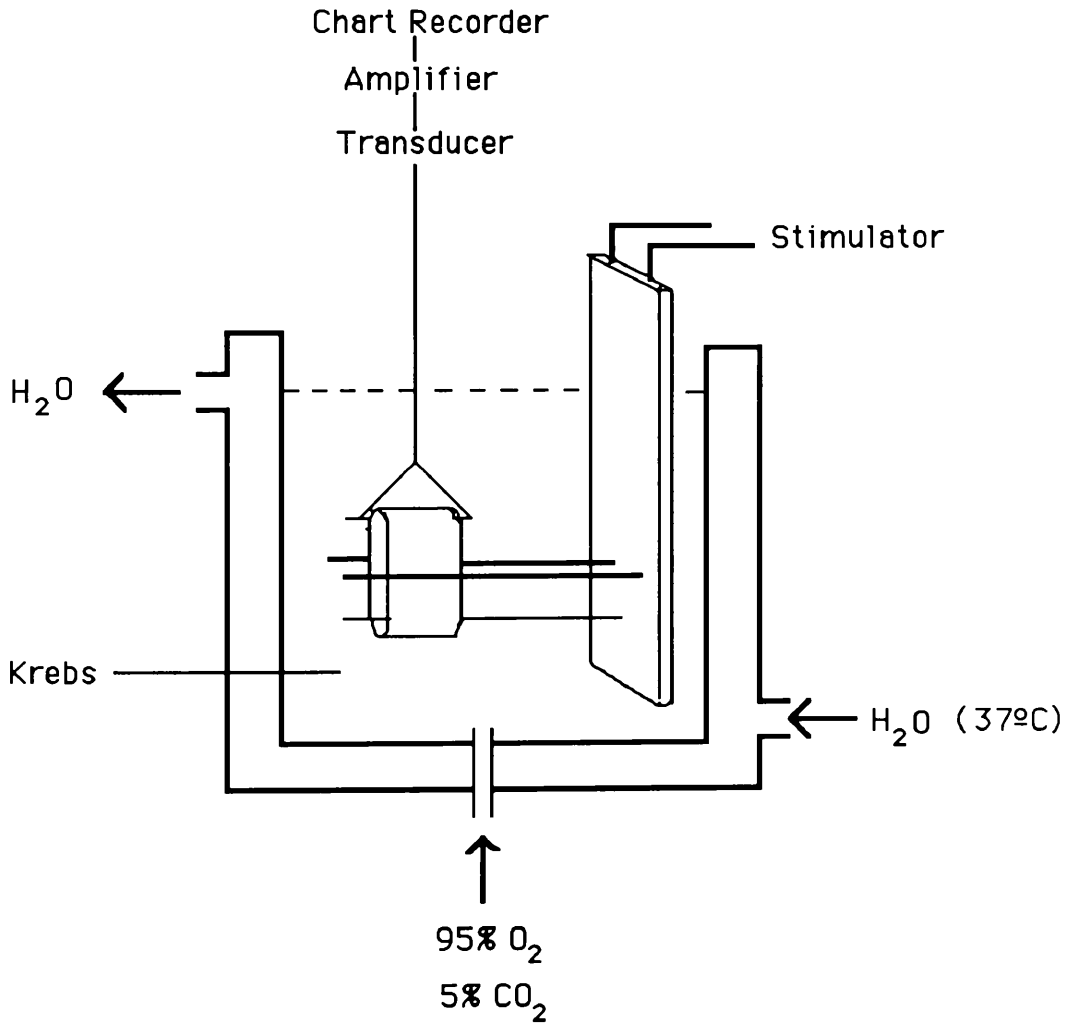


Figure 5.3 Apparatus

the response and the importance of calcium entry to the potentiation by PMA, parallel experiments were run in which the tissues were incubated with atropine or verapamil for 20 minutes prior to addition of the PMA. In all experiments paired untreated tissue rings served as time controls.

5.7 Cumulative concentration response studies

This type of protocol provides an extensive pharmacological analysis; from the response curve one can calculate the Tmax, EC_{50} & pD_2 values (*see data analysis*) which allow comparison of the sensitivity and maximum contractile response of the tissues.

Cumulative concentration response curves (CCRCs) were obtained in each tissue by increasing, by 3-fold increments, the organ bath concentration from 1nM to 30μM ACh. The threshold concentration of contraction to ACh in rabbit secondary bronchial smooth muscle was 0.03-0.1μM.

On reaching and maintaining a contraction to the maximum concentration used, the tissues were washed till they regained resting tone. Following full recovery the tissues were incubated with 1μM or 10μM PMA for a period of 45min at which point a second CCRC was completed in the presence of the phorbol ester. In all experiments time controls were included to assess the effect of time alone on the sensitivity of the preparation to ACh.

5.8 Submaximal dose studies

This type of protocol is more accurate than the CCRC study described above when less than log order changes in sensitivity are involved.

The tissues were contracted with a single sub-maximal concentration of 10μM ACh or histamine, equivalent to approximately 55% of contraction to 300μM ACh and approximately 66% of maximum contraction to histamine. On reaching a steady plateau the rings were rinsed three times in fresh Krebs and allowed to recover for 10 or 30 minutes before readdition of 10μM ACh or histamine respectively. This protocol ensured recovery of the full contractile response and was repeated until 3 successive responses differed by less than 10%. Their average value was designated 100% and all subsequent responses were expressed as a percentage. The tissues were then incubated with 1 or 10μM PMA for 45min at which point 10μM ACh or histamine was again administered. Following washout and recovery a further two contractile responses to 10μM ACh or histamine were obtained.

In experiments using antagonists, these drugs were added to the organ bath 30 minutes before the addition of the PMA. In all experiments a time control was included to examine the effect of time alone on the response of the tissue to ACh.

5.9 Antagonist studies

In order to determine the contribution of Ca^{2+} influx, Na^{+}-H^{+} exchange and cyclo-oxygenase products to the effects of PMA in the submaximal dose studies, parallel series of experiments were run in which the tissues were incubated with verapamil (10μM), nifedipine (3μM), amiloride (100μM) or indomethacin (10μM) 30min prior to the administration of PMA. The effects of the putative PKC inhibitors H7 (10μM) and staurosporine (0.5μM) were also examined.

5.10 Solutions and Drugs.

The following drugs were used in this study: acetylcholine chloride, amiloride hydrochloride, atropine sulphate, histamine diphosphate, indomethacin, 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H7), nifedipine, phorbol 12-myristate 13-acetate (PMA), staurosporine, verapamil hydrochloride (all Sigma Chemical Co.).

PMA was prepared as a 0.01M stock solution in dimethyl sulfoxide and diluted with distilled water to give final bath concentrations of 0.001 to 100μM. Indomethacin, nifedipine and BAY-K were dissolved in a minimal quantity of alcohol then diluted with distilled water. Staurosporine was disolved in a solution of distilled water and 95% ethanol (1:1 ratio). All other compounds were made up in distilled water.

Composition of Krebs-Henseleit solution (millimoles per litre):

NaCl 118.4, $NaHCO_3$ 25.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5 & Glucose 11.0.

NB. In preparation of the nominally calcium-free Krebs 2.5mM $CaCl_2$ was omitted from the solution.

5.11 Data analysis

Data were expressed as mean ± standard error of the mean (s.e.m.) and statistical significance was assessed by means of paired two-tailed Student's *t*-tests in which $p<0.05$ was considered significant. In antagonist experiments the need for multiple comparisons negated the use of Student's *t*-tests and statistical significance was therefore assessed using analysis of variance (ANOVA) at 95% significance level.

In all first CCRC, the contractile response to each concentration of ACh was expressed as a percentage of the maximal response (Tmax) obtained to ACh in that tissue. Responses to each concentration of ACh in the second CCRC were also expressed as a percentage of the Tmax obtained for the first curve in that tissue. In each experiment the EC_{50} (the concentration of agonist producing 50% of the maximal response in that curve) was determined, and the corresponding pD_2 value (the negative log of the EC_{50}) calculated. pD_2 values were then compared using the paired Student's *t*-test.

Phorbol esters have previously been shown to contract smooth muscle (*section 4.5*). In this preparation contraction was frequently seen at 100μM PMA . Our experiments were carried out at subthreshold (≤10μM PMA) concentrations. Contractile responses did occasionally occur (<10% of tissues), but where any contraction amounted to more than 0.15g that data was omitted from the calculations. In a random sample of fifty tissues incubated with PMA, three (6%) produced a rise in baseline tension and were excluded from the analysis, as were another four tissues (8%) which developed spontaneous contractions on addition of PMA.

Section 6

RESULTS

6.1 Electrical field stimulation

While there is sympathetic, parasympathetic and NANC innervation of the lung (*section 2.1*), principal nervous control of the proximal bronchi is cholinergic. Electrical field stimulation of rabbit secondary bronchi produced an immediate rapid contraction. The response was biphasic; no relaxation was evident after the initial contraction and recovery. Contractions were elicited over a range of frequencies (0.5-100Hz) using the stimulation parameters: 50V, 0.1ms pulse width, 10s train, stimulating once every 5min. A graded increase in size of contraction was seen with increasing frequency (Fig. 6.1). Stimulation at 64 Hz produced the maximal response obtained in this preparation; subsequent contractions to 80 and 100Hz produced 98% and 90%, respectively, of the contractile response to 64Hz.

6.2 Effect of phorbol ester on responses to EFS

Addition of PMA to the tissue at time zero produced a slow steady increase in the contractile response to EFS which reached a plateau after 45-60 minutes. While it is not known at which frequencies the nerves are stimulated *in vivo,* the potentiation of response was seen across a range of frequencies. At 4, 16 & 30Hz, frequencies producing 24, 65 and 88% of maximum contraction to EFS (approx. 1g; Table 6.1), 60 minutes incubation with PMA (10μM) increased responses to stimulation to 131(16)%, 134(6)% and 104(6)% respectively (Fig. 6.2), all of which were significantly greater than their time controls ($n=5-9$; $p<0.05$).

Although greatest potentiation by PMA (an increase of approximately 30%) was seen at the lower stimulation frequencies, contractile responses to 4Hz EFS were generally smaller and more variable than those obtained at 16Hz and consequently frequently failed to reach significance. Stimulation at 16Hz consistently produced responses of 0.5–1.0 grams (approximately 50% of the maximum concentraction to ACh in this

preparation, 1.32±0.15g) and this frequency was employed in subsequent experiments.

The potentiation of the contractile response by PMA was concentration dependent. The contractile response to 16Hz EFS increased steadily over 45-60 minutes after the addition of 1-100μM PMA whereas lower concentrations (0.001-0.1μM PMA) failed to elicit any significant change. Time controls showed no increase over the same period (Fig. 6.3a).

At 60 minutes into incubation 1, 10 & 100μM PMA had produced a mean increase in stimulation-induced contraction from baseline values to 124(8), 178(27) and 235(50)% respectively (Fig. 6.3b). These responses were significantly greater than the respective time control values of 77(9), 74(10) and 94(4)% ($n=5-9$; $p<0.05$).

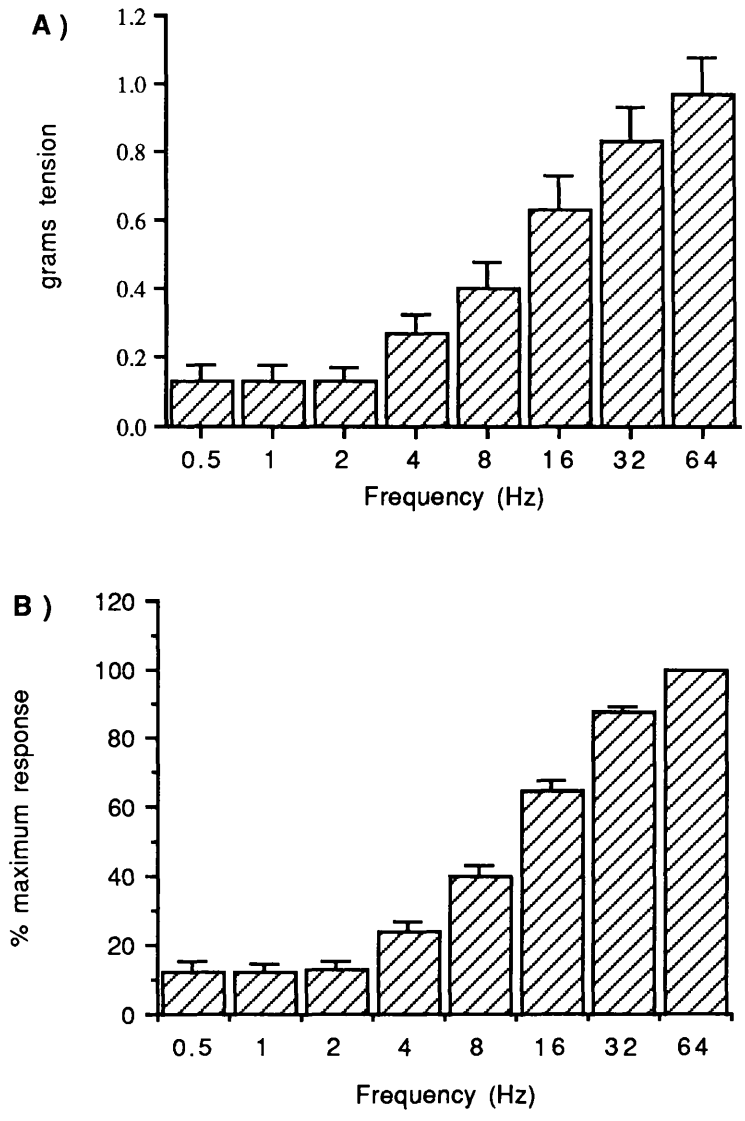


Figure 6.1

Effect of frequency on the response of rabbit secondary bronchi to EFS. Responses to 0.5–64Hz are expressed as A) as grams tension, or B) as a percentage of the maximum contraction elicited by 64Hz in each tissue.

Each bar represents the mean ± s.e.m. (n=5, x=14).

Table 6.1

Response of rabbit secondary bronchi to EFS at different frequencies.

Stimulation Frequency	Tension Produced (grams)	% Max response to EFS (64Hz)	% Max response to ACh (300μM)
4 Hz	0.27 (0.06)	24%	20%
16 Hz	0.63 (0.10)	65%	48%
30 Hz	0.83 (0.11)	88%	63%

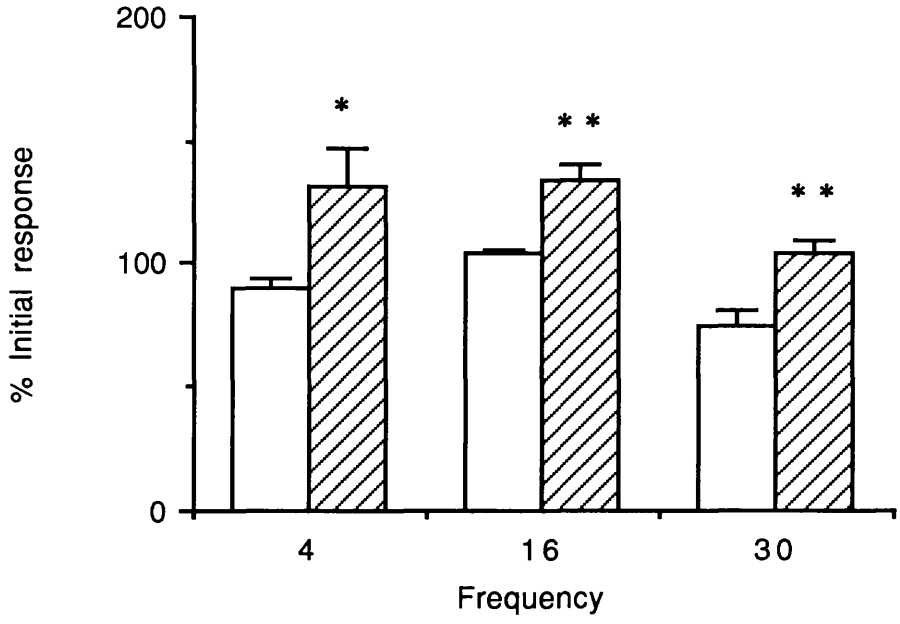


Figure 6.2

Effect of incubation with PMA on the response of rabbit secondary bronchi to EFS. Results are shown at 60 minutes into the incubation period and are expressed as a percentage of the initial response to EFS. Open bars represent time control responses to 4, 16 & 30 Hz, hatched bars show the effect of 10μM PMA. Each bar represents the mean ± s.e.m. (n=5-9). Statistically significant differences between responses in the absence and presence of PMA in paired tissues are represented by *$p<0.05$, **$p<0.01$, Student's *t*-test.

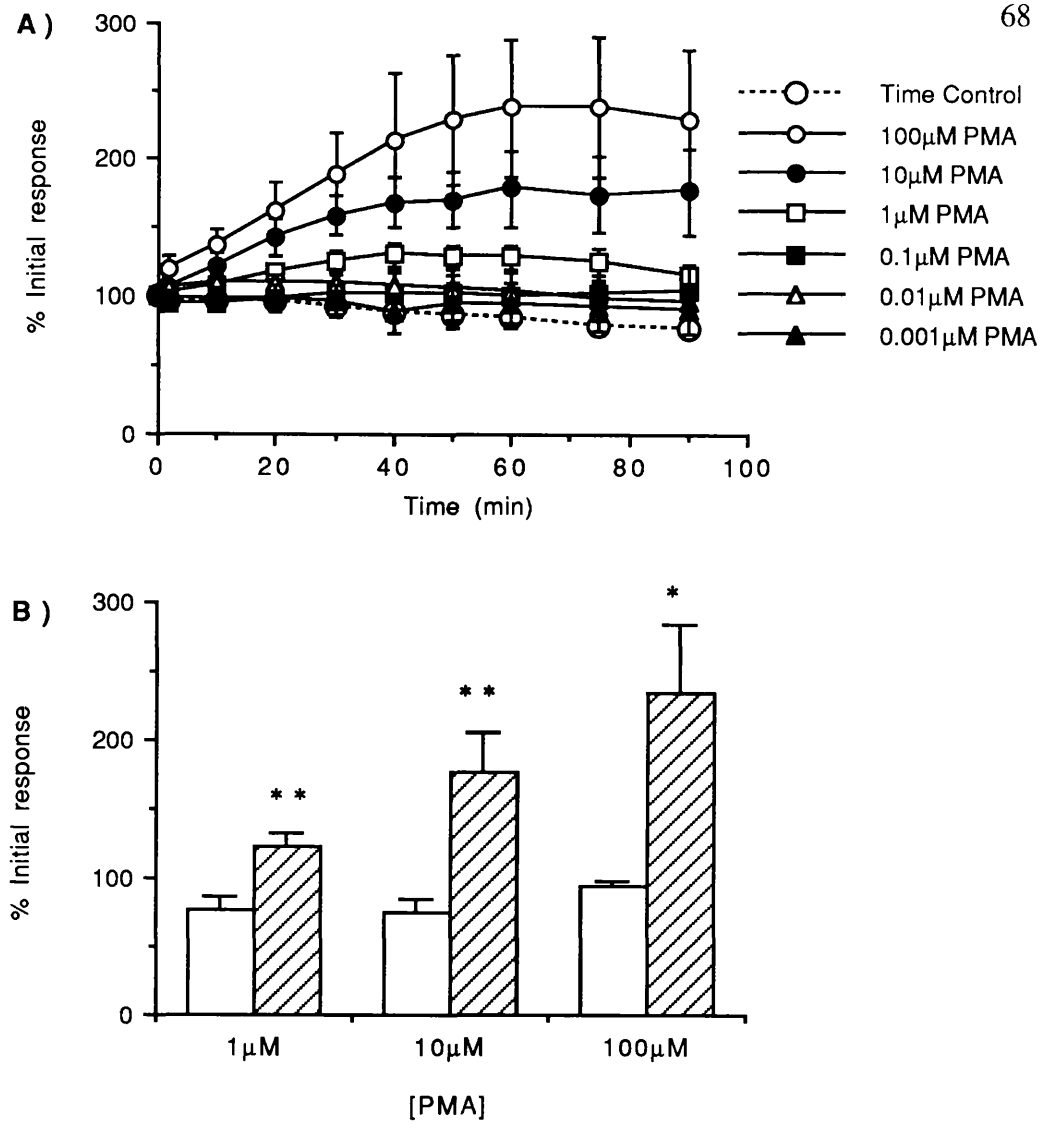


Figure 6.3

Effect of concentration on the potentiation of response to EFS of rabbit secondary bronchi. Results are expressed as a percentage of the initial response to 16Hz EFS and show A) the effect of 0.001–100μM PMA on the response to EFS over a 90 minute period, and B) the effect of 1–100μM PMA at the plateau phase, 60 minutes into the incubation period. Open bars represent time control responses to EFS, hatched bars represent test responses in the presence of PMA.

Each point represents the mean ± s.e.m. (n=7-8). Statistically significant differences between responses in the absence and presence of PMA in paired tissues are represented by *$p<0.05$, **$p<0.01$, Student's *t*-test.

6.3 Effects of antagonists on the potentiation of EFS by PMA

Atropine

Addition of 0.1μM atropine, a muscarinic antagonist, virtually abolished the response of the control tissues and also of the PMA-treated tissues to EFS at 16Hz. At 60 minutes into incubation PMA had increased the response in test tissues to 250(60)% of its initial value (Fig. 6.4). This was significantly different from time control values which had decreased to 85(5)% ($n=6$; $p<0.05$). Atropine greatly decreased the control response to EFS which was reduced, by 60 minutes, to 5(4)% ($p<0.001$). PMA failed to potentiate responses in the presence of atropine ($p>0.05$); responses remained at only 8(3)% of their initial value ($p<0.001$).

Verapamil

Incubation with verapamil (10μM) decreased the response of rabbit secondary bronchi to EFS (Fig. 6.4). Over the incubation period verapamil reduced the mean response to EFS to 48(6)%, while paired time controls showed no significant change in size at 91(6)%. PMA (10μM) produced an increase in response to EFS to 185(37)%. Pre-incubation with verapamil prevented the potentiation of response by PMA, at the 60minute time point responses to EFS in these tissues reached only 45(11)% of their initial response ($n=5, p>0.05$).

H7

Incubation with H7 attenuated the PMA-induced potentiation of response to EFS. H7 (10μM) itself significantly decreased the response from 0.82(0.1)g to 0.55(0.1)g, a decrease of 33(2.9)% (expressed as a percentage of the initial control response), while paired control tissues showed no significant change ($n=12$, $p<0.05$). Addition

of PMA (10μM) produced a significant increase in the response to EFS in both the control tissues and those pre-incubated with H7. 60 minutes incubation with PMA produced an increase of 37(8.3)% (n=5, $p<0.05$) in the control tissues and a significantly smaller increase of 15(2.6)% in H7-treated tissues. Even expressed as a percentage of the post-H7 response, the potentiation by PMA, at 17(4.1)%, was less than without H7 (Fig. 6.5).

Staurosporine

Incubation with staurosporine prevented the potentiation of response to EFS by PMA. Staurosporine (0.5 μM) itself decreased the response of rabbit secondary bronchi to EFS (Fig. 6.5); over the incubation period the mean response was significantly reduced to 54(7.2)% ($p<0.05$; n=5). PMA (10μM) produced a significant increase in response to EFS to 137(12.7)%, while paired time controls showed no change in size at 100(5.5)%. Pre-incubation with staurosporine prevented any potentiation of response by PMA, at the 60minute time point responses to EFS in these tissues reached only 50(17.4)% of their initial response (n=5,$p>0.05$).

Results of antagonist studies are summarised in tables 6.2 & 6.3, and in figure 6.6.

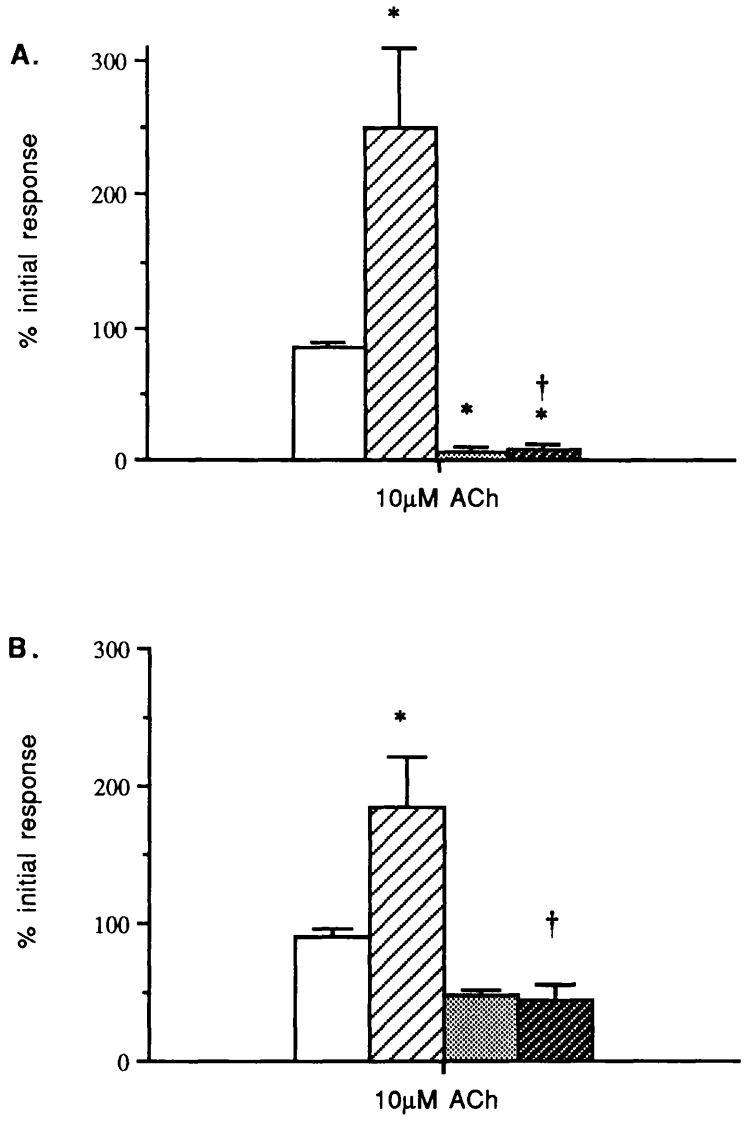


Figure 6.4

Effect of atropine and verapamil on the PMA-induced potentiation of response to EFS in rabbit secondary bronchi. Open bars represent time control responses to 16Hz EFS. Second bars represent the response to EFS at 60 minutes into incubation with 10μM PMA. Third and fourth bars represent the effect of antagonist alone, A) atropine (0.1μM) or B) verapamil (10μM), and the effect of antagonist + PMA respectively.

Responses are expressed as a percentage of the initial response obtained in each tissue. Each point represents the mean ± s.e.m. (n=5-6). Statistically significant differences between test responses and paired controls (*), between (antagonist + PMA) and PMA alone (†), and between (antagonist + PMA) and antagonist alone (#) were assessed by ANOVA, 95% confidence limits.

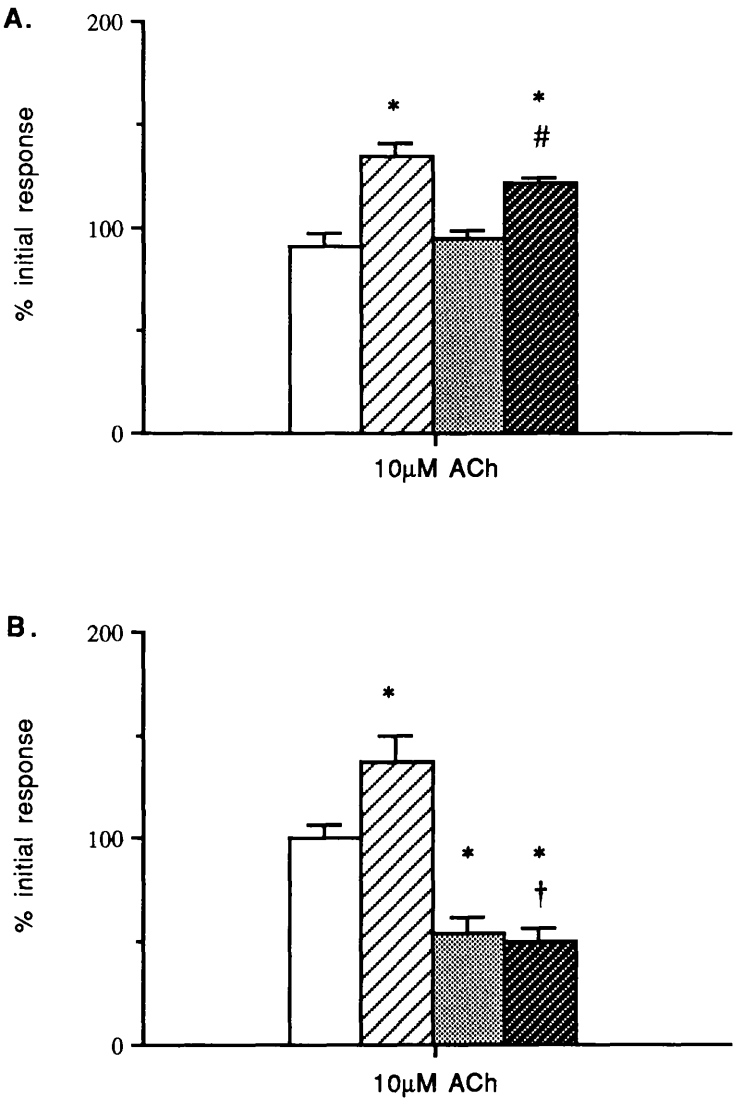


Figure 6.5

Effect of H7 and staurosporine on the PMA-induced potentiation of response to EFS in rabbit secondary bronchi. Open bars represent time control responses to 16Hz EFS. Second bars represent the response to EFS at 60 minutes into incubation with 10μM PMA. Third and fourth bars represent the effect of antagonist alone, A) H7 (10μM) or B) staurosporine (0.5μM), and the effect of antagonist + PMA respectively.

Responses are expressed as a percentage of the initial response obtained in each tissue. Each point represents the mean ± s.e.m. (n=5). Statistically significant differences between test responses and paired controls (*), between (antagonist + PMA) and PMA alone (†), and between (antagonist + PMA) and antagonist alone (#) were assessed by ANOVA, 95% confidence limits.

Table 6.2

The effect of antagonists on PMA-induced potentiation of response to 16Hz EFS

Mean (s.e.m.)% of initial response at 60 minutes into incubation period

	Atropine	*Verapamil*	*H7*	*Staurosporine*
Control	85 (5)	91 (6)	82 (4)	100 (6)
PMA	250 (60)	185 (37)	139 (12)	137 (13)
Antagonist	5 (4)	48 (3)	60 (8)	54 (7)
PMA + Antagonist	8 (3)	45 (11)	90 (6)	50 (17)

Table 6.3

The effect of antagonist on the PMA-induced potentiation of response to EFS

Significant differences at 60 minutes into incubation period

	Atropine	*Verapamil*	*H7*	*Staurosporine*
Control / PMA alone	●	●	●	●
Control / antag. alone	●	○	○	●
Control / PMA+antag.	●	○	●	●
PMA / antag.	●	●	●	●
PMA / antag. + PMA	●	●	○	●
antag. / antag. + PMA	○	○	●	○

(Significance assessed by ANOVA: ● significant difference; ○ no significant difference; 95%C.I.)

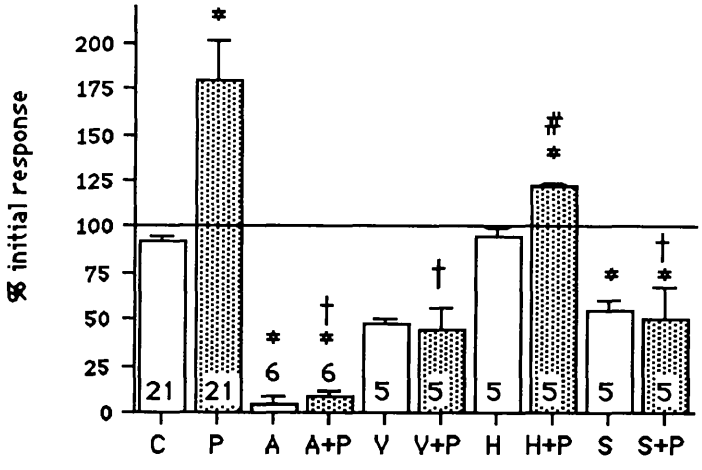


Figure 6.6

Effect of antagonists on the PMA-induced potentiation of response to 16Hz EFS. Bars represent responses to EFS in control tissues (C) and those treated with PMA (P), an antagonist (atropine [A], verapamil [V], H7[H] or staurosporine [S]), or both.

Responses are expressed as a percentage of the initial response obtained in each tissue. Bars are mean ± s.e.m., the number of experiments in each group are shown within the bars. Statistically significant differences between test responses and paired controls (*), between (antagonist + PMA) and PMA alone (†), and between (antagonist + PMA) and antagonist alone (#) were assessed by ANOVA, 95% confidence limits.

6.4 Cumulative concentration response curves to acetylcholine

The sensitivity and the maximum size of response of rabbit secondary bronchi to ACh is reproducible with time. In two cumulative concentration response curves (CCRC) to ACh in the same tissue, seperated by 45 minutes (Fig. 6.7), neither the response to the highest concentration tested (Tmax; 30μM ACh) nor the sensitivity of the tissue was altered; pD_2 values obtained for the first and second curves (5.28 (0.12) & 5.28 (0.08) respectively) were not significantly different (n=12; $p>0.05$).

6.5 Effect of PMA on ACh CCRC

Incubation with 1μM PMA 45 minutes prior to starting the second CCRC had no significant effect on the sensitivity of the tissue (pD_2 values for pre- and post-incubation curves were 5.51 (0.11) and 5.44 (0.10) respectively; n=7, $p>0.05$) or on the maximum contractile response to ACh (Tmax: 1.014 (0.255) and 0.980 (0.107) grams respectively. Fig 6.8).

Following 45 minutes incubation with 10μM PMA responses to individual concentrations of ACh (0.3, 1, 3, 10μM) within the CCRC were significantly increased in test tissues (Fig. 6.9; n=8, $p<0.05$). There was no significant increase in the contraction produced by the maximum concentration of ACh employed (Tmax: 1.337 (0.159) grams), but PMA produced a small leftward shift, increasing the mean pD_2 value of test tissues from 5.51 (0.15) to 5.80 (0.15) with the result that the second curve was significantly more sensitive than its corresponding time control (pD_2: 5.25 (0.04); $p<0.05$).

Correcting the second CCRC test figures for the small change in the control responses with time confirmed these findings (Fig. 6.10). There was no significant increase in the maximum response produced in test tissues but there was a

potentiation of response to sub-maximal concentrations of ACh (1, 3 & 10μM; n=8, $p<0.05$) and a leftward shift; test tissue pD_2 values were significantly greater than those of the time controls (5.75 (0.18) and 5.24 (0.04) respectively, $p<0.05$).

In the original experiments (Figs. 6.7-6.10) the CCRC extended from concentrations of 1nM to 30μM ACh. Higher concentrations were not initially examined due to the possibility of compromising the contractile apparatus of the preparation. Exceeding a dose of approximately 95% of maximum may do irreversible damage to the preparation (Blattner et al, 1978). However, the curves were subsequently extended to 300μM ACh in another six pairs of bronchi without any apparent damage to the test tissues. These experiments produced a similar pattern of potentiation. PMA significantly increased the response to submaximal concentrations (1, 10 & 30μM) of ACh but produced no significant increase in response to 100 or 300μM ACh and no significant shift in the pD_2 value (Fig. 6.11).

Combining all the available data (Fig. 6.12; n=14), PMA produces a potentiation of response at 0.1-30μM ACh.

6.6 *Effect of PMA on ACh CCRC in low calcium Krebs*

In contrast to the above reports, 10μM PMA did not significantly increase the responses within the ACh CCRC in nominally calcium-free Krebs (Fig. 6.13). Incubation with PMA did not produce a significant shift in pD_2 value (T1 4.97(0.14); T2 4.81(0.24)). However, PMA did prevent the rightward shift in sensitivity shown by the controls in low calcium Krebs (pD_2 values: C1 5.05(0.13); C2 4.37(0.14)). This shift was significant ($p<0.05$) and indicates a reduction in sensitivity to ACh of the control tissues. Comparing the size of the shift within individual control and test tissues a significant difference was seen (n=5, $p<0.01$). Incubation with 10μM PMA, therefore, appears to halt the desensitization of the tissue rings. Tmax was not significantly reduced by incubation in calcium free Krebs.

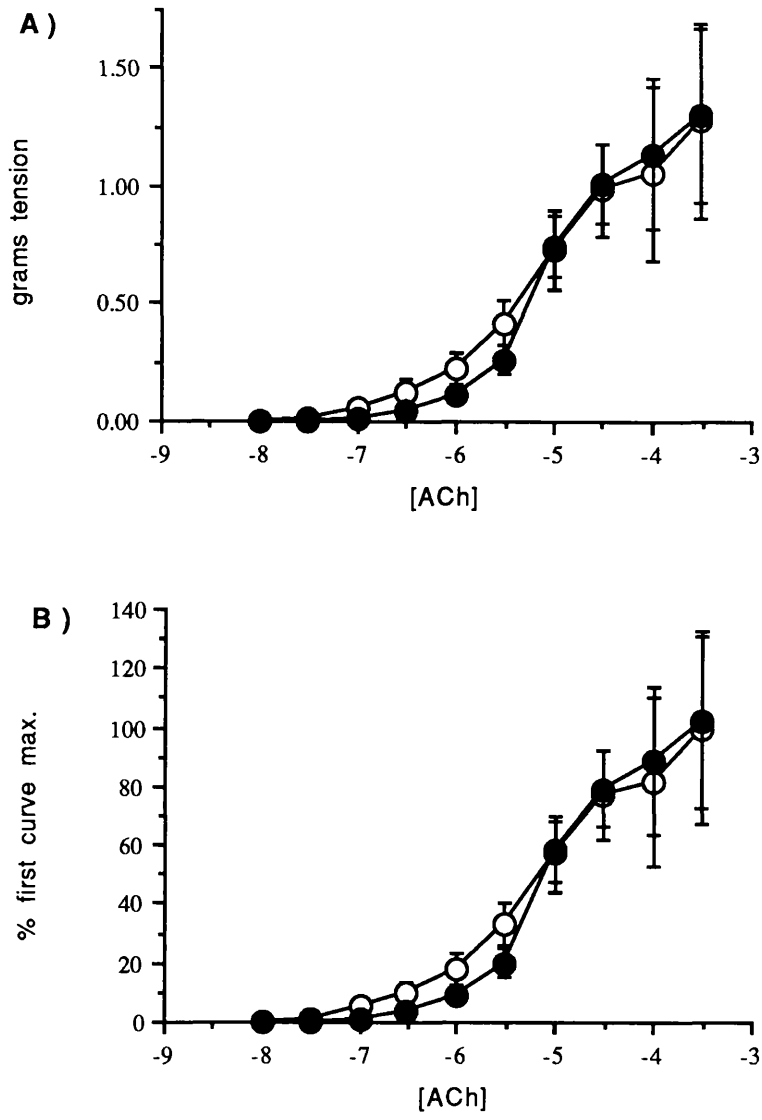


Figure 6.7

Effect of time on the response of rabbit secondary bronchi to ACh. Open symbols (○) represent initial ACh cumulative concentration response curves (CCRC) in 12 tissues. Filled symbols (●) represent the responses to a second ACh CCRC in the same tissues 45 minutes later. Responses are expressed A) as grams tension or B) as a percentage of the largest response obtained in each first curve. Statistical comparisons were made between the responses obtained in the 1st and 2nd curves at each concentration (paired t-test; n=12).

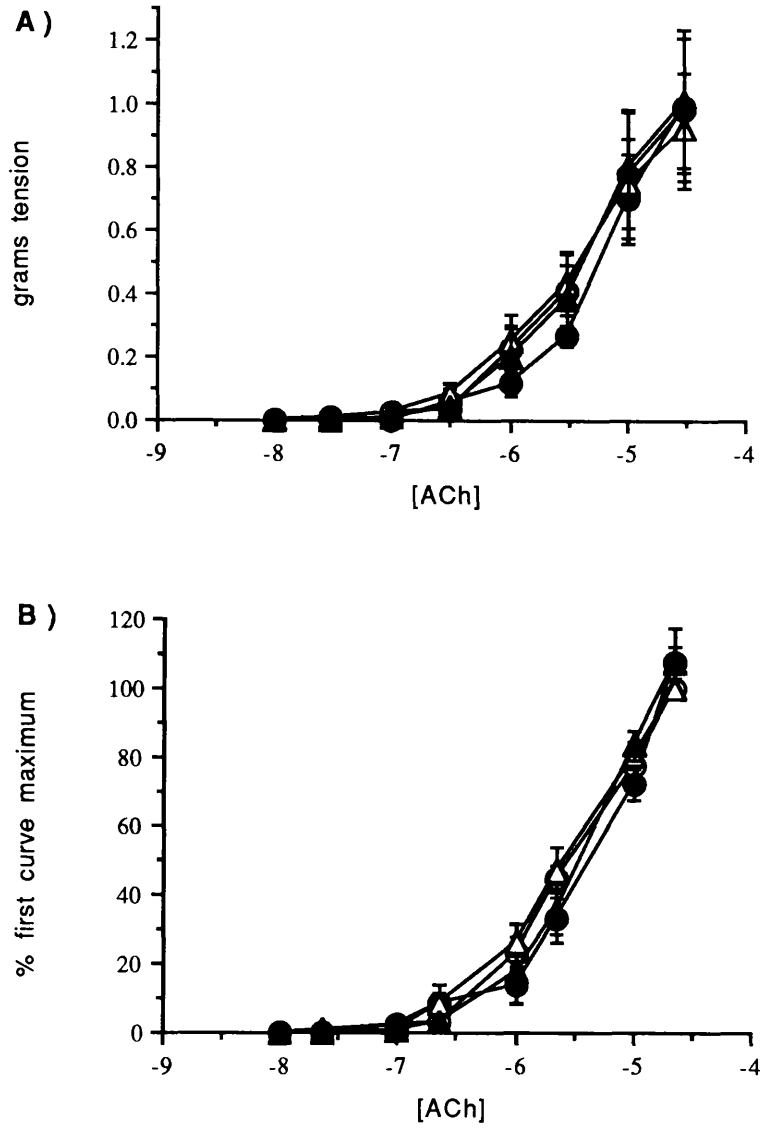


Figure 6.8

Effect of 1μM PMA on the response of rabbit secondary bronchi to ACh. Open symbols (○,△) represent responses to an ACh cumulative concentration response curve (CCRC) in two sets of paired tissues. Filled symbols represent the responses to second CCRCs in the presence (▲) or absence (●) of 1μM PMA. Responses are expressed A) as grams tension or B) as percentages of the largest response obtained in each first curve. Each point represents the mean±s.e.m. (n=7). Statistical comparisons were made between the responses obtained in the 2nd curve of paired tissues at each concentration (paired *t*-test, *$p<0.05$)

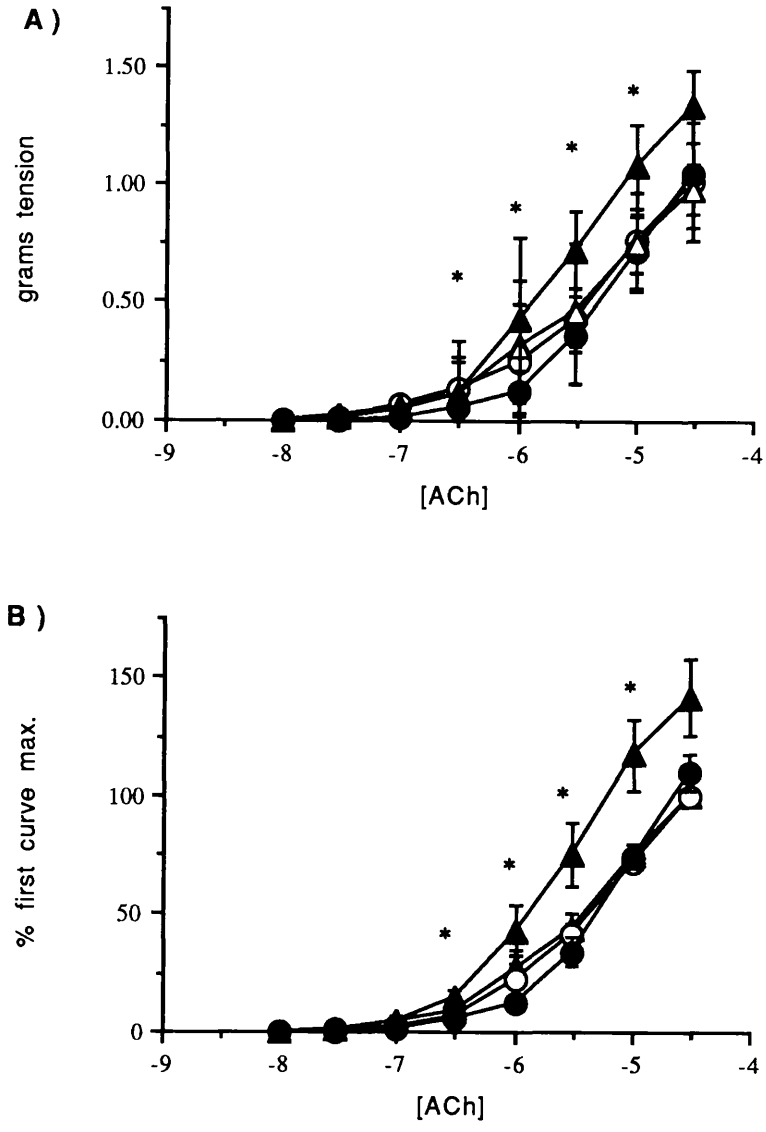


Figure 6.9

Effect of 10μM PMA on the response of rabbit secondary bronchi to ACh. Open symbols (○,△) represent responses to an ACh cumulative concentration response curve (CCRC) in two sets of paired tissues. Filled symbols represent the responses to second CCRCs in the presence (▲) or absence (●) of 10μM PMA. Responses are expressed A) as grams tension or B) as percentages of the largest response obtained in each first curve. Each point represents the mean±s.e.m. (n=8). Statistical comparisons were made between the responses obtained in the 2nd curve of paired tissues at each concentration (paired *t*-test, *$p<0.05$)

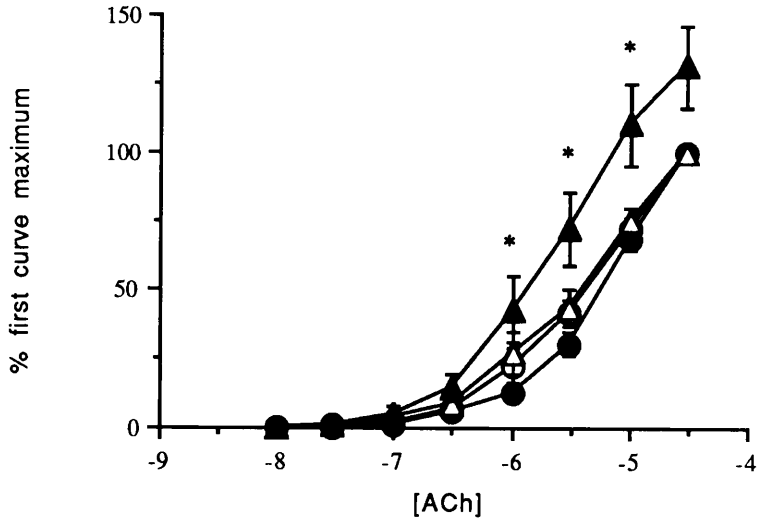


Figure 6.10

Effect of 10μM PMA on the response of rabbit secondary bronchi to ACh correcting for changes in control tissues with time. Open symbols (△,○) represent responses to an ACh cumulative concentration response curve (CCRC) in two sets of paired tissues. Filled symbols show the responses to second CCRCs in the presence (▲) or absence (●) of 10μM PMA. Results are expressed as a corrected percentage of the maximum response to ACh in the first CCRC. Each point represents the mean ± s.e.m.(n=8). Statistical comparisons were made between the responses obtained in the 2nd curves of paired tissues at each concentration (paired *t*-test; *$p<0.05$).

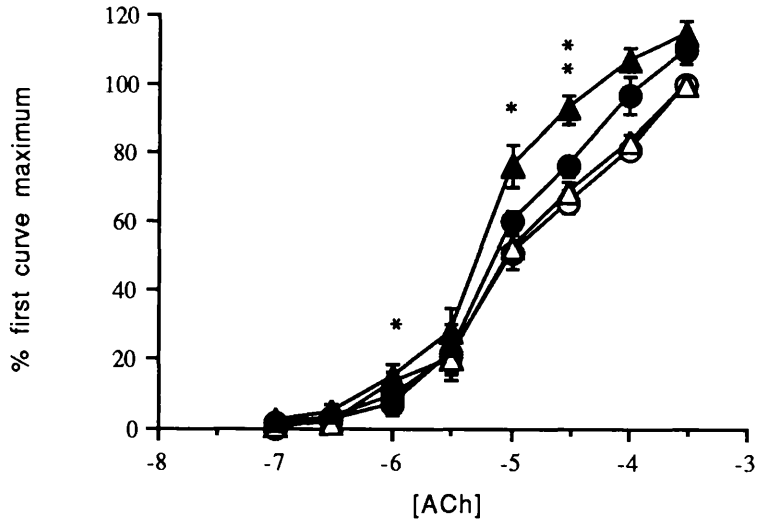


Figure 6.11

Effect of PMA on a cumulative concentration response curve (CCRC) to ACh in rabbit secondary bronchi. Open symbols represent initial control CCRCs in two sets of paired tissues (○,△). Filled symbols show the responses to second CCRCs in the presence (▲) or absence (●) of 10μM PMA. Responses are expressed as percentages of the largest response obtained in each first curve. Statistical comparisons were made between the responses obtained in the second curve of paired tissues at each concentration (paired *t*-test, *$p<0.05$, **$p<0.01$; n=6).

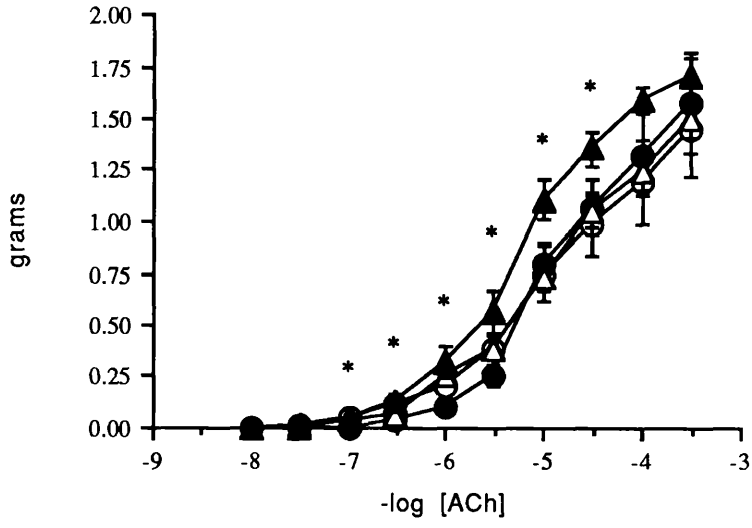


Figure 6.12

Effect of PMA on a cumulative concentration response curve (CCRC) to ACh in rabbit secondary bronchi. Open symbols represent initial control CCRCs in two sets of paired tissues (○,△). Filled symbols show the responses to second CCRCs in the presence (▲) or absence (●) of 10μM PMA. Responses are expressed as grams tension produced. Each point represents the mean ± s.e.m. (n=14). Statistical comparisons were made between the responses obtained in the second curve of paired tissues at each concentration (paired *t*-test, *$p<0.05$, **$p<0.01$).

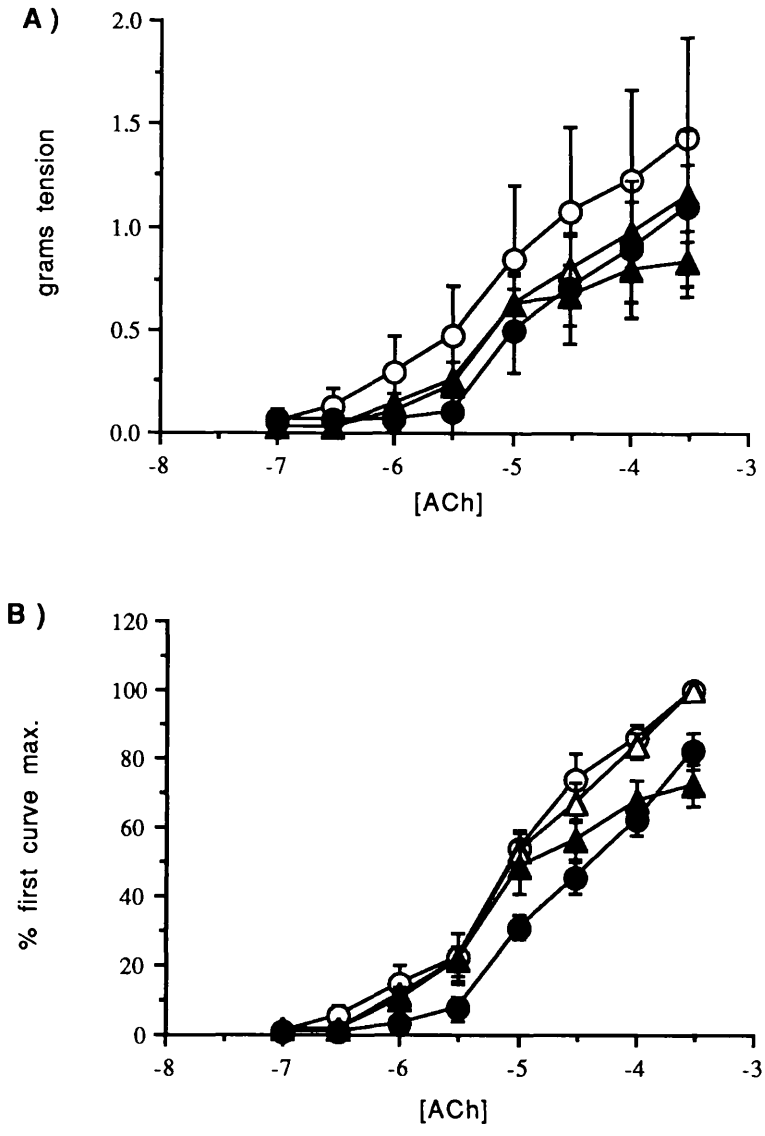


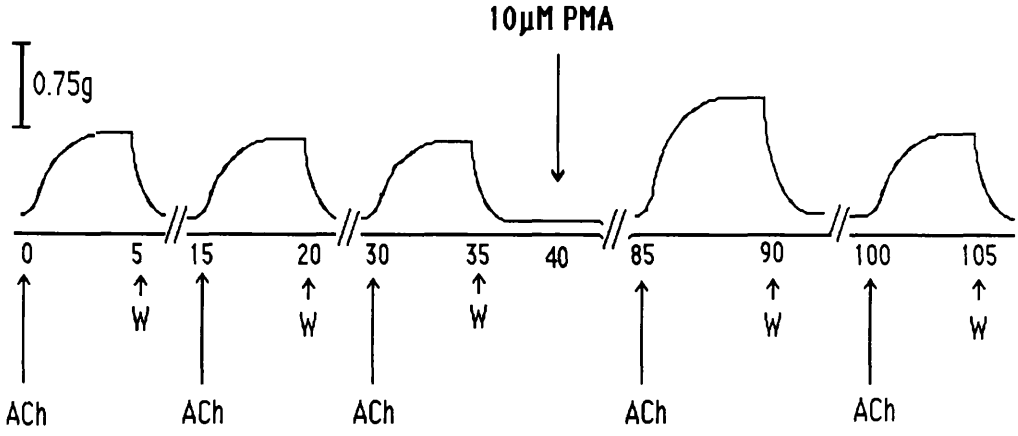
Figure 6.13

Effect of 10μM PMA on the response of rabbit secondary bronchi to ACh in a low calcium environment. Open symbols (△,○) represent responses to an ACh cumulative concentration response curve (CCRC) in two sets of paired tissues. Filled symbols show the responses to second CCRCs, in low calcium Krebs, in the presence (▲) or absence (●) of 10μM PMA. Results are expressed A) as grams tension or B) as a percentage of the largest response obtained in each first curve. Each point represents the mean ± s.e.m.(n=5). Statistical comparisons were made between the responses obtained in the 2nd curves of paired tissues at each concentration (paired *t*-test; *$p<0.05$).

6.7 *Sub-maximal dose studies: responses to ACh*

The contractile response of rabbit secondary bronchi to ACh was sustained and reproducible with time, as illustrated below. The response produced by 10μM ACh (0.743(0.11) grams) was equivalent to 56% of the maximum contraction to ACh in this tissue (1.316(0.15) grams).

Time course of the submaximal dose experiments.



6.8 *Effect of PMA on responses to ACh.*

Incubation with 10μM but not 1μM, PMA potentiated the contractile response of secondary bronchi to a single sub-maximal concentration of 10μM ACh (Fig. 6.14). 1μM PMA produced an increase in response to ACh of 39(14)% but this failed to reach significance as controls showed a slight increase of 11(6)% with time (n=6; $p>0.05$). In contrast, 45 minutes incubation with 10μM PMA increased the response to 10μM ACh to 169(20)% of its initial value which was significantly ($p<0.01$; n=8) greater than the time control 97(11)%. Following washout with fresh KH solution,

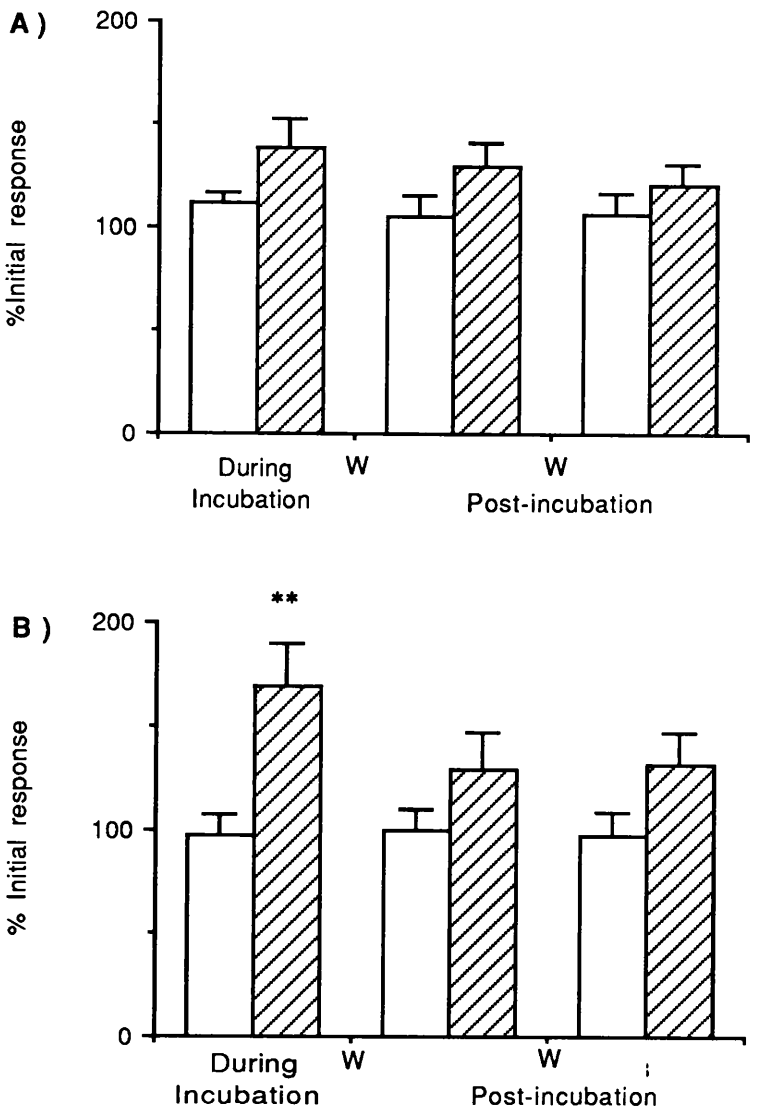


Figure 6.14

Effect of PMA on the response of rabbit secondary bronchi to a submaximal concentration of ACh. Open bars represent time control responses to 10μM ACh, hatched bars represent test responses at 45 minutes into incubation with A) 1μM or B) 10μM PMA. Responses to two subsequent contractions to 10μM ACh at 15 minute intervals are also shown.

Each point represents the mean ± s.e.m. (n=7–8). Statistically significant differences between control responses and those in the presence of PMA are represented by *$p<0.05$, **$p<0.01$, Student's *t*–test.

subsequent responses of test tissues to 10μM ACh remained raised (130 (17)% and 132 (15)% of initial response) but were not significantly greater than the time control values 100 (10)%, and 98 (11)% respectively (n=8; p>0.05).

6.9 Effect of antagonists on the potentiation of response to ACh

Nifedipine

Nifedipine (0.3μM) inhibited the potentiation of response to 10μM ACh by 10μM PMA (Fig. 6.15). In this series of 5 experiments PMA significantly increased the response of test tissues to ACh to 135(6.5)% of their initial size while controls remained steady at 102(7)% (p<0.05). Incubation with nifedipine alone had no effect on the size of reponse to ACh (100(5)%) but the dihydropyridine abolished the subsequent potentiation by PMA; test responses reached 84(13)% of their initial value which was not significantly different from the time control (p>0.05).

Verapamil

Verapamil (10μM) itself reduced the response to 10μM ACh by 38(10)% but this failed to reach significance (p>0.05, ANOVA). PMA alone significantly increased the response of test tissues to 170(18)% (n=5,p<0.05) of their initial value, while 30 minutes pre-incubation with verapamil abolished this potentiation, responses reached 59(10)% of their initial size which was (somewhat surprisingly) not significantly different from the control value of 95(6)% (ANOVA) (Fig. 6.15)

Amiloride

Amiloride (100μM) attenuated but did not significantly inhibit the potentiation of response to 10μM ACh by PMA (Fig. 6.16). In the this series of experiments (n=7),

PMA increased the response of test tissues to 155(16)% of their initial value while control tissue responses remained steady at 108(4)%. Amiloride itself reduced the response to 86(11)% and in the presence of this inhibitor the increase produced by PMA, 123(20)%, failed to reach significance. However this response was not significantly different from that of PMA alone ($p>0.05$) and may therefore have depressed rather than abolished the response to PMA.

Indomethacin

Indomethacin (10μM) had no effect on the PMA potentiation of response to ACh (Fig. 6.16). In this series of experiments, PMA produced an increase in response to 148(5)% of the initial value ($n=5$; $p<0.05$). Responses in the presence of indomethacin (122(2)%) were also significantly greater than time control values (91(8)%). In the presence of both indomethacin and PMA responses reached 157(18)% of their initial value and were significantly different from both control responses and those in the presence of indomethacin alone, but not from those in the presence of PMA alone.

H7

Incubation with H7 (10μM) had no significant effect on the response (102(9)%) to ACh. PMA produced an increase in response of test tissues to 162(18)% of initial values, which was significantly different from the mean time control value of 104(10)% ($n=7$; $p<0.05$; Fig. 6.17). The response in the presence of both PMA and H7 (137(19)%) was significantly greater than that in the presence of H7 alone, but was not significantly greater than time control values or significantly smaller than the increase with PMA alone (i.e.comparing control values with those in the presence of both drugs the results suggest H7 has abolished the PMA effect. However,

comparing H7 alone with H7 plus PMA there is evidently a PMA-induced increase in size of response despite the presence of H7)

Staurosporine

Staurosporine (0.5μM) produced a reduction in the response to ACh (Fig. 6.17). In this set of 5 experiments, staurosporine significantly reduced the response to 10μM ACh to 35 (5)% ($p<0.05$) of its initial value while PMA produced an increase to 144 (5)% ($p<0.05$), and control responses remained steady at 109 (8)% of their initial value. In the presence of both staurosporine (0.5μM) and PMA responses reached 82(8)% of their initial value, which was significantly different from those in the presence of either drug alone ($n=6$; $p<0.05$, ANOVA).

The results of the antagonist studies are summarised in tables 6.4 & 6.5 and in figure 6.18.

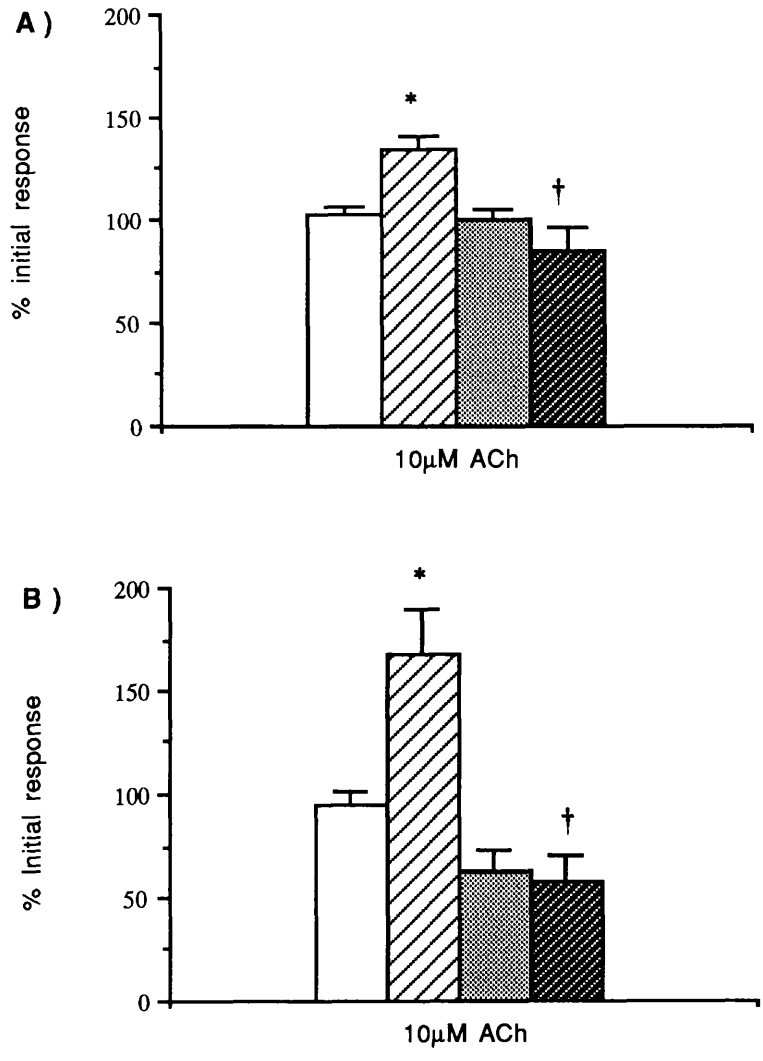


Figure 6.15

Effect of calcium channel blockade on the PMA-induced potentiation of response to ACh in rabbit secondary bronchi. Open bars represent time control responses to 10μM ACh. Hatched bars represent test responses at 45 minutes into incubation with 10μM PMA. Third and fourth bars represent the effect of antagonist alone, A) nifedipine (0.3μM) or B) verapamil (10μM), and the effect of antagonist + PMA respectively.

Each point represents the mean ± s.e.m. (n=5-6). Statistically significant differences between test responses and paired controls (*), between (antagonist + PMA) and PMA alone (†), and between (antagonist + PMA) and antagonist alone (#) were assessed by ANOVA, 95% confidence limits.

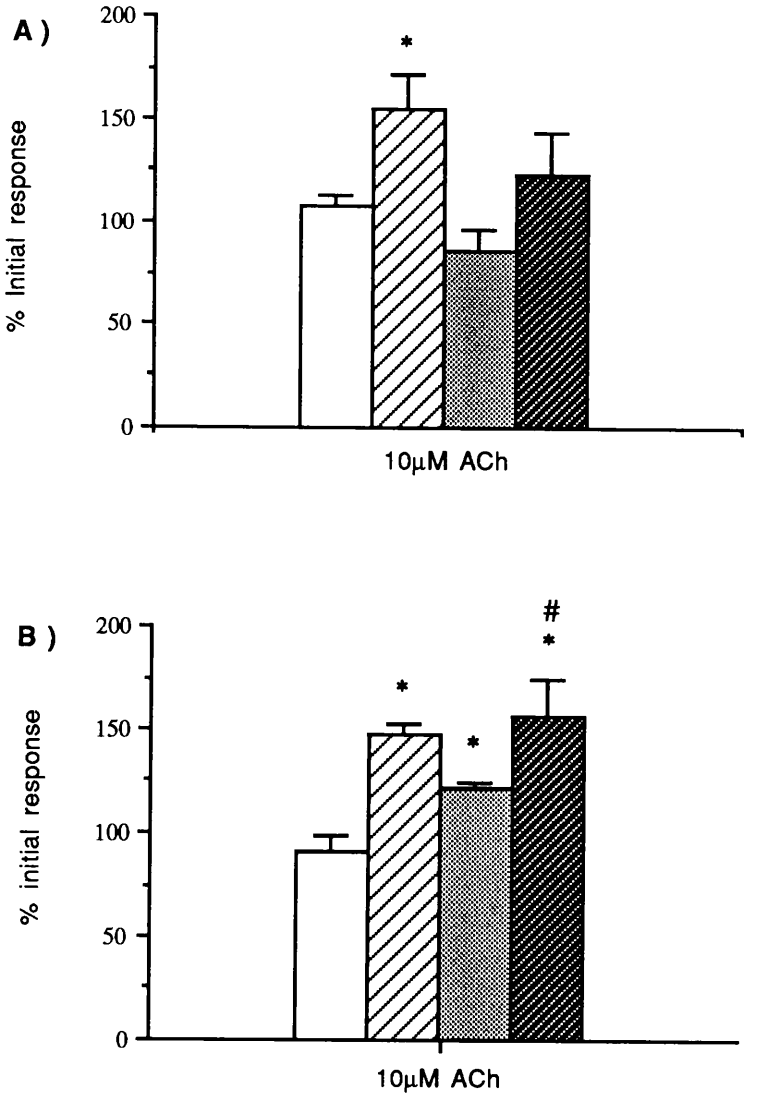


Figure 6.16

Effect of amiloride and indomethacin on the PMA-induced potentiation of response to ACh in rabbit secondary bronchi. Open bars represent time control responses to 10μM ACh. Hatched bars represent test responses at 45 minutes into incubation with 10μM PMA. Third and fourth bars represent the response in the presence of A) amiloride (100μM) or B) indomethacin (10μM), and the effect of antagonist + PMA respectively.

Results are expressed as a percentage of the initial response to 10μM ACh. Each point represents the mean ± s.e.m. (n=5-7). Statistically significant differences between test responses and paired controls (*), between (antagonist + PMA) and PMA alone (†), and between (antagonist + PMA) and antagonist alone (#) were assessed by ANOVA, 95% confidence limits.

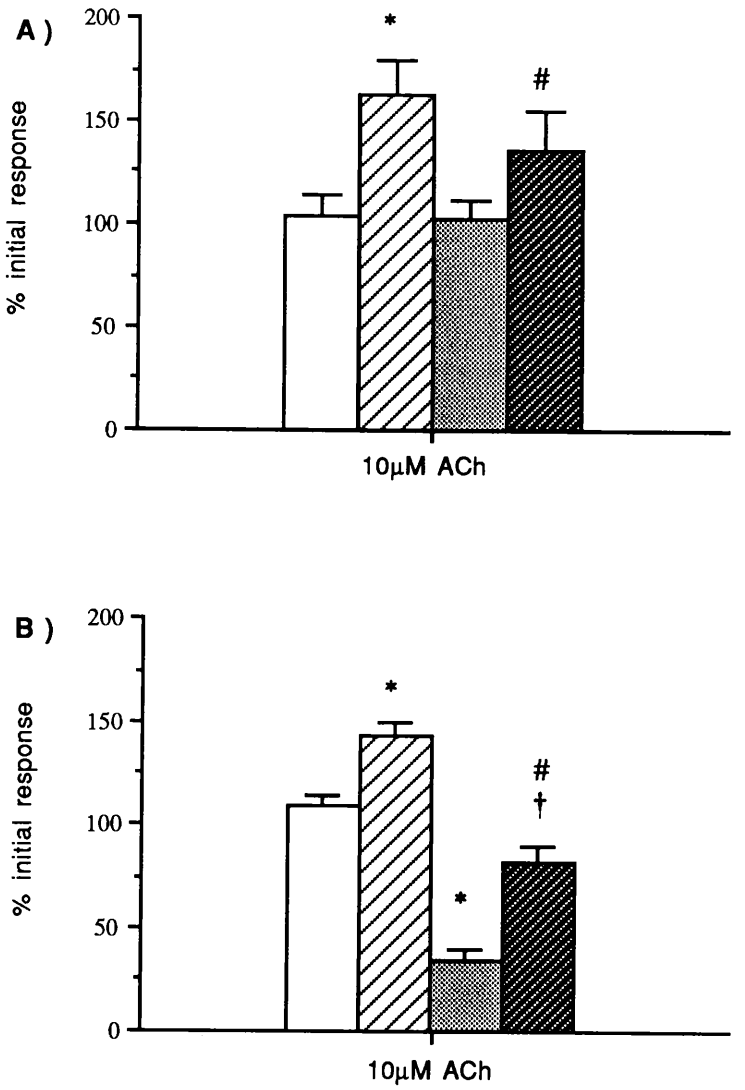


Figure 6.17

Effect of inhibition of PKC on the PMA-induced potentiation of response to ACh in rabbit secondary bronchi. Open bars represent time control responses to 10μM ACh. Hatched bars represent test responsse at 45 minutes into incubation with 10μM PMA. Third and fourth bars represent the effect of inhibitor alone, A) H7 (10μM) or B) staurosporine (0.5μM), and the effect of inhibitor + PMA respectively.

Each point represents the mean ± s.e.m. (n=5-7). Statistically significant differences between test responses and paired controls (*), between (antagonist + PMA) and PMA alone (†), and between (antagonist + PMA) and antagonist alone (#) were assessed by ANOVA, 95% confidence limits.

Table 6.4

Effect of antagonists on the potentiation of response of rabbit secondary bronchi to 10μM ACh by 10μM PMA.

Expressed as a percentage (±sem) of the initial response to 10μM ACh(n=5-7)

Antagonist	*Control*	*PMA*	*Antagonist*	*PMA+Antag.*
0.3 μM Nifedipine	102 (7)	135 (7)	100 (5)	84 (13)
10 μM Verapamil	95 (6)	170 (18)	62 (10)	59 (10)
10 μM Indomethacin	91 (8)	148 (5)	122 (2)	157 (18)
100μM Amiloride	108 (4)	155 (16)	86 (11)	123 (20)
10 μM H7	104 (10)	162 (18)	102 (9)	137 (19)
0.5μM Staurosporine	109 (4)	144 (5)	35 (5)	82 (8)

Table 6.5

Effect of antagonists on the potentiation of response of rabbit secondary bronchi to 10μM ACh by 10μM PMA.

(significant difference assessed by ANOVA test, 95% C.L.)

	Nifedipine	*Verapamil*	*Amiloride*	*Indomethacin*	*H7*	*Staurosporine*
Control / PMA alone	●	●	●	●	●	●
Control / antag. alone	○	○	○	●	○	●
Control / PMA+antag.	○	○	○	●	○	●
PMA / antag.	●	●	●	○	●	●
PMA / antag. + PMA	●	●	○	○	○	●
antag. / antag. + PMA	○	○	○	●	●	●

(● significant difference; ○ no significant difference)

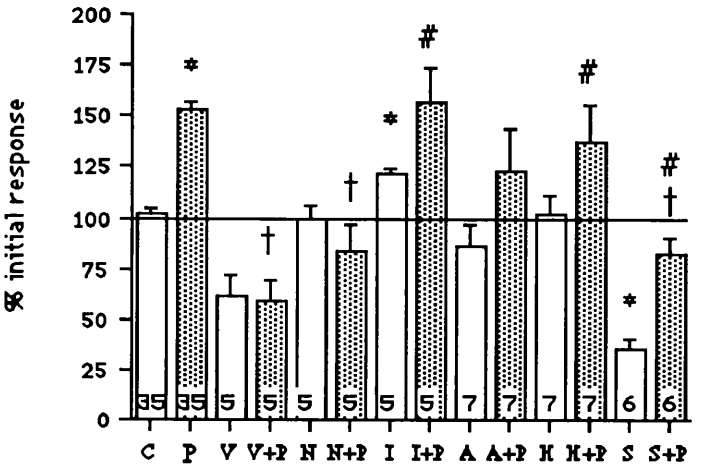


Figure 6.18

Effects of antagonists on the PMA-induced potentiation of response to 10μM ACh. Bars represent responses to ACh in control tissues (C) and those treated with PMA (P), an antagonist (verapamil [V], nifedipine [N], indomethacin [I], amiloride [A], H7[H] or staurosporine [S]), or antagonist plus PMA.

Bars are mean ± s.e.m., the number of experiments in each group is shown within the bars. Significant differences between "antagonist" and paired controls (*), between (antagonist + PMA) and PMA alone (†), and between (antagonist + PMA) and antagonist alone (#) were assessed by ANOVA, 95% confidence limits.

6.10 Sub-maximal dose studies: responses to histamine

Responses to histamine in rabbit secondary bronchi are subject to severe tachyphylaxis. In order to obtain reproducible responses tissues were thoroughly washed in fresh Krebs and allowed to rest for 30minutes between doses of histamine. Under these conditions, 10μM Histamine produced a contraction of 0.435 (0.08) grams, approximately 65% of the maximum contraction obtained to histamine in this preparation (300μM; 0.661 (0.11) grams)

6.11 Effect of PMA on response to histamine

45 minutes incubation with 10μM PMA potentiated the response to 10μM histamine in rabbit secondary bronchi (Fig. 6.19). The mean response to histamine was increased to 157(12)% of the initial response which was significantly greater than the time control, 108(3)% ($p<0.05$; $n=5$). Interestingly, this effect was not reversed by washing. Second and third responses were also significantly increased to 162(11)% and 152(13)%, with time controls remaining steady at 100(3) and 108(12)% respectively ($p<0.05$).

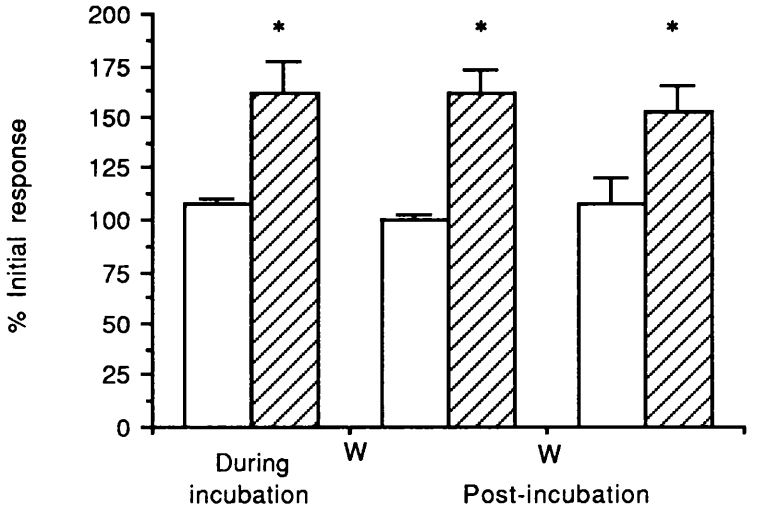


Figure 6.19

Effect of PMA on the response of rabbit secondary bronchi to a submaximal concentration of histamine. Open bars represent time control responses to 10μM histamine, hatched bars represent the effect of 10μM PMA. Responses to histamine are shown at 45 minutes into incubation with PMA and after two succesive washes in fresh Krebs solution. Bars represent the mean ± s.e.m. (n=5). Statistically significant differences between control responses and those in the presence of PMA are represented by *$p<0.05$, *$p<0.01$, Student's *t*–test.

6.12 Responses to EFS and to ACh in human bronchial muscle

Human airway smooth muscle responded to EFS (16Hz) with a biphasic contraction; no relaxation was seen. Size of response to EFS was variable but reproducible within tissues. In eight tissues the mean response was 210 (±70) mg (c.f. 630±100mg in rabbit bronchi).

Responses to ACh (10μM) were larger than to EFS, and were sustained. In eight tissues ACh produced a mean contraction of 360 (±30) mg (c.f. 743±110mg in rabbit bronchi).

6.13 Effect of PMA on responses to EFS and ACh

PMA potentiated the response to EFS at 16Hz in human airway (Fig. 6.20). The potentiation followed a similar time scale to that seen in rabbit bronchi, the responses increasing in size steadily over a period of 45-60 minutes. At 60 minutes into incubation with 10μM PMA responses in test tissues were increased to 224 (26)% of their initial value. Test tissue responses were significantly elevated ($n=5$, $p<0.01$); time control tissues produced responses of 91 (7)% of their initial value.

PMA also potentiated the response to a submaximal concentration of ACh in human airway (Fig. 6.21). Responses to 10μM ACh were increased to 260 (40)% of their initial value after 60 minutes incubation with 10μM PMA. These responses were significantly greater than their respective time control responses of 132(26)% ($n=5$, $p<0.05$). However, following washout in fresh Krebs, second and third responses did not differ significantly from control values.

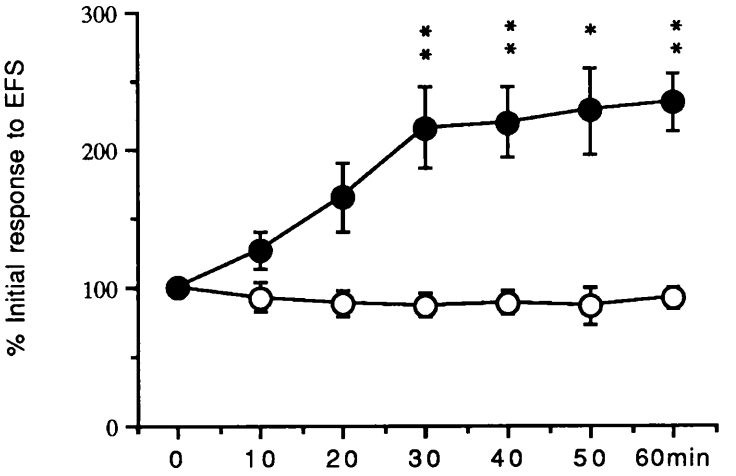


Figure 6.20

Effect of PMA on the response of human bronchi to electrical field stimulation (EFS). Open symbols represent time control responses to 16Hz EFS. Closed symbols represent test responses during incubation with 10μM PMA.

Results are expressed as a percentage of the initial response to 16Hz EFS. Each point represents the mean ± s.e.m. (n=5). Statistically significant differences between responses of paired tissues in the presence or absence of PMA are represented by *$p<0.05$, *$p<0.01$, Student's *t*–test.

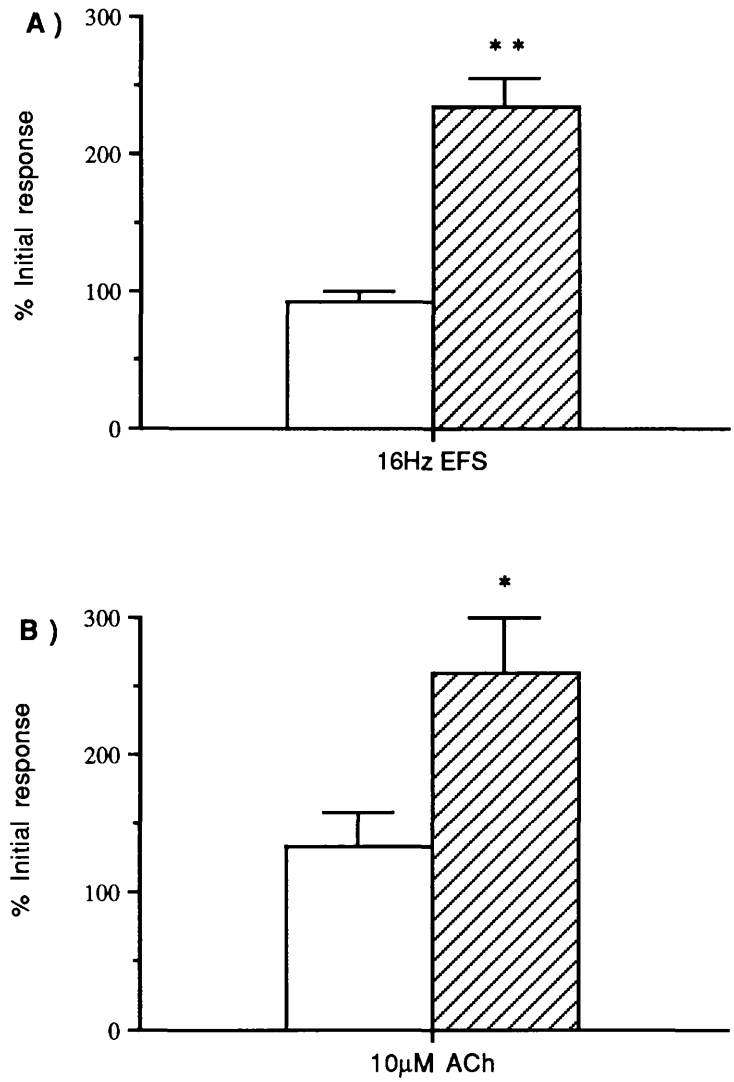


Figure 6.21

Effect of PMA on the response to of human bronchi to endogenous and exogenous ACh. Open bars represent time control responses to A) 16Hz EFS or B) 10μM ACh. Hatched bars represent test responses at 60 minutes into incubation with 10μM PMA.

Results are expressed as a percentage of the initial response to A) 16Hz EFS or B)10μM ACh. Each bar represents the mean ± s.e.m. (n=5). Statistically significant differences between responses of paired tissues in the presence or absence of PMA are represented by *$p<0.05$, **$p<0.01$, Student's *t*–test.

Section 7

DISCUSSION

7.0 Discussion

In this study PMA was found to potentiate excitatory responses in bronchial smooth muscle. The basis for the use of PMA was that it would activate smooth muscle PKC. However the phorbol esters are known to have actions that are not mediated by PKC (*section 4.6*). While indirect confirmation of the involvement of PKC in the response to PMA was provided by the inhibition of its effects by staurosporine and the more limited attenuation by H7, these compounds are not specific inhibitors of PKC and may well have actions on other cellular enzymes (*section 7.7*). Nevertheless copurification of kinase activity and phorbol ester binding has shown that PKC is the major phorbol ester receptor in cells (*section 4.5*) and the results will therefore be discussed in the context of what might be expected if facilitation by PMA were due to activation of PKC, although activation of some other, as yet unknown, system cannot be excluded.

7.1 Effect of PMA on response to EFS

At concentrations that produced no increase in baseline tension, PMA potentiated the contractile response to electrical field stimulation (EFS) in both rabbit and human airways. While it is not known at what frequency the nerves are stimulated *in vivo,* a time- and concentration-dependent potentiation of response was produced by PMA (10μM) across a range of frequencies (4, 16 & 30Hz).

Phorbol esters have been demonstrated to produce an increase in tone in vascular and airway smooth muscle preparations. However in the rabbit and human bronchial preparations this effect was rarely observed at concentrations below 100μM. In contrast, lower concentrations of PMA (0.1-10μM) produced a gradual contraction in rat and rabbit aorta (Danthuluri & Deth, 1984; Spedding, 1987), in rabbit ear arteries (Forder, 1985) and in bovine and guinea-pig tracheal smooth muscle (Park &

Rasmussen, 1989; Souhrada & Souhrada, 1989a). Rabbit bronchial smooth muscle, therefore, appears to be less sensitive to the contractile effects of phorbol esters than the above tissues. There is a large degree of variability between species and tissue preparations, however, as illustrated by the findings of Schramm & Grunstein (1989) that a phorbol ester (10μM 12-deoxyphorbol 13-isobutyrate; DPB) produced a relaxation, rather than a contraction, in rabbit trachea, and those of Menkes *et al* (1986) who demonstrated both contraction and relaxation to phorbol esters (DPB and phorbol-12,13-diacetate) in guinea pig trachea.

Following an initial latent period, PMA produced a progressive increase in the response to EFS which reached a maximum at 45-60 minutes into the phorbol ester incubation. A similar delayed action has been reported previously in the responses of vascular tissues to phorbol esters. A latent period was observed between the addition of phorbol ester and the resultant slow contraction in rat aorta (Danthuluri & Deth, 1984) and rabbit ear artery (Forder et al., 1985). In airway smooth muscle, an immediate response to PMA was seen in guinea pig trachea (Souhrada & Souhrada, 1989a). However a delay of up to 120 minutes was reported in bovine trachea (Park & Rasmussen, 1985). Other documented effects of phorbol esters, such as their inhibitory effect on α_1-adrenoceptor-mediated responses, follow a similar time scale (Danthuluri & Deth, 1984). While the delay may simply be due to the time necessary for the phorbol ester molecule to cross the plasma membran, Forder *et al* (1985) reported that agents which increased the calcium influx could significantly reduce this delay and suggested that increased levels of intracellular calcium influenced the activity of activated protein kinase C. It is possible that the concentration of calcium in a domain just below the plasma membrane is an important modulator of the activity of PKC (Rasmussen et al., 1987) and that the delayed response in rabbit bronchi may be due to a lag time in calcium influx into this submembrane region. Alternatively, it is possible that the relatively high concentration of PMA employed (10μM) immediately down-regulates the enzyme and that it is not until large amounts of phorbol ester enter

the cell that the negative effects are overridden by the positive effects. The short length of the timelag, however, does not suggest that down-regulation is taking place. Rather, it is more likely that the negative feedback of PKC on the formation of phosphatidylinositides initially opposes the positive effects on agonist-induced contraction which subsequently become dominant.

The degree of potentiation was concentration-dependent suggesting a progressively higher level of activation of the available PKC. A 1:1 relationship is postulated to exist between molecules of DAG (or PMA) and of PKC in the presence of excess phospholipid and at physiological calcium concentrations (Nishizuka, 1984, 1986; *Section 4.3*). It is therefore possible that the concentration-dependent increases in response reflect increasing numbers of activated molecules of PKC.

Significant potentiation was produced only by the higher concentrations of PMA (1-100μM) with little effect being observed at lower concentrations. As noted above, effects of phorbol esters have previously been reported at lower concentrations. However there is a large amount of variability in the potency of phorbol esters, even between preparations from the same species as demonstrated by Wagner (1987) in vascular preparations from the rabbit; while the main part of the concentration response curve was covered by 1-30nM phorbol dibutyrate (PDBu) in the basilar artery, the renal and saphenous arteries required between 30nM-3μM and 0.1-3μM PDBu respectively. However, the concentration of PMA employed (10μM) in the present study has been shown to be optimal for the contractile response in several smooth muscle preparations (Danthuluri & Deth, 1984; Chattergee & Tejada, 1986; Souhrada & Souhrada, 1989a).

That activation of PKC should increase the response to EFS initially seems inconsistent as the enzyme is understood to play a part in the sustained phase of contraction (Rasmussen & Barratt, 1984; *section 3.4*) while the response to EFS is

immediate and phasic. However, PKC is reported to increase calcium and sodium fluxes in airway smooth muscle (Souhrada & Souhrada, 1989a) and the alteration of intracellular calcium levels might itself enhance the sensitivity of the contractile proteins. Activated PKC may alternatively directly 'prime' the contractile apparatus such that the response to subsequent EFS-induced agonist binding would be enhanced. It has been suggested that activated PKC enhances the calcium sensitivity of the contractile proteins themselves (Rodger, 1986; Itoh et al., 1988) or that it might render the cell ready to function when calcium becomes available (Nishizuka, 1984).

7.2 Site of action of PMA.

Addition of the muscarinic antagonist atropine abolished control responses to EFS as well as the potentiation of response by PMA, suggesting that the potentiated response was mediated primarily through cholinergic mechanisms. Muscarinic receptors are found predominantly in proximal airways and have been identified in airway ganglia, on cholinergic nerve terminals and on airway smooth muscle (*section 2.1*). The potentiation of response to EFS by PMA could therefore result from action of the phorbol ester on the coupling of the pre- or post-junctional muscarinic receptors, or a combination of both. The potentiation was also inhibited by verapamil indicating that Ca^{2+} influx may be involved in the facilitatory effect of PMA on the contraction to EFS.

7.3 Possible pre-junctional effects

In the airways release of ACh from vagal fibres is limited by inhibitory M_2 muscarinic receptors on post-ganglionic parasympathetic nerves (Fryer & MacLagan, 1984) which have been demonstrated in humans (Minette & Barnes, 1988). An uncoupling of these pre-junctional autoregulatory receptors by PMA could

significantly increase the output of ACh upon nerve stimulation; inhibition of the M_2 receptor with the specific blocking-agent gallamine, results in a ten-fold potentiation of vagally-induced bronchoconstriction (Fryer & MacLagan, 1984).

Alternatively, PMA may block the action of an inhibitory co-transmitter. VIP is known to co-exist with ACh in cholinergic nerves, as do galanin and PHI (*section 2.1*), and reduces the contractile response to both ACh and to EFS *in vitro*, particularly at higher frequencies (Palmer et al., 1985b). All three peptides are potent bronchodilators and may act as an in-built braking system on the ACh-induced contraction of smooth muscle. An inhibition of this mechanism (e.g. of the synthesis, storage or release of these peptides), might result in an exaggerated response to cholinergic stimulation, such as that produced by PMA in these preparations.

There are many examples in the literature of phorbol ester-mediated enhancement of neurotransmitter release although these have been mainly in brain tissue; Nichols *et al.* (1987) report potentiation by phorbol esters of calcium-dependent release of all neurotransmitters studied (ACh, noradrenaline (NA), gamma-aminobutyric acid, serotonin, dopamine and aspartate) in all areas of the rat brain studied (cortex, hippocampus and corpus striatum).

Tanaka *et al.* (1986) demonstrated an increased response in the presence of PMA to Ca^{2+}-dependent depolarisation-evoked release of [^{3}H]ACh in guinea pig caudate slices. The effect was potentiated by calcium ionophore (A23187) and antagonised by H7 suggesting the increased response was mediated via PKC. This mechanism is supported by Nichols *et al.* (1987) who showed that only phorbol esters that activate PKC (β-isomers) were effective in enhancing neurotransmitter release and Daschmann *et al.* (1988) who demonstrated that the potentiation of release could be inhibited by the protein kinase inhibitors staurosporine and H7. Similarly

staurosporine, and to a lesser degree, H7, inhibited the potentiation of response to EFS by PMA in rabbit bronchi *(section 6.5)*.

PMA produced an enhancement of depolarisation-induced release of [^{3}H]NA from cultured neuronal cells (Matthies et al., 1987); however chronic phorbol ester treatment resulted in loss of the potentiating effect of PMA. This apparent desensitisation of neurotransmitter receptors has recently been studied by Dillon-Carter and Chuang (1989) who found that incubation of cultured cerebral granule cells with saturating concentrations of carbachol, histamine, NA or serotonin caused a time-dependent desensitisation of the cells to that agonist, while incubation with PMA desensitised the cells to all of the above agonists. Measurement of the PLC activity of the cells revealed that PMA attenuated the enzyme's activity and thereby down-regulated the PLC-coupled receptors. A further effect on the PI pathway was demonstrated in a recent experiment by Weiss *et al* (1989) who found that incubation with PDBu attenuated carbachol-stimulated inositol monophosphate (IP) production in cultured striatal neurons. Thus, a secondary, long term effect of PKC activation may be mediated via modulation of PLC and the subsequent inhibition of signal transduction via inhibition of the agonist-induced production of inositol phospholipids. In the rabbit airway preparation the potentiation of response to EFS by PMA was maximal at approximately 60 minutes into incubation but subsequently decreased to initial levels, or slightly below, over 120 minutes; this effect could be elicited by inhibition of the PI pathway, or by down-regulation of PKC itself.

7.4 Mechanism of pre-junctional effect

Wakade *et al.* (1985) demonstrated in [^{3}H]NA-loaded rat sympathetic neurons that the facilitatory effect of PMA on EFS-evoked NA release was unaffected by the presence of inhibitors of neuronal and extraneuronal uptake, or by removal of negative feedback inhibition by pre-synaptic α-receptors. Similarly, Allgaier *et al.*

(1987) showed that the enhanced release of NA from hippocampal slices was calcium dependent and that it was not due to an effect on the autoinhibitory feedback system as the enhancing effects of yohimbine (an α_2-antagonist) were increased in the presence of PMA, while the inhibitory effects of clonidine (an α_2-agonist) were unaffected. This group later demonstrated that the phorbol ester-induced facilitation of NA release was similarly independent of pre-synaptic changes in Na^+, K^+ or CL^- conductance (Huang et al., 1988).

The above results suggest that the facilitatory effect of PMA on EFS is not due to an uncoupling of the pre-junctional autoinhibitory M_2 receptors or to effects on neuronal or extraneuronal uptake of neurotransmitter. They indicate that PMA, probably via activation of PKC, exerts a dual effect; firstly facilitating and secondly inhibiting neurotransmitter release. PMA may act synergistically with calcium to increase neurotransmitter release; phorbol esters have been shown to enhance calcium-dependent depolarisation-induced neurotransmitter release by producing a marked decrease in the concentration of Ca^{2+} needed to provoke the release (Zurgil & Zisapel, 1985) and studies in chick sympathetic neurons suggest that activation of PKC enhances Ca^{2+} accumulation, which in turn leads to the facilitation of neurotransmitter release (Malhotra et al., 1988).

The observation in the rabbit airway preparation that verapamil inhibited the PMA-induced potentiation supports the hypotheses that calcium influx is necessary for full activation of PKC and that synergism between activated PKC and Ca^{2+} is important in the release response.

In order to examine whether PMA is having such a pre-junctional effect in the bronchial smooth muscle preparation, detailed studies of neurotransmitter output on nerve stimulation in the presence and absence of PMA would be needed. As atropine does not differentiate between receptor sub-types and blocks both M_2 and M_3

receptors, it provides no information on the site of action of PMA or on the possible involvement of a co-transmitter or regulatory neuropeptide. Alternatively, PMA could exert its effect via a post-junctional mechanism and it was decided to investigate this possibility further.

7.5 Possible post-junctional effects

The increased response to EFS in the presence of PMA could be the result of an increased number of M_3 receptors on the airway smooth muscle, an increased affinity of these receptors for ACh, or post-receptor synergism. In order to assess the contribution of post-junctional mechanisms to the PMA-induced potentiation, its effect on the direct addition of exogenous ACh to the rabbit bronchi preparation was examined. Cumulative concentration response curves (CCRC) were obtained to provide detailed information of the sensitivity and responsiveness of the preparation in the absence and presence of PMA.

Hyperresponsiveness in airway smooth muscle can be due to the muscle cell responding to a contractile agonist at lower concentrations (type I sensitivity), or producing a greater response to "normal" agonist concentrations (type II sensitivity), or both of these. PMA potentiated responses to certain concentrations within the ACh CCRC but while it produced a small shift in the the curve, it did not produce an increase in response to the maximum concentration employed. These effects are most typical of type II (also known as post-junctional or non-deviation) sensitivity and indicate a synergism beyond the receptor rather than an increase in the sensitivity or number of receptors. Post-receptor changes in excitation-contraction coupling or in the contractile apparatus itself could account for the potentiation seen; possible mechanisms include increased release of Ca^{2+} on agonist stimulation, activation of a greater number of contractile proteins or activating enzymes, increased activity of crossbridges or abnormal length-tension relationships. Similarly, this type of

response could be produced by improved cell-to-cell coupling, perhaps due to an increased number of gap-junctions. The results obtained from the rabbit preparation do not implicate Type I sensitivity in the PMA/PKC effect, thus mechanisms generating this type of sensitivity (such as depolarisation of the membrane or changes in receptor affinity or number) are unlikely to be of consequence in rabbit bronchial smooth muscle.

The shift in sensitivity of the curve was significant but very small. Under these circumstances it is often useful to examine the effect of the drug on submaximal concentrations of the contractile agonist; thus the effect of incubation with PMA on the response to 10μM ACh, one of the concentrations to which the response was increased within the CCRC, was examined. The contractile response elicited by 10μM ACh (760mg) corresponds approximately to the EC_{50} of ACh in this preparation, and was increased by approximately 70% in the presence of PMA. This effect was reversed by repeated washes in oxygenated Krebs solution, confirming that the phorbol ester was having a non-toxic, reversible effect on the smooth muscle.

The response obtained to 16Hz EFS similarly corresponds to approximately 50% of the maximum contractile response to ACh. PMA (10μM) increased the response to EFS by 34 (±6) % and that to exogenous ACh by 69 (±20) %, indicating that a substantial proportion of the PMA effect may be mediated via post-junctional mechanisms. A similar pattern was observed in human tissue substantiating these findings; following incubation with PMA, the potentiation of response to exogenous ACh was greater that that to EFS, an increase of 160 (±40)% and 124 (±26)% respectively.

The importance of post-junctional mechanisms in the phorbol ester effect was confirmed by the observation that the potentiation was not limited to cholinergic stimuli. PMA (10μM) increased the response of rabbit bronchi to a single

submaximal concentration of histamine (10μM; approximately EC_{65}) by 57%, an effect that was not significantly reversed by subsequent washing over the period tested. This result strongly supports a change in smooth muscle biochemistry rather than an effect of PMA on (two sets of) receptors. Both histamine and ACh utilize the phosphatidylinositol pathway and the second messengers I-1,4,5-P_3 and DAG (*section 4.1*) in the pharmacomechanical coupling of airway smooth muscle. PMA is a functional analogue for DAG and competitively inhibits DAG binding at its endogenous receptor. Thus PKC is strongly implicated in the development of the increase in the contractile response to exogenous and endogeous stimuli. The PMA-induced potentiation of response to EFS and to a submaximal dose of ACh was therefore examined in rabbit bronchial smooth muscle in the presence of inhibitors of PKC.

7.6 Inhibition of PKC

A number of substances are known to inhibit PKC but the specificity of most of these is not high. Inhibitors currently in use include staurosporine (Tamaoki et al., 1986), calphostin (Koybashi et al., 1989), sphingosine (Hannun et al., 1986b), the peptide inhibitor ICK-1 and inactive control NICK-1 (House & Kemp, 1987), and H7 (Hidaka & Hagiwara, 1987). Of these, H7 and staurosporine are two of the most frequently used.

The isoquinolinylsulfonamide, H7, is commonly employed as an inhibitor of PKC and has been described as both potent and specific (Hidaka & Hagiwara 1987; Anwyl, 1989; Caramelo et al., 1988; Abdel-Latif, 1986). H7 (10μM) attenuated the phorbol ester-induced electrical and contractile changes in the guinea-pig trachea (Souhrada & Souhrada, 1989a) and partially reversed PMA-induced changes in aortic smooth muscle (Carmelo et al., 1988). However it was ineffective in blocking both the contractile and relaxant effects of phorbol esters in rabbit trachea (Schramm & Grunstein, 1989). Similarly, in the rabbit bronchi preparation H7 produced only a limited attenuation of the response to PKC

activation by phorbol ester. The inhibitor appeared to dampen the increase in response to EFS produced by PMA but had no effect of the potentiation of response to a submaximal dose of ACh.

H7 acts at the ATP binding site on the hydrophilic catalytic domain of PKC (Nakadate et al., 1988; Ruegg & Burgess, 1989) which has been shown to exhibit a very high degree of homology with the ATP-binding sites of several other protein kinases (Edelman et al., 1987; Hanks et al., 1988); inhibitors acting at this site will not therefore achieve a high degree of selectivity. The lack of effect of H7 in our preparation may be due to this lack of specificity, to inappropriate incubation time or to a difficulty in penetration of the smooth muscle membrane. Species difference may also be important; it is possible that the rabbit trachea (Schramm & Grunstein, 1988) and bronchi possess an isozyme of PKC which is insensitive to H7. Alternatively, PMA may be producing its effect via mechanisms other than the activation of PKC. It was therefore of interest to determine whether a second inhibitor of PKC would produce a more pronounced inhibition of the PMA effect in the rabbit airway preparation.

Staurosporine, a microbial alkaloid produced by *streptomyces*, was discovered in 1977 by Omura *et al* and has been shown to counteract the phorbol ester-induced enhancement of neurotransmitter release (Daschmann et al., 1988). PKC has been implicated in cell growth and replication (Nishizuka, 1986); staurosporine inhibits proliferation of rabbit aorta smooth muscle cells (Matsumoto & Sasaki, 1989) and shows strong cytotoxic activity, inhibiting the growth of melanoma and leukaemia cells, which may be due to inhibition of PKC (Tamaoki et al., 1986); however, staurosporine also inhibited cyclic AMP-dependent protein kinase to the same degree as PKC (Tamaoki et al., 1986).

In the rabbit bronchial preparation staurosporine inhibited the PMA-induced potentiation of response to EFS, suggesting that the facilitatory effect of the phorbol ester is mediated by PKC. However, both staurosporine and H7 inhibit the catalytic fragment of PKC and may

not therefore be considered specific inhibitors of the enzyme, although they can be very effective in inhibiting reactions thought to be principally mediated by PKC (Garland et al., 1987; Ruegg & Burgess, 1989). The exact mechanism of action of staurosporine remains unknown, however, as its high potency is incompatible with an effect on the ATP binding site (Ruegg & Burgess, 1989); thus some unknown factor may be involved in the more effective inhibition by staurosporine than by H7 of the PMA-induced potentiation of response to EFS in the rabbit airway preparation.

Neither staurosporine nor H7 prevented the potentiation by PMA of the response to exogenous ACh. It may be that PMA is acting both pre- and post-junctionally to activate PKC, thereby increasing both the amount of neurotransmitter released by nerve stimulation and the smooth muscle response to contractile agonists, and that the pre-junctional mechanism is more sensitive to inhibitiion by staurosporine or H7. In different tissues PKC shows a considerable degree of heterogeneity and the subspecies have different kinetic properties and substrate specificities (*section 4.4*). No data on the effects of PKC inhibtors on the different subtypes of PKC are available. Higher concentrations of the two inhibitors might produce greater inhibition of the response to ACh. However, at these concentrations, enzymes such as cAMP- and CGMP-dependent kinases may also be effected (Tamaoki et al., 1986).

Several new compounds have recently become available commercially and may produce better, and more specific, inhibition of PKC than has been possible thus far with staurosporine and H7; initial studies on a modified staurosporine (Ro-31-8220; Roche Products Ltd.) suggest it may be a substantial improvement, as a tool, on these compounds. Similarly, Kase and coworkers (1987) report a novel compound, K-252b, to be up to three orders of magnitude more potent than the isoquinoline sulfonamide inhibitors and to be more selective for PKC than for cAMP-dependent or Ca^{2+}/calmodulin-dependent protein kinases. The authors describe the drug as, to their knowledge, "the most potent and selective inhibitor for PKC so far reported". However, both these drugs have a similar

structure to staurosporine and act, like staurosporine and H7, on the catalytic domain. Thus, although potent inhibitors of PKC, they may lack the specificity required to be of real use in investigating the regulatory role of the enzyme.

As PKC has separate regulatory and catalytic domains (Kishimoto et al., 1983) it is possible that compounds acting on the hydrophobic regulatory domain may prove to be more specific inhibitors of the enzyme. The protein kinase inhibitors tamoxifen, glossypol, trifluoperazine and adriamycin contain lipophilic or non-polar regions which are thought to interfere with a hydrophobic interaction between PKC and phospholipid (Nakadate et al., 1988). They may inhibit the enzyme by interfering directly with the phorbol ester binding site on the regulatory domain or with the lipid requirement for binding. It is unclear, however, whether or not these compounds will also inhibit Ca^{2+}/calmodulin-dependent enzymes (Hidaka & Hagiwara, 1987; Nakadate et al., 1988).

Calphostin C, a microbial compound derived from *Cladosporium cladosporioides* has potent cytotoxic and antitumour activities which might be due to the inhibition of PKC. Experimental evidence suggests that it too interacts with the regulatory domain of PKC (Kobayashi et al., 1989). In addition, this compound showed inhibitory effects on the binding of ^{3}[H]PDBu to PKC and might therefore be much more specific than the other available PKC antagonists.

It will be interesting to investigate the effects of these new drugs in the airway preparations where they may confirm that the phorbol ester-induced potentiation is mediated by PKC and may produce more specific inhibition of PKC than has been possible thus far.

7.7 Calcium Sensitivity of the responses

In several smooth muscle preparations the contractile response to ACh is thought to be largely independent of extracellular calcium, with the majority of the calcium for contraction coming from intracellular stores (Hurwitz et al., 1967; Raeburn et al., 1986). Conversely Black *et al.* (1986) found contraction to histamine and ACh in human bronchial smooth muscle to depend on extracellular stores, but reported that only a fraction of the calcium entry occurs via voltage-dependent channels. In the rabbit preparation the influx of extracellular calcium is important in the response to exogenous ACh; in the absence of extracellular calcium the response curve to ACh was shifted to the right. However, verapamil attenuated the response to EFS in control tissues suggesting that calcium entry through voltage-dependent channels has a role to play in the response to nerve stimulation, although it may only be a method of refilling the depleted intracellular stores.

The activity of PKC is regulated by the intracellular calcium concentration. An increase in the calcium concentration promotes the association of PKC with the membrane (*section 4.3*), and the concentration in the submembrane domain may play a major role in modulating the activity of the membrane-associated enzyme. In order to determine the contribution of calcium influx from the extracellular space to the PMA-induced potentiation of response to EFS and to exogenous ACh in rabbit bronchi, parallel series of experiments were run in which the tissues were maintained in low-calcium Krebs or were incubated with nifedipine (0.3μM) or verapamil (10μM).

The rightward shift of the ACh response curve in the low calcium environment was significantly reduced in the presence of PMA. The phorbol ester appears to halt the desensitisation, an effect which may be due to an increase in the calcium sensitivity of the contractile proteins via activation of PKC. Similarly, the potentiation of response to EFS by PMA is also dependent on the extracellular calcium, as evidenced by the inhibition of the phorbol ester effect by the calcium channel blocker verapamil. It is possible that activation

of PKC by PMA induces calcium influx, directly or indirectly, through voltage-dependent channels thereby counteracting the effects of the low calcium environment but remaining susceptible to the effects of the calcium channel blockers.

Verapamil and nifedipine block voltage-dependent calcium channels. Nifedipine modulates the channel gating by increasing the time constant for the long interval between open bursts and effectively plugs calcium channels, reducing their numbers without affecting the kinetics of activation or inactivation. In contrast verapamil has no effect on the number of calcium channels and appears to affect mainly the rate of activation, inactivation and especially recovery after inactivation of the calcium current (reviewed Hurwitz, 1986).

In rabbit secondary bronchi nifedipine prevented the PMA-induced potentiation of the response to 10μM ACh without significantly altering the control response. Verapamil itself partially inhibited the control response to ACh but subsequently prevented the potentiation of response by PMA. Verapamil also inhibited the potentiation of response to EFS. These results contrast with those of Marthan *et al* (1987), who found no effect of calcium-blocking drugs on carbachol-induced tone in human airways, and with clinical reports of a lack of effect on resting tone (Cuss & Barnes, 1985; Williams et al., 1981) and on cholinergic-induced bronchoconstriction (Roberts et al., 1986; Matthews et al., 1983). However, calcium channel-antagonists produced a relaxation of human bronchus *in vivo* (Popa et al., 1984), and *in vitro* prevented contraction to low concentrations of ACh in canine tracheal smooth muscle (1nM-1μM; Farley & Miles, 1978) and produced partial inhibition of higher doses of cholinergic agonists in human tissue (Horio et al., 1984; Raeburn et al., 1986; Roberts et al., 1986) demonstrating a degree of variability in efficacy of calcium channel antagonists in different preparations.

Nevertheless, the results suggest that, in rabbit bronchial smooth muscle, the PMA effect is largely dependent on the influx of extracellular calcium through voltage-dependent channels. The dependence of the contractile response to phorbol esters on the influx of

extracellular calcium has been reported in several tissue preparations (Danthuluri and Deth, 1984; Forder et al., 1985; Park & Rasmussen, 1985; Schramm & Grunstein, 1989; Souhrada & Souhrada, 1989a); in all cases the phorbol ester response could be attenuated by removal of calcium from the bathing solution or by blocking calcium channels. Evidence obtained in brain tissue suggests that PMA increases both the influx of calcium and its accumulation in the cell *(section 7.4)*.

The fact that the PMA potentiation can be so effectively inhibited by nifedipine and verapamil suggests a direct role for activated PKC in the regulation of calcium channels. Spedding (1987) investigated this possibility but found no evidence for such control of channel function in rat aorta or guinea pig taenia coli, considering it more likely that activated PKC influenced the influx of calcium indirectly via changes in calcium extrusion or cellular depolarisation in these preparations. However, there is no reason to believe that the same control system exists in all smooth muscles; Schramm & Grunstein (1989) observed a PKC-dependent facilitation of calcium influx through voltage-dependent channels in rabbit tracheal smooth muscle while Park & Rasmussen (1985) and Forder *et al.* (1985) note a potentiation of the effect of PMA by the calcium channel agonist BAY-K8644 in airway smooth muscle.

Thus, although several mechanisms may contribute to the potentiation process in airway smooth muscle, blocking calcium entry was completely effective in removing the PMA effect. Forder *et al.* (1985) postulate that calcium influx through voltage-gated channels acts principally to raise the cytosolic calcium concentration in a restricted domain just under the plasma membrane rather than in the bulk cytosol, resulting in up-regulation of membrane-bound PKC. It is possible that PKC is not fully active without high calcium concentrations in this submembrane region. Thus, inhibition of calcium entry into this domain may attenuate the activation of the enzyme and abolish subsequent effects such as stimulation of the Na^+-H^+ antiport (*section 7.8*).

There is, therefore, evidence that stimulation of PKC in bronchial smooth muscle leads to calcium influx through nifedipine- and/or verapamil-sensitive channels. However, at the concentrations employed in these studies, these actions do not lead to contraction in the absence of an agonist or neurotransmitter. PMA could place PKC in a state of activation which is dependent on co-activation with calcium and which may be insufficient to initiate contraction itself but could be effective in enhancing subsequent receptor-mediated contraction.

7.8 Possible mechanism of action

A. Modulation of ion flux

It has been proposed that PKC plays a role in the extrusion of Ca^{2+} immediately after its mobilisation into the cytosol, e.g. following receptor stimulation, and that the Ca^{2+}-transport ATPase is a possible target for PKC (Nishizuka, 1986). These hypotheses are substantiated by the fact that addition of phorbol esters to various cell types frequently produces a decrease in cytosolic [Ca^{2+}] concentration (Drummond et al., 1985; Albert & Tashjian, 1985). However, other evidence suggests that PKC may also be involved in the enhancement of Ca^{2+} entry (DeRiemer et al., 1985; Albert & Tashjian, 1985; Farley & Auerbach, 1986; Wakade et al., 1986).

Calcium influx into the submembrane domain could produce a high local calcium concentration which would maintain PKC in an active state and could result in the PKC-induced potentiation of Ca^{2+} influx and efflux. Measurement of the intracellular calcium concentration is usually made in the bulk cytosol. It is possible that the efflux of calcium caused by the PKC-induced increase in calcium-pumping keeps the cytosolic calcium levels low but that the concentration can reach very high levels beneath the membrane thus keeping PKC active (positive feedback) as long as the stimulus is maintained.

B. Mediator release

An increase in the production of arachidonate or its metabolites, some of which are potent bronchoconstrictors, particularly in the peripheral airways where they are as potent as histamine (Schneider & Drazen, 1980), could contribute to the increased responsiveness of bronchial smooth muscle in the presence of PMA. Such a mechanism appears to operate in human umbilical vein epithelial cells, in which PMA enhanced production of $PGF_{2\alpha}$, PGE_2 and PGD_2 (Zavoico et al., 1990).

The release of arachidonate and the synthesis of eicosanoids is related to agonist-induced phosphatidyl inositol turnover in several tissues. Phosphatidyl inositol and the poly-phosphoinositides are rich in arachidonate at the 2-position of glycerol (Majerus et al., 1988; Mitchell, 1975) and agonists shown to stimulate the release of arachidonate include muscarinic cholinergic agonists, α_2-agonists, serotonin, angiotensin II, substance P and EGF. These agonists also all produce activation of PKC.

PMA stimulates release of arachidonate and/or eicosanoids in several cell types including mast cells, macrophages and epidermal cells (Jacobson et al., 1987; Emilsson & Sundler, 1986; Ohuchi et al., 1985) and potentiates agonist- and calcium ionophore-induced release of arachidonate in platelets (Halenda & Rehm, 1987; Halenda et al., 1985) indicating a synergism between PKC-activation and the rise in intracellular calcium.

Arachidonate and its eicosanoid derivatives produce activation of PLC in intact cells (Zeitler & Handwerger, 1985), and several arachidonate products, particularly lipoxin A, activate PKC and may also be involved in the modulation of its substrate specificity (Nishizuka, 1988; Hansson et al., 1986), thus providing a possible positive feedback loop on the enzyme that initiated arachidonate production.

Indomethacin inhibits the activity of cyclo-oxygenase and thus prevents the production of thromboxanes and prostaglandins from arachidonate. In rabbit bronchial smooth muscle pre-incubation with indomethacin had no significant effect on the potentiation of response to exogenous ACh by PMA. While it is possible that, in this preparation, the effect of PMA was mediated by the lipoxygenase degradation products of arachidonate alone, these results indicate that the cyclo-oxygenase products of arachidonate metabolism do not play an important role in the increased response to cholinergic stimuli.

Indomethacin itself increased the response to a single submaximal concentration of ACh by a small but significant amount. This potentiation could be due to the removal of an inhibitory arachidonate-derived cyclo-oxygenase product(s) which previously functioned as a brake on exaggerated bronchoconstrictor responses to cholinergic stimuli. Two cyclo-oxygenase products of arachidonate metabolism, prostacyclin and PGE_2, have been shown to inhibit phosphatidylinositol breakdown and the generation of inositol phosphates (Lapetina, 1984; Takenawa et al., 1986); thus, incubation with indomethacin would prevent their usual negative modulation of the contractile process.

C. Activation of exchange mechanisms.

Souhrada and Souhrada (1989a) report that amiloride, one of the most specific and potent inhibitors of sodium entry, abolished the response of rabbit tracheal smooth muscle to phorbol esters and that the response was similarly abolished in low sodium. Findings in other smooth muscle cell systems (Besterman & Cuatrecasas, 1984; Rossoff et al., 1984; Gelfand et al., 1987) confirm that phorbol esters stimulate Na^+–H^+ exchange, resulting in sodium influx. This mechanism contributes to the regulation of intracellular pH in muscle fibres (Benos, 1982) and has been found *in vitro* to provide a substrate for PKC (Burns & Rozengurt, 1983; Rossoff et al., 1984; Moolenaar et al., 1984)

Stimulation of sodium influx might therefore be involved in the phorbol ester-induced potentiation of response to contractile stimuli and it was decided to study the effect of amiloride in the rabbit bronchial preparation. At a concentration that has been shown to exhibit near maximal inhibition of sodium transport across cell membranes (10μM; Benos, 1982), amiloride had no significant effect on the control responses to exogenous ACh but attenuated the PMA-induced potentiation. These results are consistent with the hypothesis that PKC activation in bronchial smooth muscle leads to increased activity of Na^+/H^+ exchange. This would increase the intracellular sodium concentration which could in turn result in calcium influx via Na^+/Ca^{2+} exchange, as postulated by Danthuluri and Deth (1984), and may therefore contribute to subsequent enhancement of responses to contractile stimuli.

However, the attenuation of the PMA-induced potentiation may have been due to a direct effect of the amiloride on plasma membrane ion channels or on PKC itself. Besterman *et al.* (1985) and Kleyman & Cragoe (1988) report that amiloride (0.1μM-1mM) inhibits PKC-mediated protein phosphorylation *in vitro*, and inhibits the voltage-dependent calcium channel of porcine heart sarcolemma vesicles with an IC50 of 90μM. Full elucidation of the role of Na^+/H^+ exchange in phorbol-ester mediated potentiation of response will become possible with the development of structural analogues of amiloride with increased affinity for a particular transport system (Kleyman & Cragoe, 1988).

D. Phosphorylation of cellular proteins

While the results of many groups indicate that PKC activation results in a slowly developing contractile response in tracheal and vascular smooth muscle (*section 7.1*), those obtained in the rabbit and human preparations suggest that PKC can play a role in agonist-induced responses; the contractile responses to both EFS and exogenous ACh were diminished in the presence of the PKC inhibitors H7 and staurosporine. These observations are substantiated by detailed intracellular measurements of protein

phosphorylation made by Rassmussen *et al.* (1987). This group recorded temporal changes in the pattern of protein phosphorylation induced by carbachol in tracheal smooth muscle and compared them with the pattern produced when contraction was initiated by the phorbol ester PDBu. They found that addition of carbachol produced phosphorylation of myosin light chain in less than 60 seconds, followed by a dephosphorylation to basal levels over 20 minutes, accompanied by a progressive rise in the phosphorylation of another group of cellular proteins which was subsequently maintained. These proteins included α- and β-desmin, synemin, caldesmon and four small, 18-30kDa, unidentified proteins). In contrast, addition of the phorbol ester, PDBu, resulted in a very gradual contraction over 60 minutes which reached 60% of the carbachol induced tone. At the 60 minute time point there was an increase in phosphorylation of the same group of late-phase proteins as seen 60 minutes after carbachol addition. No sustained contraction to carbachol or to PDBu, or phosphorylation of the late phase proteins was observed if extracellular calcium was removed prior to the addition of the agonists.

The above results suggest that PKC is implicated in late-phase protein phosphorylation during normal agonist action in these preparations. Via these mechanisms, inappropriate activation of PKC could produce an increase in response to endogenous and exogenous agonists.

7.9 Conclusions

The results of this study demonstrate that incubation with phorbol esters can enhance responses of airway smooth muscle to endogenous contractile agonists and suggest that this effect is mediated via activation of PKC. The findings, therefore, support the hypothesis of Obianime *et al.* (1989) that inappropriate activation of PKC in smooth muscle cells may contribute to the pathogenesis of airway hyperreactivity in asthma. Clearly PMA-induced sustained activation of airway PKC can result in exaggerated responses to calcium mobilizing agonists and this increase is at least in part due to

increased fluxes of calcium and sodium ions, and independent of prostaglandin production.

An alteration in the pattern or extent of PKC activation *in vivo* is therefore a possible mechanism for the enhanced response of airway smooth muscle, in diseases such as allergic asthma, to substances that stimulate the turnover of inositol phospholipids in their stimulus-contraction coupling. While the importance of the potentiation of response to ACh and histamine in the airways is obvious, many other airway spasmogens utilize this pathway and an abnormality in intracellular coupling would therefore produce altered responses to a wide range of mediators including substance P, serotonin, vasopressin and bradykinin as well as endothelin and platelet derived growth factor which are currently attracting much interest.

7.10 PKC and airway sensitisation

The conclusion from these studies, that PKC may be involved with bronchial hyperreactivity, is particularly interesting in the light of recently published results which suggest that exposure of airway smooth muscle cells to specific reaginic IgG_1 can activate airway PKC and that this activation is intimately involved in the response to specific antigen. Souhrada & Souhrada (1989b) report that the addition of IgG_1 to the trachea of sensitised guinea pigs produces characteristic electrical and contractile changes in the smooth muscle: an initial depolarisation and increase in the isometric force. The changes were very similar to those obtained after administration of phorbol ester (Souhrada & Souhrada, 1989a). Thus these workers suggested that the addition of IgG_1 may directly stimulate PKC (although it is possible that IgG_1 may act indirectly by stimulating turnover of inositol phospholipids and the generation of DAG). Preincubation with H7 inhibited the response to IgG_1 substantiating the probable involvement of PKC. Similarly, oral administration of another PKC inhibitor K-252a to guinea pigs resulted in

partial but significant inhibition of anaphylactic bronchoconstriction (Ohmori et al., 1988).

Thus it would appear that PKC is activated by specific antigen, is involved in the anaphylactic response and that sustained activation can result in exaggerated responses to endogenous mediators. As the response to specific antigen can be blocked by inhibitors of PKC, it is possible that the development of more specific PKC inhibitors may produce a novel and effective therapy for *in vivo* intervention in disease states such as allergic asthma. In this context it is of interest that sodium chromoglycate, a potent antiasthmatic agent of unknown action (Rafferty & Holgate, 1988; Warner, 1988), can inhibit PKC activity (Lucas & Schuster, 1987).

Section 8

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