A PHARMACOLOGICAL STUDY OF PROTEIN KINASE C ACTIVATION BY PHORBOL ESTERS IN GUINEA-PIG AIRWAYS: POSSIBLE EFFECTS ON THE INHIBITORY NON-ADRENERGIC NON-CHOLINERGIC SYSTEM

A thesis submitted to the University of London
for the degree of Doctor of Philosophy

by

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ABSTRACT

A Pharmacological Study of Protein Kinase C Activation in Guinea-Pig Airways: Possible Effects on the Inhibitory Non-Adrenergic Non-Cholinergic System

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This thesis describes the effect of phorbol esters, potent activators of the enzyme protein kinase C (PKC), on isolated guinea-pig respiratory tract tissues.

Phorbol esters evoked qualitatively different responses in lung parenchymal and tracheal strips - contraction in the lung parenchyma and relaxation in the trachea. The basis of the relaxant effect was investigated.

Relaxation occurred with the active 4β-isomer, but not the inactive 4α-isomer of phorbol dibutyrate (PDBu). The relative potencies of several active 4β-isomers, and the ability of cell permeant analogues of diacylglycerol (DAG, the endogenous PKC activator) to produce relaxation of the trachea, suggested that the response involved activation of PKC.

Was the unexpected relaxation due to a direct or an indirect mechanism? The relaxation did not appear to result from down-regulation of responses to either histamine, carbachol or contractile prostanoids. In contrast, isoprenaline-induced relaxation was inhibited by 4β-PDBu, but not 4α-PDBu. Results with the relevant mediator antagonists/enzyme inhibitors indicated that the relaxation did not involve relaxant cyclo-oxygenase products, sympathetic neurotransmitters, β-adrenoceptor stimulants or naloxone-sensitive opioids, nor the action of histamine at H₁- or H₂-receptors, the action of adenosine at A₁- or A₂-receptors or the release of epithelium-derived inhibitory factors. The relaxation was also resistant to tetrodotoxin, but was potentiated by agents said to elevate tracheal cyclic AMP levels (eg. isoprenaline and aminophylline).

One possibility which remained was that the relaxation was due to PKC-mediated release of the inhibitory non-adrenergic, non-cholinergic (NANC₁) neurotransmitter. The extent of the relaxation in the trachea to 4β-PDBu seemed to correlate with the reported distribution of functional NANC₁ innervation, VIP-immunoreactive nerve fibres and VIP-receptors in the lung. Incubation of tracheal strips with the protease inhibitors, bacitracin and aprotinin, facilitated the response to NANC₁ nerve
stimulation (by electrical field stimulation), exogenous VIP and to 4β-PDBu, suggesting a contribution of a protease-sensitive peptide to the relaxation.

The voltage-dependent Na⁺ channel activators, veratridine (VT) and aconitine (AC), are reported to cause neurotransmitter release and to produce molecular changes in synaptoneurosomes and cortical slices consistent with generation of DAG (and thus activation of PKC). In the trachea these agents caused relaxation in the presence of atropine and propranolol; and the profile of relaxation to these alkaloids in tissues taken from lower down the respiratory tract was consistent with activation of NANCᵢ mechanisms. Results obtained with inhibitors of DAG metabolism, R59022 and RHC80267, suggested that generation of DAG and its subsequent inactivation by the DAG lipase route could be involved in NANCᵢ nerve-mediated relaxation, consistent with the hypothesis that PKC activation (as by phorbol esters) is implicated in the release of the NANCᵢ neurotransmitter.

Attempts were then made to differentiate neuronal and non-neuronal relaxant responses of the trachea to 4β-PDBu by (i) desensitising to NANCᵢ nerve stimulation (using prolonged electrical stimulation and prolonged incubation with VIP or AC) or (ii) depleting neurotransmitters (with black widow spider venom or the purified toxin, αlatrotoxin) or (iii) reduction of bathing fluid temperature. The general finding was that the relaxation produced by NANCᵢ nerve stimulation, veratridine and 4β-PDBu all seemed to be inhibited by methods most likely to result in neurotransmitter depletion – in particular from NANCᵢ nerves. However, an attempt to collect and concentrate the NANCᵢ neurotransmitter released either by pharmacological or electrical stimulation failed, as did an attempt to assay for it using a cascade technique.

In conclusion, it is proposed that the relaxation of guinea-pig trachea to 4β-PDBu occurs indirectly rather than by a direct action on the smooth muscle. While the results obtained do not prove that the relaxation is due to release of the NANCᵢ neurotransmitter, most do appear to support it. However, other mechanisms for the relaxation have not been ruled out.
For my wife,

Debbie
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ABBREVIATIONS

AC Aconitine
ATP Adenosine triphosphate
BSA Bovine serum albumin
BWSV Black widow spider venom
[Ca^{2+}]_i Intracellular free calcium ion concentration
CaM Calmodulin
Cyclic AMP Adenosine 3',5'-cyclic monophosphate
Cyclic GMP Guanosine 3',5'-cyclic monophosphate
DAG 1,2-sn-diacylglycerol
DiCa
1,2-sn-dioctanoylglycerol
dil Dilution (final)
DMSO Dimethylsulphoxide
DPCPX 1,3-Dipropyl-8-cyclopentylxanthine
DTT Dithiothreitol
EDRF Endothelium-derived relaxant factor
EFS Electrical field stimulation
ELISA Enzyme-linked immunosorbent assay
EpDIF Epithelium-derived inhibitory factor
EtOH Ethanol
Expt Experiment
G-protein Guanine nucleotide regulatory protein
IP Inositol phosphate
IP_3 myo-Inositol 1,4,5-trisphosphate
IP_4 myo-Inositol 1,3,4,5-tetrakisphosphate
KH Krebs-Henseleit
αLtx αLatrotoxin
MLCK Myosin light chain kinase
MW Molecular weight
[Na^+]_i Intracellular free sodium ion concentration
NANC_e Excitatory non-adrenergic, non-cholinergic
NANC_i Inhibitory non-adrenergic, non-cholinergic
NEP Neutral endopeptidase
L-NMMA L-NG-monomethylarginine
NO Nitric oxide
OAG 1-oleoyl-2-acetylglycerol
PA Phosphatidic acid
PC Phosphatidylcholine
<table>
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<th>Description</th>
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<tr>
<td>PDA</td>
<td>Phorbol 12,13-diacetate</td>
</tr>
<tr>
<td>PDBu</td>
<td>Phorbol 12,13-dibutyrate</td>
</tr>
<tr>
<td>PDD</td>
<td>Phorbol 12,13-didecanoate</td>
</tr>
<tr>
<td>PHI</td>
<td>Peptide histidine isoleucine</td>
</tr>
<tr>
<td>PHM</td>
<td>Peptide histidine methionine</td>
</tr>
<tr>
<td>PHV-42</td>
<td>Peptide histidine valine-42</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol/phosphoinositide (see context)</td>
</tr>
<tr>
<td>PIP</td>
<td>Phosphatidylinositol 4-phosphate</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>p.m.</td>
<td>Post mortem</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate, 13-acetate</td>
</tr>
<tr>
<td>ROC</td>
<td>Receptor-operated calcium channel</td>
</tr>
<tr>
<td>t₅₀</td>
<td>Time taken to reach 50% maximum response</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>VCH</td>
<td>Venom crude homogenate</td>
</tr>
<tr>
<td>VDC</td>
<td>Voltage dependent/sensitive calcium channel</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
</tr>
<tr>
<td>VIP-IR</td>
<td>VIP-like immunoreactivity</td>
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<td>VT</td>
<td>Veratridine</td>
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PUBLICATIONS

The following publications have arisen from work described in this thesis:


The following manuscripts have been submitted for publication:


CHAPTER ONE

INTRODUCTION
INTRODUCTION

The study reported in this thesis concerns the action and characterisation of the response to phorbol esters of smooth muscle-containing tissues derived from guinea-pig lung. Initially the project was aimed at furthering the understanding of the delayed phase in asthma. The working hypothesis, which was first proposed by Dale and Obianime (1985), was that inappropriate activation of protein kinase C might contribute to the late asthmatic response. Although it is realised that this effect might not be restricted just to the airway smooth muscle, but may also involve other cell types (eg. leukocytes), the parameter chosen for recording in this study was the change in contractility.

1.1 Bronchial asthma
1.1.1 An overview

Asthma is a comparatively modern disorder, with only a few reports occurring prior to the industrial revolution. It effects approximately 5-10% of the population in industrialised countries, and there are several indications that its prevalence, severity and mortality are on the increase. This is occurring despite, and perhaps more strikingly appears to closely correlate with, increases in prescribed medication. The possibility that we are not using available therapy adequately, that improvements in therapy are required, or that current anti-asthma therapy is worsening the underlying condition and allowing it to become increasingly life threatening, should not be overlooked.

The word asthma is derived from the Greek word meaning "panting" or "breathlessness". Asthma is a disease which is heterogenous and difficult to classify. It is a recurrent respiratory syndrome which is increasingly being regarded as a collection of diseases, probably of differing aetiologies, in which there is acute reversible broncho-constriction, increased bronchial non-specific hyper-reactivity, pulmonary inflammation and altered mucociliary clearance. Other pathological changes include smooth muscle hypertrophy, mucosal and submucosal oedema, epithelial damage, formation of mucous plugs, inflammatory cell infiltrates (particularly eosinophils) and the release of a variety of pharmacologically active mediators. Indeed more recently, suggestions have been made that asthma should more correctly be
referred to as a "chronic desquamative eosinophilic bronchitis" since pulmonary eosinophilia and epithelial shedding is common to all asthmatics, even in very mild cases. The disease involves large and small airways but not alveoli. The processes leading to airflow obstruction are reversible, either spontaneously or in response to medication.

The most commonly studied variety of the disease is allergic asthma which arises from known exogenous stimuli in the form of specific antigens. Experimentally, allergic asthma comprises two major patterns of airway obstruction - immediate and delayed (Hargreave et al., 1974). The "immediate asthmatic response" is characteristically rapid in onset (>1 min post antigen challenge) and peaks within 10-30 min. It is of short duration (1-2 h) and is due mainly to spasm of bronchial smooth muscle, being rapidly reversed by bronchodilator drugs such as β-adrenoceptor agonists or theophylline. Furthermore, this phase is not associated with subsequent increases in airway reactivity (Cockcroft et al., 1977).

Asthmatics may also experience a second and more complex phase of airway obstruction, the "late asthmatic response": This usually occurs some 2-6 h post challenge and peaks within 5-12 h and, although it may occur alone, it usually follows the immediate response. This phase is in essence an acute inflammatory reaction and is associated with the infiltration of inflammatory cells, release of mediators, epithelial damage, bronchial oedema and mucous plugging mentioned above (Barnes et al., 1988; Braquet et al., 1987). Clinically, airway obstruction in the late phase may be severe, prolonged and difficult to control unless corticosteroids are employed. Acute severe asthma (status asthmaticus) can be fatal. The two main phases are clearly illustrated in figure 1.1, showing changes with time in forced expiratory volume in one second (FEV₁) - a measure of airways obstruction, after allergic challenge by inhalation of grass pollen (Cockcroft, 1983).

In addition to the late asthmatic response there normally follows a period of bronchial hyper-reactivity in which there is an increased tendency to bronchoconstriction and secretion of mucous in response to a wide variety of chemical or physical stimuli including pollen, ozone, histamine, methacholine, water and cold air. It is this phenomenon of excessive "twitchiness" of the airways, which may last for days or weeks, that is such a characteristic feature of human bronchial asthma.
Figure 1.1 Immediate and late asthmatic responses in man, following a single inhalation of grass pollen extract. The open circles represent the response on a control day following inhalation of diluent at time zero. The closed circles represent the response following inhalation of grass pollen extract at time zero. FEV$_1$ = forced expiratory volume after one second. Adapted from Cockcroft (1983).
Moreover, there is now abundant evidence that inflammation of the airways may indeed lead to bronchial hyper-responsiveness (Chung, 1986).

Although there are many different hypotheses as to the causes of asthma, one factor remains clear - there is a degree of spasm and hypertrophy of the bronchial wall smooth muscle involved, and that in the case of the delayed phase it is difficult to treat and refractory to $\beta$-adrenergic stimulation.

1.1.2 Neural versus inflammatory aetiology: Historical background

In 1885 Roy and Brown, upon vagal stimulation in canine airways, demonstrated spasm which could be prevented by atropine - a drug at that time found to be of use in the treatment of asthma. Later, in 1903, Dixon and Brodie demonstrated a similar canine response upon nasal stimulation. These early observations suggested the involvement of a cholinergic neural reflex mediating the bronchospasm, endorsing the generally held belief that asthma could be explained by neurally-mediated excessive irritability of the airways. However, in 1910, when Metzler demonstrated the phenomenon of anaphylaxis in guinea-pig and likened it to human asthma; asthma then came to be regarded as an immunologic disease. The idea of the involvement of mediator release in the pathogenesis of the disease soon gained favour over previously held theories based on neural mechanisms. More recently, however, with the realisation of the absolute complexity of the disease and the recognition of an extensive peptidergic innervation in the airways of many species, including man, asthma is increasingly being regarded as a disease in which there is a complex interplay between cells (of which many are inflammatory), chemical mediators, neurological mechanisms and environmental factors.

1.1.3 The problem with the pathogenesis of asthma and the development of adequate animal models

The lack of understanding of the pathology of the delayed phase in asthma, and the fact that the disease is peculiar to man, has meant that at present there is still no adequate valid experimental animal model for the testing and development of anti-asthmatic drugs. Indeed controversy even exists as to which of the inflammatory cells or mediators are implicated in the pathogenesis; although of all the mediators so far
ascribed a role in asthma, platelet activating factor (PAF) most closely mimics its pathology (Barnes and Chung, 1987).

The possible abnormalities responsible for bronchial hyper-reactivity and asthma can be separated into:

(i) immunological and inflammatory reactions (reviewed by Barnes and Costello, 1987).

(ii) altered neural control (see reviews by Barnes and Costello, 1987; Barnes, 1984, 1986a,b, 1987).

(iii) changes in the smooth muscle itself.

This latter category can be further separated into:

(a) airway smooth muscle hypertrophy - ie. an increase in muscle cell mass (Lopez-Vidriero and Reid, 1983).

(b) an increase in contractility (Hargreave et al., 1981).

The purpose of the present study was to investigate some of the signal transduction mechanisms which might be involved, particularly in the control of smooth muscle reactivity.

1.2 Signal transduction mechanisms in airway smooth muscle cells

Airway smooth muscle cells are exposed to many different inhibitory and excitatory hormones and mediators. The response of an individual cell to a particular mediator is dependent largely on whether the cell expresses surface receptors to that agent. In the airway smooth muscle cell there appears to be two major signalling mechanisms: changes in the intracellular calcium ion concentration, and the adenylate cyclase system. The contractile state of airway smooth muscle is probably the resultant effect of interacting excitatory and inhibitory receptors and their subsequent controlling effects on the contractile apparatus.

1.2.1 Calcium ions and contraction of airway smooth muscle

It is widely accepted that contraction of airway smooth muscle is dependent on the concentration of free (ie. ionised and unbound) calcium (Ca^{2+}) present in the myoplasm of the cell (Bolton, 1979). At rest the intracellular free calcium concentration ([Ca^{2+}]_i) is considered to be less than 0.1$\mu$M and insufficient to activate the contractile apparatus (O'Doherty et al., 1980). However, it should be noted that
although the cytosol is depicted for simplicity as having a uniform Ca\(^{2+}\) concentration, it contains Ca\(^{2+}\) gradients set up by the transport processes and binding compounds present in the maze of membranes interspersed throughout it. In marked contrast, the concentration of Ca\(^{2+}\) in the extracellular compartment is of the order of 1-2mM. Thus there exists a steep inwardly directed concentration gradient down which Ca\(^{2+}\) will tend to flow. At rest only a small amount of Ca\(^{2+}\) will gain entry across the cell membrane and into the cell. This is because the efficiency of the cell membrane in limiting the so-called "passive Ca\(^{2+}\) leak" is extremely high. The small amount of Ca\(^{2+}\) that does enter the cell, does so only slowly and is dealt with by highly efficient homeostatic mechanisms designed to limit any increase in [Ca\(^{2+}\)]\(_i\), preventing the level from rising sufficiently to permit induction of the contractile mechanisms.

The homeostatic regulation of [Ca\(^{2+}\)]\(_i\) is essential to the survival of the airway smooth muscle cell since excessively high [Ca\(^{2+}\)]\(_i\) are toxic to cellular metabolism. Essentially, there are three identifiable mechanisms that serve to regulate and/or minimise alterations in [Ca\(^{2+}\)]\(_i\) in airway smooth muscle cells (reviewed by Rodger, 1986). These are (i) a plasma membrane sodium-calcium ion exchange process (Bullock et al., 1981), (ii) a plasma membrane calcium ion efflux process (Goodman et al., 1987; Bryson and Rodger, 1987) and (iii) an intracellular sequestration process (Sands and Mascali, 1978; Hogaboom and Fedan, 1981) which is principally the responsibility of the endoplasmic reticulum under normal physiological conditions. These homeostatic mechanisms are summarised in figure 1.2. The first process, a sodium-calcium exchange, is dependent on the integrity of another plasma membrane associated pump, the electrogenic sodium pump (Na\(^+\)/K\(^+\)-ATPase), whose prime function is to remove intracellularly accumulating sodium ions and so maintain the inwardly directed sodium gradient necessary for sodium-calcium exchange. The other two Ca\(^{2+}\) removal processes are also energy dependent, in that they remove Ca\(^{2+}\) against their electrochemical gradients, energy being derived from ATP.

1.2.1a Stimulus-activation: Calcium sources and its movements

On receptor stimulation or depolarisation of airway smooth muscle there is an abrupt rise in [Ca\(^{2+}\)]\(_i\) from its resting level. The increase in
[Ca^{2+}]_{i} can only be derived from two sources: from outside the cell, or from intracellular sites such as the endoplasmic reticulum. The relative contribution of each of these two sources is "dependent on the nature and concentration of the contractile agonist and the component of the contractile response under scrutiny" (Bolton, 1979; Rodger, 1987), but remains a controversial topic.

**Extracellular calcium**

Calcium derived from the extracellular space can only gain entry to the cell once the cell membrane has been rendered permeable to it. Under physiological conditions this is achieved by the opening of specific Ca^{2+} channels in the plasma membrane through which Ca^{2+} flows down its electrochemical gradient. There are at least two types of ion channel that have been proposed: voltage-operated and receptor-operated (Bolton, 1979).

Voltage-operated or voltage-dependent calcium channels (VDC) are a population of ion channels which open as the potential across the cell membrane becomes reduced (ie. depolarised). There are three types of VDC (T, N and L) each of which has specific conductances and temporal characteristics, and is differentially influenced by agonists and antagonists. In addition they have differing tissue distributions (Meldolesi and Pozzan, 1987; Hofmann et al., 1987; Tsien et al., 1988). Only L- and T-types are thought to exist in smooth muscle, while N-types are believed to be more predominant in neuronal tissues (see review by Hofmann et al., 1987). The major characteristic of this family of ion channels is that the calcium conductance is directly related to the potential difference existing across the cell membrane (Tsien, 1983). VDCs are normally important in those cell types which exhibit action potentials. In airway smooth muscle, which has a stable resting membrane potential, action potentials are not normally observed, and the opening of VDCs is a graded phenomenon (see review by Rodger, 1987). Thus in airway smooth muscle, application of increasing concentrations of depolarising agents (eg. KCl and tetraethylammonium) results in a graded depolarisation that is well correlated with increases in contractile force. Furthermore, it can be demonstrated that these effects are the consequence of Ca^{2+} entry via VDCs, since organic Ca^{2+} channel blockers suppress markedly both the action potential discharge and
tension generation induced by tetraethylammonium (TEA) in airway smooth muscle (Kannan et al., 1983; Foster et al., 1984; Ahmed et al., 1985). Moreover, in guinea-pig trachealis, dihydropyridine VDC activators such as BAY K 8644 have been shown to amplify both calcium uptake and contractile force induced by KCl (Schramm et al., 1983; Allen et al., 1985).

In summary, there is convincing evidence for the existence of VDCs in airway smooth muscle and that they appear responsible primarily for the contractile events induced by depolarising agents such as TEA or KCl. However, there is little evidence to support the involvement of VDCs in the mechanisms underlying contraction initiated by physiologically relevant agonists such as histamine, cholinomimetics and certain arachidonate metabolites. The precise physiological relevance of these channels remains unclear.

The receptor–operated calcium channel (ROC) is envisaged as an ion channel, whose opening can be either dependent or independent of the potential across the cell membrane, but is controlled by a specific receptor(s) (Bolton, 1979). ROCs are considered to possess greater permeability to ions other than Ca\(^{2+}\). Their ionic permeability/selectivity is determined by the controlling receptor. In addition these channels are not readily susceptible to organic inhibitors of Ca\(^{2+}\) influx such as verapamil and nifedipine (Bolton, 1979). At this time there is little convincing evidence to support a clear function or role for ROCs in the control of the airway smooth muscle cell. However, without sufficient knowledge we should not casually dismiss the ROC since the inability to demonstrate its presence (or absence) in airway smooth muscle may merely reflect the lack of suitable detection techniques. With the development of such techniques it will be possible to test, for example, the hypothesis that intracellular Ca\(^{2+}\), released by different agonists, is responsible for initiation of contraction and opening of ROCs (Daniel et al., 1983). If such a situation does exist, then Ca\(^{2+}\) influx via ROCs is likely to be small since it will occur during the tonic (sustained) phase of airway smooth muscle contraction, considered to be of importance in the delayed phase of asthma, at which time the [Ca\(^{2+}\)]\(_i\) is known to be extremely low. Furthermore, this tonic phase of airway smooth muscle contraction, sustained by low level Ca\(^{2+}\) influx supposedly through ROCs, may be controlled by certain inositol phospholipid metabolites (see later).
To date, the available data on airway smooth muscle (reviewed by Rodger, 1987) do not support a function for ROCs to any significant extent in the actions underlying receptor stimulation by pharmacological agonists. However, there remains the possibility proposed by Baba and co-workers, that in airway smooth muscle there may be only one channel, the VDC, and that the suggested ROCs are simply VDCs that have been modified by agonist-receptor interaction (Baba et al., 1985, 1986). Furthermore, although there appears to be confusion as to the physiological function of these voltage-dependent channels, there also remains the possibility that they are involved merely in Ca\textsuperscript{2+} homeostasis and the maintenance of membrane potential.

In summary, although it is well established that extracellular calcium is necessary for the development of tension evoked by various receptor-mediated spasmogens (e.g. histamine, acetylcholine and leukotrienes), the evidence for the involvement of VDCs and even the existence of ROCs in airway smooth muscle responding to these agents is poor. Consequently, the precise way in which extracellular Ca\textsuperscript{2+} enters the airway smooth muscle cell, in response to such spasmogens, remains unclear.

**Intracellular calcium**

It will be appreciated that the control of ionised Ca\textsuperscript{2+} is an intricate process involving several mechanisms, some of which are illustrated in figure 1.2. Originally, the endoplasmic reticulum was believed to be the major controlling influence over cytosolic free Ca\textsuperscript{2+}. With the realisation that it could act not only as a sink for Ca\textsuperscript{2+} but also as a physiologically important source of Ca\textsuperscript{2+} in response to specific receptor stimulation, an acute interest developed into the signal transduction mechanisms responsible for the release of Ca\textsuperscript{2+} from such stores. In order for researchers to realise the full importance of the role of intracellular Ca\textsuperscript{2+} the following question needed to be addressed.

1.2.1b How does a contractile agonist, interacting with specific cell surface receptors, communicate with intracellular stores to release calcium and presumably initiate the sequence of events leading to tension development?

In recent years this question has opened up whole new areas of novel research and strategies aimed at treating not just disorders involving
smooth muscle spasm (e.g. asthma, hypertension, Raynaud's disease etc.),
but also many apparently unrelated disorders as researchers realise how
universally critical intracellular communication is to the general
functioning of almost all mammalian cells. In attempting to establish the
link several theories have been advanced, for example, $\text{Ca}^{2+}$-induced
calcium release. Evidence for such a mechanism operating in airway
smooth muscle is available (Ito and Itoh, 1984). Recently, however, much
attention has been focussed upon a possible pivotal role played by
certain membrane phospholipids, and their ability to generate second
messengers (i.e. small molecular weight molecules communicating specific
messages between activated receptors and intracellular organelles and/or
sequential processes), in the control of $\text{Ca}^{2+}$ release and maintenance of
the contractile state of smooth muscle.

Historically, this concept is based on previously unrecognised findings
by Hokin and Hokin (1953), who were the first to show that there was
rapid incorporation of the radioisotope, $^{32}\text{P}$, into phosphatidic acid (PA)
and the membrane phospholipid, phosphatidylinositol (PI), occurring in
pancreas slices after treatment with acetylcholine. This phenomenon was
later referred to as the "phospholipid effect" or the "acetylcholine-lipid
effect". Whilst this observation indicated that PI metabolism was
coupled to cholinergic receptor activation, the physiological relevance
remained unclear. Some time passed before Durell et al. (1968) and
Michell (1975), in an extensive review, proposed that stimulated
phosphoinositide metabolism may be part of receptor function and
furthermore, could be important in the action of those agonists which
raise intracellular calcium. This was later demonstrated in smooth
muscle by Dale-Brown et al. (1984). Moreover, a specific increase in PI
turnover has since been documented repeatedly in a number of tissues
including airway smooth muscle (Grandordy et al., 1986, 1987, 1988), in
response to a wide variety of neurotransmitters, hormones and many
other biologically active substances, all of which mobilise intracellular
$\text{Ca}^{2+}$ (reviewed by Michell, 1975; Hirasawa and Nishizuka, 1985). It was
soon realised, however, that the concept that PI hydrolysis was the
initial event following receptor activation, required modification following
the observation that other polyphosphoinositides are metabolised far
more rapidly than PI (Michell et al., 1981). It was noted that, via
specific kinases, PI could be rapidly converted to phosphatidylinositol
4-monophosphate (PIP) by phosphorylation, and that in turn PIP could
be rapidly phosphorylated to phosphatidylinositol 4,5-bisphosphate (PIP₂) (reviewed by Irvine, 1982; Fisher and Agranoff, 1987). The interconversion of PI to PIP can take place within the plasma membrane or in the membranes of intracellular organelles. However, the conversion of PIP to PIP₂ can only occur in the plasma membrane (Lundberg et al., 1985; Cockcroft et al., 1985). PIP₂ can be rapidly recycled back to PI by specific phosphomonoesterases, the so-called "futile cycle", and as a consequence the 4 and 5 position phosphates come into isotopic equilibrium with the ATP pool. This has been shown in tissues prelabelled with the radioisotope, ³²P (see Watson and Godfrey, 1988). PI accounts for about 5-10% of membrane phospholipids while PIP and PIP₂ are present in substantially lower quantities, each making up between 1 to 10% of the total PI pool (Downes and Michell, 1982).

It is now generally accepted that in a variety of cell types, including airway smooth muscle (Rasmussen and Barrett, 1984), in which calcium is an intracellular messenger, cell surface receptor interaction with certain agonists results in rapid diesteratic (phosphoinositidase) cleavage of PIP₂ and the subsequent generation of two putative intracellular second messengers, myo-inositol 1,4,5-trisphosphate (IP₃) and 1,2-sn-diacyl-glycerol (DAG) (Berridge, 1984). It is worth pointing out at this stage that there is probably a family of phosphoinositidases with different substrate specificities (Katan et al., 1988), and it is possible that DAG and other inositol phosphates can be derived from other phospholipids such as PI and PIP. In non-muscle tissues, DAG can also be derived from phosphatidylcholine (PC) (Farese et al., 1987). However, in studies in airway smooth muscle, decreases in PI were equimolar with increases in PA, and there was no decrease in PC or phosphatidylethanolamine pool sizes, suggesting that all or most of the increase in PA and DAG pool sizes was attributable to inositol phospholipid metabolism (Baron et al., 1984).

The contractile response of smooth muscle is a calcium-dependent process and it is becoming clear that the above signal transduction processes do operate during pharmacomechanical coupling in many smooth muscles, including airway smooth muscle. It has been proposed that IP₃ is the intracellular mediator of calcium release, particularly in response to cholinergic agonists and leukotrienes in airway smooth muscle (Hashimoto et al., 1985; Duncan et al., 1987; Grandordy et al.,
1986, 1987; Mong et al., 1987). The organelle from which IP₃ released Ca²⁺ was originally believed to be a component of the endoplasmic reticulum, but more recently it has been suggested that the Ca²⁺ comes from a novel IP₃-responsive organelle, termed calciosome (Volpe et al., 1988). In the absence of suitable antagonists, the evidence for IP₃ mediating the release of intracellular Ca²⁺ is as follows:

(i) In both canine and bovine tracheal smooth muscle there is agonist-induced breakdown of phosphoinositides and subsequent generation of IP₃ and phosphatidic acid prior to tension development (Hashimoto et al., 1985; Takuwa et al., 1986; Duncan et al., 1987).

(ii) IP₃ can increase [Ca²⁺]ᵢ and/or release stored Ca²⁺ from skinned (saponin permeabilised) canine tracheal smooth muscle (Hashimoto et al., 1985).

(iii) IP₃ is produced after ligand-receptor coupling (Reynolds and Dubyak, 1985). Furthermore, IP₃ production, in turn, correlates with the increase in [Ca²⁺]ᵢ which is independent of extracellular Ca²⁺. This was shown in a cultured airway smooth muscle cell line in which histamine was the agonist at H₁-receptors (Kotlikoff et al., 1987).

(iv) In saponin permeabilised airway smooth muscle preparations, IP₃ has been shown to elicit contractions by inducing the release of Ca²⁺ from intracellular store sites (Hashimoto et al., 1985).

Interestingly, despite substantial evidence apparently supporting a role for IP₃ in the regulation of Ca²⁺ and contractility in airway smooth muscle, there is doubt as to whether IP₃ is important in the response of both canine and guinea-pig trachea to histamine, and controversy regarding the action of cholinomimetics in these tissues (cf. Hashimoto et al., 1985 and Langlands et al., 1989). Clearly the role of other inositol phosphates may require consideration since it is widely accepted that in these tissues stimulation of both these receptor mechanisms leads to phosphoinositide turnover.

There is evidence for another water soluble inositol phosphate derived from IP₃, myo-inositol 1,3,4,5-tetrakisphosphate (IP₄), formed on agonist-induced phosphoinositide hydrolysis. Suggestions have been
made that IP₄ may function to ensure that the finite IP₃-releasable intracellular Ca²⁺ store is continuously refilled. The calcium required for this is presumably derived from the extracellular space and must enter the cell by some mechanism. In this context, suggestions support a role for IP₄ acting as an intracellular modulator controlling the refilling of the intracellular Ca²⁺ stores, possibly through an ion channel (Irvine and Moor, 1986). Such a channel may not only span the plasma membrane, but also the limiting membrane of the intracellular microsomal store (Putney, 1986) (see figure 1.2).

The other product of agonist-induced phospholipid hydrolysis, 1,2-sn-diacylglycerol (DAG), is lipid soluble and so remains in the plasma membrane. It is not regarded as being involved in [Ca²⁺]ᵢ release. Instead, it has been suggested that DAG may be more intimately involved in those events which govern tension maintenance of smooth muscle (see later), involving an effect on the enzyme protein kinase C (PKC) (Rasmussen and Barrett, 1984; Park and Rasmussen, 1985; Rasmussen et al., 1987). The major direct role for DAG is considered to be activation of PKC.

1.2.1c The protein kinase C story

Protein kinase C was originally discovered in the brain by Nishizuka (Inoue et al., 1977). It has since been found to be universally present in eukaryotic cells, including smooth muscle. Suggestions are that PKC is involved in a plethora of biological functions too numerous to list in these pages, but sharing a common mechanism by which cells sense and respond to environmental signals — one of the central issues in cell biology. Protein kinase C is one of a family of protein kinases classified according to their substrate specificities, the threonine and serine kinases. The properties of this particular enzyme, which exists in several isoenzymic forms, that distinguish it from other protein kinases are that it is dependent on Ca²⁺ and phospholipid (particularly phosphatidylycerine) for activity (Nishizuka, 1984). Indeed, it is the binding of the enzyme to membrane phosphatidylycerine which confers Ca²⁺ sensitivity to PKC in the presence of the phospholipid, DAG. DAG lowers the threshold concentration of Ca²⁺ required to activate PKC, such that it is reduced 100-fold to 1μM. PKC is normally localised to the cytosol but translocates to the endoplasmic face of the plasma
membrane in the presence of Ca\textsuperscript{2+} and DAG (Wolf et al., 1985b; Rasmussen et al., 1986). Thus DAGs have a second messenger function, through activation of PKC which in turn is considered to be an important transducer in cell signalling processes.

The evidence for DAG as a second messenger for PKC is as follows:

(i) There is agonist-induced breakdown of phosphoinositides and a concomitant sustained generation of 1,2-sn-diacylglycerol in bovine airways (Takuwa et al., 1986).

(ii) Due to its lipophilicity, DAG remains in the plasma membrane which is the site of PKC activation (Wolf et al., 1985b).

(iii) On receptor stimulation DAG is rapidly removed, terminating its action, by at least two routes of metabolism. It can either be phosphorylated by DAG-kinase to phosphatidic acid (Lapetina and Cuatrecasas, 1979) or sequentially deacylated to monoacylglycerol and glycerol (Bell et al., 1979).

(iv) In platelets, the transient appearance of DAG in the plasma membrane is always associated with PKC activation as judged by phosphorylation of a 40kDa protein, a known substrate for PKC (Kawahara et al., 1980; Sano et al., 1983).

(v) Cell permeant DAG analogues (e.g. 1-oleoyl-2-acetylglycerol and 1,2-sn-dioctanoylglycerol) mimic many of the effects of PKC activation, the presumed cellular target for phorbol esters and DAG (reviewed by Niedel and Blackshear, 1986).

(vi) DAG has been shown to activate PKC isolated from various smooth muscles (Yu, 1981).

Interest in PKC was heightened soon after it was originally described when Castagna et al. (1982) demonstrated that the tumour promoter, phorbol myristate acetate (PMA), could stimulate PKC by substituting for DAG. PKC was soon found to be the major phorbol ester "receptor" in brain and other tissues (reviewed by Ashendel, 1985; Niedel et al., 1983; Kikkawa et al., 1983). At the time phorbol esters were known to mimic the effects of numerous hormones, neurotransmitters and growth factors. The inevitable association between the phorbol ester receptor and PKC
caught the attention of many scientists and has resulted in a vast, evergrowing and fascinating literature on the role of PKC in numerous biological processes. Nearly all mammalian cells respond to phorbol esters in one or more ways. One exception being non-nucleated erythrocytes (Blumberg et al., 1983).

Phorbol esters were originally isolated from the oil of the plant, *Croton tiglium*. Phorbol 12-myristate 13-acetate (PMA) was found to be the most active, and esterification at the 12- and 13- positions on the phorboid nucleus is a consistent requirement for activity at PKC. Phorbol 12,13-dibutyrate (PDBu) and PMA are less lipophilic than phorbol 12,13-didecanoate (PDD) and therefore more easily delivered to cells. The phorbol ester binding site, presumed to be PKC, exerts marked stereoselectivity such that only the 4β-isomers are active, whilst the 4α-isomers lose their ability to mimic PKC activation (reviewed in Niedel and Blackshear, 1986). The regulation site within PKC that recognises DAG is now considered to be the "receptor" for phorbol esters (see Nishizuka, 1984). Phorbol esters unlike DAG are not rapidly metabolised (Ashendel, 1985) and so produce a more prolonged activation of PKC. In the past this was considered unphysiological. More recently, however, particularly in the maintained phase of airway smooth muscle contraction there is now evidence for sustained phosphoinositide hydrolysis and DAG production, and presumably prolonged activation of PKC (Takuda et al., 1986; Griendling et al., 1986).

What is the evidence for PKC as the phorbol ester receptor?

(i) The binding site for [³H]PDBu meets the criteria for a receptor in that binding was saturable, of high affinity, rapidly reversible, stable over time and the biological potencies of phorbol analogues correlated with their potencies as inhibitors of [³H]PDBu binding to the receptor (Driedger and Blumberg, 1980).

(ii) Castagna et al. (1982) demonstrated that PMA could substitute for DAG and activate PKC *in vitro*. Coupled with this, the profile of the PMA concentration-effect curve on PKC was reminiscent of the saturation curve for phorbol ester binding to the phorbol ester receptor.
(iii) Activation of PKC by phorbol esters displays the same structure-activity relationships seen for receptor binding (Castagna et al., 1982).

(iv) Enzyme fully stimulated by PMA or DAG can not be further activated by addition of the other activator – indicating a common mechanism for the action of DAG and PMA (see review by Niedel and Blackshear, 1986).

(v) PMA has been shown to activate PKC directly in intact cells (Sano et al., 1983; Yamanishi et al., 1983).

(vi) The phorbol ester receptor, when solubilised by detergents from mouse brain particulates, co-purifies with PKC (Niedel et al., 1983).

Without the availability of selective and potent PKC inhibitors, conclusive proof for the activation of PKC by phorbol esters within cells will be lacking. However, at present, there appears to be general agreement that PKC is the major cellular receptor for phorbol esters. Multiple forms of PKC exist which although highly homologous in their sequence, appear to be derived from distinct genes on separate chromosomes (Coussens et al., 1986; Ohne et al., 1986). Furthermore, it is possible that different subspecies of PKC are activated by, in addition to DAG, other phospholipid metabolites such as arachidonic acid and lipoxin A (Hansson et al., 1986). The possibility of differential effects of phorbol esters on different PKC isoenzymes, or even effects unrelated to PKC, must not be ruled out in the continued absence of potent and selective PKC inhibitors. For the moment, however, phorbol esters represent powerful investigative tools with which to evaluate the role of PKC in biological systems. To that end I have investigated the role of PKC in different airway smooth muscle preparations taken from guinea-pig lung.
Figure 1.2 Schematic representation of some of the proposed molecular events involved in the regulation of excitation-contraction coupling in airway smooth muscle. Note the inclusion of the "capacitance model" (Putney, 1986). Myosin is labelled "integrator" since the level of myosin phosphorylation, based on the events from signal to effector, determines the rate of ATP hydrolysis and the contractile state of the muscle. R1: cell surface receptor. R2: putative receptor for IP₄ located on ROC/second messenger-operated channel. R3: putative receptor for IP₃ located on endoplasmic reticulum (EPR)/calciosome. 1-4: calcium homeostatic mechanisms.
1.2.1d Synergistic interactions between the IP$_3$/Ca$^{2+}$ and DAG/PKC signal pathways

The concept that the two bifurcating arms of the inositol phospholipid signal pathway may interact synergistically with each other to regulate cellular processes, emerged from studies on blood platelets where secretion of 5-hydroxytryptamine, normally triggered by agonists such as thrombin, could be induced by a combination of single low concentrations of a calcium ionophore and a phorbol ester. This was first discovered by Nishizuka and colleagues (Kaibuchi et al., 1983). The calcium ionophore mimicked the effect of IP$_3$ in raising [Ca$^{2+}$]$_i$, whereas the phorbol ester activated PKC. Such combinations of low concentrations of calcium ionophore and phorbol ester have been shown to activate many different cellular processes including contraction in vascular (Rasmussen et al., 1984; Forder et al., 1985) and airway smooth muscle such as the bovine trachea (Park and Rasmussen, 1985) and guinea-pig lung parenchymal strips (Dale and Obianime, 1985, 1987; Obianime et al., 1988). In these airway smooth muscle preparations, phorbol esters cause a slowly developing and sustained contractile response (Park and Rasmussen, 1985; Dale and Obianime, 1985, 1987; Obianime et al., 1988). Similarly, contraction results with the calcium ionophore (Dale and Obianime, 1987; Obianime et al., 1988). However, when added together, using threshold concentrations, these two stimuli interact synergistically (ie. produce a greater than additive response) to produce a rapid and sustained response which is very reminiscent of that seen by surface receptor activation with carbachol (Park and Rasmussen, 1985). This has led to the suggestion that PKC may be involved in the tonic component of contraction (see later) (Park and Rasmussen, 1985).

1.3 The biochemical basis for contraction

1.3.1 Contractile structures of smooth muscle

Many of the studies on smooth muscle structure have been carried out on vascular tissue. Electron and light microscopy has established that smooth muscle is made up of myofilaments (reviewed by Somlyo and Somlyo, 1968). The myofilaments are made up of thin actin filaments and thicker myosin filaments (see Somlyo et al., 1965; Somlyo and Somlyo, 1968). Actin and myosin are the two principal proteins responsible for contraction of all types of muscle - skeletal, cardiac and smooth. These
two proteins function by forming into filaments arranged in parallel such that they can slide over each other (Adelstein and Eisenberg, 1980). The thin actin filaments are composed of two linear polymers (MW 42kDa) of globular protein wrapped together in a helical structure intertwined with another more fibrous protein, tropomyosin, whose function is still not fully understood. In contrast the thicker myosin filaments, composed of large bipolar molecules, are arranged asymmetrically in a hexameric structure. Myosin comprises one pair of heavy (MW 200kDa) chains, and two pairs of light (MW 15 and 20kDa) chains whose function is to control the binding of myosin to actin. At one end the myosin molecule is globular and possesses both the binding site for attachment to actin and the enzymatic site (myosin ATPase) which, in response to actin activation, hydrolyses ATP so providing the necessary energy to permit binding (ie. attachment) to occur (Adelstein and Eisenberg, 1980). It is at the globular head of myosin that the light chains are located. The long tail portion of the myosin molecule is responsible for its organisation into bipolar filaments. The general arrangement of the contractile proteins is summarised in figure 1.3a.

Contraction of smooth muscle is thought to occur via a mechanism similar to the sliding filament process first proposed for skeletal muscle (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954). The sliding of the thin actin filaments and thicker myosin filaments is achieved by attachment of the globular myosin heads to actin. This process is referred to as "cross-bridge formation". It is the phosphorylation of one of the myosin head light chains (the so-called p-light chain, MW 20kDa, which would normally prevent myosin and actin interaction) which commences the attachment-detachment contraction cycle of smooth muscle (see figure 1.3a). Subsequently, there follows a flexing change in the configuration of the myosin to actin and then detachment of the myosin head followed by rapid re-attachment at another site further down the actin filament. Visually, if this was possible, this cycling process driven by ATP (ATP being consumed by actin-activated myosin ATPase) might resemble the leg movements of a millipede walking along a twig. The formation of cross-bridges is believed to be important in the generation of smooth muscle tension. Furthermore, the generation of smooth muscle tension is considered to comprise two phases – phasic and tonic. The phasic component (tension development) is thought to be Ca$^{2+}$-dependent, while it is suggested that the tonic component (tension
maintenance) is due to the formation of "latch-bridges" in which there is a much lower requirement for Ca\(^{2+}\) and also a reduction in myosin phosphorylation (reviewed by Sommerville and Hartshorne, 1986; Rasmussen et al., 1987).

### 1.3.2 Mechanism of smooth muscle contraction

There is now wide acceptance that all spasmogens produce their response through an increase in cytosolic free Ca\(^{2+}\) concentration from less than 0.1\(\mu\)M to an elevated level of 1-2mM (see section 1.2.1). The increase in [Ca\(^{2+}\)]\(_i\) is believed to bring about smooth muscle contraction through three major processes/mechanisms involving different protein structures.

(i) Combination with calmodulin
(ii) Activation of myosin light chain kinase
(iii) Inhibition by caldesmon

In addition, there is another hypothesis involving yet another Ca\(^{2+}\)-dependent activator of smooth muscle contraction, namely leiitonin (Ebashi, 1980). However, the lack of consistent evidence for this particular model has led to its poor acceptance as one of the possible mechanisms by which contraction occurs in smooth muscle.

In the myosin light chain kinase (MLCK) theory of contracture in smooth muscle, an increase in [Ca\(^{2+}\)]\(_i\) leads to activation of the Ca\(^{2+}\) receptor protein, calmodulin (CaM), and activates the specific Ca\(^{2+}\)/CaM-dependent enzyme, MLCK (Adelstein et al., 1982). This in turn leads to phosphorylation of myosin light chains and initiates the contractile process (ie. a thick filament regulated process, see above and figure 1.2). Interestingly, there is evidence for direct activation of myosin ATPase by Ca\(^{2+}\). However, this is generally only observed at Ca\(^{2+}\) concentrations greater than those required to saturate CaM, and much higher than those observed in the smooth muscle cytoplasm. The physiological relevance of this effect is therefore questionable (see Kamm and Stull, 1989).

More recent evidence has demonstrated the presence of another protein, caldesmon, which is closely associated with the actin-tropomyosin-myosin domain in smooth muscle. Caldesmon derives its name from its ability to
bind CaM. In the absence of the Ca\(^{2+}\)/CaM complex caldesmon, by binding to actin-tropomyosin, inhibits cross-bridge formation and cycling. This has led to the postulate that the interaction of Ca\(^{2+}\)/CaM with caldesmon may provide a Ca\(^{2+}\)-dependent, thin filament-regulated mechanism for controlling contraction in smooth muscle (Pritchard and Moody, 1986). However, for the process to operate there is an absolute requirement for phosphorylation of the myosin light chains and therefore prefunctioning of MLCK. Although this model may account for the phasic component of smooth muscle contraction, it presupposes continued elevation of \([Ca^{2+}]_i\) for the sustained contractile response (particularly for the caldesmon theory) and the presence of phosphorylated myosin light chain. However, neither assumption has proved valid (see Rasmussen et al., 1987). Indeed, in studies where \([Ca^{2+}]_i\) has been measured, using the photoprotein aequorin as the Ca\(^{2+}\) indicator in intact smooth muscle strips, there was an immediate and rapid rise in \([Ca^{2+}]_i\), but this was only transient (2-5min) and returned to a slightly elevated value above basal (Morgan and Morgan, 1984) even though the contractile response continued to a plateau. Similarly phosphorylation of myosin light chain was only detected for 15-35min after agonist addition, and fell to basal despite the sustained mechanical response (see figure 1.3b) (Sommerville and Hartshorne, 1986). This sustained contraction, the subject of much debate and research, in the face of only transient increases in \([Ca^{2+}]_i\) and myosin light chain phosphorylation has led to the "latch-bridge" hypothesis: stress-bearing non- or slowly cycling cross-bridges (Askoy et al., 1982, 1983). Recent studies by Rembold and Murphy (1988) using a variety of agonists in vascular smooth muscle, some of which cause the muscle to enter the latch state and some of which do not, seem to indicate that latch is a consequence of phosphorylation and that the relationship between Ca\(^{2+}\) and phosphorylation depends on the agonist. However, this view is not universally accepted, for instance Morgan and co-workers (1988) and Pfitzer and co-workers (1989) report rather similar experiments with an almost opposite conclusion. It is possible that some of the current controversy over experimental results might arise from problems in the techniques for measuring phosphorylation and Ca\(^{2+}\). The use of aequorin rather than fura-2 may lead to misleading results since it has a relatively poor sensitivity to changes in Ca\(^{2+}\) concentration in the low concentration range where latch bridges are formed and broken (Ishii et al., 1988).
Figure 1.3 (a) The schematic arrangement of the major contractile proteins in smooth muscle. (b) Schematic representation of the "latch-bridge" model of smooth muscle contraction. Muscle force, intracellular calcium concentration ([Ca²⁺]ᵢ) and myosin light chain phosphorylation (MLC.P) are depicted at rest (ie. absence of agonist), during tension development and tension maintenance. Adapted from Sommerville and Hartshorne (1986).
Interest has also been centred around an additional and quite separate property of caldesmon to that discussed above. When caldesmon is added to actin, the inhibition of ATPase is associated with an abrupt increase in the affinity of smooth muscle heavy meromyosin for actin filaments (Lash et al., 1986). This property, unique to caldesmon in the thin filaments and thick filaments of smooth muscle could be related to, or even the cause of latch – a property unique to smooth muscle.

In addition, another model has been proposed to operate during latch which takes into account observations made during tonic contracture in which \([\text{Ca}^{2+}]_i\) is considered to be low. Indeed, one of the characteristic features of the latch bridge state must be an enhanced sensitivity of the contractile machinery to Ca\(^{2+}\). Exactly how this is achieved is not yet known, although a possible role for PKC has been proposed (Rodger, 1986; Rasmussen et al., 1987). PKC could well fulfill this role since in many tissues there is a sustained DAG formation, and presumably prolonged PKC activation (see earlier). Furthermore, PKC is known to function at "vanishingly small calcium concentrations" (DiVirgilio et al., 1984) in the presence of a PKC activator such as a phorbol ester, or endogenous DAG.

What is the evidence for PKC in the tonic phase of smooth muscle contraction?

(i) In bovine trachea there is sustained production of DAG, the endogenous PKC activator, induced by carbachol (Takuwa et al., 1986).

(ii) In many smooth muscle preparations phorbol esters, which activate PKC directly, produce sustained contracture (Dale and Obianime, 1985, 1987, 1989; Danthuluri and Deth, 1984; Forder et al., 1985; Park and Rasmussen, 1985).

(iii) PKC activation can increase the sensitivity of the contractile apparatus for Ca\(^{2+}\), one of the prerequisites for the latch-bridge hypothesis to operate (see above) (Jiang and Morgan, 1987; Itoh et al., 1988; Obianime et al., 1988).
(iv) In bovine trachea, the synergistic interaction of PKC activation and internal Ca$^{2+}$ mobilisation, produces a sustained contractile response which is very reminiscent of that seen with carbachol (Park and Rasmussen, 1985).

(v) Myosin light chain (Nishikawa et al., 1983), MLCK (Ikebe et al., 1985; Nishikawa et al., 1985) and actin binding proteins (Kawamoto and Hidaka, 1984) are all substrates for PKC in vitro. Further, in bovine trachea the "late phosphorylation" changes induced by carbachol are identical to those produced by the exogenous PKC activator, 4β-PDBu (Park and Rasmussen, 1986).

(vi) Intriguingly, PKC activation also activates caldesmon (Umekawa and Hidaka, 1985), which is dephosphorylated on relaxation (see earlier) (Park and Rasmussen, 1986).

It should be pointed out that the role of PKC in the tonic phase of smooth muscle contraction is not universally accepted, in fact Nishikawa et al. (1984) have suggested that PKC may be more important in relaxation. However, the weight of evidence to the contrary still remains and the role of PKC in the tonic phase of contraction has not yet been hotly disputed.

In summary, the story so far suggests two separate, but interdependent, pathways function to control contraction of airway smooth muscle. The first, the CaM/MLCK pathway, is responsible for the initial rapid development of tension to a spasmogen, and is Ca$^{2+}$ dependent. The second pathway adapts the cell to accommodate contraction at a minimum cost to itself, in terms of both energy expenditure and protection from the toxic consequences of [Ca$^{2+}$]$_i$ overload. Furthermore, there is a certain amount of evidence to suggest that this second, sustained and conservative phase involves activation of PKC.

1.4 Other modulatory effects of protein kinase C

In addition to the positive contractile action in smooth muscle, a large body of evidence has now accumulated to indicate that PKC provides negative feedback control over various steps in cell signalling. PKC
appears to be important in exerting feedback control on muscarinic receptor activation in smooth muscle. Specifically, phorbol esters inhibit the hydrolysis of PIP$_2$, as monitored by decreases in IP$_3$ production and in the transient [Ca$^{2+}$]$_i$ elevation in smooth muscle (Cotecchia et al., 1985; Baron and Coburn, 1987). This effect may be due to phorbol esters and/or PKC inhibiting PIP$_2$ hydrolysis by altering the activity of inositol phospholipid-specific phospholipase C or by an effect on the associated guanine nucleotide regulatory protein. In addition, PKC activation has been shown to stimulate both the electrogenic Na$^+$/K$^+$-ATPase pump (Moisey and Cox, 1987) and to alter the activity of membrane ion channels (Spedding, 1987). Further, PKC may also stimulate the removal of [Ca$^{2+}$]$_i$ involving an effect on the Ca$^{2+}$-transport ATPase and the Na$^+$/Ca$^{2+}$ exchanger (see review by Nishizuka, 1986). PKC is also reported to undergo autophosphorylation. The importance of this is not yet certain but may be linked to termination of the PKC signal (Wolfe et al., 1985a).

The dual actions of PKC described in this introduction, must therefore provide a versatile regulatory system that is finely tuned by the transient generation of second messengers such as DAG.

1.5 Outline of studies

The main purpose of this project was to study the effect of PKC activation, by phorbol esters, in different airway smooth muscle preparations taken from guinea-pig lung. Initially the project was to investigate the delayed phase in asthma, in which it was hypothesised that inappropriate activation of PKC could contribute (Dale and Obianime, 1985). However, in view of the unresolved controversy regarding the suitability of our tissue of study, the guinea-pig lung parenchymal strip, as a model of peripheral airway pharmacology, it was decided to study the effects of PKC activation on the lung strip in parallel with that of the trachea. Qualitatively different responses were observed to PKC activation in these two tissues (see Chapter 4) which are closely anatomically and embryologically related. This warranted further investigation. At the time there was only one report in the literature (Menkes et al., 1986) describing the unexpected relaxant effect of phorbol esters in the guinea-pig trachea and in view of results already
obtained in our laboratory, no credible explanation was offered for this curious effect. Consequently, much of the work reported in this thesis comprises a comparison of the guinea-pig lung parenchymal and tracheal strip in an attempt to understand the mechanism by which phorbol esters produce relaxation in the isolated guinea-pig trachea.
CHAPTER TWO

MATERIALS AND METHODS
METHODS

2.1 Animals

Male Dunkin-Hartley guinea-pigs (Biological Services, Joint Animal House, UCL.) of body weight 250-500g were used throughout this study. They were housed in a temperature controlled room (20°C) and given food (Special Diet Services, guinea-pig FD-1, UK.) and water ad libitum.

Guinea-pigs were killed by cervical dislocation and then exsanguinated. After thoracotomy, the trachea, heart and lungs were excised en bloc and placed into Krebs-Henseleit (KH) solution at room temperature, aerated with a mixture of 95% O₂ and 5% CO₂, pH 7.4. The composition of the KH solution was as follows (mM): NaCl 119, KCl 4.7, NaHCO₃ 25.0, KH₂PO₄ 1.1, MgCl₂ 0.56, D-glucose 11.0 and CaCl₂ 2.5 (All reagents were of analytical grade and obtained from BDH, Poole, UK.).

2.2 Organ bath studies

2.2.1 Preparation of tissues

Employing the method first described by Lulich et al. (1976), lung parenchymal strips (approx. 3x3x30mm) were cut from the distal edges of the diaphragmatic and cardiac lobes of the lung. The trachea, dissected free of the lungs prior to parenchymal strip preparation, was cleaned of unwanted connective tissue from the larynx to the carina whilst immersed in aerating KH solution at room temperature. After removal of the larynx, the tracheal tube was cut longitudinally, diametrically opposite the smooth muscle strip and then pinned out onto a cork-based polystyrene petri-dish (Sterilin, UK.) containing aerated KH solution at room temperature. Transverse strips, consisting of 2-3 cartilage bands between each cut, were then prepared from the cervical, thoracic and lower thoracic portions of the trachea. Routinely, six segments were obtained in this way, although in some experiments, 8-10 segments could be prepared (see section 2.3.2a). In preliminary experiments, however, tracheal strips were prepared by making three alternate cuts, three cartilage bands separating each cut, in a zig-zag fashion. The number of cuts was limited to three to minimise any unwanted disruption of the epithelium. Using this method, four tracheal strips were usually prepared from each animal. This particular technique was subsequently discontinued in order to maximise the number of preparations which could be obtained from each animal, and to duplicate the method of Hay.
et al. (1986), when examining the role of the airway epithelium. Great care was taken to avoid damaging the airway epithelium, which has been implicated in the modulation of airway smooth muscle responsiveness in many species including guinea-pig (Farmer et al., 1986; Goldie et al., 1986; Hay et al., 1986). However, in those experiments in which it was necessary to remove the airway epithelium, the method of Hay et al. (1986) was adopted. Briefly, the airway epithelium of alternate strips was disrupted mechanically by gentle rubbing (3-4 strokes) of the luminal surface, over both the smooth muscle and cartilage areas, with a damp cotton-tipped applicator. Unrubbed adjacent strips served as paired controls for intact epithelium. One tracheal strip from each animal was kept aside and not subjected to an experimental protocol, thereby serving as a positive control for epithelium intactness in tissues prepared using this method. On completion of each experiment the effectiveness of the procedure was ascertained by histological examination of 5µm sample rubbed and unrubbed sections after fixation in Karnovsky's fixative and staining with eosin and haematoxylin. In each case, histological examination revealed that rubbed tissues were devoid of epithelium, whereas all unrubbed tissues were intact.

Bronchial spirals were cut subsequent to lung parenchymal and tracheal strip preparation. Briefly, the remaining carinal portion of the trachea (still attached to the lungs) was bisected longitudinally and each of the lung lobes, except the left and right diaphragmatic, were then removed as close to the main bronchus as possible. A small metal seeker (diameter 1.5mm) was carefully inserted into the left or right main bronchus as far as the opening to the secondary bronchus. The surrounding parenchyma was dissected away revealing the bronchial tube which was then spirally cut. Each spiral was divided into three distinct anatomical portions – the main bronchus, hilar bronchus and non-cartilagenous secondary bronchus. Separate recordings of the hilar and secondary bronchus could then be made. Two such compounded spirals were obtained from each animal.

2.2.2 Recording of tissue responses

All tissues were rapidly mounted in organ baths containing 5ml KH solution maintained at 37°C and gassed continuously with 95% O₂ and 5% O₂, pH 7.4. Tissues were placed under 0.5g tension which was found to
be optimal for changes in response for the method of recording employed.

The responses of the lung parenchymal strip and bronchus were recorded isotonically (Hall Effect transducer, Chem-lab, UK.) connected via a silk thread, since these preparations were considered too delicate for isometric recording. Another reason for using this particular method of recording was that if significant changes in contractility represent changes in smaller airway calibre, then isotonic measurements seemed more appropriate. However, it is worth bearing in mind that in the intact lung, airway narrowing is neither isotonic or isometric since as the airway narrows the load placed on the muscle (due to the airway wall structure) and the muscle length both change (Mitchell and Sparrow, 1989). In contrast, tracheal responses were recorded isometrically (Dynamometer, UF1) – connected via a metal bar (120×0.4mm) hooked at both ends. However in a small number of preliminary experiments, where tissues were cut in a zig-zag fashion (see section 2.2.1), tracheal strips were recorded isotonically. The transducer outputs were recorded on a combination of Bryans (model 28000), Rikadenki polygraph (Model R-50) and Servoscribe (model RE-541) potentiometric flat-bed pen recorders.

In all experiments, tissues were allowed 2h in which to equilibrate during which time they were washed every 15-20min with KH solution and, for isometric recording, readjusted to the optimum level of tension prior to carrying out the experimental protocol. During re-equilibration, both the lung parenchymal strip and bronchus relaxed until a steady level of tone was achieved, while contraction occurred in the trachea.

2.2.3 Electrical field stimulation (EFS) of tissues

Electrical field stimulation (EFS) was delivered by two parallel platinum wires (25×0.25mm) placed on either side of the tissue, approximately 10mm apart (see figure 2.1a). Electrodes were connected to a stimulator (Grass, model SD9) for delivery of bipolar rectangular wave pulses. Optimum parameters for stimulation were determined in preliminary experiments, in which responses were observed over a range of voltages, pulse durations and frequencies. The properties of the
impulse delivered and the potential difference across the electrodes, used as an index of impulse quality and efficiency, was continuously monitored on an oscilloscope (Scopex, model 4S6). Trains of impulses were delivered until the response of the tissue reached a plateau and then increased without interrupting the stimulation. In this way cumulative frequency-response curves could be constructed (see figure 5.1b). On completion of each frequency-response curve, the stimulator was turned off and the tissue allowed to return to baseline without changing the bath fluid. The neural nature of responses to EFS was ascertained by incubating the tissues with tetrodotoxin (3μM) for 15-30min and repeating EFS (see figure 5.1a).

2.2.4 Construction of concentration-response curves

Cumulative concentration-response curves to exogenous agonists were constructed by adding increasing concentrations of agonist to the organ bath and allowing the resultant response to plateau between successive additions. When no response was observed a period of 5-10min, depending on the agonist, was allowed before the next addition. After completion of each concentration-response curve, tissues were either washed with KH solution and allowed to return to baseline, or papaverine (100μM) was added to the bath for calibration of the response (see below). The effect of receptor antagonists/enzyme inhibitors on the concentration-response curves was assessed either by (i) incubating parallel preparations from the same animal with the relevant drug or solvent and constructing only one concentration-response curve in addition to the histamine standard, or by (ii) constructing a concentration-response curve to the agonist before and after incubating tissues with the relevant drug or solvent for a specified period of time.

In all cases, where the agent used for pretreatment caused a change in tension level, the magnitude of agonist-induced responses was measured from the new baseline, i.e. subtracting any effect of the agent used for pretreatment.
2.2.5 Data analysis

After the equilibration period had elapsed, histamine was administered repeatedly in a cumulative fashion until consistent maximal responses were obtained using 1, 3 and 10μM histamine for the lung parenchymal strip and 10, 30 and 100μM histamine for the tracheal and bronchus preparations. The latter concentration in each case, usually resulted in the maximum contractile response. Repeated washings with KH solution returned the tissues to baseline. All subsequent contractile responses could then be expressed as a percentage of the mean maximum contractile response to histamine. In experiments in which relaxant responses were recorded, papaverine (100μM) was administered at the end of the experimental protocol in order to determine the maximum relaxant response of the tissue. Relaxation could then be expressed either as a negative percentage of the mean maximum contractile response to histamine or, more commonly, as a percentage of the maximum relaxation to papaverine.

Wherever possible, control and test preparations were taken from the same animal and tested in parallel. Data given in both the text and figures are expressed as mean ± standard error of the mean (s.e.mean). In all cases, unless otherwise indicated, the number of preparations in each group (n) represents the number of animals from which the preparations were obtained. Concentration–response curves and electric field stimulus (EFS)-response curves were fitted by eye using Sigmaplot (Jandel Scientific, Corte Madera, CA., USA.). Concentrations inducing 50% of the maximum response to each agent (EC₅₀ for excitatory responses or IC₅₀ for inhibitory responses) and EFS frequencies inducing 50% of the maximum response (EF₅₀) were determined by calculating the mean value from the linear portion of the stimulus–response curve, fitted by least squares regression analysis, obtained from individual preparations. Where the time-course of a response was investigated, experiments were analysed using the digitising software package, Sigmascan (Jandel Scientific). Data were compared by Student’s t-test for paired or unpaired observations where appropriate. Observations were considered paired when control and test responses were obtained from the same preparation. Results were considered significant when the probability level (p) was less than 0.05.
2.3 Release studies
2.3.1 Vasoactive intestinal peptide (VIP) assay
2.3.1a Tissue preparation and sample collection

Tracheal strips were removed from three animals for each of the VIP assay experiments, and cut into three equal segments. Each segment comprised 10-11 cartilage bands, cut in exactly the same way as for the organ bath studies (see section 2.2.1). Each segment, laryngeal, mid or carinal was placed into a polypropylene vial (Hughes and Hughes) containing 2ml Krebs-Henseleit (KH) solution, to which bovine serum albumin (BSA) (50mg/l) and the peptidase inhibitors bacitracin (30mg/l = 1.86U/ml) and aprotinin (100μg/l = 1.2U/ml), had previously been added. This was done according to the plan shown below. Tissues were maintained at 37°C, gassed with a mixture of 95% O₂ and 5% CO₂.

```
          LARYNX
         Laryngeal (L)
               --
               Mid (M)
               --
         Carinal (C)
         CARINA

     VIAL VIAL VIAL
     1     2     3
     L     M     C
GUINEA-PIG 1
     M     C     L
GUINEA-PIG 2
     C     L     M
GUINEA-PIG 3
```

On completion of all dissections, 2ml KH solution containing BSA and protease inhibitors was pipetted into fresh polypropylene vials. The tissues were then transferred to these vials in the order shown above, aerated and maintained at 37°C. After 30min had elapsed vial 1 received 40μl distilled water (containing 1%v/v DMSO) - the control for vial 2. Vial 2 received 4β-PDBu (10μM) in 40μl distilled water (containing 1%
DMSO). At this stage no additions to vial 3 were made. All vials were then left to aerate for 60 min at 37°C. After which, each vial was centrifuged (800gx2 min) (Gallenkamp, Labspin), the supernatant removed and placed into fresh polypropylene vials. The tissues were blotted and weighed. To vial 3, 4β-PDBu (10 μM) in 40 μl distilled water (containing 1% DMSO) was then added. The final DMSO concentration did not exceed 0.02% v/v. Samples were kept on ice and taken for assay.

2.3.1b VIP-immunoreactivity quantification

Vasoactive intestinal peptide (VIP) was quantified using an inhibition enzyme-linked immunosorbent assay (ELISA). For each incubation, the wells of a Dynatech polystyrene micro-ELISA plate (Dynatech, Laboratories, Inc., Alexandria, Va.) contained 100 μl of solution. The plate was coated with 0.1 μg/ml VIP in carbonate-bicarbonate buffer, pH 9.6, containing 0.2% sodium azide, by incubating for 18-24 h at 4°C. VIP standards were prepared using the same buffer as used for the samples. The coated ELISA plates were washed three times in phosphate buffered saline containing 0.05% Tween 20 (PBS-Tween), pH 7.4, to which 0.1% gelatin had been included to prevent non-specific binding. The plates were emptied by inversion. Sample or standard 50 μl aliquots were added to each well (each sample was assayed in triplicate) followed by 50 μl of antiserum raised in rabbits to natural porcine VIP (RIA UK. Ltd., Washington, UK.) diluted 1:75000 in the sample buffer. The plates were washed three times in PBS-Tween. Goat anti-rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase (Sigma) at a dilution of 1:500 in PBS-Tween containing 0.1% gelatin, was added to each well. The plates were then incubated in a humidity chamber for 2 h at 37°C. After which they were washed three times in PBS-Tween, and once in 0.1 M glycine containing 1 mM magnesium chloride and 1 mM zinc chloride, pH 10.4. p-Nitrophenyl phosphate (Sigma) (1 mg/ml in the above buffer) was added and left for 4 h at 20-22°C to develop colour. The absorbance was read in a Titertek multiscan automatic spectrophotometer at 405 nm. The standard absorbance readings were plotted against VIP concentration (0-10000 pg/well) on logarithmic graph paper to give a straight line, fitted by least squares regression analysis, from which the sample VIP values were obtained (see figure 8.1). Results were expressed as picomoles of VIP per gram wet weight of tissue.
2.3.2 Collection and concentration of the putative inhibitory non-adrenergic, non-cholinergic (NANC) peptide neurotransmitter

2.3.2a Tissue preparation and sample collection

The trachea was removed from four male Dunkin-Hartley guinea-pigs (200-400g). Each trachea was divided into approximately 10 equal segments and placed onto the lower electrode plate which was cemented into the base of a polypropylene vial (Hughes and Hughes, UK.) containing 2ml KH solution, to which the peptidase inhibitors, bacitracin (30mg/l = 1.86U/ml) and aprotinin (100μg/l = 1.2U/ml) had previously been added. Tissues were maintained at 37°C and gassed with a mixture of 95% O₂ and 5% CO₂. Tissues were then washed twice at 15min intervals in KH solution containing the protease inhibitors. After 10min re-equilibration had elapsed, a further 4min was allowed prior to removal of the bathing solution. This sample of bathing fluid is referred to as the "Prestimulus sample" and was placed into fresh polypropylene vials and stored frozen at -75°C until required. The tracheal tissues were then washed twice as previously explained, and after another period of 10min had elapsed, subjected to electrical field stimulation (EFS) for 4min using appropriate parameters prior to removal of another sample of bathing fluid. This second sample was also transferred to a polypropylene vial and stored at -75°C, and is referred to as the "stimulus" sample. EFS was delivered by two parallel platinum foil plate electrodes placed 10mm apart, above and below the tissues. The electrodes were connected and monitored as explained in section 2.2.3. Where the pharmacological stimulus, aconitine, was used in place of EFS, the 4min prestimulation and stimulation periods were extended to 10min and the prestimulation sample contained the solvent, dimethylsulphoxide (DMSO) at the appropriate concentration. At the end of the experiment tissues from each animal were placed into an oven (70°C) for 24h, removed periodically for weighing, and their dry weight recorded.

2.3.2b Concentration of samples of bathing fluid

After collection of samples from eight animals (4 animals × 2 experimental days) all samples were thawed and then kept on ice. Concentration was attempted using Sep-Pak C₁₈ cartridges (Waters, UK.). Two cartridges were used for each concentration procedure, one for "stimulus" samples
and the other for "prestimulus" samples. The cartridges were first "cleaned and wetted" by passing approximately 30ml absolute methanol (4°C) through each of the columns, followed by an equivalent volume of KH solution (4°C) in 5ml aliquots. At this stage the prestimulus samples could be passed slowly through the appropriately labelled cartridge and the effluent pooled, whereupon it was passed through the cartridge once more. This process of "double pass" was repeated for the other cartridge with the stimulus samples.

Finally, the samples were eluted off the cartridges, using analytical grade absolute methanol (3ml at 4°C) passed through the cartridges in 1ml aliquots (approx. 1ml/min). The samples were then dried off under nitrogen gas and taken up in 500μl deionised distilled water containing the anti-oxidant, dithiothreitol (1mM). Test and control (ie. stimulus and prestimulus) samples could then be investigated for effects on an appropriate assay tissue (see Chapter 8).

2.3.3 Superfusion-cascade assay

Tracheal tissue from one animal was prepared as for the putative NANC\textsubscript{i} peptide collection and concentration assay (see above). However, one tracheal segment taken from the cervical portion of the trachea, the portion of the tissue more sensitive to the effects of NANC\textsubscript{i} nerve stimulation (Coburn and Tomita, 1973), was set up for isometric recording. The remaining 8–9 tracheal segments were loosely packed into the modified syringe arrangement shown in figure 2.1b, and bathed in KH solution containing protease inhibitors, gassed with a mixture of 95% O\textsubscript{2} and 5% CO\textsubscript{2}, pH 7.4. The temperature of the syringe effluent was periodically monitored and adjusted to ensure all tissues were maintained at 37°C. The volume of the space in the syringe containing the tracheal segments was 1ml. The flow rate for the perfusing KH solution was controlled by a peristaltic pump (Gilson, Minipuls 2) and routinely set at 2ml/min, but could be varied using the flow rate calibration graph shown in figure 2.2. Preliminary studies demonstrated that using this flow rate the time delay from the packed tracheal segments, housed in the syringe, to the assay tissue was less than 6 seconds. Electrical field stimulation (EFS) was delivered by two parallel platinum foil plates (8x5mm) placed either side of the tissue (approx-
imately 12mm apart) in the syringe assembly (figure 2.1b). Electrodes were connected, and impulses monitored, as described in section 2.2.3.

Drugs could be delivered either by superfusion directly over the assay tissue (O.T.) or through the syringe arrangement (T.S.). Drugs were normally delivered to the tissues by replacing the superfusing KH solution with a KH solution containing an appropriate concentration of drug for two minutes, or until the response stabilised. Washing was achieved by replacing the drug-containing KH solution with fresh KH solution.

2.4 General points

In all experiments every effort was made to "time-match" control and test preparations, and to control for drug additions either by solvent control and/or volume readjustment, where the solvent was water. Drug additions were usually made using automatic pipettes (Gilson Pipetman), which were routinely calibrated. The volume added to the organ bath did not exceed 2%v/v of the final volume, and where the solvent was DMSO or ethanol the final concentration in the organ bath did not exceed 0.06%v/v, unless otherwise indicated. At this concentration no effect on the basal tone of any preparation was observed, nor was there any effect on the histamine-induced responsiveness.

In experiments where peptides were used, organ baths were coated prior to the experiment with the siliconising agent, dimethyldichlorosilane solution (BDH, UK.), in order to minimise adherence of the peptides to the glass. Peptides were dissolved in sterile water containing the anti-oxidant, dithiothreitol (1mM), and stored in aliquots at -20°C in polypropylene vials. All further manipulations of peptides were also carried out at 4°C in polypropylene vials.
Figure 2.1 (a) Platinum wire electrode assembly used for Electrical field stimulation (EFS) in isolated organ bath studies. (b) Modified syringe assembly used for superfusion-cascade experiments (see Chapter 8). Note that the syringe was placed into a water jacket (37°C, not shown).
Figure 2.2 Calibration graph used to obtain a known flow rate from the peristaltic pump (Gilson, Miniplus 2) used in superfusion cascade experiments (see Chapter 8). The line was fitted using least squares regression analysis.
2.5 Materials

The drugs used in this study were obtained from the following sources, and dissolved in the solvent shown. Where two solvents occur (e.g. DMSO, DDW), a stock solution (50mM) was made in the first, and all subsequent dilutions made in the second.

<table>
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<tr>
<th>COMPOUND</th>
<th>SOLVENT</th>
<th>SOURCE</th>
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</thead>
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<tr>
<td>A23187</td>
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<td>Calbiochem</td>
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<td>Aconitine</td>
<td>DMSO</td>
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</tr>
<tr>
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<td>DDW</td>
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<tr>
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<td>DDW</td>
<td>Koch-Light</td>
</tr>
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<td>Bacitracin</td>
<td>DDW</td>
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<td>Sigma, UK.</td>
</tr>
<tr>
<td>Cadmium chloride</td>
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</tr>
<tr>
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<td>Cimetidine</td>
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<td>Gift from Dr.C.Hoyle, Anatomy Dept, UCL.</td>
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<tr>
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<tr>
<td>Isoprenaline sulphate</td>
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<td>Wellcome</td>
</tr>
<tr>
<td>Chemical Name</td>
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<td>Source 2</td>
</tr>
<tr>
<td>---------------------------------------------------</td>
<td>---------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>MDL 12330A</td>
<td>EtOH</td>
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</tr>
<tr>
<td>Mepyramine maleate</td>
<td>DDW</td>
<td>May &amp; Baker, UK.</td>
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<td>Methylene blue</td>
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<tr>
<td>L-N^G-monomethylarginine (L-NMMA)</td>
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<td></td>
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<tr>
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<tr>
<td></td>
<td></td>
<td>Dr.J.Rivier, Salk Inst.,</td>
</tr>
<tr>
<td></td>
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<td>Veratridine</td>
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</tr>
</tbody>
</table>

All reagents were of analytical grade.
CHAPTER THREE

A PHARMACOLOGICAL EVALUATION OF THE GUINEA-PIG LUNG
PARENCHYMAL STRIP AS A MODEL OF PERIPHERAL AIRWAY REACTIVITY:

Comparison of lung strip and trachea
Summary

1. Histamine evoked an immediate concentration-dependent contraction in both the lung parenchyma and trachea which was observed throughout the entire project in over 250 animals (eight tissues from each animal). In both respiratory tract tissues this response was mepyramine-sensitive and so was considered to be mediated primarily by $H_1$-receptors. However, in some parenchymal strip preparations ($n=2$) a cimetidine-sensitive relaxation occurred to histamine, presumably mediated by $H_2$-receptors. These preparations were excluded from the study.

2. Carbachol also caused an immediate concentration-dependent contraction in both respiratory tract preparations. This effect was blocked by the muscarinic receptor antagonist, atropine.

3. Noradrenaline produced an immediate concentration-dependent relaxation in both the lung strip and trachea, which was propranolol-sensitive and so considered to be mediated primarily by $\beta$-adrenoceptors.

4. In the presence of cocaine (neuronal uptake inhibitor) noradrenaline was more potent in central airways (trachea) than in the lung periphery (parenchyma). Neuronal uptake was therefore considered to be more important for the removal of exogenous noradrenaline in the trachea than lung parenchyma.

5. Relaxation of the trachea to noradrenaline appeared to involve an opposing contractile component, which was sensitive to phentolamine and thus possibly mediated by $\alpha$-adrenoceptors, but which was not observed in the lung periphery. However, inhibition of uptake (by phentolamine) might also explain this result.

6. The results are discussed in light of the reported localisation of receptors and nerves within the lung, and suggest that the pulmonary vasculature does not appear to be involved in relaxation of guinea-pig lung parenchyma to noradrenaline. However, they do not resolve the controversy regarding the use of this preparation as a model of peripheral airway pharmacology. For this reason it was decided to study the response of both the trachea and lung parenchyma in parallel.
3.1 Introduction and background

Originally, the techniques available for the investigation of direct drug effects on smooth muscle *in vitro* were open (Akcasu, 1959) and closed (Castillo and DeBeer, 1947) ring tracheal chain preparations, and changes in intraluminal pressure recorded from whole trachea (Farmer and Coleman, 1970). These were not considered to be ideally representative of the situation observed in the intact lung *in vivo*. Measurements of lung compliance or airways resistance suggested that the principal site of action of certain drugs and immunologically-released mediators, in hyper-reactive airway syndromes, was at the level of the fine peripheral airways (Drazen and Austen, 1974). Direct evidence for such an action required a different sort of *in vitro* preparation of the airways. It was proposed that the lung parenchymal strip might be a suitable model whereby the effects of drugs on the peripheral airways could be assessed *in vitro* (Lulich *et al.*, 1976).

Mammalian lung parenchyma is a morphologically complex tissue, which may contain as many as forty different cell types. Some of these cells are directly involved in force-generation such as bronchial wall airway smooth muscle, microvascular smooth muscle, alveolar duct smooth muscle and actin-containing interstitial smooth muscle cells (myofibroblasts). Other cell types, not directly involved in force-generation, include a wide variety of inflammatory cells such as macrophages, neutrophils, eosinophils, mast cells, platelets, lymphocytes etc., all of which can release mediators, and some of which can generate toxic oxygen free-radicals, and in turn all are capable of altering pulmonary mechanics. Since the lung parenchymal strip contained all these different cell types, it was considered that it would be useful in assessing those drugs capable of modifying immunologically-induced mediator release, and would be more representative of the *in vivo* situation in asthma.

Lung parenchymal strips from a variety of species including man (Hanna *et al.*, 1981), guinea-pig (Chand and DeRoth, 1979), dog (Chand *et al.*, 1979) and rat (Burns and Doe, 1978) have been studied. Recently however, due to the complexity of this tissue, its status as a model of peripheral airway pharmacology has been challenged. Controversy exists as to the relative contribution of the major force-generating cell types to the final response on agonist stimulation. Kapanci *et al.* (1974), based
on observations in rat, monkey and human tissue, have suggested that the alveolar interstitial cells are the primary target for mediators and neurotransmitters since 50% of these alveolar cells contained bundles of fibrils (30-80Å in diameter). Furthermore, immunofluorescent techniques have demonstrated binding of sera containing anti-actin antibodies to alveolar interstitial cells in rat lung. However, it was not established whether these cells can contract or relax to specific stimuli (Goldie et al., 1982). Drazen and Schneider (1978) suggested that these alveolar interstitial cells and/or alveolar duct smooth muscle, but not the vascular or bronchial smooth muscle, were responsible for the contractions in guinea-pig lung parenchymal strip produced by histamine and carbachol. The basis for this conclusion was that the responsiveness of the tissue to histamine and carbachol was similar in ultra-thin lung strips shown to contain few or no conducting airways or vasculature. However, it should be kept in mind that the responsiveness of these components may be of relatively minor influence in lung strips of the thickness more usually used in experiments. Later in 1981, Clayton et al. studying the effect of the α-adrenoceptor antagonist, phentolamine, in transmurally stimulated reserpine- and non-reserpine-affected guinea-pig lung parenchyma, suggested that vascular smooth muscle contributed to the tissue response, since it is the vasculature which possesses α-adrenoceptors and not bronchial wall airway smooth muscle (O'Donnell et al., 1978). This assumption is of major importance to the study described in this chapter.

In view of the uncertainty, Goldie et al. (1984) suggested that the lung parenchymal strip was not an adequate model of peripheral airway pharmacology and its use as such should be discontinued. However, the recording of various parameters in vivo such as dynamic compliance (indicative of small airway reactivity) by changes in both vascular and airway smooth muscle, indicates that the net contractility of the parenchymal strip is mostly due to these two smooth muscle types (Goldie et al., 1982). This, together with good correlation between in vitro and in vivo results coupled with ease and simplicity of preparation, has led to its continued use (Bertram et al., 1983).

The experiments reported in this chapter aimed to resolve some of the controversy surrounding the use of this preparation as a model of peripheral airway pharmacology, strengthening the on-going argument
for the non-involvement of the pulmonary microvasculature in the response of the guinea-pig parenchymal lung strip to various broncho-active agents. This was undertaken by (i) comparing the behaviour of agents known to have differential effects on airway and vascular smooth muscle, and (ii) assessing the relative contribution of α- and β-adrenoceptors and functional neuronal uptake sites (coupled with their known anatomical distributions) to the relaxant response of noradrenaline in both central and peripheral lung tissues. The latter lends itself to an indirect assessment of the extent to which the pulmonary vasculature elements contribute to the response of the lung strip.

3.2 Experimental Protocol

Four tracheal and four lung parenchymal strips were removed from each animal and prepared for isotonic recording (see previous chapter), and were studied in parallel. All tissues were exposed initially to histamine (see section 2.2.5), the maximum contractile response determined, and then returned to baseline by washing with KH solution. After a re-equilibration period (40 min), paired lung strip and tracheal test tissues were each incubated for 30 min with either cocaine, propranolol or phentolamine which were present throughout the experiment. The remaining two tissues, one lung strip and one tracheal strip, served as controls receiving a control volume of solvent. Tissues were then precontracted with histamine prior to administering increasing concentrations of noradrenaline cumulatively. This was done only once for each tissue. Relaxation to noradrenaline was compared to the maximum relaxation achievable by papaverine (100 μM) at the end of the experiment.
3.3 Results

3.3.1a Histamine: comparison in lung parenchyma and trachea

Histamine caused an immediate concentration-dependent contractile response in the guinea-pig lung strip, which was maximum at 10μM. The EC50 was 1±0.09μM (n=4), in agreement with observations made by Drazen and Schneider (1978). Histamine also evoked concentration-dependent contraction in the tracheal strip (EC50 9.7±0.2μM, n=4), which was maximal at 100μM. Throughout this project tissues were tested with histamine and virtually all contracted. However, in a small number (n=2) of lung parenchymal strips, histamine (1μM) caused relaxation which was readily reversed by the H2-receptor antagonist, cimetidine (10μM). Any tissue displaying this relaxant response to histamine at basal tone, in the absence of an H2-receptor antagonist, was immediately excluded from the study. The contractile response to histamine, in both tissues, was abolished by the H1-receptor antagonist, mepyramine (1μM).

3.3.1b Carbachol: comparison in lung parenchyma and trachea

Like histamine, carbachol induced an immediate contractile response in both tissues. The EC50 was 0.4±0.08μM (n=11) for the lung parenchyma, and 0.1±0.03μM (n=3) in the trachea. The maximum response of both tissues to carbachol occurred at 100μM and, in the lung parenchyma, reached 88.6±3.9% (n=11) of the maximum contractile response to histamine. The effect of carbachol in both tissues was blocked by the muscarinic receptor antagonist, atropine (1μM).

3.3.2 Noradrenaline

3.3.2a Comparison in the lung parenchyma and trachea

Guinea-pig lung parenchymal and tracheal strips were initially precontracted with 3 and 30μM histamine. These were submaximal concentrations which evoked a sustained contracture reaching 80±1.4% and 78±1.7% of the maximum histamine response respectively. Over the period required to complete the noradrenaline cumulative concentration-response curve (approx. 20–25min), the spontaneous waning of the sustained histamine (3μM) response was 11.3±0.2% in three lung parenchymal strips taken from separate animals. While no such waning of the sustained histamine (30μM) response was observed in the trachea over the same time period. Initially only qualitative data was sought
and so the spontaneous reduction in precontracting tone of the lung strip was considered acceptable.

Cumulative additions of noradrenaline (10nM-100μM) evoked concentration dependent relaxation in the lung parenchymal and tracheal strip (figure 3.1). The extent of the noradrenaline-induced relaxation, determined by the maximum relaxation to papaverine (100μM), was 94.1±2.6% (n=3) in the trachea and 49.7±4.4% (n=3) in the lung parenchymal strip (see also figure 3.1).

3.3.2b Effect of cocaine, propranolol and phentolamine on the relaxation of lung parenchyma and trachea to noradrenaline

The results are shown in figure 3.1 and table 3.2. In both tissues propranolol, a non-selective β-adrenoceptor antagonist, and phentolamine, a non-selective α-adrenoceptor antagonist, were used to assess the contribution of the α- and β-adrenoceptors to the relaxation produced by noradrenaline. Cocaine was used to assess the contribution of neuronal uptake to the removal of exogenous noradrenaline. In the trachea, both cocaine (10μM) and phentolamine (3μM) caused a marked parallel leftward shift of the noradrenaline concentration-response curve, without any change in its maximum (see figure 3.1). However, no such shift was observed for the lung parenchyma, although phentolamine (3μM) appeared to steepen the concentration-response relationship to noradrenaline. Propranolol (0.03μM) caused a marked parallel rightward shift to noradrenaline in both the tracheal and lung parenchymal strip.

In the trachea statistically significant changes in the mean IC₅₀ values for relaxation induced by noradrenaline were obtained to each of the inhibitors, while in the lung strip only propranolol significantly altered the mean IC₅₀ value. Table 3.2 summarises the changes in mean IC₅₀ values for relaxation produced by noradrenaline in both respiratory tract preparations, by each of the three inhibitors.
Figure 3.1. Results of experiments showing the effect of cocaine (10μM) (●), phentolamine (3μM) (△) and propranolol (0.03μM) (▲) on relaxation produced by noradrenaline (O) in (a) guinea-pig tracheal and (b) lung parenchymal strip preparations. All tissues were precontracted with histamine. Relaxation is expressed as a percentage of the maximum relaxation induced by papaverine (100μM). Points represent mean ± s.e.mean for n=3-4. Isotonic recording.
**Results OBTAINED with:**

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<th>Mediator</th>
<th>AIRWAY SMOOTH MUSCLE (eg. trachea)</th>
<th>COMPOUNDED SMOOTH MUSCLE TISSUE (eg. lung strip)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine</td>
<td>Contraction</td>
<td>Contraction</td>
</tr>
<tr>
<td>Carbachol</td>
<td>Contraction</td>
<td>Contraction</td>
</tr>
<tr>
<td>Noradren.</td>
<td>Relaxation</td>
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**Results EXPECTED with:**

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<tbody>
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<td></td>
<td>(eg. arteriole/venule)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IC₅₀ (µM)</th>
<th>LUNG STRIP</th>
<th>TRACHEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA Control</td>
<td>0.48±0.09</td>
<td>2.9±0.2</td>
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</tr>
<tr>
<td>NA + Phentolamine (3µM)</td>
<td>1.1±0.35</td>
<td>0.86±0.07  ***</td>
<td></td>
</tr>
<tr>
<td>NA + Propranolol (0.03µM)</td>
<td>14±4 *</td>
<td>9.3±0.36   ***</td>
<td></td>
</tr>
<tr>
<td>NA + Cocaine (10µM)</td>
<td>0.43±0.03</td>
<td>0.21±0.02  ***</td>
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</tr>
</tbody>
</table>

**Table 3.1.** The results obtained to some selected pharmacological agents on tracheal and lung parenchymal strip preparations compared with the results reported to occur in pulmonary vascular smooth muscle.

**Table 3.2.** Comparison of the effects of phentolamine, propranolol and cocaine pretreatment on relaxation to noradrenaline (NA) in the guinea-pig lung parenchyma and trachea. Tissues were exposed to the inhibitors for 30min prior to precontraction with histamine and the construction of a single cumulative concentration response curve to noradrenaline. * p<0.05, *** p<0.001 compared to the response in the absence of inhibitors using Student’s t-test for unpaired observations. Figures represent mean ± s.e.mean for n=3–4. Isotonic recording.
3.4 Discussion

As previously indicated (see section 2.2.2) the response of the lung parenchyma was recorded isotonically, since it is too delicate for isometric recording. To facilitate direct comparison, tracheal responses were also recorded isotonically. Quantification of responses was achieved using a standard by which all subsequent responses could be clearly expressed. For this purpose the maximum response to histamine was chosen to represent all subsequent contractile responses, since it gave consistent, reproducible and readily reversible contractions in both tissues. Furthermore, the use of a standard meant that the assessment of responsiveness was little influenced by the amount or orientation of contractile elements within an individual preparation, but rather by differential activity of the contractile elements to pharmacological agents (Drazen and Schneider, 1978). For this reason papaverine (100μM) was also chosen as a standard by which all relaxant responses could be expressed.

The contractile response to histamine and carbachol obtained in both the guinea-pig lung parenchyma and trachea was consistent with the findings of Drazen and Schneider (1978), who demonstrated that there was little difference in the relative responsiveness to these agents in ultra-thin parenchymal strips of varying amounts of identifiable conducting airways and microvasculature, determined by histological sectioning. They reported that despite varying densities of conducting airways and microvasculature, all parenchymal strips studied possessed large quantities of alveolar duct smooth muscle, which is abundant in guinea-pig lung parenchyma (Miller, 1921). In view of the similarity of responsiveness to pharmacological agents in lung strips of varying thickness, it seemed reasonable to suggest that alveolar duct smooth muscle or other alveolar contractile elements, such as the pulmonary alveolar septae interstitial cells, were mainly responsible for contractions seen to histamine and carbachol. However, whether this is also the case in lung strip of more usual thickness is not certain.

The relaxation of the lung parenchymal strip to large concentrations of noradrenaline, described in this study and by others also in guinea-pig (Chand and DeRoth, 1979), and in cat (Lulich et al., 1976) is interpreted as firm evidence for the non-involvement of the pulmonary micro-
vasculature in the mediation of responses to spasmogenic agents (Lulich et al., 1976). However, noradrenaline in lung strips from a variety of other species including horse and rabbit (Chand and DeRoth, 1979) caused contractile responses. This may, in part, represent an overwhelming contribution of the pulmonary microvasculature and/or contractile interstitial cells in the pulmonary alveolar septae of these species (Kapanci et al., 1974). Interspecies differences do not appear to be resolvable on the basis that lung strips of differing size from animals of different sizes will contain different amounts and proportions of various contractile components. In fact, lung strips from animals of quite different sizes, eg. guinea-pig, rabbit, cat and dog, respond to a wide variety of agonists in qualitatively similar ways (see Goldie et al., 1984). Further, in lung strips which respond to 5-hydroxytryptamine (5-HT), the determinant appears to be the ratio of vascular to bronchial smooth muscle. Those strips which contracted to 5-HT were shown to possess over four times more vascular than airway smooth muscle. However, in strips which relaxed to 5-HT, there was only twice as much vascular to bronchial smooth muscle. Relative differences in the parenchyma content did not appear to influence the qualitative response to 5-HT (see review by Goldie et al., 1984).

O'Donnell et al. (1978) have shown by fluorescence histochemistry that there was a progressive decrease in the density of adrenergic innervation to airway smooth muscle, from the laryngeal end of the trachea, which was densely innervated, to the terminal bronchioles, which contained only occasional fibres. This was despite the existence of a dense adrenergic innervation to pulmonary blood vessels in the guinea-pig lung (Cech, 1964; Hebb, 1964). These results have been confirmed using a more discriminative technique, utilising an antibody to the noradrenaline synthesising enzyme, dopamine β-hydroxylase (Sheppard et al., 1983).

Comparison of the IC50 values for relaxation to noradrenaline in both tracheal and lung parenchymal strip preparations in the presence of cocaine, suggests a decrease in responsiveness to noradrenaline in going from central airways to the lung periphery. This presumably reflects a reduction in the number of functional receptors stimulated by noradrenaline, possibly correlating to a reduction in adrenergic innervation (O'Donnell et al., 1978). This is also reflected in the effect
of cocaine, where potentiation of relaxation to noradrenaline by cocaine was observed only in the trachea. This is also suggestive of neuronal uptake sites being in close proximity to the β-adrenoceptors mediating relaxation (see later). Clearly, in the trachea neuronal uptake is still an important mechanism for the removal of exogenous noradrenaline. This does not appear to be the case in the lung periphery, since cocaine had little effect on the noradrenaline-induced relaxation of parenchymal strip. However, in the lung periphery, noradrenaline removal may be largely dependent upon other processes such as extraneuronal sites and/or neuronal uptake adjacent to the vasculature which may play only a minor role in the relaxant response to noradrenaline. It is worth noting that Zaagsma et al. (1983) did not observe potentiation of the response to noradrenaline in the guinea-pig lung parenchymal strip after inhibition of extraneuronal uptake with cortisone, lending further support for the non-involvement of the microvasculature in this response.

Radioligand binding studies and more recently autoradiographic techniques, have demonstrated a high density of β-adrenoceptors associated with airway smooth muscle from the trachea down to the terminal bronchioles of the lung periphery (Rugg et al., 1978; Barnes et al., 1982). Further, autoradiographic studies have also shown that greater than 90% of all β-adrenoceptors in lung are localised to the alveolar walls (Barnes et al., 1982). The marked right-ward shift of the noradrenaline concentration-response curve in both tissues, in the presence of the non-selective β-adrenoceptor antagonist, propranolol, clearly demonstrates that the relaxation of the lung parenchyma and trachea is primarily mediated by β-adrenoceptors. Zaagsma et al. (1983) using selective β₁- and β₂-adrenoceptor antagonists, demonstrated that the noradrenaline-induced relaxation of guinea pig trachea was mediated primarily by a mixed β₁- and β₂-adrenoceptor population, while the relaxation in the lung parenchyma was mediated principally by an homologous β₂-adrenoceptor population, of which most are localised to the alveolar walls (see above). In dog tracheal smooth muscle, while β₂-adrenoceptors predominate, 20% of β-adrenoceptors are of the β₁-subtype (Barnes et al., 1983). Functional studies of the same tissue in vitro show that relaxation to exogenous β-agonists is mediated by β₂-receptors, but relaxation to sympathetic nerve stimulation is mediated by β₁-adrenoceptors. These findings are consistent with the hypothesis
that $\beta_1$-adrenoceptors are regulated by sympathetic nerves (neuronal $\beta$-receptor), whereas $\beta_2$-receptors are regulated by circulating adrenaline (hormonal $\beta$-receptors). Further evidence for this idea is provided in the guinea-pig trachea, which has a sparse sympathetic innervation, where binding studies suggest that only 15% (approx.) of $\beta$-adrenoceptors are of the $\beta_1$-subtype (Carswell and Nahorski, 1983). Further, in the cat trachea, which has a dense sympathetic nerve supply, relaxation to $\beta$-agonists is mediated predominantly by $\beta_1$-receptors, whereas in the parenchymal lung strip, which contains bronchioles devoid of sympathetic nerves, responses are mediated by $\beta_2$-receptors (Lulich et al., 1976). A similar situation is reported in guinea-pig (see above, Zaagsma et al., 1983).

The potentiation of the response to noradrenaline in the trachea by the non-selective $\alpha$-adrenoceptor antagonist, phentolamine, may represent an "unmasking" of a functionally antagonistic contractile component to the noradrenaline response, mediated by $\alpha$-adrenoceptors. In the parenchymal strip preparation, phentolamine caused a small reduction in the response to low concentrations of noradrenaline ($< 0.3\mu M$) which did not achieve statistical significance, while at higher concentrations of noradrenaline phentolamine was without effect. The reason for the lack of effect in the parenchyma, when considering the high density of $\alpha$-adrenoceptors associated with the pulmonary vasculature (Phillips et al., 1985), may reflect the proposed main action of noradrenaline to be on the airway smooth muscle, facilitating the argument for the non-involvement of the pulmonary vasculature in the responsiveness of the parenchyma to certain spasmogens and relaxants. A similar effect with phentolamine has been reported in the cat lung strip (Lulich et al., 1976). However, this cannot of course completely eliminate some possible contribution of the pulmonary vasculature to the final response, particularly when considering the occasional cimetidine-sensitive relaxant effect of histamine in the lung parenchymal strip, which was observed in this study. This is presumed to involve $H_2$-receptors, which have been identified in the guinea-pig parenchyma by receptor binding using $[^3H]$tiotidine (Foreman et al., 1985). An alternative explanation for the effects seen with phentolamine in both the trachea and lung parenchyma could be inhibition of exogenous noradrenaline uptake, which is considered to be more important in the upper airways than in the periphery (Zaagsma et al., 1983), and should be borne in mind when
considering the data for phentolamine. However, it is unlikely to occur as a result of conventional re-uptake mechanisms, since cocaine and cortisone are reported to be ineffective in potentiating relaxation to noradrenaline in lung parenchyma (Zaagsma et al., 1983).

In summary, the evidence thus far suggests that relaxation of guinea-pig lung parenchyma to noradrenaline is mediated primarily by β-adrenoceptors, of which most are localised to the alveolar walls of the terminal bronchi and are not associated with adrenergic nerve fibres at the vasculature. Further, α-adrenoceptors, presumably localised to vascular tissue (Phillips et al., 1985), do not appear to play a major role in the relaxation of the lung strip to noradrenaline. The evidence appears to be in agreement with the statement made by Chand and DeRoth (1979), that noradrenaline-induced relaxation of the guinea-pig parenchymal strip constitutes reasonably strong evidence for the non-involvement of the pulmonary vasculature - indicating that the response of the lung parenchyma could be due largely to other force-generating elements such as bronchial smooth muscle. However, when considering the approach used and the results obtained in this study, it should be borne in mind that it may be an over-simplification to suggest that responses of central airways and vascular smooth muscle to agonists will reflect their effects in the peripheral airways and blood vessels within the parenchymal strip. Be that as it may, until the development of techniques which allow the direct investigation of responses of individual small airways and blood vessels, as well as alveolar septae dissected from lung parenchyma, the true detailed pharmacological properties of the lung strip will remain uncertain.

The results obtained could not confirm adequately the possibility that the response of the lung strip was due predominantly to bronchial smooth muscle elements. Consequently, it was decided to study the response of both the trachea and lung parenchyma in parallel.
CHAPTER FOUR

A PRELIMINARY INVESTIGATION OF THE MECHANISM
BY WHICH PHORBOL ESTERS PRODUCE RELAXATION IN
THE GUINEA-PIG TRACHEA
Summary

1. Phorbol esters evoked qualitatively different responses in central and peripheral lung tissues excised from guinea-pig. Contraction occurred in lung parenchyma, while relaxation occurred in the trachea. However, under depolarising conditions, phorbol esters caused contraction in the trachea which was prevented by organic blockers of voltage-sensitive Ca$^{2+}$ channels. The basis for the relaxant effect of phorbol esters in the guinea-pig trachea was investigated.

2. In the trachea the relaxant effect of phorbol esters appeared to be associated primarily with PKC activation. This was demonstrated by the lack of relaxant activity seen with the 4α-isomer of PDBu, the relative potencies of the active 4β-phorbol esters and the ability of cell permeant DAG analogues to mimic the effect of phorbol ester stimulation in the trachea and lung parenchyma.

3. The relaxant effect of phorbol esters in the trachea did not appear to result from down-regulation of responses to histamine, carbachol or contractile cyclo-oxygenase products. In contrast, tracheal relaxation to isoprenaline was inhibited by 4β-PDBu. 4α-PDBu had no effect.

4. The possibility that the relaxant effect of phorbol esters was an indirect one involving release of mediators was tested. However, relaxation was unaffected by a wide variety of enzyme inhibitors/mediator antagonists. Luminal airway epithelium removal also failed to prevent the relaxation of the trachea to 4β-PDBu.

5. A limited interaction between 4β-PDBu and agents which elevate cyclic AMP was noted in the trachea.

6. The possibility that relaxation of the trachea to phorbol esters could result from PKC-mediated release of a relaxant neurotransmitter(s), possibly from inhibitory non-adrenergic non-cholinergic (NANC) nerves, was raised for later testing.
4.1 Introduction and background

In many smooth muscle preparations, phorbol esters cause a sustained contraction (see Danthuluri and Deth, 1984; Dale and Obianime, 1985, 1987; Obianime et al., 1988; Forder et al., 1985). However, in a small number of other smooth muscle preparations, phorbol esters not only cause relaxation, but reverse also receptor-mediated contraction. In those tissues which relax to phorbol esters - guinea-pig ileum, rat uterus and guinea-pig trachea (Baraban et al., 1985; Menkes et al., 1986; Huang et al., 1987; Sasaguri and Watson, 1989, 1990) - the relaxant response has been attributed to a direct effect on the smooth muscle. However, it seemed unlikely that one preparation of guinea-pig respiratory tract smooth muscle should respond to phorbol esters by an intrinsically different mechanism from that of another taken from further down the bronchial tree, but which has an identical embryological derivation.

In this chapter the following question was addressed: Was the unexpected relaxation due to the tracheal smooth muscle itself responding directly to phorbol esters in an intrinsically different manner from that of the lung parenchyma, or was it due to an indirect effect involving the release of mediators which might cause relaxation either directly or indirectly?

The initial approach was to reassess the observations of Menkes et al. (1986), who first reported the relaxant effect of phorbol ester stimulation in guinea-pig trachea, and to evaluate their conclusions. This was followed by experiments aimed at determining whether the relaxant response to phorbol esters occurred indirectly involving the release of a relaxant mediator. To do this, a range of selective receptor antagonists and enzyme inhibitors was examined on the relaxation of the guinea-pig trachea to the phorbol ester, 4β-phorbol 12,13-dibutyrate (4β-PDBu).
4.2 Experimental protocol

In experiments in which the relative potency of different phorbol esters was investigated, four tracheal strips were removed from each animal and studied in parallel.

The protocol used for experiments described in section 4.3.3 is shown diagramatically in figure 4.5. For experiments described in section 4.3.4, four to six tracheal strips were removed from each animal and incubated with each inhibitor for 30min prior to the construction of a single cumulative concentration-response curve to 4β-PDBu. One tissue was not exposed to the drug and served as a control to which an appropriate concentration of solvent was added. Where cyclo-oxygenase inhibitors were investigated, tissues were precontracted with histamine to restore tone.

In experiments in which the role of the luminal epithelium was under scrutiny, paired rubbed and unrubbed tissues were first exposed to increasing concentrations of histamine in a cumulative fashion. After re-equilibration (40min) tracheal tissues were precontracted with an equieffective concentration of histamine (EC₆₅ intact 10μM; denuded 3μM) prior to exposure to 4β-PDBu. At the end of the experiment papaverine (100μM), the relaxation of which is reported not to be affected by epithelium removal in guinea-pig (Farmer et al., 1986), was added to the organ bath to determine the maximum relaxation of the tissues. All tissues were examined histologically as described in section 2.2.1.
4.3 Results
4.3.1 Comparison of the response of the guinea-pig trachea and lung parenchyma to phorbol esters

The phorbol ester, 4β-phorbol 12,13-dibutyrate (4β-PDBu) produced contraction of lung parenchymal strip preparations (confirming the reports of Dale and Obianime, 1985, 1987), but relaxation of tracheal strip preparations (confirming the reports of Menkes et al., 1986). This differential effect occurred at both intrinsic (figures 4.1a and b) and agonist-induced tone (figure 4.2).

Due to the relatively prompt onset of action of 4β-PDBu (2-3min) observed in both tissues, peaking at 15-30min, it was feasible to construct a cumulative concentration-response curve. This is illustrated in figure 4.1, in which the active phorbol esters 4β-PDBu and 4β-phorbol 12,13-diacetate (4β-PDA) are compared in the trachea with the inactive ester, 4α-PDBu (figures 4.1b, c and d). The mean cumulative responses to 4β-PDBu, in the lung parenchyma and trachea, are shown in figure 4.3a.

As indicated in figure 4.1 this relaxant effect of phorbol esters was not restricted to just 4β-PDBu. A similar relaxant response was observed to 4β-PDA and 4β-phorbol 12-myristate 13-acetate (4β-PMA). Over the concentration range investigated (1nM-10μM) with the phorbol esters, the maximum relaxation to 4β-PDBu occurred at 3μM and was 80±6% (mean ± s.e.mean, n=3) of the maximum response to papaverine (100μM), while the maximum relaxation to 4α-PDBu was only 18±5.9% (n=3). The maximum response to 4β-PDA also occurred at 3μM and was similar (70±7.5%, n=3) to the maximum relaxant response to 4β-PDBu, while 4β-PMA did not achieve a maximum at 10μM - the highest concentration investigated. The relative potency of each of the active phorbol esters was 4β-PDBu > 4β-PDA > 4β-PMA. The mean IC₅₀ values for relaxation in the trachea evoked by the phorbol esters are given in table 4.1.

Results obtained from a single experiment in which parenchymal and tracheal strips were incubated with the DAG analogues, OAG (300μM) and diC₈ (300μM) demonstrated small transient (10min, peaking at approximately 3-6min) responses which were immediate in onset and consistent with phorbol ester stimulation in both tissues, i.e. contraction in
parenchyma (OAG 10.8% of histamine maximum contraction; diC<sub>a</sub> 4.2%) and relaxation in the trachea (OAG 21.6% of papaverine maximum relaxation; diC<sub>a</sub> 7.2%). In the same experiment 4β-PDBu (1μM) produced relaxation (90%) in the trachea, and contraction in the lung parenchyma (117%). However, lack of potency and solvent limitations precluded further investigation of these analogues.

4.3.2 Effect of depolarising agents and of a calcium ionophore on tracheal relaxation to 4β-PDBu

In the presence of KCl (60mM), ouabain (10μM), A23187 (3μM) or under conditions of reduced bathing temperature (16°C, see also section 7.3.6b), the relaxation of the trachea to 4β-PDBu was converted to contraction (figure 4.4). This effect with KCl confirmed results reported by Menkes et al. (1986), while the effect with ouabain and with reduced bathing temperature confirmed reports by Huang et al. (1987). Under each of these conditions plasma membrane depolarisation and influx of Ca<sup>2+</sup> through voltage-dependent Ca<sup>2+</sup> channels is believed to occur, since the contraction was prevented by organic Ca<sup>2+</sup> channel blockers such as diltiazem and nifedipine (Menkes et al., 1986; Huang et al., 1987). In addition, verapamil (10μM) was also effective in preventing phorbol ester-induced contraction of the guinea-pig trachea.

4.3.3 Is the relaxation to 4β-PDBu due to PKC-mediated inhibition of the receptor-linked signal transduction mechanism for endogenous or exogenous spasmogens?

Menkes et al. (1986) had suggested that the relaxant response of the trachea to phorbol ester stimulation involved protein kinase C-mediated inhibition of the stimulus-activation coupling mechanisms responsible for agonist-induced contraction, and for endogenous spasmogens responsible for maintaining basal tone. This hypothesis was investigated by testing the effect of phorbol ester stimulation in the trachea on the subsequent histamine- and carbachol-induced contractions, and also on the relaxation to isoprenaline. The results are presented in figures 4.5, 4.6, 4.7 and table 4.2.
In all experiments 4β-PDBu (1μM) produced an immediate and sustained relaxation from intrinsic tone which was maximal and amounted to 89±2.2% (n=5) of the maximum relaxation to papaverine (100μM). At this concentration, 4β-PDBu was without effect on the histamine cumulative concentration-response curve elicited soon after (15min) addition of the phorbol ester (figures 4.5b, 4.5d, 4.6a) or after a more prolonged exposure (85min) (figure 4.7b). Likewise, the response to carbachol was unchanged (figure 4.7c). Similarly, no effect on the response to histamine was seen with 4α-PDBu (1μM), the inactive isomer (figure 4.5c, 4.5e, 4.6b). In contrast, however, 4β-PDBu (1μM) caused inhibition of relaxation to isoprenaline, demonstrated as a right-ward shift of the concentration-response curve (figure 4.5b, 4.6c); 4α-PDBu was without effect (figure 4.5c, 4.6d). A significant increase in the mean IC_{50} value for relaxation to isoprenaline was obtained with 4β-PDBu, while 4α-PDBu was again without effect (table 4.2).

4.3.4 Does the tracheal relaxation to 4β-PDBu involve the release of mediators?

The results presented in this section (figures 4.8-4.11) represent a detailed pharmacological study in which the relevant specific mediator antagonists and enzyme inhibitors were employed to assess the possible contribution of a variety of mediators to the relaxation of the trachea evoked by 4β-PDBu.

Pretreatment (30min) of the trachea with the following receptor antagonists/ enzyme inhibitors failed to prevent relaxation to 4β-PDBu. For propranolol (10μM), guanethidine (10μM), tetrodotoxin (1μM) and naloxone (10μM), see figure 4.8; for aspirin (30μM), indomethacin (3μM), cimetidine (10μM) and mepyramine (1μM), see figure 4.9; and for aminophylline (10μM) and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 3μM) see figure 4.10. In addition, disruption of the luminal airway epithelium (see section 2.2.1) failed to prevent the relaxation to 4β-PDBu in the trachea (figure 4.10a). However, removal of airway epithelium was able to potentiate the contractile response to histamine (figure 4.10b). This was demonstrated as a left-ward shift of the concentration-response curve in which there was no change in the maximum extent of contraction, confirming observations by Hay et al. (1986). The mean EC_{50} value for contraction to histamine was 4.2±0.86μM in intact
preparations and differed significantly (p<0.05, unpaired t-test) from the value, 1.7±0.39μM, in denuded preparations.

In a small number of guinea-pigs, control tracheal strips responded to 4β-PDBu with an initial concentration-dependent contraction, before the more usual relaxation. This can best be seen in figure 4.9a and b. However, its occurrence was too infrequent to facilitate a systematic study of the mechanism involved.

4.3.5 Interaction of 4β-PDBu with agents said to increase cyclic AMP in the trachea

The results are shown in figure 4.12. In preliminary experiments on the trachea, in which a near maximum relaxant concentration (see figure 4.5 and 4.6) of isoprenaline (10nM) was used to rapidly reverse the initial histamine standard contractile response, it was noted that the subsequent relaxation induced by 4β-PDBu was potentiated. This was demonstrated as a left-ward shift of the concentration-response curve and also as an increase in the maximum extent of relaxation. In tissues exposed to isoprenaline, the maximum extent of relaxation to 4β-PDBu was -239±51% (n=3) of the maximum contractile response to histamine. However, in those tracheal strips which were not exposed to isoprenaline after responding to the histamine standards, the maximum extent of relaxation to 4β-PDBu was only -93±6.5% (n=3). This potentiation of the maximum relaxant response to 4β-PDBu achieved statistical significance (p<0.01, unpaired t-test) and was apparent despite a 60min re-equilibration period and several washes with KH solution prior to the addition of cumulative concentrations of 4β-PDBu (figure 4.12a). The mean IC50 value for 4β-PDBu was 177.8±20nM (n=5) in tissues not exposed to isoprenaline (10nM) and 63±15nM (n=4) in those tracheal strips which were exposed to the relaxant. The potentiation of the mean IC50 value reached statistical significance (p<0.05, unpaired t-test).

It seemed that similar results were obtained with a threshold concentration of aminophylline (1μM), itself causing -9.4±3.6% (n=4) of the maximum contractile response to histamine, which appeared to cause potentiation of the maximum relaxant response to 4β-PDBu from -152±19% (n=3) of the maximum contractile response to histamine in the absence of aminophylline, to -213±23% (n=3) in its presence (see figure 4.12b).
However, this did not reach statistical significance (p>0.05, unpaired t-test). This was also the case for the mean IC₅₀ values for relaxation to 4β-PDBu (p>0.05, unpaired t-test) (IC₅₀ 120±20nM untreated preparations; 70±10nM in aminophylline-treated preparations, n=3-4).

Further investigation demonstrated that pretreatment with salbutamol (30nM), in tracheal tissues precontracted with carbachol (1μM), appeared to potentiate the maximum relaxation of the trachea induced by 4β-PDBu (figure 4.12c). However, the increase was not statistically significant (p>0.05, unpaired t-test) (IC₅₀ 2.3±0.9μM, maximum 4β-PDBu response 9.6±1.1% of papaverine maximum in untreated preparations; IC₅₀ 0.11±0.04μM, maximum response 15.3±3.2% in salbutamol, 30nM, treated preparations, n=3-4). 4β-PDBu, at a concentration which produced little effect on the carbachol-induced precontracting tone (100nM), had no effect on the relaxation of the trachea to salbutamol (p>0.05, unpaired t-test) (IC₅₀ 130±27nM, maximum response 51±10% of the papaverine maximum; IC₅₀ 155±3nM, maximum response 52±5% in 4β-PDBu-treated tissues, n=3). A similar negative result (p>0.05, unpaired t-test) was obtained when pretreating with a larger concentration of 4β-PDBu (300nM), which itself caused a small relaxant response (6.3±3.7% of the maximum relaxation to papaverine, n=3) (IC₅₀ 151±3.5nM, maximum relaxant response to salbutamol in the presence of 4β-PDBu 48±4%, n=3) (figure 4.12c).
Figure 4.1. The response of central and peripheral lung tissues to increasing concentrations of phorbol esters given cumulatively. (a) The response of the lung parenchyma to 4β-phorbol 12,13-dibutyrate (4β-PDBu). (b) The response of the trachea to 4β-PDBu. (c) The response of the trachea to 4α-PDBu. (d) The response of the trachea to another phorbol ester, 4β-phorbol 12,13-diacetate (4β-PDA). Responses were obtained from the same animal and are representative of all experiments. Recording was isotonic, W = wash, P = papaverine.
Figure 4.2. The response of guinea-pig (a) tracheal and (b) lung parenchymal tissues precontracted with histamine (Hist) and Carbachol (Carb) to 4β-phorbol 12, 13-dibutyrate (4β-PDBu). Traces are representative of at least three separate experiments. Responses to each spasmogen were obtained from tissues removed from the same animal. Isotonic recording. W = wash.
Figure 4.3. Cumulative concentration-response curves to selected phorbol esters. (a) Concentration-response relationship to $4\beta$-PDBu showing relaxation in the trachea (O) (n=15-16) and contraction in the lung parenchyma (●) (n=19-22). (b) Concentration-response relationship for relaxation in the trachea by different phorbol esters. $4\beta$-Phorbol 12, 13-dibutyrate ($4\beta$-PDBu) (●), $4\beta$-phorbol 12,13-diacetate ($4\beta$-PDA) (■), $4\beta$-phorbol 12-myristate 13-acetate ($4\beta$-PMA) (▲) and $4\alpha$-PDBu (O) were each added to the tissues at intrinsic tone. Data represent mean of three separate experiments. Vertical bars show s.e.mean.
Figure 4.4. Reversal of the relaxant response in the trachea to 4β-PDBu (a) by depolarising agents (b-d) or under conditions of reduced bathing temperature (16°C) (e). Traces are representative of at least three separate experiments (except e, where n=1). Hist = histamine, P = papaverine (100μM), W = wash. Isometric recording.
Figure 4.5. Protocol used to assess the effects of 4α- and 4β-PDBu on the subsequent histamine-induced contraction (d and e) and isoprenaline-induced relaxation (b and c) of the guinea-pig trachea. Comparisons could be made within tissues since there was no time-dependent change in the sensitivity to histamine or isoprenaline (a). Traces are representative of five separate experiments. W = wash, P = papaverine (100μM). Isometric recording.
Figure 4.6. The effect of 4α- (b and d) and 4β-PDBu (a and c) on the response of the guinea-pig trachea to histamine (a and b) and isoprenaline (c and d). Responses were obtained either in the absence (○) or presence (●) of 4α-/4β-PDBu. For protocol see figure 4.5. Data represent mean of 3-5 separate experiments. Vertical bars represent s.e.mean. Isometric recording.
Figure 4.7. The effect of prolonged incubation (85min) with 4β-PDBu (1μM) on (b) the response of the trachea to histamine (Hist) and (c) the response to carbachol (Carb). The upper trace (a) demonstrates that there was no change in responsiveness to histamine during the experiment. Traces are representative of three separate experiments. W = wash  Isometric recording.
Figure 4.8. The effect of (a) propranolol (10μM, n=4), (b) guanethidine (10μM, n=3), (c) tetrodotoxin (1μM, n=3) and (d) naloxone (10μM, n=3) on the cumulative concentration-response curve to 4β-PDBu in guinea-pig trachea. All inhibitors were preincubated for 30min prior to exposure to 4β-PDBu. Responses were obtained in the absence (O) and presence (●) of the inhibitors. Vertical bars represent s.e.mean. In (b) and (d) responses were recorded isometrically and expressed as a percentage of the maximum relaxation to papaverine (100μM) which was determined at the end of each experiment.
Figure 4.9. The effect of cyclo-oxygenase inhibition in the trachea with (a) aspirin (30µM, n=3) and (b) indomethacin (3µM, n=5), and histamine receptor blockade with (c) cimetidine (10µM, n=3) and (d) mepyramine (1µM, n=3) on the cumulative concentration-response relationship to 4β-PDBu. All inhibitors were preincubated for 30min prior to addition of 4β-PDBu. Responses were obtained in the absence (O) and presence (●) of inhibitors. Points represent mean ± s.e.mean. All responses were recorded isotonically, except in (c) where isometric recording was used.
Figure 4.10. The effect of epithelium removal on (a) relaxation to 4β-PDBu (n=4) and (b) contraction to histamine (n=4) in the guinea-pig trachea. In (a) and (b) responses were obtained in either epithelium intact (○) or denuded (●) preparations. In (a) tissues were precontracted with histamine (EC$_{65}$ intact 10μM; denuded 3μM). In (c) the effect of aminophylline (10μM, n=3) was tested on the response of the trachea to 4β-PDBu (1μM). While in (d) the effect of dipropyl-cyclopentylxanthine (DPCPX, 3μM, n=3) (●) was tested on the cumulative concentration-response curve to 4β-PDBu (○). * p<0.05, ** p<0.01 compared to the response in epithelium intact preparations to denuded preparations using student's t-test for unpaired observations. Points represent mean ± s.e.mean. All responses were recorded isometrically.
Figure 4.11. The response to 4β-PDBu (0.1 and 1μM) in histamine (Hist) -precontracted guinea-pig tracheal strip exposed to (a) aspirin (30μM) and (b) indomethacin (3μM). Responses are representative of three separate experiments. Isotonic recording. W = wash.
Figure 4.12. Effect of agents said to elevate intracellular cyclic AMP levels in tracheal strips on the relaxation to 4β-PDBu. (a) Comparison of tracheal strips exposed (●) (n=5) and not exposed to isoprenaline (10nM) (Ο) (n=4) after the initial response to the histamine standard. (b) Cumulative concentration–response curves to 4β-PDBu (Ο) (n=3), aminophylline (■) (n=4) alone and to 4β-PDBu in the presence of a threshold relaxant concentration of aminophylline (1μM) (●) (n=3). (c) Effect of salbutamol (30nM, n=4) (●) on the relaxant response to 4β-PDBu (Ο) and to salbutamol (□) in the presence of 4β-PDBu (300nM, n=3) (■). In (c) tissues were precontracted with carbachol (1μM). Points represent mean ± s.e.mean. Isotonic recording.
<table>
<thead>
<tr>
<th>Phorbol ester</th>
<th>IC$_{50}$ (µM)</th>
<th>Concentration ratio$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4β-PDBu</td>
<td>0.12 ± 0.05</td>
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<tr>
<td>4β-PDA</td>
<td>0.21 ± 0.02</td>
<td>1.75</td>
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<tr>
<td>4β-PMA</td>
<td>2.0 ± 0.1</td>
<td>16.7</td>
</tr>
</tbody>
</table>

Table 4.1 Mean IC$_{50}$ values for relaxation in the trachea induced by three different active phorbol esters.

$^1$ Concentration ratio, calculated as the inverse of the IC$_{50}$ value for 4β-PDBu divided by the IC$_{50}$ value for each of the remaining phorbol esters.

Figures represent mean ± s.e.mean of n=3. Isotonic recording.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Histamine EC₅₀ (µM)</th>
<th>ratio¹</th>
<th>Isoprenaline IC₅₀ (µM)</th>
<th>ratio¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0.002% DMSO)</td>
<td>12.2 ± 1.3</td>
<td>1.0</td>
<td>2 ± 0.08</td>
<td>1.0</td>
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<td>4β-PDBu (1µM)</td>
<td>14.1 ± 1.6</td>
<td>1.2</td>
<td>12.4 ± 1.8 ***</td>
<td>6.2</td>
</tr>
<tr>
<td>Control (0.002% DMSO)</td>
<td>11.3 ± 1.4</td>
<td>1.0</td>
<td>1.8 ± 0.04</td>
<td>1.0</td>
</tr>
<tr>
<td>4α-PDBu (1µM)</td>
<td>12 ± 2.1</td>
<td>1.1</td>
<td>1.7 ± 0.05</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Table 4.2. Effect of 4α- and 4β-PDBu on the mean EC₅₀ and IC₅₀ values for contraction and relaxation in the guinea-pig trachea to histamine and isoprenaline respectively. ***p <0.001 compared to responses within preparations before addition of 4α- or 4β-PDBu, paired Student's t-test. Figures represent mean ± s.e.mean of n = 4-5.

¹ Concentration ratio, calculated as the IC₅₀ or EC₅₀ value in the presence of phorbol ester divided by the IC₅₀ or EC₅₀ value in the absence of phorbol ester.
4.4 Discussion

Phorbol esters evoke qualitatively different responses in guinea-pig peripheral and central lung tissues - contraction in the lung parenchymal strip (Dale and Obianime, 1985, 1987) and relaxation in the trachea (Menkes et al., 1986). The tissues are anatomically and embryologically closely related, and so there was a need to understand the mechanism of the unexpected relaxant response in the trachea.

Initially, it was important to assess whether the relaxation of the trachea to phorbol esters involved activation of protein kinase C or an unrelated non-specific effect. Structurally, the active 4β-phorbol esters possess a relatively polar head group (the phorbol moiety) with lipophilic ester substituents in the 12- and 13-positions of the phorboi nucleus. Thus to some extent they represent non-ionic detergents. To distinguish between "receptor"-mediated activities of phorbol esters and non-specific effects, due possibly to their membrane perturbing detergent-like structures, inactive 4α-isomers can be used as a negative control. In the absence of potent and selective inhibitors, three pieces of evidence suggest that the relaxant effect of phorbol esters in the trachea observed in this project, involved PKC.

The first was the relative inability, even at high concentrations, of the inactive 4α-isomer of PDBu to evoke relaxation in the trachea. The 4α-isomer differs from the 4β-isomer only in the spacial arrangement of the hydroxy functional group at position 4 on the phorbol ring. However, this isomeric change alters the conformation of the cyclopentenone group and 4-hydroxy group in a manner sufficient to reduce substantially its ability to activate PKC. This is despite their otherwise closely matched structural features and overall lipophilicity.

Secondly, all three active esters were able to relax the trachea in a concentration-dependent manner, exhibiting parallel concentration-response curves, suggesting a common mechanism. The order of potency of these agents both on isolated PKC and in whole cell systems has been reported to be 4β-PMA > 4β-PDA > 4β-PDBu (Castagna et al., 1982). However, in the trachea the rank order was 4β-PDBu > 4β-PDA > 4β-PMA, which is the inverse of their lipid solubility. A similar finding was reported for phorbol esters in guinea-pig lung parenchymal strip (Dale
and Obianime, 1987) and ileal longitudinal strip also from guinea-pig (Sasaguri and Watson, 1990). It seems likely, therefore that the inhibitory effect of the phorbol esters could be influenced by their ability to diffuse through the tissue layers, a process which will favour the more hydrophilic esters (eg. PDBu), while the more lipophilic esters (eg. PMA) may become trapped by a lipid "sink", limiting their access to PKC. For this reason, coupled with its relatively prompt onset of action and that cumulative concentration-response curves could be constructed, 4β-PDBu was used throughout this study.

The final piece of evidence, supporting the action of 4β-phorbol esters at PKC in the trachea, was the apparent ability of the cell permeant DAG analogues, OAG and diC₈, to mimic the qualitative effects of phorbol ester stimulation in both respiratory tract tissues.

Menkes et al. (1986) attributed the phorbol ester-induced relaxant response of the trachea to a direct effect, involving PKC-mediated inhibition of the signal transduction mechanisms responsible for both basal tone maintenance and agonist-induced contraction. Indeed, in support of this conclusion, phorbol esters have been reported to inhibit agonist-induced PIP₂ turnover in tracheal smooth muscle (Baron and Coburn, 1987), which could conceivably lead to relaxation. However, the results reported in this chapter indicated that this explanation cannot account for the relaxation since it was still possible to produce marked agonist-induced contractions to histamine and carbachol either 15min or some 85min after the addition of a maximum relaxant concentration of the phorbol ester. This suggested that PKC had not inhibited the receptor-mediated signal transduction mechanism to histamine and carbachol. Furthermore, Menkes et al. (1986) also suggested that the relaxation to phorbol esters from intrinsic tone involved a similar inhibition of the signal transduction mechanisms responsible for basal tone maintenance. Tracheal tone maintenance in guinea-pig is thought to be mediated primarily by contractile prostanoids such as prostaglandin F₂₀ and thromboxane A₂ (Orehek et al., 1975). However, 4β-PDBu was still capable of producing marked relaxation in precontracted tissues which had been exposed to concentrations of cyclo-oxygenase inhibitors, aspirin and indomethacin, known to maximally inhibit the enzyme (see figure 4.11).
PKC activation is known to down-regulate responses to various stimulants in several cell types (see review by Sibley and Lefkowitz, 1985). In guinea-pig lung parenchymal strips precontracted with the phorbol ester, 4β-PMA, the potency of the β-adrenoceptor receptor agonist, isoprenaline, was markedly reduced (Dale and Obianime, 1985). This observation led these authors to hypothesise that inappropriately activated PKC may be involved in the delayed phase of asthma, in which β-adrenoceptor agonists are refractory. In this study the phorbol ester, 4β-PDBu, in the trachea appeared to share this action of 4β-PMA on the relaxant response to isoprenaline, which was not seen with 4α-PDBu - the inactive isomer, suggesting that the effect involved PKC. Moreover, this inhibition of the relaxant action of isoprenaline was observed in the same tracheal strips in which the contractile response to histamine was shown to be unaffected by PKC activation. However, the phorbol ester did not inhibit relaxation to other relaxants such as salbutamol or aminophylline, which are believed to have similar signal transduction mechanisms to isoprenaline (ie. they increase the intracellular cyclic AMP concentration).

Consideration of these results together with the evidence implicating a positive action of PKC in the proposed "latch-bridge" state of maintained tension in smooth muscle, suggested that it was unlikely that phorbol esters produced relaxation in the trachea by a direct effect on the smooth muscle. It seemed more likely that the relaxant effect of phorbol esters on the guinea-pig trachea was an indirect one, particularly as it was possible to convert relaxation induced by 4β-PDBu to a sustained contraction in the presence of KCl (Menkes et al., 1986) and other depolarising agents (Huang et al., 1987). Under these conditions, the contraction was sensitive to calcium channel blockers such as verapamil, diltiazem and nifedipine (Menkes et al., 1986). The explanation of this contractile effect would appear to be that depolarisation of smooth muscle opens voltage-dependent calcium channels and that the subsequent increase in [Ca^{2+}]_i synergises with PKC which has been activated by phorbol esters, and thus causes contraction. Synergism of this nature, in smooth muscle, has been reported in several studies (Rasmussen et al., 1984; Dale and Obianime, 1987; Obianime et al., 1988).
The trachea possesses many different cell types known to influence smooth muscle reactivity. Phorbol esters are known to release many biologically active substances, some of which could mediate relaxation. These substances could originate from a variety of cell types within the trachea, such as inflammatory, endocrine, neuronal or even epithelial.

Phorbol esters could cause the release of a neurotransmitter substance, possibly from ganglionic sites, by depolarising inhibitory non-adrenergic, non-cholinergic nerve fibres, or causing the release of endogenous catecholamines and/or associated co-transmitter(s) from sympathetic adrenergic nerve endings, particularly since phorbol esters have been reported to depolarise neurones (Rang and Ritchie, 1987, 1988) and to evoke noradrenaline release (Wakade et al., 1985; Allgaier et al., 1986, 1987). Phorbol esters have also been reported to mobilise neuroendocrine-derived naloxone-sensitive opioids (Schultzberg et al., 1979). However, pretreatment with a voltage-sensitive sodium channel blocker (tetrodotoxin), β-adrenoceptor antagonist (propranolol) or an adrenergic-neurone blocking agent (guanethidine) failed to prevent relaxation of the guinea-pig trachea to 4β-PDBu. This implies that nerve depolarisation and the release of neurotransmitters from sympathetic adrenergic neurones, including the action of endogenous catecholamines acting at β-adrenoceptors, does not appear to be involved in the response of the trachea to 4β-PDBu. Further, the failure of the opioid receptor antagonist, naloxone, to prevent relaxation of the trachea to 4β-PDBu suggests that the release of naloxone-sensitive endogenous opioids such as enkephalins are similarly not involved. However, the role of naloxone insensitive opioids cannot be excluded.

In addition, it has been reported that phorbol esters can mobilise arachidonic acid (Levine and Hassid, 1977) leading to the synthesis and release of relaxant prostanoids such as prostaglandin E2 and prostaglandin I2 (PGI2, prostacyclin). However, the relaxant response of the trachea to 4β-PDBu was not prevented by the cyclo-oxygenase inhibitors, aspirin and indomethacin. This was not reflected in the findings of Raeburn (1989) (see later).

Phorbol esters are known to evoke histamine release from mast cells (Blackwell et al., 1985; Cantwell and Foreman, 1987) which are found in abundance in the trachea. Histamine is a potent spasmogen at histamine
H₁-receptors in airway smooth muscle, and a potent relaxant at H₂-receptors (Drazen et al., 1979). However, in the present study both cimetidine, an H₂-receptor antagonist, and mepyramine, an H₁-receptor antagonist, failed to prevent relaxation to 4β-PDBu in the trachea, indicating the non-involvement of mediators at these receptors in this response. Furthermore, the lack of effect of aminophylline (10μM) a specific adenosine A₁- and A₂-receptor antagonist, suggested that it was unlikely that the relaxant effect of 4β-PDBu involved the release of adenosine acting primarily at the A₂ receptor. A role for adenosine at A₁-receptors was also considered unlikely to be involved in this response, since the selective A₁-receptor, dipropylcyclopentylxanthine (DPCPX) failed to prevent relaxation of the trachea to 4β-PDBu.

Mechanical removal of the luminal airway epithelium has been shown to alter the reactivity of the underlying smooth muscle to a variety of spasmogenic and relaxant agonists in vitro (reviewed by Farmer, 1987; Goldie et al., 1990). This phenomenon was first documented in canine bronchus (Flavahan et al., 1985) and has been confirmed in many other species including guinea-pig (Goldie et al., 1986; Hay et al., 1986). The mechanism by which the airway epithelium modulates airway smooth muscle reactivity in vitro is uncertain and the subject of debate (Farmer, 1987; Goldie et al., 1990). It has been suggested that the epithelium functions as a diffusion barrier (Holroyde, 1986). The epithelium may also represent a site for uptake or metabolism of bronchoactive agents reducing their effective concentration at the smooth muscle layer (see Goldie et al., 1990). Another explanation is that the airway epithelium may release one or more inhibitory factors, either basally or in response to certain agonists, which are capable of attenuating airway responsiveness (see Farmer, 1987). Superfusion-cascade experiments failed to demonstrate the release of a relaxant from airway epithelium (Holroyde, 1986). The explanation for this lack of effect may lie in the possibility of preferential abluminal release of an epithelium-derived inhibitory factor (EpDIF), or of dilution and rapid degradation of the putative inhibitory factor(s). This has encouraged the use of a "sandwich" preparation, originally described by Furgott and Zawadski (1980) for studies of endothelium-derived relaxant factor, which in turn has facilitated the demonstration of an EpDIF in response to antigen in sensitised guinea-pigs (Hay et al., 1987). The possibility that 4β-PDBu was able to relax the trachea by releasing the putative
airway epithelium-derived inhibitory factor(s) was investigated. It was found that mechanical removal of the luminal epithelium (confirmed by histological examination) failed to prevent relaxation induced by 4β-PDBu, suggesting that the mechanism for the relaxant action of the phorbol ester does not involve the release of an EpDIF. Further, it is also unlikely that the epithelium posed a diffusion or metabolic barrier to 4β-PDBu since epithelium removal did not modify either the rate or extent of the response. These observations are in direct conflict with a report by Raeburn (1989) in which the relaxation of the guinea-pig trachea by 4β-PDBu was held to be epithelium-dependent. It was found that on removal of the epithelium, contraction resulted with 4β-PDBu. In addition to this observation, Raeburn (1989) reported that the relaxant effect of 4β-PDBu in intact preparations was abolished by cyclooxygenase inhibition with indomethacin (3μM). Again this was not found in this present study (see section 4.3.4 and figure 4.11). The reason for these discrepancies is not apparent, but is unlikely to result from differences in methodology which were carefully matched with those of Hay et al. (1986).

The observation that relaxation of the trachea to 4β-PDBu, after the response to the histamine standards, was potentiated by the non-selective β-adrenoceptor agonist, isoprenaline, was intriguing. This phenomenon occurred despite frequent washing of the tissues with KH solution during the re-equilibration period (60min) prior to addition of 4β-PDBu to the organ bath. The explanation for this effect is unclear, but may represent a "carry-over" effect involving sustained elevated residual cyclic 3',5'-adenosine monophosphate (cyclic AMP) levels and subsequent interaction with 4β-PDBu. Irvine et al. (1986) demonstrated that the phorbol ester, 4β-PMA, could inactivate (by phosphorylation) the cyclic AMP-dependent phosphodiesterase in hepatocytes leading to an accumulation of cyclic AMP. Similarly aminophylline and the β2-adrenoceptor agonist, salbutamol, potentiated the relaxant effect of 4β-PDBu in this study. However, it is recognised that this effect was weak and the concentration of aminophylline used in this study (1μM) is unlikely to be effective in inhibiting the cyclic AMP-dependent phosphodiesterase since much higher concentrations are generally required to inhibit this enzyme completely (see Polson et al., 1978). More recent evidence does, however, suggest close correlation between inhibition of certain forms of smooth muscle phosphodiesterase by
xanthine drugs and their relaxant effect on canine trachealis (Polson et al., 1985). It appears that a particular pool of intracellular cyclic nucleotides may be increased by the action of xanthine drugs and that the relaxant effect of these agents may, after all, be due to activation of a cyclic AMP-dependent kinase.

One possibility not eliminated by the results obtained at this stage was that relaxation to 4β-PDBu could result from a PKC-mediated release of a relaxant neurotransmitter(s), possibly from inhibitory non-adrenergic, non-cholinergic (NANC) nerve fibres. However, if the relaxant effect of 4β-PDBu was to involve the release of such a relaxant, then the potentiation of the response to 4β-PDBu by agents elevating cyclic AMP might result either from post-junctional events (ie. synergism between two or more relaxants at the smooth muscle) or from modulation of, for example, neurotransmitter release. Further, in the guinea-pig trachea, Jackson et al. (1988) have provided evidence that the relaxant action of another phorbol ester, 4β-PDA, occurs as a result of elevated cyclic AMP levels.

The evidence for the above hypothesis involving the release of a NANC neurotransmitter is presented in the following chapter, and much of the remaining work of this project was aimed to test its validity in understanding the relaxant action of phorbol esters in the guinea-pig trachea.
CHAPTER FIVE

COMPARISON OF THE RELAXANT RESPONSE OF 4β-PDBu WITH ELECTRICALLY-EVOKED NANC₁ NERVE STIMULATION AND A PUTATIVE NANC₁ NEUROTRANSMITTER IN GUINEA-PIG TRACHEA
Summary

1. The effect of 4β-PDBu was tested at various levels of the respiratory tract. 4β-PDBu caused relaxation of both the trachea and hilar bronchus, but in the case of the latter it was much less marked. However, in lung parenchyma and secondary bronchus, contraction occurred to 4β-PDBu which was much less marked in the bronchus. The extent to which relaxation occurred seemed to correlate with the reported distribution of functional NANCi innervation, VIP-immunoreactive nerve fibres and receptors for VIP in the airways.

2. Tracheal relaxation produced by 4β-PDBu bore some similarity in extent and in the slope of the stimulus-response relationship to that induced by VIP and stimulation of NANCi nerves. Other similarities were also encountered: Combined incubation of the protease inhibitors, bacitracin and aprotinin, facilitated the relaxant response of the trachea to NANCi nerve stimulation, exogenous VIP and 4β-PDBu. However, this was not paralleled in the presence of the neutral endopeptidase (NEP) inhibitor, phosphoramidon. In the combined presence of the proteases, chymotrypsin and papain, there was potentiation to high concentrations of 4β-PDBu (although statistical significance was not achieved), and marked attenuation of the response to VIP and NANCi nerve stimulation.

3. Relaxation to 4β-PDBu was unaffected by a putative VIP-receptor antagonist. However, the antagonist also failed to prevent NANCi nerve-, and more importantly, VIP-induced relaxation. Different VIP receptors or the presence of metabolic barriers may explain its ineffectiveness.

4. Methylene blue, a guanylate cyclase inhibitor, failed to prevent relaxation produced by NANCi nerve stimulation or VIP, but was marginally effective against high concentrations of 4β-PDBu (>0.3μM); although this was not statistically significant.

5. L-N⁵-Monomethylarginine was ineffective against 4β-PDBu- and NANCi nerve-induced relaxation. The formation of nitric oxide from L-arginine is unlikely to contribute to the relaxation of the guinea-pig trachea produced by 4β-PDBu or NANCi nerve stimulation.
5.1 Introduction and background

The autonomic innervation of the airways consists of afferent (sensory) and efferent (motor) pathways. Afferent innervation arises from the sensory ganglia of the vagus nerve or from dorsal root ganglia of the spinal cord. Its main function is to provide sensory information to the central nervous system (CNS) required for the control of bronchomotor tone and breathing pattern under physiological and pathological conditions; and carries impulses from lung irritant, stretch and chemoreceptors. The major function of the efferent innervation is to modulate airway smooth muscle tone, and certain other processes such as mucus secretion, airway epithelium transport, vascular permeability and blood flow in the bronchial circulation. The efferent innervation of the airways consists not only of the classical opposing parasympathetic (cholinergic) and sympathetic (adrenergic) pathways, but also non-adrenergic, non-cholinergic (NANC) pathways. NANC innervation can be excitatory (NANC<sub>e</sub>) or inhibitory (NANC<sub>i</sub>). Using selective pharmacological intervention, it is possible to demonstrate each of these efferent pathways in guinea-pig airways in vitro (Coburn and Tomita, 1973; Grundstrom et al., 1981; Taylor et al., 1984; see also figure 5.1a); although evidence suggests that human airway smooth muscle lacks a functional sympathetic (adrenergic) innervation (Davis et al., 1982).

In the previous chapter the relaxation to the phorbol ester, 4β-PDBu, was not affected by adrenergic neurone blockade with guanethidine and β-adrenoceptor blockade with propranolol. Empirically, if the relaxant effect of 4β-PDBu in the guinea-pig trachea is in fact caused by the release of a neurotransmitter, it could be described as an inhibitory non-adrenergic response (see below).

The inhibitory non-adrenergic non-cholinergic system

Innervation and function

The existence of an inhibitory innervation to the airway smooth muscle of many species, after adrenergic and cholinergic blockade, has been demonstrated in both isolated airway preparations and in whole animals. Electrical field stimulation (EFS) of the guinea-pig tracheal preparation causes a biphasic response: an initial contractile phase followed by relaxation (see figure 5.1a). The initial contractile component is atropine-sensitive, while both phases are blocked by tetrodotoxin (TTX).
The contraction is potentiated by anti-cholinesterases (eg. figure 5.2), indicating that it is due to excitatory cholinergic nerves. However, in the presence of atropine, EFS causes a TTX-sensitive relaxation which is only partially reduced by propranolol, the majority of the inhibitory response being unaffected. This particular response is considered to represent the activation of NANC$_i$ pathways (Coburn and Tomita, 1973; Coleman and Levy, 1974; Taylor et al., 1984). Similarly, it has been shown that after cholinergic and adrenergic blockade, nicotine causes relaxation of the isolated guinea-pig trachea, which is blocked by TTX and also by hexamethonium, suggesting that it is mediated by the activation of nicotinic receptors on post-ganglionic efferent nerve fibres (Jones et al., 1980). In vivo, in cats and guinea-pigs, electrical stimulation of the vagus produces bronchodilatation which is resistant to both adrenergic and cholinergic blockade (Chesrown et al., 1980; Diamond and O'Donnell, 1980).

The extent to which the NANC$_i$ system mediates bronchodilatation at differing levels down the tracheobronchial tree varies between species. In cats, using tantalum bronchography, bronchodilatation in response to vagal nerve stimulation occurs at all airway levels down to the small bronchioles, but is more predominant in larger airways (Matsumoto et al., 1985). However, in isolated guinea-pig airways, NANC$_i$ responses to EFS can only be demonstrated in the trachea; the bronchi (main and hilar) producing only contractile responses (Grundstrom et al., 1981).

*The neurotransmitter*

Although the existence of a NANC$_i$ innervation to airway smooth muscle is well established, there is considerable controversy as to the precise identity of the neurotransmitter mediating NANC$_i$ responses, which may vary between species.

Purine nucleotides have been implicated as neurotransmitters in the gastrointestinal and genitourinary tracts (Burnstock, 1972) and it was therefore suggested that NANC$_i$ "nerves" might be purinergic (Satchell, 1984). Guinea-pig tracheal smooth muscle is relaxed by the purine, adenosine, and in addition, its related nucleotides, adenosine mono-, di- and triphosphates (Coleman and Levy, 1974; Satchell, 1984), thus mimicking the relaxant response induced by NANC$_i$ nerve stimulation by EFS. Furthermore in guinea-pig trachea, dipyridamole (adenosine uptake inhibitor) and adenosine deaminase (adenosine catabolising enzyme) have
been reported to produce responses consistent with adenosine mimicking NANC\textsubscript{i} nerve stimulation by EFS (Coleman, 1976; Satchell, 1984). However, there is now a great deal of evidence which argues against a purine nucleotide as the neurotransmitter of NANC\textsubscript{i} nerves in airways. For example, dipyridamole failed to potentiate NANC\textsubscript{i} relaxation to EFS in several other species including human (Davis \textit{et al.}, 1982), bovine (Cameron \textit{et al.}, 1983) and feline airways (Itoh and Takeda, 1982). Moreover in guinea-pig, blockade of the adenosine receptor responsible for adenosine-induced tracheal relaxation with NPC205, a potent and selective antagonist, failed to prevent NANC\textsubscript{i} responses to EFS (Ellis and Farmer, 1989a). Similar results have been obtained with other adenosine receptor antagonists such as aminophylline (Davis \textit{et al.}, 1982; Cameron \textit{et al.}, 1983; Karlsson and Persson, 1984), or after desensitisation to purines (Ito and Takeda, 1982), incubation with adenosine deaminase (Ellis and Farmer, 1989a) and in the presence of a maximum relaxant concentration of adenosine (Karlsson and Persson, 1984).

The discovery of a large number of bronchoactive and vasoactive peptides in the lung, coupled with electron microscopy studies showing "p-type" (peptide-containing) granules, in addition to cholinergic and adrenergic vesicles, in pulmonary nerve endings (Laitinen \textit{et al.}, 1985) has led to the suggestion that NANC\textsubscript{i} responses may be mediated by "peptidergic" nerves. Of the several peptides isolated from the lung only vasoactive intestinal peptide (VIP), so called due to its discovery in bovine small intestine (Said and Mutt, 1970), and the related peptide, peptide histidine isoleucine (PHI), relax airway smooth muscle and are thus the only major candidates for the NANC\textsubscript{i} neurotransmitter. Peptide histidine methionine (PHM) is the human counterpart for PHI. VIP is a 28 amino acid residue peptide which may co-exist with PHI in airway nerves (Lundberg \textit{et al.}, 1984a). PHI contains 27 amino acid residues and exhibits various structural and amino acid sequence homologies with VIP, but has an NH\textsubscript{2}-terminal histidine and a COOH-terminal isoleucine, whereas PHM has a COOH-terminal methionine. Both VIP and PHI/M are derived from the same precursor molecule, prepro-VIP, since they are all expressed on the same precursor gene (Itoh \textit{et al.}, 1983). In addition, another peptide, peptide histidine valine-42 (PHV-42), has been isolated from prepro-VIP (Yiangou \textit{et al.}, 1987).
Figure 5.1 (a) Characterisation of the response of guinea-pig trachea to EFS (S). The lower tracing shows the effect of phentolamine (Phentol), propranolol (prop) and tetrodotoxin (TTX) and atropine on the biphasic response induced by EFS. The upper tracing shows that the responsiveness to EFS (S) did not change with time during the experiment. Note that volume additions were controlled for with Krebs-Henseleit solution (KHS). Traces are representative of at least three experiments. (b) Example of a typical record showing the response of the guinea-pig trachea to EFS, delivered cumulatively in the presence of atropine (1μM) and propranolol (1μM). Hist = histamine, W = wash, Papav = papaverine (100μM). Isometric recording.
Figure 5.2 Enhancement of the contractile component of the response of the guinea-pig trachea to EFS by the anticholinesterase inhibitor, eserine (0.5μM). The left-hand panel shows a representative trace, while the right-hand panel shows the effect of eserine on the mean response to EFS in 5 experiments. Vertical bars represent s.e.mean. Hist = histamine, W = wash. Isometric recording.
What is the evidence that this family of peptides may mediate NANC\textsubscript{i} responses in guinea-pig airways?

(i) VIP fulfills the criteria of a neurotransmitter (Giachetti \textit{et al.}, 1977; Said \textit{et al.}, 1980).

(ii) VIP-like immunoreactive (VIP-IR) nerve fibres are present in the vagus (Lundberg \textit{et al.}, 1978) and one method of demonstrating NANC\textsubscript{i} responses \textit{in vivo} is by electrical vagal stimulation (Diamond and O'Donnell, 1980).

(iii) VIP-IR nerve fibres are found in close association with tracheal airway smooth muscle (Polak and Bloom, 1982).

(iv) VIP is a potent relaxant of airway smooth muscle in several species (see Barnes, 1986b) and mimics the relaxant effect of NANC\textsubscript{i} nerve stimulation by EFS, being released into the bathing medium in a frequency-dependent and TTX-sensitive fashion (Matsuzaki \textit{et al.}, 1980). In addition, PHI/M relaxes guinea-pig tracheal smooth muscle, although it is less potent than VIP (Christofides \textit{et al.}, 1984; Ellis and Farmer, 1989b). PHI/M co-exists and, in the cat submandibular gland, is co-released with VIP (Lundberg \textit{et al.}, 1984a,b). PHV-42 is even more potent than VIP or PHI in guinea-pig trachea (Yiangou \textit{et al.}, 1987).

(v) Specific anti-sera to VIP (Matsuzaki \textit{et al.}, 1980; Ellis and Farmer, 1989a) and PHI (Ellis and Farmer 1989a) reduced the NANC\textsubscript{i} responses to EFS in the guinea-pig trachea.

(vi) VIP mimics the electrophysiological changes in bovine and feline airway smooth muscle elicited by NANC\textsubscript{i} nerve stimulation (Cameron \textit{et al.}, 1983; Ito and Takeda, 1982).

(vii) Desensitisation of airway smooth muscle to exogenous VIP leads to a reduction in NANC\textsubscript{i} responses to EFS in cats (Ito and Takeda, 1982) and guinea-pigs (Venugopalan and O'Malley, 1988).
However, a number of findings argue against such a role for VIP in mediating NANC\textsubscript{i} responses.

(i) NANC\textsubscript{i} relaxation of guinea-pig trachea persists in the presence of a maximally effective concentration of exogenous VIP (Karlsson and Persson, 1984). In a similar study (Ellis and Farmer, 1989a) the NANC\textsubscript{i} response was greatly attenuated, but not abolished, in the presence of a maximally effective concentration of VIP suggesting that while VIP may account at least in part for NANC\textsubscript{i} responses, other transmitters may be released on NANC\textsubscript{i} stimulation by EFS.

(ii) Similarly, in guinea-pig trachea the protease, chymotrypsin, which abolished the response to exogenous VIP and PHI, only reduced the response to NANC\textsubscript{i} stimulation to EFS by 30%, suggesting that the predominant enzyme-resistant component to the response may not be due to VIP or a related peptide (Ellis and Farmer, 1989b). The possibility that the lack of inhibition may have resulted from the failure of the enzyme to reach the sites of VIP release in sufficient concentrations to degrade the peptide is unlikely, but should not be overlooked (see discussion, this chapter).

Further confirmation of a role for VIP in mediating these NANC\textsubscript{i} responses must await the availability of selective and potent VIP-receptor antagonists.

\textit{VIP and PHI/M receptors}

VIP produces its relaxant effects by activation of specific high affinity receptors localised primarily on airway smooth muscle. The response is unaffected by \(\beta\)-adrenoceptor antagonists, and by indomethacin and tetrodotoxin. Like \(\beta\)-agonists, VIP and PHI stimulate adenylate cyclase activity in target cells leading to an accumulation of cyclic AMP in the trachea (Fransden \textit{et al}., 1978) and other lung tissues from several species (Robberecht \textit{et al}., 1982). Autoradiographic studies in human and guinea-pig lung have demonstrated VIP receptors in several cell types, including the smooth muscle layer of the larger central airways, but not of small airways (Carstairs and Barnes, 1986). These findings are consistent with functional studies in human airways in which VIP is a poor relaxant in small airways compared to larger airways, although isoprenaline relaxes both small and large airways to an equal extent (Palmer \textit{et al}., 1986).
The distribution of PHI receptors throughout rat, guinea-pig and feline lung, parallels that of VIP (Christofides et al., 1984). This is to be expected since PHI is localised to VIP-IR nerve fibres and is co-released with VIP (Lundberg et al., 1984a,b). PHI/M may activate the same receptor as VIP, although there is evidence to suggest that it may activate different receptors (Palmer et al., 1986).

Recently, it has become apparent that there could be yet another candidate for the transmitter mediating NANC\textsubscript{i} responses. In the central nervous system (CNS) there is increasing evidence for a neuromodulatory/neurotransmitter role for a highly labile substance described, by vascular physiologists, as endothelium-derived relaxing factor. This nitric oxide (NO)-like substance is released through activation of glutamate receptors in the cerebellum (Garthwaite et al., 1988) and it is suggested that this substance has a role in neurotransmission. Further evidence suggests a neurotransmitter role for NO released from NANC\textsubscript{i} fibres in the anococcygeus muscle of the mouse and rat (Gibson et al., 1989; Gillespie et al., 1989) and in canine gastrointestinal tissue (Bult et al., 1990). For the moment, the identity of the NANC\textsubscript{i} neurotransmitter in the guinea-pig trachea remains uncertain.

The aim of this chapter was to understand more fully the relaxant action of 4β-PDBu in the trachea and to investigate the hypothesis that the relaxation of the trachea to 4β-PDBu involves the release of a relaxant neurotransmitter, particularly from NANC\textsubscript{i} nerve fibres. Since the identity of the NANC\textsubscript{i} neurotransmitter is at present not known, this was undertaken by comparing the relaxant response to 4β-PDBu not only with a putative NANC\textsubscript{i} neurotransmitter, VIP, but also with electrically-evoked nerve stimulation in the presence of atropine and propranolol - considered to represent the release of the NANC\textsubscript{i} neurotransmitter (eg. Coburn and Tomita, 1973).

Finally, it is recognised that the term, "NANC nerve fibre" is a misnomer and implies an anatomically distinct sub-division of the autonomic nervous system, when in fact this is not the case and "NANC" should more correctly be regarded as a mechanism of neuronal transmission which is shared by both parasympathetic and sympathetic nerve fibres.
5.2 Experimental protocol

In the guinea-pig trachea, EFS was applied continuously (0.1ms, 100:18V) over a range of frequencies (0.25-20Hz). When the response to a particular frequency plateaued the frequency was then increased. At 20Hz, the stimulator was switched off and tension allowed to return to the prestimulation level, which usually occurred within 10-15min (see figure 5.1b). At this point, in experiments involving phosphoramidon (10µM), capsaicin was added.

The effect of the various receptor antagonists/enzyme inhibitors was investigated on responses to EFS, VIP and 4β-PDBu by incubating paired parallel tissues for a specified period with the drug or vehicle prior to precontraction with histamine (approx. EC₅₀). Relaxation was then induced in a cumulative fashion to 4β-PDBu, VIP or EFS. Atropine (1µM) and propranolol (1µM) were present throughout all procedures involving EFS. In all experiments except where stated, the peptidase inhibitors, bacitracin (30µg/ml = 1.86U/ml) and aprotinin (1.2U/ml) were present in the KH solution for 30min prior to precontraction with histamine (10µM) and construction of cumulative stimulus-response curves to the relaxants. Relaxant responses were compared to the relaxation induced by a maximally effective concentration of papaverine (100µM) which was added at the end of each experiment.
5.3 Results

5.3.1 The response to 4β-PDBu at different levels of the guinea-pig tracheobronchial tree

The results are shown in figure 5.3. The response of preparations of trachea, hilar and secondary bronchus, and of lung parenchyma were compared. Relaxation occurred to 4β-PDBu (1μM) both in histamine-precontracted (approx. EC₇₅) trachea and hilar bronchus. The relaxation was more marked in the trachea than in the hilar bronchus (78.6±2.5% reversal of the histamine contraction in the trachea and 27±2% reversal in the hilar bronchus, n=3). However, in both the secondary bronchus and lung parenchyma, contraction resulted to 4β-PDBu (1μM). The response of the secondary bronchus to 4β-PDBu being weaker than that seen in the lung parenchyma (135±8% of the histamine maximum contraction in the parenchyma and 30±4% in the secondary bronchus, n=3).

5.3.2 Comparison of the concentration-response relationship for relaxation in the guinea-pig trachea to 4β-PDBu, VIP and NANCᵢ nerve stimulation

In the presence of atropine (1μM) and propranolol (1μM), EFS of guinea-pig trachea, precontracted with histamine (10μM), induced an inhibitory response which was dependent on pulse duration, voltage across the electrodes and frequency of stimulation, and was inhibited by tetrodotoxin (TTX, 1-3μM) (see figure 5.1a). The optimum parameters for stimulation, determined in preliminary experiments, were elicited by a pulse duration of 0.5ms and a potential difference of 100V at source and 15-18V across the electrodes. Maximum relaxation to EFS occurred at a frequency of 10-20Hz (figures 5.1a and 5.4b). These parameters and conditions were used routinely for EFS of the guinea-pig trachea. VIP produced concentration-dependent relaxation in histamine (10μM)-precontracted tracheal strips (IC₅₀ 5.3±0.7nM, n=12) achieving maximum at 0.3μM (99±0.9% of the maximum relaxation to papaverine, 100μM) (figure 5.4). Similar concentration-dependent relaxations were observed with 4β-PDBu (IC₅₀ 21.3±4.2nM, n=12) reaching maximum at 1μM (81±3.9% of the papaverine maximum) (figure 5.4b). The concentration ratio for VIP compared to 4β-PDBu, calculated as the IC₅₀ for VIP-induced relaxation divided by the IC₅₀ for 4β-PDBu, was 4.0.
5.3.3 Modulation of relaxation by peptidase inhibitors in the guinea-pig trachea to NANCᵢ nerve stimulation, VIP and 4β-PDBu

5.3.3a Effect of bacitracin and aprotinin

The results are shown in figure 5.5. Bacitracin (30μg/ml = 1.86U/ml) and aprotinin (1.2U/ml) used in combination potentiated relaxation in guinea-pig trachea induced by NANCᵢ nerve stimulation, exogenous VIP and 4β-PDBu. This was demonstrated as a left-ward shift of the frequency-response for NANCᵢ nerve stimulation induced by EFS, and in the concentration-responses for exogenous VIP and 4β-PDBu. The mean EF₅₀ value for relaxation evoked by NANCᵢ nerve stimulation was enhanced significantly (p<0.05, unpaired t-test) by bacitracin and aprotinin (EF₅₀ 2±0.03Hz in untreated preparations; EF₅₀ 1.2±0.2Hz in treated tissues, ratio 1.7, n=3). Bacitracin and aprotinin also enhanced significantly (p<0.001, unpaired t-test) the maximum extent of relaxation to EFS at 10Hz (91.6±0.86% of the maximum relaxation to papaverine in untreated tissues; 99.7±0.3% in protease inhibitor-treated preparations, n=3).

Bacitracin and aprotinin significantly reduced (p<0.05, unpaired t-test) the mean IC₅₀ value for relaxation induced by exogenous VIP (IC₅₀ 20.9±5.4nM in untreated tissues, IC₅₀ 3.6±0.7nM in treated preparations, concentration ratio, 5.8, n=3), but were without effect (p>0.05, unpaired t-test) on the maximum relaxation induced by VIP (88.5±2.8% of the maximum relaxation to papaverine in untreated tissues; 96±3.3% in treated preparations, n=3).

Similarly, bacitracin and aprotinin significantly reduced the mean IC₅₀ value for relaxation induced by 4β-PDBu (IC₅₀ 34.6±2.6nM in untreated tissues; 15.4±1.4nM in treated preparations, concentration ratio 2.2, p<0.01, unpaired t-test, n=3), but were without effect (p>0.05, unpaired t-test) on the magnitude of the maximum relaxation induced by 4β-PDBu (74.8±4.1% in untreated preparations; 84±3% in treated tissues, n=3).

5.3.3b Effect of phosphoramidon

The results are shown in figure 5.6. Phosphoramidon (10μM) produced variable effects on the relaxation in the guinea-pig trachea to NANCᵢ nerve stimulation, exogenous VIP and 4β-PDBu. In all of the preparations studied phosphoramidon had no effect on either basal tension (p>0.05, unpaired t-test) or the response to histamine (10μM) used for precontraction (52.2±4% of the maximum contractile response to histamine in 2μM DTT-treated control...
preparations; 54.1±4.2% in phosphoramidon-treated tissues, p>0.05, unpaired t-test, n=4).

Phosphoramidon had no effect (p>0.05, unpaired t-test) on the mean EF50 value for relaxation induced by EFS, in the presence of atropine (1μM) and propranolol (1μM) (EF50 1.9±0.25Hz in 2μM DTT-treated control preparations; 1.36±0.14Hz in phosphoramidon-treated tissues, n=4), and was also without effect on the maximum extent of relaxation to EFS at 20Hz (92.5±2.6% of the papaverine maximum in 2μM DTT-treated control tissues, 93±2.2% in phosphoramidon-treated tissues, n=4). However, phosphoramidon significantly (p<0.01, unpaired t-test) attenuated the relaxation to NANC_j stimulation by EFS at 2 and 3Hz (see figure 5.6b). In addition, phosphoramidon significantly (p<0.01, unpaired t-test) potentiated the contractile response to capsaicin (10μM) (20.4±4% of the maximum contraction to histamine in 2μM DTT-treated control tissues; 48.9±6% in phosphoramidon-treated preparations, n=4) (not shown in figures).

Phosphoramidon also potentiated relaxation (left-ward shift of the concentration-response curve) of the guinea-pig trachea to exogenous VIP (IC50 5.6±1nM in 2μM DTT-treated control tissues; 2.27±0.38nM in phosphoramidon-treated preparations, concentration ratio 2.5, p<0.05, unpaired t-test, n=4), but was without effect on the magnitude of the maximum relaxant response to VIP (98.4±1% control vs. 99.4±0.4% phosphoramidon-treated tissues, n=4).

Phosphoramidon was without effect (p>0.05, unpaired t-test) on the relaxation of the trachea produced by 4β-PDBu (IC50 25.5±4.8nM in 2μM DTT-treated control tissues; 31.6±5nM in phosphoramidon-treated preparations, n=4) and was also ineffective on the magnitude of the maximum relaxant response to 4β-PDBu (76.8±5.4% control vs. 78.7±6.8% phosphoramidon-treated tissues, n=4).

5.3.4 Modulation by proteases of relaxation induced by NANC_j nerve stimulation, VIP and 4β-PDBu in the guinea-pig trachea

The results are shown in figure 5.7. αChymotrypsin (5U/ml) and papain (5U/ml) used in combination attenuated markedly the relaxant response of guinea-pig trachea to NANC_j nerve stimulation and exogenous VIP, but were largely without effect on the relaxation produced by 4β-PDBu.

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Addition of α-chymotrypsin and papain to tracheal tissues produced variable effects at basal tension, which were not altered by the inclusion of atropine (1μM) and propranolol (1μM) into the protocol. In two experiments relaxation from baseline was observed (37.6% and 12.4% of the maximum relaxation to papaverine), while in one other experiment contraction occurred, which was 9% of the maximum response to histamine. However, despite this disparate effect of the proteases, their effects on the relaxants were entirely reproducible. Furthermore, the proteases did not significantly (p>0.05, unpaired t-test) alter the response to histamine (10μM) used for precontraction (56.6±2.9% of the histamine maximum contraction in control tissues, 53.6±5.2% in protease-treated preparations, n=3).

α-Chymotrypsin and papain inhibited NANCi nerve-mediated relaxation. This was demonstrated as a rightward shift of the frequency-response curve (mean IC₅₀ 2±0.03Hz in untreated tissues; 3.78±0.16Hz in protease-treated tissues, p<0.001, unpaired t-test, n=3) and a reduction in the magnitude of the maximum relaxant response (91.6±0.86% control vs. 70.3±1.45% in protease-treated tissues, p<0.001, unpaired t-test, n=3). α-Chymotrypsin and papain also significantly (p<0.01, unpaired t-test) reduced the duration of relaxation to EFS, such that after cessation of stimulation at 20Hz the time taken for 50% recovery was 390±43s in control preparations and 126±24s in protease-treated preparations (n=3). In addition, the proteases attenuated markedly the relaxant response to exogenous VIP (IC₅₀ 20.9±5.4nM in untreated preparations; >1μM in protease-treated tissues, n=3), such that maximum relaxation was not achieved.

In contrast the relaxation to 4β-PDBu in the presence of the proteases was slightly potentiated, seen as a leftward shift of the concentration-response curve (IC₅₀ 34.6±2.6nM in control preparations; 20±3.9nM in treated tissues, p<0.05, unpaired t-test, n=3) and an increase in the magnitude of the maximum relaxation (74.8±4.1% of the maximum relaxation to papaverine in control tissues; 95.6±2.3% in protease-treated tissues, p<0.05, unpaired t-test, n=3).
5.3.5 Effect of \([\text{[4C1-D-Phe}^6,\text{Leu}^{17}\text{]}\text{VIP}}\) on relaxation in guinea-pig trachea induced by NANC\(_i\) nerve stimulation, VIP and 4\(\beta\)-PDBu

The results are shown in figure 5.8. \([\text{[4C1-D-Phe}^6,\text{Leu}^{17}\text{]}\text{VIP}}\) (25\(\mu\text{M}\)) was ineffective in preventing relaxation in the trachea to NANC\(_i\) nerve stimulation, 4\(\beta\)-PDBu or exogenous VIP. \([\text{[4C1-D-Phe}^6,\text{Leu}^{17}\text{]}\text{VIP}}\) evoked an immediate, but transient contractile response from basal tone which amounted to 21.6\(\pm\)4.7\% of the maximum contraction to histamine, but was without effect (p>0.05, unpaired t-test) on the response to histamine (10\(\mu\text{M}\)) used for precontraction (50\(\pm\)0.88\% of the histamine maximum in 5\(\mu\text{M}\) DTT-treated control preparations; 53\(\pm\)4\% in \([\text{[4C1-D-Phe}^6, \text{Leu}^{17}\text{]}\text{VIP}}\)-treated tissues, n=3).

\([\text{[4C1-D-Phe}^6,\text{Leu}^{17}\text{]}\text{VIP}}\) (25\(\mu\text{M}\)) had no significant effect (p>0.05, unpaired t-test) on relaxation induced by NANC\(_i\) nerve stimulation (EF\(_{50}\) 1.2\(\pm\)0.2Hz, maximum relaxation 99.7\(\pm\)0.3\% 5\(\mu\text{M}\) DTT-treated control tissues; EF\(_{50}\) 0.93\(\pm\)0.08Hz, maximum relaxation 95\(\pm\)1.9\% \([\text{[4C1-D-Phe}^6,\text{Leu}^{17}\text{]}\text{VIP}}\)-treated preparations), exogenous VIP (IC\(_{50}\) 3.6\(\pm\)0.7nM, maximum relaxation 96\(\pm\)3.3\% 5\(\mu\text{M}\) DTT-treated control tissues; IC\(_{50}\) 2.6\(\pm\)1.3nM, maximum relaxation 99\(\pm\)0.3\% \([\text{[4C1-D-Phe}^6,\text{Leu}^{17}\text{]}\text{VIP}}\)-treated tissues) or by 4\(\beta\)-PDBu (IC\(_{50}\) 15.5\(\pm\)1.4nM, maximum relaxation 84\(\pm\)3\% 5\(\mu\text{M}\)-DTT-treated control tissues; IC\(_{50}\) 14.5\(\pm\)2.9nM, maximum relaxation 93\(\pm\)6.2\% \([\text{[4C1-D-Phe}^6,\text{Leu}^{17}\text{]}\text{VIP}}\)-treated tissues). Similar results were found with 10\(\mu\text{M}\) \([\text{[4C1-D-Phe}^6, \text{Leu}^{17}\text{]}\text{VIP}}\).

5.3.6 Effect of adenylate and guanylate cyclase inhibitors on relaxation produced in guinea-pig trachea by NANC\(_i\) nerve stimulation, VIP and 4\(\beta\)-PDBu

5.3.6a Effect of an adenylate cyclase inhibitor

The results are shown in figure 5.9. MDL 12330A (30\(\mu\text{M}\)) (cis-N-(2-phenylcyclopentyl) azacyclotridec-1-en-2-amine monohydrochloride) evoked an immediate small sustained contractile response in the trachea which amounted to 8\(\pm\)0.6\% of the histamine maximum contraction, but was without effect (p>0.05, unpaired t-test) on the response to histamine (10\(\mu\text{M}\)) used for precontraction (57\(\pm\)5\% of the maximum contraction to histamine in 0.03\% etOH-treated control preparations; 47\(\pm\)3.5\% in MDL 12330A-treated tissues, n=3).
MDL 12330A significantly (p<0.05, unpaired t-test) inhibited NANC\textsubscript{i} nerve-mediated relaxation, demonstrated as a reduction in the magnitude of the maximum relaxation to EFS at 20Hz (97±4% of the papaverine maximum in 0.03% etOH-treated control preparations; 72±8% in MDL 12330A-treated tissues, n=3). However, this attenuation was not reflected in the mean EF\textsubscript{50} values (0.79±0.16Hz in 0.03% etOH-treated control vs. 0.41±0.06Hz in MDL 12330A-treated, p>0.05, unpaired t-test, n=3).

Similarly, MDL 12330A was without effect on the mean IC\textsubscript{50} values for relaxation, or on the maximum extent of relaxation, induced by VIP (IC\textsubscript{50} 4.89±1nM, maximum relaxation 99.5±0.5%, 0.03% etOH-treated control preparations; IC\textsubscript{50} 2.25±0.43nM, maximum relaxation 99.7±0.3% MDL 12330A-treated tissues, p>0.05, unpaired t-test, n=3). However, MDL 12330A did potentiate significantly (p<0.05, unpaired t-test) the relaxant response to 4β-PDBu (68.7±4.3% of the maximum relaxation to papaverine in 0.03% etOH-treated tissues; 94.6±0.3% in MDL 12330A-treated preparations), but was without effect (p>0.05, unpaired t-test) on the mean IC\textsubscript{50} value for relaxation (12.7±4.2nM 0.03% etOH-treated vs. 3.58±1.6nM MDL 12330A-treated). However, the threshold 4β-PDBu concentration (1nM) was significantly potentiated (p<0.05, unpaired t-test) by MDL 12330A.

5.3.6b Effect of a guanylate cyclase inhibitor

The results are shown in figure 5.10. Methylene blue (10μM) induced an immediate sustained contractile response (45.9±5% of the maximum response to histamine, n=3), but had no significant effect (p>0.05, unpaired t-test) on the response to histamine (10μM) used for precontraction (63±6% of the maximum histamine response in untreated tissues; 66.9±4.7% methylene blue-treated). Methylene blue had no effect on relaxation (p>0.05, unpaired t-test) induced by NANC\textsubscript{i} nerve stimulation (EF\textsubscript{50} 2.3±0.4Hz, maximum relaxation 92.7±1.7% untreated; EF\textsubscript{50} 1.9±0.03Hz, maximum relaxation 86±5.8% methylene blue-treated, n=3) or relaxation to exogenous VIP (IC\textsubscript{50} 11.4±2.5nM, maximum relaxation 87±4% untreated; IC\textsubscript{50} 18±3nM, maximum relaxation 93.7±1.6% methylene blue-treated, n=3). However, methylene blue did appear to reduce the maximum extent of relaxation to 4β-PDBu (74.4±1.3% untreated; 59.4±8% methylene blue-treated, n=3) but this did not
achieve statistical significance. In addition, the mean IC$_{50}$ value for relaxation to 4β-PDBu was also unchanged (64.4±4nM untreated; 59.5±3nM methylene blue-treated, n=3).

5.3.7 Effect of inhibition of nitric oxide formation on relaxation in guinea-pig trachea produced by NANC$_i$ nerve stimulation and 4β-PDBu

The results are shown in figure 5.11. The guanidino substituted arginine analogue, L-NG-monomethylarginine (L-NMMA) (300μM), produced an immediate and sustained atropine (1μM)-resistant contractile response (17.5±2.9% of maximum histamine response in untreated tissues, 12±1% in atropine-treated preparations, p>0.05, unpaired t-test, n=3-4). L-NMMA was without effect on the response to histamine (10μM) used for precontraction (46.7±2.6% of maximum histamine response in untreated tissues, 53.6±7% in L-NMMA-treated preparations, p>0.05, unpaired t-test, n=3).

L-NMMA was without effect (p>0.05, unpaired t-test) on relaxation induced by NANC$_i$ nerve stimulation (EF$_{50}$ 2.4±0.05Hz, maximum relaxation 86.3±2.5% untreated; EF$_{50}$ 2.7±0.1Hz, maximum relaxation 91±2.7% L-NMMA-treated preparations, n=3) or 4β-PDBu (IC$_{50}$ 25.4±5nM, maximum relaxation 90±1% untreated; IC$_{50}$ 14.9±1.3nM, maximum relaxation 85±8% L-NMMA-treated preparations, n=4).
Figure 5.3 Comparison of the effects of 4β-PDBu at different levels of the guinea-pig tracheobronchial tree. All tissues were precontracted with histamine (approx. EC₇₀). Traces are representative of at least three separate experiments. W = wash, hist = histamine. Isotonic recording.
Figure 5.4 (a) Representative trace showing relaxation in guinea-pig trachea to increasing concentrations of exogenous VIP (in this case precontracted with histamine). For similar traces for NANC_i nerve stimulation and 4β-PDBu, see figures 5.1b and 4.4a respectively. W = wash, P = papaverine (100μM), Isometric recording. (b) Comparison of the relaxant response of the trachea to NANC_i nerve stimulation (■), VIP (●) and 4β-PDBu (▲). NANC_i nerve activation was induced by EFS (0.5ms, 100:18V, 0.25-10Hz) in the presence of atropine (1μM) and propranolol (1μM). Peptidase inhibitors, bacitracin (30μg/ml = 1.86U/ml) and aprotinin (1.2U/ml), were present throughout. All tissues were precontracted with histamine (10μM). Points represents mean ± s.e.mean of n=12-13. Isometric recording.
Figure 5.5 Effect of the peptidase inhibitors, bacitracin (30μg/ml = 1.86 U/ml) and aprotinin (1.2U/ml), on relaxation of the guinea-pig trachea to (a) NANC<sub>i</sub> nerve stimulation by EFS (0.5ms, 100:18V, 0.25-20Hz) in the presence of atropine (1μM) and propranolol (1μM), (b) exogenous VIP and (c) 4β-PDBu. Responses were obtained either in the absence (○) or presence (●) of the peptidase inhibitors. All tissues were precontracted with histamine (10μM). Points represent mean ± s.e.mean of n=3. Isometric recording.
Figure 5.6 Effect of the peptidase inhibitor, phosphoramidon (10 μM × 15 min), on the relaxation of the guinea-pig trachea to (a) NANC i nerve stimulation by EFS (0.5 ms, 100:17 V, 0.25-20 Hz) in the presence of atropine (1 μM) and propranolol (1 μM), (b) exogenous VIP and (c) 4β-PDBu. Responses were obtained either in the absence (O) or presence (●) of phosphoramidon. All tissues were precontracted with histamine (10 μM). Points represent mean ± s.e.mean of n=4. ** p<0.01 compared to the response in the absence of phosphoramidon using Student’s t-test for unpaired observations. Isometric recording. Bacitracin (1.86 U/ml) and aprotinin (1.2 U/ml) were present throughout.
Figure 5.7 Effect of the proteases, achymotrypsin (5U/ml×10min) and papain (5U/ml×10min), on relaxation of the guinea-pig trachea to (a) NANC\textsubscript{i} nerve stimulation by EFS (0.5ms, 100:18V, 0.25-20Hz) in the presence of atropine (1\textmu M) and propranolol (1\textmu M), (b) exogenous VIP and (c) 4\beta-PDBu. Responses were obtained in the absence (O) or presence (●) of the proteases. All tissues were precontracted with histamine (10\textmu M). Points represent mean ± s.e.mean of n=3. Isometric recording. Bacitracin and aprotinin were not included in these experiments.
Figure 5.8 Effect of the VIP receptor antagonist, [4Cl-D-Phe⁶,Leu¹⁷]VIP (25µM×30min), on relaxation of the guinea-pig trachea to (a) NANCᵢ nerve stimulation by EFS (0.5ms, 100:17V, 0.25–20Hz) in the presence of atropine (1µM) and propranolol (1µM), (b) exogenous VIP and (c) 4β-PDBu. Responses were obtained in the absence (○) or presence (●) of [4Cl-D-Phe⁶,Leu¹⁷]VIP. All tissues were precontracted with histamine (10µM). Points represent mean ± s.e.mean of n=3. Isometric recording. Bacitracin (1.86U/ml) and aprotinin (1.2U/ml) were present throughout.
Figure 5.9 Effect of the adenylate cyclase inhibitor, MDL 12330A (30μM×30min), on relaxation of the guinea-pig trachea to (a) NANC\textsubscript{i} nerve stimulation by EFS (0.5ms, 100: 15V, 0.25-20Hz) in the presence of atropine (1μM) and propranolol (1μM), (b) exogenous VIP and (c) 4β-PDBu. Responses were obtained in the absence (O) or presence (●) of MDL 12330A. All tissues were precontracted with histamine (10μM). Points represent mean ± s.e.mean of n=3. * p<0.05, **p<0.01 compared to responses in the absence of MDL 12330A using Student's t-test for unpaired observations. Isometric recording. Bacitracin (1.86U/ml) and aprotinin (1.2U/ml) were present in (b).
Figure 5.10  Effect of the guanylate cyclase inhibitor, methylene blue (10μM×30min), on relaxation of the guinea-pig trachea to (a) NANC\textsubscript{i} nerve stimulation by EFS (0.5ms, 100:18V, 0.25-20Hz) in the presence of atropine (1μM) and propranolol (1μM), (b) exogenous VIP and (c) 4β-PDBu. Responses were obtained in the absence (O) or presence (●) of methylene blue. All tissues were precontracted with histamine (10μM). Points represent mean ± s.e.mean of n=3. Isometric recording. Bacitracin (1.86U/ml) and aprotinin (1.2U/ml) were present in (b).
Figure 5.11 Effect of the nitric oxide synthesis inhibitor, L-NMMA (30μM×15min), on relaxation in the guinea-pig trachea to (a) NANC_i nerve stimulation by EFS (0.5ms, 100:18V, 0.25-20Hz) in the presence of atropine (1μM) and propranolol (1μM) and (b) 4β-PDBu. Responses were obtained in the absence (O) and presence (●) of L-NMMA. Points represent mean ± s.e.mean of n=3-4. In (a) tissues were precontracted with histamine (10μM). Bacitracin and aprotinin were not present in these experiments. Isometric recording.
5.4 Discussion

This chapter reports experiments which investigated the possibility that the relaxant effect of phorbol esters in the guinea-pig trachea might involve PKC-mediated release of a relaxant neurotransmitter(s), particularly from NANC\textsubscript{i} nerves. The rationale for this approach is outlined below and relies partly on evidence already accumulating in the literature, together with some of the findings reported in this study.

The first piece of evidence is derived from the literature and demonstrates a loose correlation between the ability of certain tissues to relax to phorbol esters and the existence of a NANC\textsubscript{i} innervation. For example, NANC\textsubscript{i} nerves can be demonstrated in guinea-pig ileum (Schultzberg \textit{et al.}, 1980), rat uterus (Papka \textit{et al.}, 1985) and guinea-pig trachea (see Introduction, this chapter), which all relax to phorbol esters (Baraban \textit{et al.}, 1985; Menkes \textit{et al.}, 1986; Sasaguri and Watson, 1989, 1990). On the other hand, in those tissues in which phorbol esters cause contraction such as the rat aorta (Danthuluri and Deth, 1984) and guinea-pig lung parenchyma (Dale and Obianime, 1985), few if any NANC\textsubscript{i} nerve fibres are present. Functional NANC\textsubscript{i} innervation can usually be demonstrated by tissue relaxation to EFS after adrenergic and cholinergic blockade, and in other tissues (eg. rat intestine, Belai \textit{et al.}, 1990) by the release of the putative NANC\textsubscript{i} neurotransmitter, VIP, on electrical stimulation. In the present study, careful dissection of the main bronchus in guinea-pig demonstrated that the level at which the relaxant response to the phorbol ester, 4\beta-PDBu, became contractile occurred where the cartilagenous hilar bronchus gave way to the non-cartilagenous secondary bronchus which in situ, is surrounded by lung parenchyma. In isolated guinea-pig airways NANC\textsubscript{i} responses to EFS can only be demonstrated in the trachea, with EFS of the hilar bronchus producing just contractile responses (Grundstrom \textit{et al.}, 1981). The possibility remains that NANC\textsubscript{i} responses may still be present in guinea-pig hilar bronchus, but are masked by the predominant NANC\textsubscript{c} response to EFS, and that they could account for the reduced, but still present, relaxant response to 4\beta-PDBu – assuming a degree of selectivity of 4\beta-PDBu for NANC\textsubscript{i} nerves. Whereas in the secondary bronchus the relative sparsity of VIP-IR NANC\textsubscript{i} nerves (Lundberg and Saria, 1987) might account for the small, but nevertheless present, contractile response to 4\beta-PDBu, considered to be a direct effect on the airway smooth muscle (see previous chapter).
It is of interest that the putative NANC$_i$ neurotransmitter, VIP, has an effect which is similar to 4$\beta$-PDBu in potency, magnitude of relaxation and slope. In addition, the slope and magnitude of relaxation induced by NANC$_i$ nerve stimulation by EFS, resembles that of VIP. These effects may well be fortuitous but tentatively suggest a common mechanism for relaxation of the guinea-pig trachea to these agents. Further, VIP is known to cause cyclic AMP accumulation in guinea-pig trachea (Fransden et al., 1978), and a synergistic elevation of cyclic AMP occurs with VIP when combined with the cyclic AMP-dependent phosphodiesterase inhibitor, aminophylline — one of the criteria used to implicate cyclic nucleotides in the functional response to a particular agonist. A similar synergism, although weak, appears to occur with 4$\beta$-PDBu in aminophylline-treated tracheal tissues (see section 4.3.3) suggesting that, as with VIP, some degree of interaction between 4$\beta$-PDBu and cyclic AMP could occur. This has also been suggested by Jackson et al. (1988), who recorded changes in tracheal cyclic AMP content in response to phorbol ester stimulation.

As touched upon in the previous chapter, there is now a great deal of apparently incontrovertible evidence, which is continuously expanding, to implicate PKC activation (by phorbol esters and cell-permeant DAG analogues) in the release of a variety of neurotransmitters (reviewed in detail in Miller, 1986; Kaczmarek, 1987; El-Fakahany et al., 1988); with well over one hundred reports in central nervous system (CNS) preparations — far too many to include here. In the CNS, autoradiographic techniques have shown most PKC to occur in neuronal tissue (Worley et al., 1986) with high concentrations of immunoreactive PKC being localised to the presynaptic terminals (Wood et al., 1986). Moreover, this neuronal PKC is reported to phosphorylate some of the presynaptic membrane-associated proteins which are believed to be involved in the exocytotic release of neurotransmitter from nerve terminals (Wang et al., 1988, 1989; Perin et al., 1990).

In the periphery, it has been reported that a phorbol ester and calcium ionophore were able to synergise causing the vesicular release of noradrenaline and acetylcholine from the nerve terminals of enteric neurones in guinea-pig small intestine (Tanaka et al., 1984; Hashimoto et al., 1988). Moreover, in the rat ileum longitudinal muscle strip, a tissue which also receives a NANC$_i$ innervation, PKC activation (by 4$\beta$-PDBu) has been shown to enhance the release of the putative NANC$_i$ neurotransmitter, VIP (Belai et al., 1990).
The story so far suggests that PKC not only occurs in postjunctional tissues but also prejunctionally in nerves, and that it may be important in controlling neuronal function, in particular neurotransmitter release. In addition, this proposed role for PKC is not limited just to the CNS, but may also operate in peripheral neurones, extending to NANC$_i$ mechanisms. With this information in mind, and the observation of the sequential loss of relaxation to $4\beta$-PDBu tending towards peripheral airways, it seemed possible that the "pen-trace" recording of the guinea-pig trachea in response to phorbol esters could be resultant of at least two effects: (i) An *indirect* effect of the phorbol ester involving the release of a potent relaxant, possibly from NANC$_i$ nerve terminals (figure 5.12a), and (ii) a *direct* contractile effect of the phorbol ester on the airway smooth muscle. Under normal conditions, the first effect would predominate over the second. However, under conditions in which the smooth muscle [Ca$^{2+}$]$_i$ is raised (whereupon phorbol esters cause contraction) it is the *direct* effect on the smooth muscle which would now predominate (figure 5.12b); the increased [Ca$^{2+}$]$_i$ synergising with activated PKC. A similar synergism between PKC activators and raised [Ca$^{2+}$]$_i$ has been shown in isolated bovine trachea (Park and Rasmussen, 1985) and in guinea-pig tracheal and lung parenchymal strip preparations (Menkes *et al.*, 1986; Obianime *et al.*, 1988). An alternative explanation of why $4\beta$-PDBu synergises with KCl, but reverses the contraction to carbachol and histamine, could lie in the presence of a second intracellular Ca$^{2+}$ store, discrete from that used by agonists acting via drug-receptor interaction, which is only made accessible to $4\beta$-PDBu under conditions such as depolarisation with KCl. This mechanism has been proposed to account for resting tone and KCl-induced contractions in guinea-pig trachealis (Raeburn and Rodger, 1987).

It is becoming increasingly clear that the effects of many vasoactive and bronchoactive peptides including the putative NANC$_i$ neurotransmitter, VIP, are regulated by enzymatic degradation. Accordingly, any hypothesis of the release of a relaxant which may be peptidergic in nature, could be tested by investigating the possible effects of various proteases and peptidase inhibitors. In this study the effects of three peptidase inhibitors, bacitracin (aminopeptidase inhibitor), aprotinin (αchymotrypsin, trypsin, plasmin and kallikrein inhibitor) and phosphoramidon (neutral endopeptidase inhibitor) were investigated on the relaxation of guinea-pig trachea produced by NANC$_i$ nerve stimulation by EFS, VIP and $4\beta$-PDBu. Combined incubation with bacitracin and aprotinin potentiated the relaxation
Figure 5.12 Schematic representation of the proposed mechanism to explain the dual effects of phorbol esters in the guinea-pig trachea. In (a) the predominant effect of the phorbol ester (4β-PDBu) is *indirect* and involves the hypothesised neuronal PKC-mediated release of a potent (NANC$_i$) relaxant which subsequently acts on the airway smooth muscle. In (b) a *direct* effect of the phorbol ester on the smooth muscle predominates, in which the [Ca$^{2+}$]$_i$ is raised by depolarisation (K$^+$). Under these conditions, synergism between raised [Ca$^{2+}$]$_i$ and activated smooth muscle PKC overcomes any indirect effect of the phorbol ester, leading to contraction.
produced by EFS, VIP and 4β-PDBu raising the possibility that enzymatic
degradation by aminopeptidases and/or aprotinin-sensitive proteases may
modulate the tracheal responsiveness induced by NANC_i nerve stimulation,
supporting the involvement of VIP in NANC_i responses, and further
providing some evidence for the release of a "VIP-like" substance, possibly
from NANC_i nerve terminals. Although the effects of bacitracin and
aprotinin were not studied independently, a recent report by Ellis and
Farmer (1989b) has shown that aprotinin alone did not influence responses
to EFS or to exogenous VIP, suggesting that neither the NANC_i
neurotransmitter or VIP are inactivated to any appreciable extent by
endogenous aprotinin-sensitive peptidases. By elimination, it is possible
therefore that the potentiation of the relaxant effect to EFS and VIP may
be mediated primarily by bacitracin-sensitive aminopeptidases. However, no
such firm conclusion can be drawn regarding 4β-PDBu, although its effects
appear to be closely correlated with that of NANC_i nerve stimulation and
VIP with these particular peptidase inhibitors.

The potentiation of the capsaicin-sensitive contraction of guinea-pig
bronchi to EFS by phosphoramidon, a neutral endopeptidase inhibitor (NEP;
EC 3.4.24.11), has prompted the suggestion that NEP may be important in
the regulation of neuronally released contractile tachykinins (Djokic et al.,
1989). In this study phosphoramidon significantly potentiated the relaxant
response to VIP, an effect which has also been reported by Liu et al.
(1987), but was found to be ineffective against the magnitude of the
relaxation produced by EFS or 4β-PDBu. The potentiation of the response
to exogenous VIP by phosphoramidon (10μM) seen in the present study
(concentration ratio 2.5) was not as marked as that reported by Rhoden
and Barnes (1989) (concentration ratio 3.9). The explanation for this is
most likely to be that in the experiments presented in this chapter, other
peptidase inhibitors (bacitracin and aprotinin) were also present, such that
the shift in the concentration-response curve to VIP by phosphoramidon
appears reduced. However, damage to the airway epithelium and loss of
the NEP activity could also explain this discrepancy (see later). The
effectiveness of phosphoramidon on VIP-induced relaxation, together with
its lack of effect on NANC_i nerve stimulation and 4β-PDBu, tends to
question the role of VIP in mediating the response to NANC_i nerve
stimulation in the guinea-pig trachea, but maintains the similarity in the
response between NANC_i nerve- and 4β-PDBu-evoked relaxation. However,
since there is substantial evidence in favour of a role for VIP as an NANC_i
neurotransmitter (see Introduction, this chapter), other explanations for this disparity may exist. Problems relating to accessibility to, or ineffectiveness of phosphoramidon at the sites of release of NANC_i neurotransmitter are unlikely, since in the same tracheal preparations in which NANC_i nerve stimulation was studied, the inhibitor significantly potentiated the contractile response to capsaicin. In another study, phosphoramidon was able to potentiate the contractile response of guinea-pig bronchi evoked by NANC_e stimulation (Djokic et al., 1989). One factor which may explain this discrepancy relates to the localisation of NEP in tracheal tissues. EFS-evoked and, according to our hypothesis, neuronal PKC-evoked release of the NANC_i neurotransmitter(s) from nerve terminals contained within the smooth muscle layers is probably very localised. Modulation of such responses, by enzymatic degradation, must therefore occur proximal to the neuroeffector sites. Consequently, if NEP is not localised to this area in sufficient concentrations it may not play a role in the degradation of the NANC_i neurotransmitter or of endogenously released VIP which may involve activation of neuronal PKC. This is consistent with the findings of Rhoden and Barnes (1989) who suggested that the response of guinea-pig trachea to exogenous VIP is modulated primarily by NEP localised to airway epithelium rather than smooth muscle, a view shared by Farmer and Togo (1990). The physiological relevance of this observation is unclear. It is possible that endogenous peptides (VIP, PHI/M, PHV-42) may not serve as substrates for NEP.

It is of interest to note that the NANC_i response in the presence of phosphoramidon was consistently attenuated (reaching statistical significance) at 2 and 3Hz. The reason for this is not clear, but conceivably could involve functional antagonism brought about by EFS-induced release of contractile tachykinins, protected from degradation by NEP inhibition. Inclusion of a tachykinin-receptor antagonist and/or use of capsaicin-treated tissues might well resolve this unexpected effect of phosphoramidon on the NANC_i response to EFS in the trachea.

In feline trachea the protease, a-chymotrypsin (which acts at amino-acyl bonds), abolished the relaxation to exogenous VIP, while the response to NANC_i nerve stimulation was unaffected (Altiere and Diamond, 1985). The most immediate explanation of this effect was that VIP, or related peptides, may not mediate the NANC_i responses. An alternative explanation was that endogenously-released VIP may not be susceptible, for whatever reason, to
degradation by α-chymotrypsin. However, using the guinea-pig tracheal preparation, Ellis and Farmer (1989a) recently investigated the effect of two proteases, α-chymotrypsin and papain (which acts at arginine, lysine and glycine residues), on the neurogenic NANC\textsubscript{i} response and on the relaxation to exogenous VIP, one of the putative NANC\textsubscript{i} neurotransmitters in this tissue. In the present study, these two proteases were also examined, but in combination, on the response to NANC\textsubscript{i} nerve stimulation, exogenous VIP and in addition, 4β-PDBu. The proteases were used together, and at a concentration 2.5 fold greater than in the study by Ellis and Farmer (1989a), to maximise their effect. This concentration was sufficient to abolish or greatly reduce the relaxant response of the trachea to exogenous VIP. In the present study and that reported by Ellis and Farmer (1989a), NANC\textsubscript{i} responses were significantly attenuated, but could not be abolished, suggesting that although the NANC\textsubscript{i} response in guinea-pig trachea may, at least in part be mediated by VIP (and/or PHI in the study by Ellis and Farmer), a substantial (approx. 65%) protease-resistant component to NANC\textsubscript{i} nerve stimulation was apparent. The mediator(s) responsible for this protease-resistant response may still be peptidergic, but not available for degradation by the proteases. However, since these proteases have a broad spectrum of peptide substrates it may also be possible that the mediator responsible is not a peptide (Ellis and Farmer, 1989a). This may also reflect the lack of effect of the proteases on the 4β-PDBu response, which could involve the selective release of a protease-resistant relaxant.

The evidence thus far suggests that relaxation in the trachea to 4β-PDBu could be mediated, at least in part, by a "peptide-like" relaxant which is not a substrate for NEP. In addition, Rhoden and Barnes (1989) have suggested that in guinea-pig trachea, a component of the response to EFS in the presence of atropine and propranolol, may involve the release of a TTX-resistant epithelium-derived relaxant. There remains the possibility that this relaxant may also be a peptide serving as a substrate for the proteases, and which may be reflected in the component of the NANC\textsubscript{i} response which is sensitive to these enzymes, but which is not observed with 4β-PDBu which could act selectively on NANC\textsubscript{i} nerves. In view of these recent observations by Rhoden and Barnes (1989), it might be of use to examine the effect of the proteases on the NANC\textsubscript{i} response in epithelium denuded preparations to determine the contribution, if any, of the airway epithelium to the protease-sensitive component of the NANC\textsubscript{i} response.
The residual component of the NANC$_i$ response in the presence of the proteases is unlikely to be mediated by degradation fragments of VIP since the activity of exogenous VIP, even at high concentrations, was greatly reduced by the proteases. Currently, there appears to be little information on the biological activity of the peptide fragments. That which is available suggests that the fragments of chymase-treated VIP do not possess significant biological activity or ability to interact with specific high affinity VIP binding sites (Staun-Olsen et al., 1986). In addition, Ellis and Farmer (1989a) have provided indirect evidence to suggest that reduced accessibility of these two proteases, which have similar molecular weights, to the site of NANC$_i$ neurotransmitter release is unlikely to account for their inability to abolish such responses. This was because both proteases decreased the magnitude of the NANC$_i$ response to the same extent, but papain had little or no effect on the duration of the response. However, more direct evidence is lacking.

The reason for the small protease-induced potentiation of the maximum response to 4β-PDBu is unclear, although the release by 4β-PDBu of a protease-sensitive contractile peptide such as substance P or a related tachykinin could account for this effect, particularly at higher concentrations of the phorbol ester. The lack of effect of any of the peptidases or peptidase inhibitors on the response to histamine used for precontraction, suggests that it is unlikely that any of the observations were due to non-specific effects of the agents investigated, although it may have been more appropriate to test the proteases against a relaxant such as isoprenaline.

The most direct approach to establish a role for VIP in NANC$_i$- or 4β-PDBu-mediated responses is to determine the effect of a potent and selective VIP-receptor antagonist. [4Cl-D-Phe$_6$,Leu$_{17}$]VIP, a VIP analogue, has been reported to inhibit competitively in the micromolar concentration range, VIP-induced cyclic AMP accumulation and amylase release from guinea-pig pancreatic acini (Pandol et al., 1986). However in the guinea-pig trachea, the antagonist failed to prevent relaxation to NANC$_i$ nerve stimulation, 4β-PDBu and, perhaps more disappointingly, to VIP itself. Similar negative results for NANC$_i$ nerve stimulation and VIP have been reported in guinea-pig (Ellis and Farmer, 1989b) and feline airways (Thompson et al., 1988). There are several explanations for the lack of effect of the antagonist. The most immediate is that VIP receptors
mediating relaxation to VIP in the trachea differ from those mediating pancreatic amylase release. Alternatively, diffusion barriers may prevent the antagonist gaining access to the VIP receptor in sufficiently high concentrations. This is unlikely since it is an analogue of VIP and of similar size and molecular weight. However, metabolic barriers may exist. VIP is metabolised by NEP, possibly localised to the airway epithelium (Rhoden and Barnes, 1989; Farmer and Togo, 1990), and also to some extent by other proteases in guinea-pig airways. Similarly, [4Cl-D-Phe\(^6\),Leu\(^{17}\)]VIP might also undergo degradation, precluding its access to VIP receptors in sufficient concentration. This is unlikely to occur by bacitracin- and aprotinin-sensitive catabolic routes since these inhibitors were already present in this study. However, inhibition of NEP was not employed in this particular study, when investigating the effect of the VIP antagonist, or in any of the reports in the literature. It would be interesting to repeat these experiments in the presence of phosphoramidon and/or in epithelium-denuded tissues.

For the moment, the unavailability of selective and potent antagonists to VIP receptors in the airways precludes definitive identification of VIP as the neurotransmitter mediating the response of NANC\(_i\) nerves, or investigation of its release by 4β-PDBu from presynaptic sites. Alternative approaches must be sought.

The post-junctional, post-receptor mechanism mediating neurogenic relaxation can be compared to the mechanism of relaxation of the putative NANC\(_i\) neurotransmitter, VIP, and in turn for 4β-PDBu. Thus if the NANC\(_i\) neurotransmitter, VIP and 4β-PDBu each share a common mechanism of action (elevation of cyclic AMP), then this should be supported by an inhibitor of adenylate cyclase - the enzyme responsible for generation of cyclic AMP on receptor stimulation. VIP is known to elevate cyclic AMP levels in the trachea (Fransden et al., 1978) and the evidence supporting a similar mechanism for 4β-PDBu has already been discussed (see above and Chapter 4). However, to date there do not appear to be any reports in the literature where cyclic nucleotide levels have been measured directly in response to NANC\(_i\) nerve stimulation in guinea-pig trachea. However, in a recent study (Rhoden and Barnes, 1990) the effect of two phosphodiesterase (PDE) inhibitors, SK&F 94120 (a cyclic AMP-PDE inhibitor) and zaprinast (a cyclic GMP-PDE inhibitor), on relaxation of guinea-pig tracheal smooth muscle to NANC\(_i\) nerve stimulation and VIP has been reported. These
authors have suggested that cyclic AMP may mediate relaxation of guinea-pig tracheal smooth muscle in response to NANCi nerve stimulation and VIP. However, no direct measurements of cyclic nucleotide levels were made.

In the present study MDL 12330A was investigated on the tracheal relaxation produced by NANCi nerve stimulation, VIP and 4β-PDBu. MDL 12330A is a lactam-imine which has been reported to inhibit hormone-stimulated and basal adenylate cyclase activity of rat liver preparations, but which was ineffective against ouabain-sensitive Na+/K+-ATPase activity (Siegel and Weich, 1976) or voltage-dependent Na+, K+ or T-Ca2+ channels (Rampe and Triggle, 1990). In addition, methylene blue, a putative inhibitor of soluble and guanylate cyclase (Gruetter et al., 1981) was investigated.

MDL 12330A significantly attenuated the magnitude of the response to NANCi nerve stimulation at submaximal and maximal frequencies, consistent with suggestion that cyclic AMP may mediate relaxation in this tissue to NANCi nerve stimulation (Rhoden and Barnes, 1990). However, the reverse was seen with 4β-PDBu - ie. potentiation. This also occurred with VIP at 0.3 and 1nM, although MDL 12330A was without effect on the other concentrations of VIP investigated. This latter effect, in the absence of direct measurements of tissue cyclic AMP levels, is not consistent with the proposed role of this inhibitor, assuming VIP receptor activation in this tissue does indeed involve activation of adenylate cyclase and generation of cyclic AMP. In view of the obvious difficulty of interpreting these effects and the lack of effect of the inhibitor on VIP-induced relaxation, it was decided that MDL 12330A could not be used as a reliable adenylate cyclase inhibitor in our system. This was emphasised by recent electrophysiological and biochemical findings in GH3 endocrine cells, where MDL 12330A blocked slow Ca2+ channels at a site allosterically linked to the 1,4-dihydropyridine site (Rampe et al., 1987).

In bovine retractor penis muscle and rat anoccygeus muscle, neurogenic NANCi responses are blocked by inhibitors of guanylate cyclase and are associated with an increase of the tissue content of cyclic guanosine 3',5'-monophosphate (cyclic GMP), but not cyclic AMP, suggesting that in these tissues NANCi responses are not mediated by VIP and may involve a cyclic GMP-dependent process (Bowman et al., 1982; Bowman and Drummond, 1984; see Gillespie et al., 1990). In addition, evidence is becoming increasingly available to suggest that nitric oxide (NO) mediates its relaxant
effects through activation of the guanylate cyclase (see Palmer et al., 1987) and that it may be important in mediating the cyclic GMP-dependent NANC\textsubscript{i} relaxation in both mouse and rat anococcygeus muscle (Gibson et al., 1989; Gillespie et al., 1990) and canine gastrointestinal tissue (Bult et al., 1990). However, methylene blue was without effect on the relaxation in the trachea to NANC\textsubscript{i} nerve stimulation or exogenous VIP, suggesting that these responses are not mediated by guanylate cyclase. This is in contrast to the observations of Ko and Lai (1988) who reported some degree of inhibition of the NANC\textsubscript{i} component to EFS in the guinea-pig trachea with another proposed inhibitor of guanylate cyclase, oxyhaemoglobin. In contrast, methylene blue produced a small non-significant attenuation of high concentrations (0.3–1μM) of 4β-PDBu. It is possible that guanylate cyclase may be activated at higher concentrations of the phorbol ester, but is unlikely to account for the greater proportion of the relaxation occurring at the lower concentrations of 4β-PDBu. The lack of effect of L-NMMA, a competitive inhibitor of the NO generating system, when used at a concentration shown to be maximally effective in inhibiting acetylcholine-induced endothelium-dependent relaxation in the rabbit aorta (Rees et al., 1989), suggests that NO synthesis from L-arginine and release from NANC\textsubscript{i} nerves is unlikely to account for the relaxation to EFS and 4β-PDBu in the guinea-pig trachea.

It should be borne in mind that the effects of the adenylate and guanylate cyclase inhibitors in this study may result not only from post-junctional inhibition, but also prejunctional or non-smooth muscle effects. In particular, responses to EFS represent a balance between NANC\textsubscript{e}, occasionally present in the trachea (Grundstrom et al., 1981), and NANC\textsubscript{i} components. A similar argument, according to our hypothesis must also apply to 4β-PDBu. The effects of the adenylate or guanylate cyclase inhibitors could therefore be due to differential post-junctional and/or differential prejunctional effects on both NANC\textsubscript{i} and NANC\textsubscript{e} mechanisms. The routine use of capsaicin depletion or a neurokinin antagonist may have simplified the situation. It may have been beneficial to supplement these experiments with results obtained from the use of selective cyclic AMP-dependent and cyclic GMP-dependent phosphodiesterase inhibitors had they been available. Other approaches involving direct measurements of cyclic nucleotides might also prove fruitful.
In conclusion, it has been shown that relaxation of the guinea-pig trachea by 4β-PDBu seems to be associated with the previously reported distribution of NANC\(_i\) innervation in guinea-pig airways. Furthermore, these results are consistent with relaxation of the guinea-pig trachea to 4β-PDBu being due, at least in part, to the release of a cyclic GMP-independent relaxant, possibly a peptide from NANC\(_i\) neurones. However, they do not by any means prove that this is so.
CHAPTER SIX

INVESTIGATION INTO POSSIBLE MECHANISMS OF RELEASE OF
THE NANC\textsubscript{i} NEUROTRANSMITTER IN GUINEA-PIG TRACHEA
Summary

1. The effect of two voltage-dependent sodium channel activators, veratridine (VT) and aconitine (AC), was examined on the guinea-pig tracheal strip, secondary bronchus and lung parenchyma. Both alkaloids produced dual effects in the trachea: a sustained maximum relaxation; and in the presence of eserine, a sustained contraction which was sensitive to both atropine and tetrodotoxin (TTX) and probably due to the release of neuronal acetylcholine. The relaxation could be said to be due to the release of a NANCj neurotransmitter since it occurred both in the presence and absence of atropine and propranolol, and was not modified by guanethidine. Relaxation to the alkaloids did not occur in the secondary bronchus or lung parenchymal strip, correlating with the reported distribution of functional NANCj innervation.

2. TTX reduced both the rate and extent of relaxation to VT, and was marginally less effective on the extent of relaxation to AC. These effects were partially reversed by cadmium (Cd2+) which, in the absence of TTX, did not alter the tracheal relaxation to either alkaloid or to NANCj nerve stimulation induced by EFS. However, there remained a TTX- and Cd2+-resistant component to the alkaloid-induced relaxation. All contractile responses to the alkaloids in the trachea were abolished by TTX suggesting that, while relaxation to the alkaloids may involve both TTX-sensitive and -resistant mechanisms, contraction was mediated predominantly by a TTX-sensitive mechanism.

3. Removal of the luminal airway epithelium, or incubation with cyclooxygenase inhibitors, or L-NMMA or methylene blue suggested that it was unlikely that the relaxation of the trachea to the alkaloids involved epithelium-derived inhibitory factors, relaxant eicosanoids or generation of nitric oxide from L-arginine. In addition, it seemed that the relaxation was unlikely to occur by a cyclic GMP-dependent mechanism.

4. In the presence of proteases, the relaxant response of the trachea to both alkaloids was reduced to 69% of the control response, leaving a substantial protease-resistant component to the response.
5. The inhibitors of DAG metabolism, R59022 and RHC80267, potentiated relaxation of the trachea to NANC\textsubscript{i} nerve stimulation. Potentiation of relaxation to exogenous VIP occurred only with R59022 (a DAG kinase inhibitor), whilst no effect was observed with RHC80267 (a DAG lipase inhibitor). In contrast, RHC80267 potentiated relaxation in the trachea to 4\beta-PDBu, which did not occur with R59022. To the extent that these inhibitors are specific for DAG metabolising enzymes, these results imply that DAG generation and its subsequent inactivation by the lipase route of metabolism, may be involved in NANC\textsubscript{i} nerve-mediated relaxation.

6. The PKC inhibitors, staurosporine and Ro 31-8220, at the concentrations used, were ineffective against relaxation of the trachea produced by NANC\textsubscript{i} nerve stimulation, exogenous VIP, aconitine and 4\beta-PDBu. Thus no conclusions could be drawn as to the role of PKC in these responses.
6.1 Introduction and background

In 1985, Batty et al. reported that in rat cerebral cortical slices, depolarisation induced by veratrine was accompanied by increased inositol phosphate accumulation. Further, in preparations of guinea-pig cerebral cortical synaptoneurosomes, other agents which cause sodium ion (Na\(^+\)) influx through voltage-dependent channels, such as veratridine, aconitine, batrachotoxin and various scorpion toxins also caused phosphoinositide (PI) turnover, measured by inositol phosphate accumulation (Gusovsky et al., 1986, 1987). It is now widely accepted that PI breakdown generates the endogenous PKC activator, diacylglycerol (DAG), so that PKC activation could be a consequence of agents raising intracellular Na\(^+\) levels, such as veratridine and aconitine. Further, cell permeant DAGs have been shown to stimulate neurotransmitter (dopamine) release from rat brain striatal synaptoneurosomes, an effect attributable to PKC activation (Nelson-Davis et al., 1990). The precise mechanism responsible for Na\(^+\) or Na\(^+\) channel modulation of PI turnover is unclear, but may involve interaction with guanine nucleotide regulatory proteins and/or mobilisation of Ca\(^{2+}\), either from intracellular or extracellular sources (Gusovsky et al., 1986) and in turn leading to activation of a calcium-dependent phosphodiesterase (phospholipase C) and the subsequent breakdown of PI.

Accordingly, the effects of the two alkaloids veratridine (VT) (derived from a suborder of the plant family Lilicacae) and aconitine (AC) (derived from Aconitum napellus and structurally unrelated to VT) were examined on the guinea-pig trachea. Ulbricht (1969) suggested that VT depolarised excitable cells by directly altering the voltage-sensitivity of the sodium channel such that a significant proportion of the channel population was active at the resting membrane potential, allowing influx of Na\(^+\). AC has since been found to operate through a similar mechanism (reviewed by Catterall, 1980). Both VT and AC are lipid-soluble toxins and, in neurones, often cause the release of neurotransmitters (Catterall, 1980). This approach of investigating the effect of the alkaloids in guinea-pig airways, was also prompted by the observations of Besson et al. (1983) who reported VT-induced release of the putative NANC\(_j\) neurotransmitter, VIP, from the lamina propria of rat jejunoo-ileum - a tissue containing VIP-like immunoreactive (VIP-IR) nerve fibres (Schultzberg et al., 1980).
The experiments reported in this chapter were designed to investigate possible mechanisms responsible for release of the NANC\textsubscript{i} neurotransmitter in guinea-pig trachea, and to discover the extent to which PKC activation might be involved in this process.

The contribution of PKC to the release process of the NANC\textsubscript{i} neurotransmitter from NANC\textsubscript{i} nerves was assessed by two different approaches. Initially, the effect of inhibitors of DAG metabolism (R59022 and RHC80267) was investigated on the tracheal relaxation induced by electrical stimulation of NANC\textsubscript{i} nerves, the putative NANC\textsubscript{i} neurotransmitter, VIP, and 4\beta-PDBu. This investigation was followed by a study of the effect of two putative PKC inhibitors, staurosporine and Ro 31-8220. In addition, staurosporine was also investigated on the response of the trachea to AC.

DAG, transiently generated on PI breakdown, is considered to be inactivated by at least two enzymatic pathways: phosphorylation by DAG kinase to phosphatidic acid, and deacylation by DAG lipase. The relative importance of these two routes probably varies between tissues and species studied. R59022 is reported to inhibit DAG kinase in intact platelets leading to enhanced PKC activity. The IC\textsubscript{50} for DAG kinase was 3.8\mu M with 80% inhibition of the enzyme occurring at 10\mu M (DeChaffoy de Courcelles et al., 1985). RHC80267 is reported to be a potent and selective inhibitor of DAG lipase in platelets, with an IC\textsubscript{50} of 4\mu M (Sutherland and Amin, 1982). In the same study this inhibitor was shown to be inactive against phospholipase C at 300\mu M, and to cause only 15% inhibition of phospholipase A\textsubscript{2}.

The microbial product, staurosporine, is reported to be a potent inhibitor of serine- and threonine-specific protein kinases, showing selectivity for PKC with an IC\textsubscript{50} of 3nM in a rat brain isolated PKC assay (Tomaoki et al., 1986), although a value of 10nM has also been reported (Davis et al., 1989). However, as yet there are no biochemical reports in the literature describing the effect of this inhibitor on PKC isolated from airway smooth muscle preparations. Ro 31-8220 is one of a series of newly developed compounds structurally related to staurosporine, which are reported to be potent and selective inhibitors of PKC in various isolated (IC\textsubscript{50} 8nM for isolated rat brain PKC) and whole cell systems (compound 3 in Davis et al., 1989). Both
staurosporine and Ro 31-8220 compete with ATP at PKC, and in this study were used at concentrations well in excess of their quoted IC₅₀ values in isolated PKC assays.

6.2 Experimental protocol

In a small number of preliminary experiments, it was found that solubility and solvent limitations precluded the construction of a cumulative concentration-response relationship to the alkaloids. In view of this shortcoming it was decided to study the response of the tissues to concentrations of VT and AC reported to cause maximal Na⁺-influx (Gusovsky et al., 1987), and which also produced maximum relaxation of the trachea. The remaining experiments were carried out as stated in section 2.2.
6.3 Results

6.3.1 Characterisation of the response to veratridine and aconitine at different levels of the guinea-pig tracheobronchial tree

The response produced by veratridine (VT) and aconitine (AC) at 30μM, a concentration reported to evoke near maximum Na⁺ in influx in guinea-pig cerebral cortical synaptoneuroosomes (Gusovsky et al., 1987), varied at different levels down the tracheobronchial tree. The response to VT in the trachea, secondary bronchus and lung parenchymal strip is shown in figure 6.1. Similar results were obtained with AC.

6.3.1a Guinea-pig trachea

According to the conditions employed, VT and AC were able to produce both contraction and relaxation in this tissue. Each of these responses was marked and sustained (see figure 6.2a to d).

Alkaloid induced relaxation

The relaxation produced by both alkaloids was prompt in onset (<30s) and resulted in near maximum relaxation of the tracheal preparation (figure 6.2a and c). The extent of relaxation was unaffected (p>0.05, unpaired t-test) by pretreatment with propranolol (1μM, n>3), or atropine (1μM, n>3) in combination with propranolol (figure 6.2, right-hand panel). In three experiments, pretreatment (20min) with guanethidine (10μM) was also without effect (p>0.05, unpaired t-test) on the relaxation to either VT or AC (figure 6.3a). Similarly, pretreatment (30min) of the tracheal strips with the cyclo-oxygenase inhibitors aspirin (30μM) or indomethacin (3μM) failed (p>0.05, unpaired t-test, n=3) to prevent relaxation to the alkaloids (figure 6.3b). Removal of the luminal airway epithelium in the trachea also failed (p>0.05, unpaired t-test, n=3-4) to prevent the relaxation to either VT or AC (figure 6.3c).

In contrast, the relaxation produced by the alkaloids was modified in the presence of tetrodotoxin (TTX). However, the mechanism of action of the two alkaloids appeared to differ, since although TTX caused a marked and comparable reduction in the rate of relaxation to the alkaloids (mean t₀ for VT 81.7±7.8s in control tissues, 580±140s in TTX-treated, p<0.05, unpaired t-test, n=3; t₀ for AC 78.4±8.6s in control tissues, 540±23s in TTX-treated preparations, p<0.001, unpaired t-test, n=3) (figure 6.4a and
b), the extent of relaxation was only reduced ($p<0.01$, unpaired t-test) in the case of VT, while AC remained unaltered.

The attenuation by TTX of the maximum extent of tracheal relaxation to VT, and the rate of relaxation to both alkaloids, was partially reversed by the divalent metal ion, cadmium ($Cd^{2+}$) (200$\mu$M). $Cd^{2+}$ per se, produced relaxation from intrinsic tracheal tone which was not altered ($p>0.05$, unpaired t-test) by atropine (1$\mu$M) and propranolol (1$\mu$M) in combination (44.3±8% of the maximum relaxation produced by papaverine in control tissues; 45±6.3% in atropine- and propranolol-treated tissues, n=3) or by TTX (3$\mu$M) (44.3±8% in control tissues; 44.8±8% in TTX-treated tissues, n=3). In addition $Cd^{2+}$ (200$\mu$M) alone, and when in combination with TTX, was without effect ($p>0.05$, unpaired t-test) on the tracheal response to histamine (10$\mu$M) used for precontraction (49.2±3.7% of the histamine maximum contraction in control tissues; 39±6% in $Cd^{2+}$-treated preparations, 44.8±8% in $Cd^{2+}$ and TTX-treated preparations, n=3). Similarly, $Cd^{2+}$ was also without effect ($p>0.05$, unpaired t-test) on the rate or extent of relaxation to either VT (mean $t_{50}$ 81.7±7.8s, maximum relaxation 93±1.4% in control tissues; $t_{50}$ 95.3±6s, maximum relaxation 94.6±1.6% in $Cd^{2+}$-treated preparations, n=3) or AC (mean $t_{50}$ 74.9±9s, maximum relaxation 85.4±1.6% in control tissues; $t_{50}$ 78.4±8.6s, maximum relaxation 91.6±2.5% in $Cd^{2+}$-treated preparations, n=3) and on the relaxation to NANC$_i$ nerve stimulation (figure 6.4).

L-$N^G$-monomethylarginine (L-NMMA) (300$\mu$M), a guanidino substituted arginine analogue, which prevents the formation of nitric oxide from L-arginine (Rees et al., 1989), was also unable to prevent the relaxant effect of the two alkaloids in the trachea (figure 6.5a). Similar findings were observed with the guanylate cyclase inhibitor, methylene blue (10$\mu$M) (figure 6.5b).

The extent of relaxation to both VT and AC (in the presence of atropine and propranolol) was reduced to 69% of the control value by combined preincubation (10min) with the proteases, papain (5U/ml) and a-chymotrypsin (5U/ml) (figure 6.5c). However, no effect was observed on the rate of relaxation to VT or AC (mean $t_{50}$ for VT 81.7±7.8s in control tissues, 90±5s in protease-treated tissues; $t_{50}$ for AC 74.9±9s in control tissues, 85±3s in protease-treated preparations, $p>0.05$, unpaired t-test, n=3).
Alkaloid induced contraction

As indicated above, the action of the two alkaloids did not appear to be identical. This was emphasised further in tracheal tissues pretreated with eserine (0.5μM), where AC caused marked relaxation followed by contraction; while VT caused an initial contraction followed by relaxation and then a further, more marked contraction (n>6) (figure 6.2b and d, 6.6a and b). This phenomenon with eserine was observed in all experiments. In tracheal tissues taken from at least five animals, eserine (0.5μM) per se evoked a delayed, but sustained contractile response (46±6% of the maximum contractile response to histamine). In each case the resultant contraction seen with eserine was subtracted from the alkaloid-induced response. All contractions to the alkaloids, evoked in the presence of eserine, were found to be both TTX (3μM)- and atropine (1μM)-sensitive (figure 6.6). In three experiments, atropine (1μM) given prior to the alkaloids caused total inhibition (figure 6.2, right-hand panel) and when given after, caused complete reversal of the contraction to both VT and AC (figure 6.2 and 6.6, left-hand panels). TTX (3μM) again reduced the rate of response to both alkaloids, but only reduced (p<0.01, unpaired t-test) the extent of relaxation to VT (figure 6.6).

6.3.1b Guinea-pig secondary bronchus

For these studies only VT (30μM) was investigated. Tissues were precontracted with histamine (10μM) which caused 41.6±3.8% of the histamine maximum contraction. VT (30μM) evoked a small contractile response (18.3±1.2% of the histamine maximum contraction, n=3). This response, once stable, was not reversed by atropine (1 and 3μM) (see figure 6.1). VT was not investigated at intrinsic tone on this tissue.

6.3.1c Guinea-pig lung parenchyma

In all experiments (n=4) neither VT or AC (30μM) had any relaxant effect on this tissue. However, some degree of contraction, which was atropine-sensitive (figure 6.1, results for AC not shown), was observed — amounting to 8±1% of the histamine maximum response for VT, and 6±0.9% for AC. Eserine (0.5μM) was without effect on this preparation.
6.3.2 Effect of inhibitors of DAG metabolism

R59022 and RHC80267 in their own right produced concentration-dependent relaxation of the trachea from intrinsic tone, which was rapid in onset (<1min) and plateaued within 7-12min. However, RHC80267 appeared more efficacious than R59022 (see figure 6.7a). In view of this relaxation the inhibitors were tested on the contractile response to histamine, which was used to restore tracheal tone.

6.3.2a Effect of inhibitors of DAG metabolism on histamine-induced tracheal contraction

RHC80267 potentiated the contraction in guinea-pig trachea induced by histamine. This was seen as a left-ward shift of the concentration-response curve and a marked increase in the maximum extent of contraction. However, no effect was observed at lower concentrations (<3µM) of histamine. Significant decreases (p<0.05, paired t-test) in mean EC$_{50}$ values for contraction to histamine were obtained at 3 and 30µM RHC80267 (table 6.1), and significant increases in the maximum contractile response to histamine (100µM) were observed at each concentration of the DAG lipase inhibitor investigated (figure 6.7b).

In contrast, R59022 inhibited the contraction in the trachea produced by histamine. This was seen as a marked right-ward shift of the concentration-response curve and a substantial depression in the maximum extent of contraction. The depression of the maximum contractile response was so marked that a mean EC$_{50}$ value for histamine could only be determined at 3µM R59022. The response to histamine failed to reach 50% maximum of the control response when higher concentrations of the inhibitor (10 and 30µM) were tested (figure 6.7c).

6.3.2b Effect of inhibitors of DAG metabolism on tracheal relaxation produced by NANC$_{i}$ nerve stimulation, exogenous VIP and 4β-PDBu

In view of the relatively small relaxation produced at intrinsic tracheal tone by R59022, and its marked inhibitory effect on the contraction to histamine, relaxant responses in experiments using this inhibitor to NANC$_{i}$ nerve stimulation, VIP and 4β-PDBu were recorded from intrinsic tone. In contrast, however, in order to determine whether any reduction in relaxation induced by RHC80267 could be due to an enhancement in the level of precontraction, concentrations of histamine
were selected which produced a contraction (in the presence of varying concentrations of RHC80267) similar to that produced by 10µM histamine in the absence of the inhibitor. These concentrations were determined from the data presented in figure 6.7b such that there was no significant difference (p>0.05, paired t-test) in the contraction to histamine in the presence of 10µM RHC80267 (contraction induced by 10µM histamine 54.7±4% in 0.02% DMSO-treated control tissues; contraction induced by 6.3µM histamine 57±3% in RHC80267-treated tissues, n=3) or 30µM RHC80267 (contraction induced by 10µM histamine 65.9±4% in 0.02% DMSO-treated tissues; contraction induced by 3.5µM histamine 68±6% in RHC80267-treated preparations, n=3).

Both inhibitors of DAG metabolism caused significant (p<0.05, unpaired t-test) potentiation of relaxation induced by NANC\textsubscript{i} nerve stimulation; seen as a left-ward shift of the frequency-response curve (mean EF\textsubscript{50} 1.49±0.25Hz in control tissues vs. 0.74±0.07Hz in R59022-treated preparations; EF\textsubscript{50} 2.1±0.3Hz vs. 1.13±0.07Hz in RHC80267-treated tissues, n=3) (figure 6.8a and 6.9a). However, a significant (p<0.001, unpaired t-test) potentiation of relaxation to VIP (left-ward shift of the concentration-response curve) was also seen with R59022 (IC\textsubscript{50} 5.7±0.2nM control vs. 0.69±0.05nM in R59022-treated preparations, n=3) which did not occur with RHC80267 (IC\textsubscript{50} 10.4±2.7nM control vs. 13±2nM in RHC80267-treated preparations, n=3) (figure 6.8b and 6.9b). Conversely, RHC80267 significantly (p<0.01, unpaired t-test) potentiated relaxation of the tracheal to 4β-PDBu (IC\textsubscript{50} 17±1.5nM control vs. 4.7±1.4nM in RHC80267-treated tissues, n=3), which was not seen with R59022 (IC\textsubscript{50} 21.2±7nM control vs. 27±8nM in R59022-treated tissues, p>0.05, unpaired t-test, n=3) (figures 6.8c and 6.9c). The maximum extent of relaxation to each of the relaxants was unaffected by the concentrations of the DAG metabolic inhibitors investigated. R59022 was also studied at 3µM, but was without effect on any of the relaxants investigated. Curiously, the potentiation observed with RHC80267 at 10µM to each of the relaxants, did not occur at 30µM.
6.3.3 Effect of PKC inhibitors on tracheal relaxation produced by NANC nerve stimulation exogenous VIP, 4β-PDBu and Aconitine (AC)

The results are shown in figures 6.10 and 6.11. The PKC inhibitors, staurosporine and Ro 31-8220, at the concentrations investigated, both caused an immediate and sustained relaxation from intrinsic tracheal tone which plateaued after 8-10 min (staurosporine, 35nM 15.5±5% of the maximum relaxation produced by papaverine; Ro 31-8220, 10μM 26±3.6%, n=3). The inhibitors could therefore be tested at intrinsic tone since they produced little effect in their own right.

Staurosporine (35nM) and Ro 31-8220 (10μM) were ineffective (p>0.05, unpaired t-test) in preventing NANC nerve-induced relaxation of the trachea (EF50 2.4±0.4Hz in control tissues vs. 2.2±0.3Hz in staurosporine-treated preparations; EF50 2.2±0.2Hz in control tissues vs. 1.75±0.4Hz in Ro 31-8220-treated preparations, n=3). Similarly, both inhibitors had no significant effect (p>0.05, unpaired t-test) on relaxation to exogenous VIP (IC50 8.5±0.8nM in control tissues vs. 4±1nM in staurosporine-treated preparations; IC50 3.1±0.75nM in control tissues vs. 2.6±0.48nM in Ro 31-8220-treated tissues, n=3) or 4β-PDBu (IC50 12±1nM in control tissues vs. 12.8±4nM in staurosporine-treated preparations; IC50 12.7±2.5nM in control preparations vs. 11.6±2nM in Ro 31-8220-treated tissues, n=3). The maximum extent of relaxation to each of the relaxants was unaffected by the putative PKC inhibitors (figure 6.10 and 6.11). Staurosporine was also investigated at 75 and 100nM. However, at these concentrations intrinsic tracheal tone could not be maintained and fell to near maximum relaxation (n=3). Artificial elevation of tone with histamine was similarly unsuccessful, and in all experiments (n=3) the response to histamine could not be maintained and fell to total relaxation after 60-85 min. In two experiments staurosporine given cumulatively (0.035-1μM) did not reverse relaxation induced by 4β-PDBu (1μM). In addition, AC (30μM) which caused total relaxation in the presence of atropine (1μM) and propranolol (1μM), was not reversed by staurosporine (35 and 100nM) given either after the relaxation had plateaued, or 10 min before addition of the alkaloid (n=3).
Figure 6.1 Comparison of the effect of veratridine (VT) (30μM) at different levels of the tracheobronchial tree. All tissues were precontracted with histamine (approx. EC_{50}). Traces are representative of at least 3-4 separate experiments. W = wash, hist = histamine.
Figure 6.2 Left-hand panel: Representative traces showing the effect of eserine (0.5μM) (see b and d) on the relaxant response of the trachea to a single application of either veratridine (VT) shown in (a) or aconitine (AC) shown in (c). Both alkaloids were used at 30μM and propranolol (1μM) was present throughout. Hist = histamine, Pap = papaverine (100μM), W = wash.

Right-hand panel: Mean responses of six experiments (of which one is shown in the left-hand panel) showing the effect of eserine (Es) on the control relaxation to the alkaloids. Note the complete reversal of contraction by atropine (A) (1μM) when given after (left-hand panel) or 30min before the alkaloids (see right-hand panel, A+Es). Propranolol (P) (1μM) was present in all experiments shown, except for the first two columns in this figure. Data are presented as mean ± s.e.mean. Isometric Recording.
Figure 6.3 The relaxant response of the trachea to both veratridine and aconitine after (a) pretreatment (20 min) with guanethidine (Guan, 10 µM), (b) pretreatment (30 min) with the cyclo-oxygenase inhibitors, aspirin (Asp, 30 µM) and indomethacin (Indo, 3 µM) and (c) removal of luminal airway epithelium. In (a) and (c) relaxation was recorded from intrinsic tone, while in (b) tracheal tone was restored with histamine (10 µM). Atropine (1 µM) and propranolol (1 µM) were present in all experiments (n=3–4). Isometric recording.
Figure 6.4 The results of three experiments in the trachea, in which the effects of cadmium (Cd\(^{2+}\), 200\(\mu\)M) and tetrodotoxin (TTX, 3\(\mu\)M) were compared on the rate and extent of relaxation to (a) veratridine (VT) and (b) aconitine (AC). The effect of Cd\(^{2+}\) (200\(\mu\)M) on NANC\(_{1}\) nerve-induced relaxation induced by EFS (100:18V; 0.5ms, 0.25-20Hz) is shown in (c). Atropine (1\(\mu\)M) and propranolol (1\(\mu\)M) were present in all experiments, and all tissues were precontracted with histamine (10\(\mu\)M). Data are presented as mean ± s.e.mean of n=3. Isometric recording.
Figure 6.5 The relaxant response of the trachea to veratridine and aconitine after (a) preincubation (15min) with L-\(\text{NG}\)-monomethylarginine (L-NMMA) (300\(\mu\text{M}\)), (b) pretreatment (15min) with methylene blue (10\(\mu\text{M}\)) and (c) combined preincubation for 10min with the proteases, \(\alpha\)-chymotrypsin (5U/ml) and papain (5U/ml). None of the inhibitors affected the response to histamine (10\(\mu\text{M}\)) used for precontraction. Atropine (1\(\mu\text{M}\)) and propranolol (1\(\mu\text{M}\)) were present in all experiments (\(n=3\)). ** \(p<0.01\) compared to the response in the absence of proteases using Student's t-test for unpaired observations. Isometric recording.
Figure 6.6  

Left-hand panel: Representative traces showing abolition of the contractile response of the trachea to (a) veratridine (VT) and (b) aconitine (AC) in the presence of TTX (3μM) (c and d). Responses shown were obtained from the same animal. Both alkaloids were used at 30μM and propranolol (1μM) was present throughout. Hist = histamine, pap = papaverine (100μM), W = wash.

Right-hand panel: Mean responses showing the effect of TTX on the alkaloid-induced relaxant and contractile (Es) responses of the trachea. Note TTX significantly reduced (** p<0.01 compared to responses in the absence of TTX using unpaired Student's t-test, n=3) the relaxant response to VT. All experiments were carried out in the presence of propranolol (1μM). Data are presented as mean ± s.e.mean of n=3.
Figure 6.7 Effect of the inhibitors of DAG metabolism, R59022 and RHC80267, on (a) intrinsic tracheal tone. Effect of (b) RHC80267 and (c) R59022 on the contraction of guinea-pig trachea to histamine. In (b) and (c) responses were obtained in the absence (O) and presence of 3μM (●), 10μM (■) or 30μM (▲) R59022 and RHC80267 after 15min preincubation. Data are presented as mean ± s.e.mean of n=3-5 animals. * p<0.05, ** p<0.01, ***p<0.001 compared to the response in the absence of the inhibitors using Student's t-test for paired observations. Isometric recording.
Figure 6.8 Effect of R59022 (10μM×15min) on relaxation of the guinea-pig trachea to (a) NANCᵢ nerve stimulation by EFS (0.5ms, 100:17V, 0.25-20Hz) in the presence of atropine (1μM) and propranolol (1μM), (b) exogenous VIP and (c) 4β-PDBu. Bacitracin (1.86U/ml) and aprotinin (1.2U/ml) were present in (b). Responses were obtained in the absence (O) and presence (●) of R59022 from intrinsic tone. Points represent mean ± s.e.mean of n=3. Isometric recording.
Figure 6.9  Effect of RHC80267 (10μM×15min) on relaxation of the guinea-pig trachea to (a) NANC<sub>i</sub> nerve stimulation by EFS (0.5ms, 100:16V, 0.25–20Hz) in the presence of atropine (1μM) and propranolol (1μM), (b) exogenous VIP and (c) 4β-PDBu. Bacitracin (1.86U/ml) and aprotinin (1.2U/ml) were present in (b). Responses were obtained in the absence (○) and presence (●) of RHC80267 precontracted with 10 and 6.3μM histamine respectively. Points represent mean ± s.e.mean of n=3. Isometric recording.
Figure 6.10 Effect of staurosporine (35nM×10min) on relaxation of the guinea-pig trachea to (a) NANC<sub>i</sub> nerve stimulation by EFS (0.5ms, 100:18V, 0.25-20Hz) in the presence of atropine (1μM) and propranolol (1μM), (b) exogenous VIP and (c) 4β-PDBu. Bacitracin (1.86U/ml) and aprotinin 1.2U/ml) were present in (b). Responses were obtained in the absence (〇) and presence (●) of the inhibitor from intrinsic tone. Points represent mean ± s.e.mean of n=3. Isometric recording.
Figure 6.11 Effect of Ro 31-8220 (10μM×20min) on relaxation of the guinea-pig trachea to (a) NANC<sub>i</sub> nerve stimulation by EFS (0.5ms, 100:18V, 0.25-20Hz) in the presence of atropine (1μM) and propranolol (1μM), (b) exogenous VIP and (c) 4β-PDBu. Bacitracin (1.86U/ml) and aprotinin (1.2U/ml) were present in (b). Responses were obtained in the absence (O) and presence (●) of the inhibitor from intrinsic tone. Points represent mean ± s.e.mean of n=3. Isometric recording.
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<td>R59022 (30μM)</td>
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Table 6.1 Effect of inhibitors of DAG metabolism on contraction of guinea-pig trachea to histamine. * p<0.05, *** p<0.001 compared to responses in the absence of inhibitors of DAG metabolism using Student's t-test for unpaired observations. Figures represent mean ± s.e.mean of n=12-13 for control (0.01%v/v DMSO) values and n=4-5 for values obtained in the presence of inhibitors. Isometric recording.
6.4 Discussion

As indicated in the previous chapter there are several lines of evidence which implicate VIP and/or the related peptide, PHI, as a neurotransmitter mediating at least part of the NANC<sub>1</sub> response in guinea-pig trachea. VIP produces a prolonged relaxation of isolated airway smooth muscle preparations which is unaffected by adrenergic blockade, and which mimics the time-course of NANC<sub>1</sub> responses in vitro and in vivo (Barnes, 1988). Results which were comparable with NANC<sub>1</sub> nerve stimulation, and to some extent with the relaxant effect of VIP and PHI in tracheal smooth muscle, were obtained in this study to the alkaloids, veratridine (VT) and aconitine (AC). Furthermore, in the secondary bronchus and lung parenchyma, where there are relatively few VIP receptors and VIP-IR nerve fibres to the bronchiolar smooth muscle (see Barnes, 1986b), the alkaloids failed to produce relaxation. Similarly, the density of cholinergic innervation decreases in smaller airways, possibly reflecting the reduction in the atropine-sensitive component of the response of the alkaloids in the bronchial and parenchymal preparations. The lack of responsiveness of the lower airway preparations to VT and AC may also indicate that the predominant action of these alkaloids is neuronal and does not involve a direct effect on the smooth muscle in which there are very few voltage-activated Na<sup>+</sup> channels. Moreover, sodium influx in smooth muscle is usually associated with contraction of the muscle and not relaxation. Further, in guinea-pig ileum, which has a similar embryological derivation to lung tissue, Na<sup>+</sup> channels are found only on neurones and not on smooth muscle (Gerschon, 1967).

Relaxation of the trachea to both VT and AC was unaffected by cyclo-oxygenase inhibition, luminal airway epithelium removal and inhibition of the nitric oxide-generating system. Thus it seemed unlikely that the relaxation induced by either alkaloid in the trachea involved the release of relaxant eicosanoids, epithelium-derived inhibitory factor(s) or the generation of nitric oxide from L-arginine. The possibility that nitric oxide could be generated by other synthetic routes must remain a possibility. Nitric oxide is a potent activator of guanylate cyclase and has been postulated to account for the NANC<sub>1</sub> relaxation seen in the mouse and rat anoococcygeus muscle (Gibson et al., 1989; Gillespie et al., 1989), and in canine gastrointestinal tissue (Bult et al., 1990). However, combining the lack of effect of L-NMMA with the
ineffectiveness of methylene blue, a putative soluble and particulate guanylate cyclase inhibitor (Gruetter et al., 1981), in preventing the relaxant response of the trachea to both VT and AC substantiates further the argument that nitric oxide is unlikely to be involved in this response. Furthermore, pretreatment of the trachea with propranolol or guanethidine indicated that it was unlikely that adrenergic neurotransmitters acting at β-adrenoceptors, or that relaxants co-released with noradrenaline, were important in mediating the response of this tissue to VT or AC.

Addition of a specific cholinesterase inhibitor, eserine, to the isolated airway tissues caused a substantial increase in tracheal tone, which was not observed in the lung parenchyma. This implies either a degree of spontaneous cholinergic bronchomotor tone in the trachea or direct release of acetylcholine by eserine. These mechanisms do not appear to operate in the lung periphery, once more reflecting the view that the density of cholinergic innervation appears to decrease in smaller airways (Barnes, 1986b). Addition of the alkaloids after eserine, converted the previously observed relaxation in the trachea to contraction. However, the profile of contraction to VT was not identical to that of AC and it was evident that there was a qualitative difference in the way in which the two alkaloids caused this unexpected response. The reason for this anomaly is not clear. What is clear is that the contraction seen in the trachea to both VT and AC in the presence of eserine, was mediated by neuronally-released acetylcholine acting at cholinergic muscarinic receptors, since it was abolished by both TTX and atropine. Further, since the contraction to the alkaloids was completely reversed by atropine, it appeared that the response in the trachea was entirely due to acetylcholine, and that tachykinins and other spasmogens were not involved. However, this may not reflect the situation in the secondary bronchus, where atropine-resistant capsaicin-sensitive NANCe responses tend to predominate, and in which VT caused only contraction which was not blocked by atropine.

It is of interest to note that contraction in the trachea to both alkaloids appeared so dependent on the presence of eserine. It is possible that the relaxation involved a component of acetylcholine release which was rapidly inactivated and insufficient to overcome the predominant inhibitory effect seen to both alkaloids, and that only when the
concentration of acetylcholine was allowed to increase sufficiently (ie. after cholinesterase inhibition) did the atropine-sensitive contracture become evident. This may also explain the delay experienced when recording this excitatory response, which occurred after the initial relaxant action of the alkaloids, particularly in the case of AC.

TTX reduced both the rate and extent of the VT-induced relaxation (and also contraction, see later) in the trachea, suggesting that a TTX-sensitive sodium channel population was at least partially responsible for the relaxation. However, the relaxation was not abolished by TTX, and so the contribution to the response by a mechanism which was either unrelated to sodium channel activation, or due to an effect on a TTX-resistant population of sodium channels, was examined. Indeed, Gusovsky et al. (1987) had provided evidence for such a population of TTX-resistant sodium channels which were sensitive to blockade by the divalent metal ion, cadmium (Cd²⁺). Cd²⁺ is also reported to block (non-selectively) voltage-dependent Ca²⁺ channels (see Watson et al., 1990). The former possibility was explored in the trachea, to attempt to account for the TTX-resistant component of the alkaloid-induced response. Cd²⁺ alone, did not affect the rate or extent of the alkaloid-induced relaxation, correlating with similar results obtained to NANCᵢ nerve stimulation. This suggested that the mechanisms responsible (eg. Ca²⁺ influx) for the release and/or action of the NANCᵢ neurotransmitter were not affected to any appreciable extent by the concentration of Cd²⁺ used. However, when used in combination with TTX, Cd²⁺ appeared to reverse partially the TTX-induced reduction in rate and extent of relaxation to both alkaloids; instead of blocking the residual TTX-resistant component. It is possible that Cd²⁺ might compete with TTX for the same binding site (without altering its properties) - presumably the TTX-sensitive, voltage-dependent sodium channel, although other sites cannot be ruled out.

Examination of the data obtained with TTX demonstrated that both the rate and magnitude of relaxation to VT was reduced, while only the rate of relaxation seemed to be reduced with AC. The reason for this difference is not clear, but once again emphasises an apparently subtle difference in the mode and/or kinetics of action of these two alkaloids. It is possible that if the alkaloids (by persistent activation) were able to modify the sodium channel population, they might render the majority
insensitive to TTX which might also increase the permeability of the modified channels to larger cations such as calcium - triggering neurotransmitter release.

Combined incubation of the tissues with the proteases, papain and α-chymotrypsin, reduced the relaxant response in the trachea to both alkaloids. The extent of the reduction (69% from control) to both VT and AC was remarkably similar to results obtained by Ellis and Farmer (1989b), who reported a protease-induced reduction (to 65%) in the maximum relaxant response to NANCi nerve stimulation by EFS after adrenergic and cholinergic blockade, observed also in the present study (see figure 5.7 and Discussion, Chapter 5). Again in accord with results of Ellis and Farmer (1989b), there remained a substantial protease-resistant component to NANCi nerve stimulation which was also apparent with the alkaloids. Further, the concentration of the proteases used in the present study, and that of Ellis and Farmer, was sufficient to attenuate dramatically or abolish the response of the trachea to putative NANCi peptide neurotransmitters, such as VIP and PHI, when applied exogenously.

Taken together, the results so far suggest that in guinea-pig trachea, these alkaloids may cause the release of the inhibitory NANC neurotransmitter(s) (accounting for the relaxation) and of acetylcholine (accounting for the contraction), but not of excitatory NANC neurotransmitters (although this may not be the case in the bronchus) or sympathetic adrenergic neurotransmitters. Moreover, there is now evidence for the co-localisation within post-ganglionic nerve fibres (and co-release) of the putative NANCi neurotransmitter, VIP and PHI, with acetylcholine (see Barnes, 1986b). Consequently, the dual effects of these alkaloids might suggest further that in guinea-pig trachea they activate selectively a particular nerve fibre population.

How might these alkaloids bring about the release of the NANCi neurotransmitter?

The scheme proposed by Gusovsky et al. (1986, 1987), in which Na⁺ influx upon activation of voltage-dependent sodium channels (both TTX-sensitive and -insensitive) induced by VT and AC, lead to an associated phosphoinositide (PI) turnover, provided an important clue to
this question. PI breakdown would cause the generation of DAG, the endogenous PKC activator, so that PKC activation could be a consequence of the two alkaloids. In the guinea-pig trachea it seemed possible (see above discussion) that these two alkaloids could bring about the release of the NANC<sub>i</sub> neurotransmitter, and it is hypothesised that phorbol esters (activating PKC) produce tracheal relaxation which is consistent with the release of the NANC<sub>i</sub> neurotransmitter(s) (see previous chapter). The evidence implicating neuronal PKC in the regulation of neurotransmitter release is substantial and has been discussed in the previous chapter.

In this study the inhibitors of DAG metabolism, R59022 and RHC80267, were investigated to test indirectly the contribution of PKC to the NANC<sub>i</sub> nerve-mediated response of the trachea. Both inhibitors of DAG metabolism potentiated the relaxant response of the trachea to NANC<sub>i</sub> nerve stimulation, suggesting that DAG, presumably via PKC activation, could be involved in NANC<sub>i</sub> nerve-mediated relaxation. This effect could result from prejunctional enhancement of the release of the neurotransmitter, or postjunctional facilitation of the action of the neurotransmitter. Thus in the absence of the precise identity of the NANC<sub>i</sub> neurotransmitter in guinea-pig airways, and quantification of its release, it was necessary to investigate also the effect of both inhibitors on the relaxation produced by the putative NANC<sub>i</sub> neurotransmitter, VIP, which is considered to act at postjunctional receptors which are not directly linked to PI hydrolysis. Further, in the absence of any direct evidence to the contrary, any potentiation in the NANC<sub>i</sub> nerve-mediated response, not reflected in the response to exogenous VIP, could represent a facilitation of the release of the NANC<sub>i</sub> neurotransmitter. This was the case with RHC80267, the DAG lipase inhibitor, suggesting that PKC activation may be important in neurotransmitter release from electrically stimulated NANC<sub>i</sub> nerves. Moreover, the mechanism could involve DAG generation, possibly from PI, which might then be deacylated primarily by the lipase route of metabolism. The reason for the marked enhancement of VIP-induced relaxation of the trachea in the presence of R59022 is not clear, but may represent a post-junctional effect unrelated to DAG kinase inhibition.

RHC80267 potentiated relaxation of the trachea to 4β-PDBu, which was not seen with R59022. These results were intriguing since one would
not expect to see enhancement of the phorbol ester response in the presence of inhibitors of DAG metabolism, since phorbol esters are considered to act down-stream of DAG metabolism. The explanation for this unexpected enhancement may reside with recent reports in which phorbol esters can in their own right mobilise DAG from phosphatidylcholine (Daniel et al., 1986). In addition it has been proposed that the phorbol ester, PMA, can be metabolised by DAG metabolising enzymes (Cabot, 1984). These effects could of course occur either pre- or postjunctionally, tending to complicate the interpretation of the RHC80267-induced enhancement of relaxation in the trachea to 4β-PDBu.

Similar constraints must also apply to the use of PKC inhibitors. However, in this study it was not possible to substantiate the involvement of DAG (possibly metabolised by the lipase route) in the response of the trachea to NANCı nerve stimulation, since both PKC inhibitors, staurosporine and Ro 31-8220, were ineffective in preventing tracheal relaxation induced by either exogenous VIP, or NANCı nerve stimulation by EFS or aconitine, or surprisingly, 4β-PDBu. The immediate interpretation of this result would be that PKC activation is not necessary for the release of the NANCı neurotransmitter, if it were not for the fact that the inhibitors had no effect on the relaxation of the trachea to 4β-PDBu. This was unexpected, particularly as staurosporine is reported to be a highly potent inhibitor of PKC, showing greater than 10-fold selectivity for PKC than the cyclic AMP- and cyclic GMP-dependent protein kinases (Tomaoki et al., 1986). However, this degree of selectivity is not reflected in a recent review by Ruegg and Burgess (1989) despite general agreement in the potency of this inhibitor. Another possibility for the lack of effect of these structurally related PKC inhibitors may be that PKC in the trachea (both neuronal and non-neuronal) is not susceptible to these inhibitors at the concentrations used. However, a more likely explanation may lie in poor accessibility of the inhibitors to the enzyme due to surface dilution effects and/or enzymatic inactivation of the inhibitors.

It is of interest to note that the DAG lipase inhibitor, RHC80267, caused a marked potentiation of contraction of the trachea to histamine. This effect was seen on both the mean EC₅₀ value (particularly at 3 and 30μM) and on the magnitude of the maximum contraction to histamine. One obvious explanation for this effect is that DAG, generated as a
result of histamine H₁-receptor-mediated PI hydrolysis in the tracheal smooth muscle (Grandordy et al., 1987), is enzymatically inactivated by the DAG lipase route. In contrast, the DAG kinase inhibitor, R59022, suppressed markedly the contraction of the trachea to histamine in an apparently non-competitive manner. This is consistent with the reported property of R59022 being a histamine H₁-receptor antagonist (De Chaffoy de Courcelles et al., 1985), making it unsuitable for the investigation of intracellular signalling in response to histamine. A similar finding has been reported by Lippe and Holzer (1989) in guinea-pig intestinal smooth muscle.

In conclusion, the relaxant profile of the two voltage-dependent sodium channel activators, VT and AC, in guinea-pig airways reflects closely the component of electrical stimulation which is attributable to NANCᵢ nerve activation and the release of the NANCᵢ neurotransmitter(s). Further, the release mechanism may involve the generation of DAG, the endogenous PKC activator, and its subsequent metabolism by the DAG lipase route. This is consistent with the hypothesis that relaxation of the guinea-pig trachea to phorbol esters might involve PKC-mediated release of a neurotransmitter from NANCᵢ nerve endings.
CHAPTER SEVEN

DIFFERENTIATION OF NEURONAL AND NON-NEURONAL RESPONSES TO 4β-PDBu IN THE GUINEA-PIG TRACHEAL STRIP
Summary

1. Various techniques were employed in attempts to differentiate neuronally- and non-neuronally-mediated relaxation of guinea-pig trachea to NANC₃ nerve stimulation, voltage-sensitive Na⁺ channel activators and to phorbol esters.

2. In tracheal tissues treated with black widow spider venom in the form of either the venom crude homogenate (VCH) or its purified toxin, α-latrotoxin (αltx), the relaxation to NANC₃ nerve stimulation, veratridine and (in one experiment) 4β-PDBu, was reduced while the contractile response to histamine remained unaltered.

3. In tracheal tissues exposed to capsaicin, relaxation to both 4β-PDBu and NANC₃ nerve stimulation by EFS was enhanced implying that sensory nerve neuropeptides released by EFS or 4β-PDBu may modulate NANC₃ nerve- and/or 4β-PDBu-induced responses.

4. In tracheal tissues treated with the voltage-sensitive Na⁺ channel activators, veratridine and aconitine, NANC₃ nerve-induced relaxation was inhibited. However, the response to 4β-PDBu was unaffected.

5. Desensitisation to VIP in tracheal tissues could not be demonstrated using maximally effective frequencies of EFS stimulation (10Hz) for prolonged periods (3h), or with supermaximal relaxant concentrations of VIP (2μM) or aconitine (30μM). However, prolonged EFS (in the presence of atropine and propranolol) reduced the subsequent frequency–response relationship to NANC₃ nerve stimulation and the concentration–response relationship to 4β-PDBu, implying that tracheal relaxation to 4β-PDBu was in part dependent on the functioning of NANC₃ nerves.

6. Reduced bathing fluid temperature (16°C) converted the relaxation of the trachea to 4β-PDBu into contraction. Cold storage (4°C) of tracheal tissues (24h and 48h post mortem) inhibited NANC₃ nerve-mediated responses recorded at 37°C, but had no effect on the relaxant response to 4β-PDBu.

7. The results presented in this chapter demonstrate that relaxation of the guinea-pig trachea in response to NANC₃ nerve stimulation, veratridine and 4β-PDBu was reduced by methods most likely to result in neurotransmitter depletion, particularly from NANC₃ nerves. However, no conclusions could be made regarding the proposed role of VIP in these responses.
7.1 Introduction and background

In order to delineate further the contribution of neuronal elements to the relaxant action of phorbol esters in the guinea-pig trachea, experiments were designed to differentiate neuronal and non-neuronal (e.g. smooth muscle) responses. In the absence of appropriate potent and selective antagonists for the putative NANC\textsubscript{i} neurotransmitter receptor, three indirect approaches were adopted: (i) neurotransmitter depletion using the venom and purified toxin of the black widow spider, (ii) desensitisation to NANC\textsubscript{i} nerve-mediated responses either by prolonged EFS, or prolonged incubation with exogenous VIP (a putative NANC\textsubscript{i} neurotransmitter) or with aconitine (whose relaxant action may involve release of the NANC\textsubscript{i} neurotransmitter, see previous chapter), and (iii) overnight cold storage or reduced bathing fluid temperature. It is realised that a degree of "mechanistic overlap" probably occurs between these approaches, particularly in respect to depletion and desensitisation. In view of this the above categories should not be regarded as rigid and are merely intended for clarity of approach.

7.1.1 Neurotransmitter depletion

7.1.1a Black widow spider venom and αlatrotoxin

The crude extract of the venom glands of the mature European female black widow spider (BWSV), *Latrodectus mactans tredecimguttatus*, contains several toxic moieties and has been shown to be active *in vitro* in several different innervated tissues from vertebrates (reviewed by Hurlbut and Ceccarelli, 1979). In 1958, Cantore found that its addition to the organ bath caused contraction of the rabbit ileum preparation, which was found to be reversible on washing and atropine-sensitive. Repeated administration of the venom was tachyphylactic, while the response to exogenous acetylcholine remained unchanged. Electrophysiological studies on the rat isolated superior cervical ganglion have shown that the venom depresses postganglionic action potentials evoked by preganglionic stimulation, while eliciting discharges of asynchronous action potentials from the unstimulated ganglion. Further, addition of the venom to unstimulated ganglia previously loaded with radiolabelled choline, caused a 50% decrease in the labelled acetylcholine content (Paggi and Rossi, 1971). Therefore, early experimental investigation demonstrated the ability of the venom, after repeated electrical stimulation, to release neurotransmitter substances to the point of
depletion. This extends to catecholamine depletion in nerve fibres of
the mouse iris muscle (Frontali, 1972) and to the release of \( \gamma \)-amino-
butyric acid from cerebral cortex which, in the case of the latter, was
also associated with depletion of tissue synaptic vesicle content (see
Tzeng and Siekevitz, 1979a).

Fractionation of the crude venom has revealed that many of the
pharmacological, electrophysiological and physiological effects of the
venom reside in the action of a high molecular weight (130kDa) protein,
devoid of enzymic (protease/phospholipase) activity, which subsequently
has been called \( \alpha \)latrotoxin (\( \alpha \)ltx). \( \alpha \)ltx differs from many other known
toxins with respect to its selectivity and specificity of action. The
effects of \( \alpha \)ltx are exclusive to vertebrate synapses where it induces
massive stimulation of asynchronous exocytosis; ie. fusion of synaptic
vesicles with the presynaptic plasma membrane (confirmed by freeze-
fracture electron microscopy, see Tzeng and Siekevitz, 1979a), followed
by quantal release of their segregated neurotransmitters. Outside
synapses, \( \alpha \)ltx is largely inactive. This property differentiates \( \alpha \)ltx from
those toxins which act on ion channels (eg. VT and AC) and receptors
(eg. \( \beta \)bungarotoxin) because these latter structures have a wide
distribution in different cell types, and in different regions of the
plasma membrane of individual cells.

Clinically, the symptoms of a bite from the spider in man and other
mammals suggest that the main target for the toxin is the somatic and
autonomic nervous system. The initial effects are excitatory (see
Hurlbut and Ceccarelli, 1979). There are no inhibitors of the action of
\( \alpha \)ltx except for some divalent cations of cobalt and cadmium.

Electrophysiological recordings have demonstrated the ability of the
purified toxin to activate large membrane conductances due to the
opening of discrete cation channels of low specificity, since they were
permeable to both monovalent and divalent cations. However, these
cation channels appear not to be sensitive to organic blockers of
voltage-dependent Na\(^+\) (tetrodotoxin), K\(^+\) (tetraethylammonium) or Ca\(^{2+}\)
(verapamil) channels, used at optimally selective concentrations
(reviewed in Scheer et al., 1985). It was suggested that \( \alpha \)ltx was able
to insert itself across the plasma membrane lipid bilayer allowing marked
influx of monovalent and divalent cations along with massive depolar-
isation leading to the asynchronous release of neurotransmitter. However, this model did not account for the strict specificity of αltx for only a few cellular targets. Indeed, no effect on ion transport could be observed with the toxin in muscle, whereas cerebral cortical synaptoneurosomes (rat, bovine and guinea-pig) were massively affected (see Scheer et al., 1985). Further suggestions lead to the demonstration that specific high affinity binding sites for αltx, αltx receptors, exist in many if not all cellular systems sensitive to the toxin (Tzeng and Siekevitz, 1979b). The αltx receptor is recognised as an integral membrane protein (protease-sensitive and readily solubilised) and appears not to be found in non-nerve systems (e.g. platelets, muscle and liver cells) (see Scheer et al., 1985).

In this study, both the crude venom homogenate (VCH) and the purified toxin, αltx, were employed in attempts to deplete the trachea of neurotransmitters, particularly from NANC_i nerves, prior to examining the relaxant response to EFS, VT and 4β-PDBu. The rationale being that treatment with the venom extract/toxin should diminish the response to NANC_i stimulation by EFS, and - according to the working hypothesis - also to VT and 4β-PDBu.

7.1.1b Capsaicin

Capsaicin, the major pungent ingredient of hot peppers of the plant genus Capsicum, has been used extensively as an experimental tool in studies on peptide-containing sensory neurones. Capsaicin selectively activates a population of vagal C-fibre primary afferents in the airways (see Widdicombe, 1981) and initiates the release and subsequent depletion (at high concentrations) of bioactive peptides including substance P-immunoreactivity (IR), calcitonin gene-related peptide-IR (Lundberg et al., 1983) and neurokinin A (Buck and Burks, 1986). However, histochemical and biochemical evidence suggests that in adult animals, cholinergic, VIP-ergic and adrenergic nerve fibres remain intact after capsaicin treatment (reviewed by Buck and Burks, 1986).

This apparent selectivity of capsaicin for particular neurones is most likely to reside in the functional make-up of their plasma membrane structure and/or the ease of access of capsaicin to the free nerve
endings of those cells. Capsaicin is a relatively lipophilic molecule and is thought to alter membrane fluidity and/or ion permeability, particularly Ca\textsuperscript{2+}, leading to impulse initiation and release of sensory nerve peptides. Other cellular effects of capsaicin may include damage to the microtubule system, preventing retrograde axonic transport of trophic factors (reviewed in Buck and Burks, 1986).

For this study capsaicin was used to investigate the possible contribution of sensory neurones to the relaxant response of the trachea to NANC\textsubscript{i} nerve stimulation and 4\beta-PDBu.

7.1.2 Desensitisation

This is yet another method in a series of indirect approaches which, in the absence of appropriate receptor antagonists, can be used to investigate the role of putative neurotransmitters in nerve-mediated responses. As indicated previously (Chapter 5, Introduction) variable results have been obtained using this particular approach. Itoh and Takeda (1982) found that relaxation of feline trachea to nerve stimulation was much reduced by partial desensitisation to exogenous VIP, a putative neurotransmitter for the nerve-mediated response under investigation. In the guinea-pig, however, one study has shown that exogenous VIP did not effect the magnitude of the neurogenic inhibition; nor was the relaxant response to exogenous VIP altered by "prolonged" (12 min) maximal (10-20 Hz) nerve stimulation (Karlsson and Persson, 1984). However, the authors did not present data to show that the conditions used were sufficient to cause auto-desensitisation - ie. attenuation of the response to VIP itself. More recently, in another study using the guinea-pig tracheal pouch preparation, some degree of auto- and cross-desensitisation to exogenous VIP and cross-desensitisation to NANC\textsubscript{i} nerve stimulation was demonstrated (Venugopalan and O'Malley, 1988). However, auto-desensitisation to NANC\textsubscript{i} nerve-mediated relaxation was not investigated. In each case the extent of desensitisation was not marked. In a more rigorous study (Ellis and Farmer, 1989a), a more prolonged (3 h) incubation of the isolated guinea-pig tracheal strip preparation with a supermaximal relaxant concentration (3 \textmu M) of VIP, resulted in a 40% reduction in the magnitude of the inhibitory response to NANC\textsubscript{i} nerve stimulation. Disappointingly however, within the constraints of the
experimental conditions used, no data was presented demonstrating auto-desensitisation to exogenous VIP- or NANC$_i$ nerve-mediated relaxation.

The experiments reported in this study were designed to demonstrate desensitisation of the tracheal strip preparation to VIP, using conditions of prolonged incubation with maximally effective relaxant concentrations of exogenous VIP and AC, and in addition, prolonged maximal inhibition with EFS. Furthermore, where possible, the study also aimed to examine the dependency of the tracheal relaxation to 4β-PDBu on functional NANC$_i$ nerve-mediated responsiveness.

7.1.3 Reduced temperature

It has long been recognised that, in isolated preparations removed from homoiothermic mammals, reduced bathing fluid temperature (15–20°C) attenuates or even abolishes neuronally-mediated responses. It has also been noted that although the rate of response to direct agonists is reduced, the magnitude can be maintained (Day and Vane, 1963). For experiments reported in this section, it was hypothesised that if the predominant effect of the phorbol ester was neuronal, then under conditions of reduced bathing fluid temperature the relaxant response to 4β-PDBu in the trachea would be attenuated.

In summary, each of these additional approaches was employed to provide a more direct method, particularly with neuronal depletion, with which to test the hypothesis that the relaxant action of phorbol esters in the trachea involved a component which was due to PKC-mediated release of a relaxant, particularly from NANC$_i$ nerves.
7.2 Experimental protocol

Preparation of L. mactans venom crude homogenate

The method of Frontali et al. (1972) was used. Briefly, five venom sacs (Sigma, UK) were added to 500μl saline (0.9%v/v) at 4°C, in a 100μl (filled to 500μl) glass pestle and mortar. The venom sacs were ground to a fine suspension by approximately 20 twists/strokes of the pestle until all the material was dispersed. The suspension was then centrifuged (900g x 15min) at 4°C and the supernatant carefully removed and aliquoted (50μl) into polypropylene vials prior to storage at -20°C until required. The supernatant was then referred to as the "venom crude homogenate" (VCH). This protocol was used in order to make the most effective use of the VCH where a concentration of 50μl venom supernatant/ml of tissue bathing solution (equivalent to approx. 75μg protein or 1 gland/ml) could be achieved in the organ bath. This had been shown to deplete catecholamines from guinea-pig gut mesentery (see Hurlbut and Ceccarelli, 1979). However, in order for the tissues to be completely immersed, a minimum bathing volume of 2ml was required so that only half the optimal required crude venom concentration (1:40 dilution) could be used. Control tissues received 50μl (1:40 dilution) saline (0.9%v/v) according to the protocol scheme in figure 7.1. Where overnight incubation (4°C) with VCH or αltx was required to produce "depletion", tissues remained under tension (0.5g).

Desensitisation

For experiments in which there was prolonged stimulation of NANCi nerves by EFS, or incubation with VIP and aconitine (AC), protease inhibitors (bacitracin 1.86U/ml and aprotinin 1.2U/ml) and protein synthesis inhibitors (cycloheximide 50μg/ml and actinomycin D 10μg/ml) were present in the Krebs-Henseleit (KH) solution for the duration of the experiment. Furthermore, atropine (1μM) and propranolol (1μM) were also present where EFS and AC were investigated. Three pairs of tracheal tissues were set-up for isometric recording as described in section 2.2. One tissue from the first pair was subjected to EFS (0.5ms, 100:18V, 10Hz) for 3h, while one of the preparations in each of the two remaining pairs received either VIP (2μM) or AC (30μM). Dithiothreitol (4μM) and DMSO (0.06%v/v) served as controls for VIP and AC respectively. After 3h had elapsed, all tissues were precontracted with
histamine (10μM) (in the case of the first pair of tissues this was done only after the stimulator had been switched off) and then exposed to cumulative additions of either VIP (0.1-100nM) or 4β-PDBu (1nM-1μM), or subjected to EFS (0.5ms, 100:18V. 0.25-20Hz). At the end of the experiment maximum relaxation was determined with papaverine (100μM).

Cold storage and reduced bathing fluid temperature

Where the effect of cold storage on the tracheal response to NANCi nerve stimulation was studied, six transverse tracheal strips were prepared from the cervical and thoracic portions of the tracheal tube (see section 2.2). Two strips were set-up immediately for isometric recording, while two other tracheal strips were placed into 20ml aerated KH solution and stored at 4°C for 24h post mortem (p.m.). The remaining two strips were handled in the same fashion, but stored at 4°C for 48h p.m. When required, each pair of tissues was first placed into 20ml aerated KH solution at room temperature for 1h, prior to mounting in the organ bath (37°C).

Where reduced bathing fluid temperature was required, temperature was maintained using a refrigeration unit (Grant, model CC15) plumbed into the thermostatically-controlled water circulation circuit.
7.3 Results

7.3.1 Effect of *L. mactans* venom crude homogenate

The results presented below represent a single preliminary experiment in which the effect of the venom crude homogenate (VCH) was investigated in the trachea (figure 7.1 and 7.2). Further experiments were not carried out, since it was considered that the purified toxin would be of more use. However, it is worth comparing the results obtained with the VCH to those obtained with the purified toxin.

Addition of the venom crude homogenate (1:40 dilution, see section 7.2) to the organ bath caused relaxation which was prompt in onset (<30sec), sustained (>150min) and maximum in extent. On day 1 of the experiment, the atropine-sensitive component of the tracheal response to 10sec trains of EFS (0.5ms, 100:18V, 40Hz) every 30min, was used as an index for the effectiveness of the VCH in reducing cholinergic nerve responses (see figure 7.1a and b). However, over a period of 160min no reduction in the response to EFS with time was seen in either saline control or VCH-treated tissues (figure 7.1a and b, day 1; figure 7.2a), although some reduction in magnitude of contraction was seen in VCH-treated preparations.

After 18h at 4°C (without washing) and a further equilibration period (approx. 2h) to 37°C, during which the bathing fluid (KH solution) was replaced only once, the magnitude of the contractile response to histamine (10-100μM) was found to be unchanged from day 1 in both control and VCH-treated tissues (figure 7.2b). However in VCH-treated preparations, the atropine-sensitive contractile response to EFS was reduced (figure 7.1a and b, day 2; figure 7.2c). All tissues were then precontracted with histamine (10μM). The response to NANC_i nerve activation (ie. EFS in the presence of atropine and propranolol) after VCH exposure was reduced to 58% of the control relaxation at 10Hz, and to 33% at 20Hz. However, the reduction in relaxation to 4β-PDBu (1μM) was only 18% of the control in VCH-treated preparations (figure 7.2d).

7.3.2 Effect of α-Latrotoxin

For this study only two experiments are presented. This was due to the large quantity of purified toxin required for each experiment (approx. 20μg) relative to the total stock concentration (150μg), the
length of time required for each experiment and the limited shelf-life of the purified toxin used in this study (approx. 10-12 days at -20°C). A similar protocol was adopted to that used in the VCH study. However, two additional tracheal tissues were included, in which the relaxant effect of veratridine (VT) was investigated in the presence and absence of the toxin.

Addition of α-Latrotoxin (2.4μg/ml; total 4.8μg) to the organ bath caused relaxation of tracheal tissues which was immediate in onset (<10sec), sustained (210min) and maximum in extent, but was more rapid in rate of relaxation than the VCH (cf. figure 7.1 and 7.3). The magnitude of the atropine-sensitive component to EFS was not reduced with time on day one in the αltx-treated preparations over a period of 210min (figure 7.4a). However on day 2, after 18h at 4°C, this component of the tracheal response to EFS was reduced to less than half of the mean control response (figure 7.4b) (in one of two experiments it was abolished). In each case, the mean maximum response to histamine (100μM) on day 1 in control tissues (saline) was unchanged when compared on day 2 (day 1 saline control 100%; day 2 105.7±3%, mean ± range, n=2). Furthermore on day 2, αltx was also without any marked effect on the maximum contractile response to histamine (105.7±3% of the histamine maximum on day 2 control saline-treated tissue; 101.3±2% αltx-treated, mean ± range, n=2).

The extent of the mean response to NANCi nerve activation (EFS in the presence of atropine and propranolol) was reduced by 38% after prolonged treatment with the toxin (figure 7.3a and b, day 2; figure 7.4c). Similarly, treatment with αltx markedly reduced the mean relaxant response to VT at 30μM and 50μM (figure 7.3e and f, day 2; figure 7.4d), while the mean relaxant response to 4β-PDBu (1nM-10μM) in the trachea was largely unaltered after prolonged exposure to the toxin (figure 7.4e). However, in one of the two experiments presented the concentration-response relationship to 4β-PDBu was inhibited (shown in figure 7.3c and d, day 2). The value of this single observation is obviously limited.

In two further experiments, αltx (2.4μg/ml; total 4.8μg) was found to be ineffective in preventing the relaxation of the trachea to either NANCi nerve stimulation or to VT. The toxin was similarly without effect when used at a higher concentration (3.5μg/ml; total 7μg).
7.3.3 Effect of capsaicin on basal tracheal tone and relaxation to 4β-PDBu and NANC\textsubscript{1} nerve stimulation.

The results are shown in figures 7.5 and 7.6. Capsaicin (10μM) caused an immediate contraction (63.6±4.4% of the maximum contractile response to histamine, n=6) in the guinea-pig trachea, which rapidly waned to a point below the pre-capsaicin baseline (52.2±14% of the maximum relaxation to papaverine, n=6), plateauing approximately 45 min after addition of capsaicin to the organ bath. Replacement of the bathing fluid with fresh KH solution at either the contractile peak ("peak contraction") or relaxant peak ("peak relaxation") to capsaicin resulted in an immediate return to baseline (see figure 7.5). The contractile response to capsaicin was tachyphylactic, since addition of capsaicin for a second time resulted in a marked depression of the peak contractile response (1st exposure 63.6±4.4% of the maximum contractile response to histamine; 2nd exposure 3.9±0.8%, p<0.001, paired t-test, n=6). However, the peak relaxant response was unchanged (1st exposure 52.2±14% of the maximum relaxation to papaverine; 2nd exposure 86±6%, p>0.05, paired t-test, n=6).

The relaxant effect of 4β-PDBu was then investigated in tracheal tissues which had been twice exposed to capsaicin, in each case washed either at the peak contractile or peak relaxant response. In all tissues exposed to capsaicin, the cumulative concentration-response curve to 4β-PDBu was shifted to the left, when compared to control tissues which had been exposed only to the relevant concentration of diluent (ethanol, 0.02%V/v) and not to capsaicin (IC\textsubscript{50} 71.7±13nM in control tissues; 16±2.2nM in tissues exposed to capsaicin washed at the peak contraction, p<0.05, unpaired t-test; 9.3±2.9nM in preparations exposed to capsaicin washed at the peak relaxation, p<0.01, unpaired t-test, n=3-4). The magnitude of the maximum relaxation to 4β-PDBu was unaffected by either method of treatment with capsaicin (see figure 7.6a and b). Exposure to capsaicin (washed at peak contraction) potentiated also the relaxant response to NANC\textsubscript{1} nerve stimulation evoked by EFS in the presence of atropine (1μM) and propranolol (1μM) (EF\textsubscript{50} 1.8±0.03Hz in untreated preparations; 0.6±0.02Hz in capsaicin-exposed preparations, p<0.001, unpaired t-test, n=3). However, unlike the results obtained to 4β-PDBu, the magnitude of the maximum relaxation to NANC\textsubscript{1} nerve stimulation (20Hz) was significantly (p<0.01, unpaired t-test) enhanced.
(85±2% of the maximum relaxation produced by papaverine in control tissues; 97±1% in capsaicin-treated tissues, n=3) (figure 7.6c). The effect of capsaicin treatment washed at the peak relaxation was not investigated on NANC₃ nerve-induced relaxation.

7.3.4 Effect of veratridine and aconitine on tracheal relaxation induced by NANC₃ nerve stimulation and 4β-PDBu

Both veratridine (VT) and aconitine (AC) at 30μM caused prompt relaxation of the guinea-pig tracheal strip which was maximal (VT 99±0.5% of the maximum relaxation to papaverine, n=7; AC 97.7±2.3%, n=3) and consistent with data presented in the previous chapter. After 30min incubation of the trachea with the alkaloids, repeated washing (every 10min) with fresh KH solution containing atropine (1μM) and propranolol (1μM), returned the tissues to baseline after 60-90min.

Both VT and AC inhibited tracheal relaxation to NANC₃ nerve stimulation (induced by EFS in the presence of atropine and propranolol) to the extent that, in the presence of the alkaloids, an EF₅₀ value could not be determined. However, both the slope and magnitude of relaxation was clearly reduced, particularly in the case of AC (see figure 7.7a and b). In contrast, exposure to VT (30μM×30min) did not affect the relaxation of the trachea to 4β-PDBu (IC₅₀ 11.6±4nM, maximum relaxation 86±8% of the relaxation produced by papaverine in control 0.06% etOH-treated tissues; IC₅₀ 13±4nM, maximum relaxation 97.7±1.2% in VT-treated preparations, p>0.05, unpaired t-test, n=3) (figure 7.7c).

7.3.5 Effect of prolonged maximal NANC₃ nerve stimulation, VIP and AC on tracheal relaxation to VIP and 4β-PDBu

Relaxation to prolonged (3h) maximal NANC₃ nerve stimulation produced by EFS (0.5ms, 100:18V, 10Hz) achieved 93.1±4% (n=6) of the maximum relaxation to papaverine and was sustained throughout. Prolonged (3h) incubation of the tracheal tissues with a supermaximal relaxant concentration of exogenous VIP (2μM) produced relaxation which was similarly sustained and achieved 100±0% (n=4) of the maximum relaxation to papaverine. AC (30μM) also produced maximal relaxation (97.7±1.9%, n=4) of the tracheal preparation, which was also sustained throughout the 3h pretreatment period. In each case after 3h had elapsed, the sustained relaxation to EFS, VIP or AC was readily reversed by
histamine (10μM) which was used to restore tone in the preparation. The contraction produced by histamine was maintained.

7.3.5a Effect on tracheal relaxation to exogenous VIP

The results are shown in figure 7.8. Prolonged stimulation of NANC_i nerves by EFS (0.5ms, 100:18V, 10Hz×3h) produced inhibition of the subsequent relaxation to NANC_i nerve stimulation (0.5ms, 100:18V, 0.25-20Hz) in the trachea. This was seen as a right-ward shift of the frequency-response curve (EC_{50} 1.5±0.08Hz in control tissues; EF_{50} 3.53±0.72Hz in preparations after prolonged EFS, p<0.05, unpaired t-test, n=3) and a reduction in the maximum extent of relaxation at 20Hz (93.7±1.2% of the maximum relaxation produced by papaverine in unstimulated tissues; 71.7±8.9% in stimulated preparations, p<0.05, unpaired t-test, n=3) (see figure 7.8a). However, this apparent inhibition of relaxation to NANC_i nerve stimulation after prolonged activation of NANC_i nerves was not reflected in the responsiveness of the trachea to the putative NANC_i neurotransmitter, VIP (IC_{50} 4.5±1nM, maximum relaxation 96.3±2% in unstimulated control tissues; IC_{50} 6±1.8nM, maximum relaxation 91.5±4.3% in preparations after prolonged EFS, p>0.05, unpaired t-test, n=3) (see figure 7.8b). Similarly, no effect on relaxation to VIP was seen in the trachea after prolonged exposure to a supermaximal relaxant concentration of VIP (2μM) (IC_{50} 12.8±1.1nM, maximum relaxation 90.4±1.6% in 4μM DTT-treated control tissues; IC_{50} 7±3nM, maximum relaxation 88.1±1.3% in preparations after prolonged exposure to VIP, p>0.05, unpaired t-test, n=3) (see figure 7.8c). In contrast, some degree of inhibition of tracheal relaxation to VIP was seen after prolonged incubation with aconitine (30μM) although this did not achieve statistical significance (p>0.05, unpaired t-test) (IC_{50} 7.6±1.8nM, maximum relaxation 94.5±3.2% in 0.06% DMSO-treated control tissues; IC_{50} 31.3±9.4nM, maximum relaxation 81.8±7.3% in preparations after prolonged exposure to AC, n=3) (see figure 7.8d).

7.3.5b Effect on tracheal relaxation to 4β-PDBu

The results are presented in figure 7.9. Prolonged NANC_i nerve stimulation by EFS significantly inhibited relaxation in the trachea produced by 4β-PDBu. This was seen as a right-ward shift of the concentration-response curve to 4β-PDBu (IC_{50} 14.4±2.9nM in unstimulated control tissues; IC_{50} 32.4±3.8nM in preparations after
prolonged EFS, p<0.01, unpaired t-test, n=3) and a reduction in the maximum extent of relaxation to the phorbol ester (99±1.3% of the maximum relaxation to papaverine in unstimulated control tissues; 78.2±4% in preparation after prolonged EFS, p<0.01, unpaired t-test, n=3) (see figure 7.9a). Surprisingly, in tracheal tissues exposed to prolonged incubation (3h) with either VIP (2μM) or AC (30μM), relaxation to 4β-PDBu was enhanced. However, this observation is based on the result of a single experiment and should be interpreted with caution (figure 7.9b and c).

7.3.6 Effect of cold storage and reduced bathing fluid temperature on tracheal responsiveness to EFS and 4β-PDBu

7.3.6a Cold Storage

The results are shown in figure 7.10. Tracheal responsiveness to NANC \textsubscript{i} nerve stimulation and 4β-PDBu was assessed at approximately 5, 24 and 48h post mortem (p.m.). Frequency-dependent relaxation to EFS (0.5ms, 100:15V, 0.25–10Hz) in the presence of atropine (1μM) and propranolol (1μM) could be readily observed at 5h p.m. At 24h p.m. relaxation to NANC \textsubscript{i} stimulation was still present, but was reduced (approx. 2 fold at the IC\textsubscript{50}). However, at 48h p.m. tracheal responsiveness to NANC \textsubscript{i} nerve-stimulation was greatly reduced (see figure 7.10), while the responsiveness to histamine (100μM) was unchanged (5h p.m. 680±60mg; 24h p.m. 690±70mg; 48h p.m. 740±10mg, mean ± s.e.mean of four observations - 2 from each of two tissues on each experimental day). 4β-PDBu (1μM) produced relaxation in the trachea 5h p.m. which was still evident at 24 and 48h p.m. with no marked change in extent of relaxation. These observations were obtained from a single animal.

7.3.6b Reduced bathing fluid temperature

The results of a single experiment are shown in figure 4.4e, Chapter 4. Reduction of the bathing fluid temperature to 16°C resulted in conversion of the usual relaxant response to 4β-PDBu (1μM) in the guinea-pig trachea into contraction, confirming observations of Huang et al. (1987). For this reason further replicates were not obtained.
Figure 7.1 Protocol to investigate the effect of black widow spider venom crude homogenate (VCH) (1:40 dilution) in guinea-pig trachea over a 2 day period. Day 1 shows the response to 10s trains of EFS (0.5ms, 100:16V, 40Hz) every 30min (▲) in the (a) absence and (b) presence of VCH. In the remaining tissues, changes from intrinsic tone in the (c) absence and (d) presence of VCH were monitored for 160min. After which, without washing, all tissues were maintained at 4°C for 18h. On Day 2 a period (100min) of temperature re-equilibration to 37°C was allowed, followed by washing with fresh KH solution. The responsiveness to histamine (10-100µM) given cumulatively was then assessed in all tissues. After washing and a further re-equilibration period, the response to EFS (as for Day 1) was reassessed in the (a) control and (b) VCH-exposed tissues. All tissues were then precontracted with histamine (H, 10µM) and the response to EFS (0.5ms, 100:18V) at 10 and 20Hz in the presence of atropine (1µM) and propranolol (1µM) was examined in (a) control and (b) VCH treated preparations. In the remaining tissues a single concentration (1µM) of 4β-PDBu was examined in (c) control and (d) VCH-treated preparations. Maximum relaxation was determined on addition of papaverine (P, 100µM) at the end of the experiment. Isometric recording.
Figure 7.2 Quantitative data from a single experiment shown in figure 7.1. (a) Changes in the atropine-sensitive component of the tracheal response to EFS (0.5ms, 100:18V. 40Hz) trains (10sec) every 30min. Responses were obtained in the absence (○) and presence (●) of venom crude homogenate (VCH) at 1:40 dilution. VCH was added at time zero and left in the bath throughout Day 1. (b) Maximum response to histamine (100μM) in the absence and presence of VCH on day 1 and 2. All responses are expressed as a percentage of the maximum response to histamine (100μM) on Day 1 in the saline-treated control tissues. (c) Response to EFS (0.5ms, 100:18, 40Hz) on day 2, obtained from a control (saline) and VCH-treated tissue. S1 and S2 represent two 10s trains of EFS 30min apart. (d) Relaxant response in the trachea to NANC fiber nerve stimulation by EFS (0.5ms, 100:18V) at 10Hz and 20Hz, and to 4β-PDBu (1μM) in saline control and VCH-treated preparations (histamine precontracted). Isometric recording.
Figure 7.3 Protocol used to investigate the effect of α-latrotoxin (αLtx, 2.4μg/ml, total 4.8μg) on the guinea-pig trachea. For tissues a–d, see legend to figure 7.1 and read αLtx for VCH. The lower two traces on day 1 are identical to (c) and (d). However on day 2, veratridine (VT), 30 and 50μM was examined in (e) control and (f) αLtx-treated preparations. In addition, propranolol (1μM) was present also in these two tissues. Isometric recording.
Figure 7.4 Effect of a Latrotoxin (aLtx, 2.4 μg/ml total 4.8 μg) on various responses of the guinea-pig trachea. (a) Changes in the atropine-sensitive component of the tracheal response to EFS (0.5ms, 100:18V, 40Hz) trains (10s) every 30min. Responses were obtained in the absence (O) and presence (●) of purified toxin which was added at t=15min and left in the organ bath throughout day 1. (b) Effect of the toxin on the mean response to EFS on day 2, in control (saline) and aLtx-treated tissues. S₁-S₃ represent 10s trains of EFS, 30min apart. Note the responses are represented as a percentage of the maximum contractile response to histamine (100 μM). (c) Response to NANC_i nerve stimulation on day 2 by EFS (0.5ms, 100:18V, 0.25-20Hz) in the presence of atropine (1 μM) and propranolol (1 μM) in saline control and aLtx-treated tissues. (d) Relaxation of the trachea to veratridine (VT) in the presence of propranolol (1 μM) on day 2, in control saline and aLtx-treated tissues. (e) Relaxant response to 4β-PDBu in control saline (O) and aLtx-treated (●) preparations. In (c), (d) and (e) tissues were precontracted with histamine (10 μM). Results are presented as mean ± range of 2 experiments. Isometric recording.
Figure 7.5 Comparison of repeated capsaicin (10μM) exposure on intrinsic tracheal tone. Tissues were either washed (W) at the peak contractile or peak relaxant response after initial exposure to capsaicin (C₁/open columns). This was repeated with a second exposure to capsaicin (C₂/shaded columns). The upper trace shows the response of two tracheal tissues to the two methods of capsaicin treatment and is representative of 6 experiments. The histogram shows the mean ± s.e.mean of 6 experiments. Hist = histamine, Papav = papaverine (100μM). Isotonic recording.
Figure 7.6 Effect of repeated exposure to capsaicin (10μM) on the relaxation of the guinea-pig trachea induced by (a and b) 4β-PDBu and (c) NANCι nerve stimulation by EFS (0.5ms, 100:18V, 0.25-20Hz) in the presence of atropine (1μM) and propranolol (1μM). In (a and c) capsaicin was washed out at the peak contraction whereas in (b) it was removed after "peak relaxation" was achieved. Responses were obtained either in the absence of (O) or after exposure (●) to capsaicin. Points represent mean ± s.e.mean of n=4-6. Protease inhibitors were absent from these experiments. Isotonic recording.
Figure 7.7 Effect of the sodium channel activators (30μM), veratridine (a and c) and aconitine (b), on the relaxation of guinea-pig trachea produced by NANC_i nerve stimulation by EFS (0.5ms, 100:17V, 0.25-20Hz) and 4β-PDBu. All tissues were incubated (10min) with atropine (1μM) and propranolol (1μM) and precontracted with histamine (10μM). Responses were obtained in either the absence of (O) or after a 30min exposure (●) to the alkaloids. Points represent mean ± s.e.mean of n=3-7. Protease inhibitors were absent from these experiments. Isometric recording.
Figure 7.8 Comparison of guinea-pig tracheal responsiveness to relaxation induced by EFS (0.5ms, 100:18V, 0.25-20Hz) and VIP after prolonged (3h) stimulation/exposure to (a and b) EFS (10Hz), (c) VIP (2μM) and (d) aconitine (AC, 30μM). Responses were obtained in the absence of (O) or after prolonged treatment (●). Points represent mean ± s.e.mean of n=3. Responses in (a) and (b) were obtained within the same control or treated preparation. All tissues were precontracted with histamine (10μM) and the protease inhibitors, bacitracin (30μg/ml=1.86 U/ml) and aprotinin (1.2U/ml) were present throughout. In addition to atropine (1μM) and propranolol (1μM), the protein synthesis inhibitors, cycloheximide (50μg/ml) and actinomycin D (10μg/ml), were also present throughout. Isometric recording.
Figure 7.9 Comparison of guinea-pig tracheal responsiveness to 4β-PDBu after prolonged (3h) stimulation to (a) EFS (0.5ms, 100:18V, 10Hz), and prolonged (3h) incubation with (b) VIP (2µM) and (c) aconitine (AC, 30µM). Responses were obtained in the absence (O) or after prolonged treatment (●). In (a) points represent mean ± s.e.mean of n=3 separate experiments, while in (b) and (c) points represent data from a single experiment. Experimental conditions are as described in legend to figure 7.8. Isometric recording.
Figure 7.10 A single experiment showing effect of cold storage (4°C) on tracheal relaxation to (a) NANC$_i$ nerve stimulation by EFS (0.5ms, 100:15V, 0.25-10Hz) in the presence of atropine (1μM) and propranolol (1μM), and (b) single concentration of 4β-PDBu (1μM). Responses were obtained 5h post mortem (p.m.) (O, open column), 24h p.m. (●, cross-hatched column) and 48h p.m. (■, shaded column). At the beginning of each experimental day tissues were allowed 90min equilibration in fresh KH solution at 37°C prior to recording responses. All tissues were precontracted with histamine (10μM). Isotonic recording.
7.4. Discussion

This chapter reports experiments in the trachea which were designed to differentiate between phorbol ester responses on neuronal and non-neuronal tissues (ie. smooth muscle). These were carried out in an attempt to test further the working hypothesis of phorbol ester-induced release of a NANC\textsubscript{i} neurotransmitter(s). Three main approaches were used: (i) neurotransmitter depletion, (ii) desensitization to a putative NANC\textsubscript{i} neurotransmitter and (iii) reduced bathing fluid temperature and cold storage. Each of these methods aimed to prevent or attenuate neuronally-mediated tracheal responses in order to determine the extent to which relaxation in the trachea to 4\beta-PDBu was dependent on functional NANC\textsubscript{i} mechanisms.

Depletion was attempted using the venom crude homogenate (VCH) and the purified toxin, αLatrotoxin (αltx), of the mature European female black widow spider. In addition, the pungent ingredient of hot peppers, capsaicin, and the voltage-sensitive Na\textsuperscript{+} channel activators, veratridine (VT) and aconitine (AC), were also investigated in the trachea.

For "depletion" to occur (in our experiments a specific reduction in the atropine-sensitive component of the response of the trachea to EFS), both the VCH and αltx required overnight incubation (4°C) with the tracheal tissues. However, many of the reported neurotransmitter release and depletion effects of the venom (and purified toxin) in isolated cells and tissues, are often recorded within minutes (eg. Cantore, 1958; Vicentini and Meldolesi, 1984). The reason for this discrepancy is not apparent, but the initial sustained relaxation to both VCH and αltx, which in the case of αltx was particularly rapid, could involve the release of a neuronally-derived relaxant, but not depletion which may require more prolonged treatment, as indicated by the experiments reported in this chapter. It is interesting to note that in rat pheochromocytoma (PC12) cells the toxin, which is known to bind with high affinity to surface sites (the αltx receptor) causing Ca\textsuperscript{2+} influx and neurotransmitter (dopamine) release, directly caused (ie. not a consequence of depolarisation) a rapid accumulation of inositol phosphates (Vicentini and Meldolesi, 1984); which is considered to indicate phosphoinositide breakdown. This of course, could generate the endogenous PKC activator, diacylglycerol, so that PKC activation might
also be a consequence of αltx. These observations could implicate further the DAG/PKC pathway in the control of neurotransmitter release (discussed in Chapter 5), particularly from NANCi nerve fibres (see Discussion, previous chapter), since both VCH and αltx attenuated relaxation to NANCi nerve stimulation and, in the case of αltx, to veratridine. This latter observation supports the proposed neuronal action of this alkaloid (discussed in the previous chapter), particularly at NANCi nerves, since this effect occurred in the presence of atropine and propranolol. However, in only one of two experiments (see figure 7.3) was the purified toxin effective against relaxation of the guinea-pig trachea to 4β-PDBu. The lack of replicates for these experiments obviously undermines their value but, as preliminary experiments, the results are worth considering and suggest that αltx may be a useful investigative tool with which to study the role of NANCi mechanisms in isolated airway preparations. Further, the effects of both VCH and αltx are unlikely to be non-specific since the direct contractile response to histamine was unchanged after treatment with the toxin. However, it is acknowledged that relaxants such as VIP or isoprenaline may have served as more appropriate controls with which to test the specificity of the toxin in the trachea.

In this study capsaicin was investigated to assess the contribution of sensory nerves to the relaxant response of the guinea-pig trachea to NANCi nerve stimulation and 4β-PDBu. Capsaicin caused an immediate contraction which rapidly waned, eventually plateauing below the original baseline. Repeated addition of capsaicin to the organ bath demonstrated the familiar tachyphylaxis of the peak contractile response. However, no such reduction in the relaxant phase was seen, and although histochemical evidence has suggested that nerve fibres having immunoreactivity for VIP are unaffected by capsaicin, and that while the identity of the NANCi neurotransmitter is unknown, it was considered that the relaxant component of the response to capsaicin might involve release of such a neurotransmitter. Accordingly, the relaxant effect of 4β-PDBu was studied in tracheal tissues twice exposed to capsaicin, washed either at the peak contractile or once the inhibitory phase had plateaued. In both cases the relaxant response to 4β-PDBu was potentiated. A similar response was observed to NANCi nerve stimulation by EFS after repeated exposure to capsaicin (washed at the peak contraction) suggesting that neuropeptides released from sensory nerves
by EFS or 4β-PDBu may modulate NANC\textsubscript{j} nerve- and/or 4β-PDBu-induced responses. This suggests that relaxation of the guinea-pig trachea to 4β-PDBu could involve a neuronal (especially NANC\textsubscript{j} nerve fibres) mechanism, although it is recognised that this similarity may be fortuitous and does not necessarily imply a common mechanism. Enhancement of the NANC\textsubscript{j} response to EFS in the guinea-pig trachea by capsaicin, has recently been reported in a communication to the British Pharmacological Society (Stretton et al., 1989). It should be pointed out that these authors also reported a similar enhancement to exogenous VIP after sensory nerve depletion, but not to isoprenaline, suggesting that a component of the facilitation of the NANC\textsubscript{j} nerve-mediated relaxation by EFS may involve a selective effect on the coupling of post-junctional VIP receptors. This too, cannot be ruled out in explaining the similarity of the response to 4β-PDBu. However, the importance of the enhancement to VIP will only be fully explained when the identity of the NANC\textsubscript{j} neurotransmitter in the guinea-pig trachea is known.

Veratridine (VT) and aconitine (AC), voltage-dependent Na\textsuperscript{+} channel activators, were able to substantially inhibit tracheal responses to NANC\textsubscript{j} nerve stimulation by EFS. However, it was not clear whether this was due to a reduction in the quantal release of the NANC\textsubscript{j} neurotransmitter by preventing the propagation of action potentials along the neurone, or depletion of the neurotransmitter from the nerve endings. It is therefore possible that the ineffectiveness of VT against relaxation of the trachea produced by 4β-PDBu, may be interpreted not only as an effect of the phorbol ester on non-neural mechanisms, but also as an effect in neuronal tissues which is down-stream of the voltage-sensitive Na\textsuperscript{+} channel to which the alkaloids bind with high affinity (see Catterall, 1980).

In another approach, desensitisation to NANC\textsubscript{j} mechanisms was attempted using prolonged NANC\textsubscript{j} nerve stimulation evoked by EFS in the presence of atropine and propranolol, or by prolonged incubation with a putative NANC\textsubscript{j} neurotransmitter such as VIP, or by prolonged incubation with AC which it is hypothesised causes the release of the NANC\textsubscript{j} neurotransmitter(s) (see Discussion, previous chapter). These experiments were carried out in the presence of peptidase inhibitors (bacitracin and aprotinin) in order to limit proteolytic degradation of any peptide neurotransmitters released on NANC\textsubscript{j} nerve stimulation, and also in the
presence of protein synthesis inhibitors (cycloheximide and actinomycin D) in order to limit the synthesis of peptides such as PHV-42 believed to be a precursor of the putative NANC$_i$ neurotransmitters, VIP and PHI/M (see Yiangou et al., 1987, Chapter 5).

Ellis and Farmer (1989a) reported a 40% reduction in the magnitude of the relaxant response to NANC$_i$ nerve stimulation in the guinea-pig trachea after prolonged (3h) incubation with a maximally effective relaxant concentration of the putative NANC$_i$ neurotransmitter, VIP. This protocol was also adopted for experiments reported in this chapter. However, in contrast to the study of Ellis and Farmer (1989a), no attenuation of the responsiveness to NANC$_i$ nerve stimulation by EFS (ie. cross-desensitisation) was obtained in the present study. This was not considered to be altogether surprising since no reduction in the response to exogenous VIP (ie. auto-desensitisation) was seen! However, a 25% reduction in the magnitude of the relaxation to NANC$_i$ nerve stimulation was observed after prolonged maximal NANC$_i$ nerve activation (ie. auto-desensitisation), the profile of which bore some similarity to the inhibitory effect seen with the proteases, a-chymotrypsin and papain, on the relaxation of the trachea to NANC$_i$ nerve activation by EFS (see section 5.3.4).

The failure to produce desensitisation in the trachea to VIP does not, however, refute or advance the argument for the involvement of VIP in a neurotransmitter role for NANC$_i$ nerve-mediated responses (Karlsson and Persson, 1984; Ellis and Farmer, 1989a,b), but merely suggests that the protocol used was inadequate for the purpose of the experiment - to produce desensitisation. However, this failure occurred in the same tissues in which prolonged EFS did little to alter tracheal responsiveness to exogenous VIP, but caused inhibition of the relaxation to NANC$_i$ nerve stimulation by EFS (ie. auto-desensitisation). This implied that, if VIP was involved in NANC$_i$ nerve-mediated responses, the change in tracheal responsiveness to NANC$_i$ nerve stimulation by EFS could be a prejunctional phenomenon (ie. depletion), since prolonged incubation with VIP did not change the responsiveness to NANC$_i$ nerve-induced relaxation or to VIP itself; the latter is presumed to act post-junctionally. Consequently, the apparent desensitisation to NANC$_i$ nerve-mediated relaxation by prolonged EFS is more likely to represent incomplete depletion of the NANC$_i$ neurotransmitter, rather than post-junctional
receptor desensitisation. Further, it was under these conditions that the responsiveness to 4β-PDBu was reduced, an effect which was consistent with one of the two experiments obtained with αltx, where neuronal depletion was investigated (see above). However, since only one experiment was carried out after prolonged exposure to VIP or AC, in which the tracheal relaxation to 4β-PDBu was investigated, no conclusion regarding the potentiation of the response to the phorbol ester could be drawn. It is tempting to speculate that if AC does release the NANCi relaxant neurotransmitter, and if this is VIP in this tissue, then the potentiation of the response to the phorbol ester may involve interaction with cyclic AMP (either directly or indirectly) (see Discussion, Chapter 4), which is known to be elevated by VIP in the guinea-pig trachea (Fransden et al., 1978).

Huang et al. (1987) reported that at low temperature (16°C), the familiar relaxant response of the guinea-pig trachea to phorbol esters became contractile. If the effect of the phorbol ester was predominantly an indirect one (ie. not on the smooth muscle) as hypothesised, this finding (confirmed in Chapter 4, figure 4.4) might not be very surprising, since the effect of reduced bathing fluid temperature is known to be more pronounced on neuronal function than on smooth muscle (Day and Vane, 1963), and cooling the tissue might tip the balance (discussed in Chapter 5, see figure 5.12) from a possible indirect effect involving the release of a NANCi nerve relaxant, in favour of a direct effect on smooth muscle PKC. There are, however, other explanations which might account for this qualitative change in the response to 4β-PDBu, for example inhibition of the Na+/K+ ATPase pump. These will be considered in more detail in Chapter 9. The results obtained after cold storage of tracheal tissues are of limited value due to the small number of replicates and the inability to determine whether inhibition of NANCi nerve-mediated relaxation after 24h and 48h post mortem, was due to blockade of action potential propagation along the neurone and/or the extent to which nerve terminals were depleted of neurotransmitter (susceptible to release by the phorbol ester).

In summary, no firm conclusions can be drawn from the results presented in this chapter, since the number of replicates was often deficient. However, relaxation of the guinea-pig trachea caused by EFS (in the presence of atropine and propranolol) and 4β-PDBu was inhibited
by methods which were likely to cause depletion of NANC\textsubscript{i} nerves (ie. treatment with VCH/\textalpha{}ltx and prolonged EFS in the presence of atropine and propranolol) supporting the hypothesis that relaxation in the guinea-pig trachea to 4\beta{}-PDBu could at least in part be dependent on functionally intact NANC\textsubscript{i} nerves, although other nerve mechanisms cannot be fully excluded. In addition, the relaxant response to VT was reduced by more than 50\% in preparations treated with \textalpha{}ltx, endorsing the neuronal nature of the response to the alkaloid in the trachea. However, no conclusions can be made as to the proposed role of VIP in NANC\textsubscript{i} nerve-, AC- or 4\beta{}-PDBu-mediated relaxation.
CHAPTER EIGHT

RELEASE STUDIES OF THE NANC₁ NEUROTRANSMITTER
IN GUINEA–PIG TRACHEA: A PILOT INVESTIGATION
Summary

1. Various techniques were employed in attempts to collect or quantify the NANC_1 neurotransmitter released on NANC_1 nerve stimulation by EFS, by voltage-sensitive Na^+ channel activators or by activation of PKC by 4β-PDBu.

2. In two experiments an ELISA technique was used in an attempt to measure changes in VIP-like immunoreactivity (VIP-IR) after incubation of tracheal strips with 4β-PDBu. No difference could be detected in the bathing medium of tracheal tissues incubated with and without 4β-PDBu.

3. A procedure was designed to collect and concentrate the NANC_1 neurotransmitter released from guinea-pig trachea, where the perfusate was tested on isolated tracheal strips. No detectable difference could be demonstrated between unstimulated samples and samples stimulated by EFS (using various stimulating parameters). When aconitine was used as a stimulus instead of EFS, the response of the tracheal assay preparation could not be distinguished from that of aconitine itself.

4. In another set of experiments, a superfusion-cascade apparatus was employed, where EFS was used to release the NANC_1 neurotransmitter into the bathing medium superfusing the assay tissue. No response of the assay tissue could be detected.

5. Since this was merely a pilot study, and various problems in methodology were encountered, no firm conclusions could be drawn from the results obtained in this chapter.
8.1 Introduction and background

In yet another approach to test the working hypothesis of phorbol ester-induced release of the NANC$_i$ neurotransmitter(s), it was decided to try to measure the release of this unidentified species. This was attempted initially using electrical field stimulation (EFS) - a method considered to release the NANC$_i$ neurotransmitter(s) (see Chapter 5). Aconitine was also studied since it was hypothesised that this alkaloid might cause the release of a relaxant from NANC$_i$ nerves (see Discussion, Chapter 6). Eventually it was hoped to demonstrate release of a relaxant, possibly from NANC$_i$ nerve fibres, using the PKC activator, 4ß-PDBu.

The first method was to measure VIP-like immunoreactivity (VIP-IR) using an enzyme-linked immunosorbent assay (ELISA). ELISA is an heterogenous enzyme assay based upon principles employed in radioimmunoassay, where after incubation of antigen (ie. VIP) and antibody, the antigen-antibody complexes formed are separated from free antigen and antibody by one of a number of different techniques and the activity in one or both of these fractions can be determined. In ELISA this is detected by the activity of an enzyme conjugated to the antibody complex and measured by the colourimetric change of $p$-nitrophenyl phosphate enzyme-dependent hydrolysis. ELISA has the advantage of antigen versatility, while requiring little knowledge of enzymology.

In another approach, an attempt was made to collect and concentrate biologically active material from guinea-pig trachea after EFS, using parameters reported to cause accumulation of VIP-IR in the tissue bathing medium (Matsuzaki et al., 1980). Finally, a cascade apparatus was employed to assay for release of a relaxant from guinea-pig trachea during EFS because it was thought, on the basis of a study by Garthwaite et al. (1988), that the NANC$_i$ neurotransmitter might be identical or similar to EDRF/NO, and would therefore have a transient existence.

The experiments reported in this chapter comprise a pilot study in which it was hoped to develop an assay system with which to quantitate the relaxant species released, possibly from NANC$_i$ nerves, by EFS and, according to the working hypothesis, the voltage-sensitive Na$^+$ channel activators, veratridine and aconitine, and the PKC activator, 4ß-PDBu.
8.2 Experimental protocol

The experimental designs used in this chapter are described in detail under section 2.3, "Release Studies".

8.3 Results

8.3.1 Effect of 4β-PDBu on release of VIP-like immunoreactivity from guinea-pig trachea

The results are shown in figure 8.1 and table 8.1. Data were obtained from two experiments in which tracheal strips from three animals were incubated in three vials according to the plan described in section 2.3.1a. Animal body weights between experiments, were not significantly different (experiment 1, 426±3g; experiment 2, 456±8g, p>0.05, unpaired t-test, n=3) and were considered matched. In both experiments (see figures 8.1a and b) standard calibration curves were linear over the range 10,000-50pg immunoreactive VIP per well of the microtitre plate used in the experiment. All test and control samples fell within this range. However, in both experiments no significant difference (p>0.05, unpaired t-test) could be detected between control (vial 1) and 4β-PDBu-stimulated (vial 2) samples (see table 8.1). Further, no significant difference (p>0.05, unpaired t-test) was detected in VIP-IR accumulation between control or test samples when compared to vial 3, in which tissues were incubated for 60min as in vial 1, but were removed prior to addition of 4β-PDBu (10μM). This served as an additional control with which to test the effect of the phorbol ester on the ELISA assay.

Curiously, there was a marked (>30 fold) quantitative difference in VIP-IR accumulation between the two experiments. Further, in the second experiment the change in optical density (at 450nM) in a sample of bathing medium not exposed to phorbol ester, or incubated with the tracheal tissues, was not significantly different (p>0.05, unpaired t-test) from any of the values obtained for any of the other samples (ie. vials 1-3).
8.3.2 Assay of the putative NANC\textsubscript{i} peptide neurotransmitter

The results are shown in figures 8.2 to 8.5. Each sample was generated under stated parameters, using tracheal tissues removed from eight guinea-pigs (see section 2.3.2a). Samples were tested on isolated tracheal strips from each of another four animals. These latter tissues are referred to as "assay tissues". When no response to a particular "stimulus" sample (generated from eight animals) was observed in an individual assay tissue, it was considered to be the fault of the stimulating parameters used to generate the sample, which were fixed for each group of eight animals, and not that of the assay tissue which was routinely shown to be responsive to NANC\textsubscript{i} nerve stimulation and/or VIP. For this reason figures 8.2 to 8.4 show essentially similar experimental data. However, in each case the stimulating parameters to EFS were varied between separate "stimulus" samples - each derived from eight animals (see figure legends for details).

Figure 8.2 shows the effect of two samples, "prestimulus" and "stimulus", when added to the assay tissue at final dilution of 1:50 in a tissue bathing volume of 5ml. The "stimulus" sample was generated by EFS (2ms, 100:18V, 20Hzx4min). Note that the "stimulus" sample caused a weak relaxation (30% of the maximum relaxation to papaverine) of the assay tracheal tissue. This was not seen with the "prestimulus" sample, despite relaxation to NANC\textsubscript{i} nerve activation by EFS (0.5ms, 100:18V, 10Hz) being observed readily (85% of the maximum relaxation to papaverine) in another assay tissue from the same animal.

In another experiment (see figure 8.3), a similar comparison was made where the "stimulus" sample was generated at a lower frequency (10Hz), but which had previously been shown in organ bath studies to produce maximal relaxation to NANC\textsubscript{i} nerve activation (eg. see figure 5.4). In addition, a reduced (2ml) bathing volume was used to maximise the effective concentration of biologically active material in the samples. However, both the "prestimulus" and "stimulus" samples produced concentration-dependent relaxation at the two dilutions investigated, which was similar in extent and rate in both samples (Prestimulus 1:167 13%, 1:50 34%; stimulus 1:167 9%; 1:50 26% of the maximum relaxation to papaverine, 100\mu M). Further, the relaxation produced to the 1:50 dilution of both samples was reversible and reproducible (see figure 8.3). Both assay tissues responded with near maximum relaxation to
NANC\textsubscript{i} nerve stimulation by EFS (0.5ms, 100:18V, 10Hz) and to exogenous VIP (0.3\textmu M), a putative NANC\textsubscript{i} peptide neurotransmitter.

Figure 8.4 shows an experiment similar to that above, in which the "stimulus" sample was generated by EFS using parameters (0.5ms, 100:17V, 10Hz\times4min), which in organ bath studies, produced relaxation which was abolished by TTX (eg. see figure 5.1b). The tissue bathing volume was 2ml. Both "prestimulus" and "stimulus" samples produced concentration-dependent, but comparable relaxation in the tracheal assay tissue (prestimulus 1:67 15%, 1:50 36%; stimulus 1:167 22%; 1:50 44% of the maximum relaxation to papaverine, 100\textmu M), while in both tissues NANC\textsubscript{i} nerve stimulation by EFS produced near maximum relaxation (see figure 8.4).

In one experiment (not shown) absolute methanol (3ml) was passed through a Sep-Pak C\textsubscript{18} cartridge (previously "cleaned and wetted" with Krebs-Henseleit (KH) solution, see section 2.3.2b) and dried-off under nitrogen gas before being taken up in 500\textmu l deionised distilled water containing dithiothreitol (1mM). Addition of this "blank" sample to the assay tissue at a final dilution of 1:100 produced no effect on the intrinsic tone of the preparation. In addition, a sample "spiked" with VIP (0.5\textmu M), and subjected to the same concentration procedure described in section 2.3.2b, was examined on the guinea-pig trachea (1:50 dilution). Relaxation, which was comparable in extent to that produced by maximally effective EFS in the presence of atropine (1\textmu M) and propranolol (1\textmu M), was observed. Furthermore, the rate of relaxation to the spiked sample was slowed in the presence of the proteases, \alpha-chymotrypsin (5U/ml) and papain (5U/ml) (t\textsubscript{50} control 95s, protease-treated 1203s), while the extent of relaxation appeared unaltered.

In figure 8.5, the "stimulus" sample was generated using aconitine (30\textmu M) incubated with tissues from eight animals for 10min (see section 2.3.2.a). Addition of the "stimulus" sample (1:100 dilution) to the assay tissue (guinea-pig trachea) produced total relaxation in the presence of atropine (1\textmu M) and propranolol (1\textmu M), which was not seen with the "prestimulus" sample (1:100 dilution), but which was similar to the relaxation produced by aconitine (30\textmu M). However, in the presence of eserine (0.5\textmu M) (and propranolol, 1\textmu M), the "stimulus" sample produced a biphasic response - relaxation followed by a sustained atropine-sensitive
contraction, which bore more than a passing resemblance to the profile of response observed with aconitine (cf. figure 8.5c and d).

In all experiments, during the sample concentration procedure (see section 2.3.2b), it was noted that when the methanol had evaporated to dryness, an off-white powder remained which was readily soluble in aqueous solution. Although no measurements of mass were made, there appeared to be more powder in the "stimulus" samples compared to the "prestimulus" samples. Moreover, the powder was absent in the "blank" sample (see above).

In all experiments the ratio of mean body weight (grams) to tracheal wet weight (milligrams) within each group of eight animals was always within the range 10-17.

8.3.3 Superfusion-cascade assay

The results are shown in figures 8.6 and 8.7, and represent two experiments in which an attempt was made to detect the presence of a relaxant in the effluent from the syringe arrangement (see figure 2.1b) after EFS. Atropine (1μM) and propranolol (1μM) were present throughout both experiments to prevent the action of acetylcholine and β-adrenoceptors stimulants on the assay tissue. In this particular tissue, EFS was not attempted due to the difficulty in arranging stimulating electrodes proximal to the assay tissue during superfusion.

The results in figure 8.6 demonstrated that at a flow rate of 2ml/min no relaxant activity in the effluent of the syringe arrangement could be detected by the assay tissue after EFS of the tracheal tissues in the syringe. This situation did not improve despite increasing the flow rate through the syringe (T.S) to 3ml/min. The assay tissue was, however, responsive to VIP applied directly (O.T), bypassing the syringe.

In the second experiment (figure 8.7) similar results to the first were obtained to EFS despite a flow rate of 4ml/min. Curiously, when the assay tissue was treated with ouabain (10μM) and tone maintained with histamine (10μM), both applied directly to the assay preparation (O.T), the phorbol ester, 4β-PDBu (100nM), produced relaxation when passed through the syringe (ie. T.S) containing the loosely packed tracheal preparations.
Figure 8.1 Data obtained from two experiments (a and b) showing calibration curves for determination of VIP-like immunoreactivity released from 4β-PDBu (10μM)-stimulated guinea-pig tracheal strips measured by ELISA. Curves were fitted to data points indicated (*) using least squares regression analysis. Data points represent mean ± s.e.mean of triplicate determinations. Sample absorbances were read directly from the above plots and converted to pmoles of VIP-like immunoreactivity per gram wet weight of tracheal tissue in each sample.
Figure 8.2 The effect of two concentrated samples, (b) prestimulus (prestim) and (c) stimulus (stim) at a final dilution of 1:50 on guinea-pig trachea. The stimulus sample was generated by EFS (2ms, 100:18V, 20Hz×4min) of tracheal strips removed from eight guinea-pigs and subjected to the collection and concentration procedure described in section 2.3.2. The assay tissues were bathed in 5ml KH solution containing atropine (1µM), propranolol (1µM) and the protease inhibitors, bacitracin (1.86U/ml) and aprotinin (1.2U/ml). Tissue (a) demonstrated that the assay tissue was responsive to NANC\textsubscript{1} nerve stimulation by EFS (0.5ms, 100:18V, 10Hz). Hist = histamine, W = wash, dil = final dilution of sample in the organ bath, P = papaverine (100µM). Isometric recording.
Figure 8.3 Comparison of two concentrated samples, (a) prestimulus ("prestim") and (b) stimulus ("stim") on the guinea-pig trachea. The stimulus sample was generated by EFS (2ms, 100:18V, 10Hz×4min) of tracheal strips removed from eight guinea-pigs and subjected to the collection and concentration procedure outlined in section 2.3.2. The above assay tissues were bathed in 2ml KH solution containing atropine (1μM), propranolol (1μM) and the protease inhibitors, bacitracin (1.86U/ml) and aprotinin (1.2U/ml). Note that both assay tissues responded to NANC$i$ nerve stimulation by EFS (0.5ms, 100:18V, 10Hz) and to exogenous VIP (0.3μM). Hist = histamine, W = wash, dil = dilution, P = papaverine (100μM). Isometric recording.
Figure 8.4 Comparison of two concentrated samples, (a) prestimulus ("prestim") and (b) stimulus ("stim") on the guinea-pig trachea. The stimulus sample was generated by EFS (0.5ms, 100:17V, 10Hz×4min) of tracheal strips obtained from eight animals and subjected to the collection and concentration procedure outlined in section 2.3.2. The above assay tissues were bathed in 2ml KH solution containing atropine (1μM), propranolol (1μM) and the protease inhibitors, bacitracin (1.86U/ml) and aprotinin (1.2U/ml). Note that both assay tissues were reactive to NANCi nerve stimulation by EFS (0.5ms, 100:17V, 10Hz). Hist = histamine, W = wash, dil = dilution, P = papaverine (100μM). Isometric recording.
Figure 8.5 Comparison of two concentrated (a) prestimulus ("prestim") and stimulus ("stim") samples with (c) aconitine (30μM) on guinea-pig trachea. A similar comparison for the (b) stimulus sample and (d) aconitine is shown in the presence of eserine (0.5μM). The stimulus sample was generated by incubation (10min) of tracheal strips from eight guinea-pigs with aconitine (30μM), while in the prestimulus sample tissues were incubated with DMSO (0.06%v/v). All samples were then subjected to the collection and concentration procedure outlined in section 2.3.2. Each of the above assay tissues was bathed in 2ml KH solution containing propranolol (1μM) and the protease inhibitors, bacitracin (1.86U/ml) and aprotinin (1.2U/ml). Hist = histamine, W = wash, dil = dilution, P = papaverine (100μM). Isometric recording.
Figure 8.6 The effect of EFS and VIP on guinea-pig trachea. A modified 10ml polypropylene syringe (perfused with Krebs-Henseleit solution at the flow rates shown) was packed with tracheal strips removed from a single guinea-pig. These tissues were subjected to EFS (0.5 and 1ms, 100:18V, 10Hz) and the effluent from the syringe (T.S.) allowed to superfuse a single tracheal strip (from the same animal) in a cascade. Drugs could be applied directly over the assay tissue (O.T.). Horizontal bars indicate the time (min) for which drugs or EFS were administered to the assay tissue. Atropine (1μM) and propranolol (1μM) and the protease inhibitors, bacitracin (1.86U/ml) and aprotinin (1.2U/ml) were present throughout. Hist = histamine, Pap = papaerine. Isometric recording.
Figure 8.7 The effect of EFS and 4β-PDBu (in the presence of ouabain) on guinea-pig trachea. EFS (0.5, 1 and 2 ms, 100:18 V, 30 Hz) was delivered to the syringe (packed with tracheal strips from the same animal). As indicated in figure 8.6, the effluent from the syringe (T.S.) was allowed to superfuse the assay tracheal tissue. 4β-PDBu (0.1 μM) was also passed through the syringe (T.S.) and allowed to superfuse the assay tissue, over which ouabain (10 μM) was administered directly over the tissue (O.T.). Atropine (1 μM) and propranolol (1 μM) and the protease inhibitors, bacitracin (1.86 U/ml) and aprotinin (1.2 U/ml) were present throughout. Hist = histamine, P = papaverine (100 μM). Isometric recording.
Table 8.1 Changes in VIP-like immunoreactivity (VIP-IR), determined by ELISA, in samples of bathing medium incubated (30min) either with (vials 1 and 2) and without (vial 3) guinea-pig tracheal strips or with (vial 2 and 3) and without (vial 1) 4β-PDBu (10μM). Data are given as mean ± s.e.mean of triplicate determinations from two separate experiments (Expt. 1 and 2) expressed as picomoles of VIP per gram wet weight of tracheal tissue in each vial. In each experiment tissues were obtained from three animals. For experimental details see section 2.3.1, where each vial corresponds to that given above. Note, in vial 3, tissues were present throughout the incubation period, but were removed prior to addition of the phorbol ester. Protease inhibitors (bacitracin 1.86U/ml) and aprotinin 1.2U/ml) were present in all samples throughout. Student's t-test for unpaired observations was employed for comparisons where p<0.05 was considered significant.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>VIP-IR (pmoles/g tissue) in samples vials</th>
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<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
</tr>
<tr>
<td>&quot;Vial 1&quot; tissues</td>
<td>5.87±0.68</td>
</tr>
<tr>
<td>+ 0.02% DMSO</td>
<td></td>
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<tr>
<td>&quot;Vial 2&quot; tissues</td>
<td>5.11±0.84</td>
</tr>
<tr>
<td>+ 4β-PDBu</td>
<td></td>
</tr>
<tr>
<td>&quot;Vial 3&quot; No tissues</td>
<td>5.32±1.17</td>
</tr>
<tr>
<td>+ 4β-PDBu</td>
<td></td>
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</tbody>
</table>
8.4 Discussion

In the previous chapters of this thesis, experiments were designed which made use of indirect classical organ bath techniques available to the pharmacologist in order to test the hypothesis that relaxation of the guinea-pig trachea to phorbol esters might involve PKC-mediated release of a relaxant neurotransmitter, particularly from NANCᵢ neurones. However, this concluding chapter of results reports a series of preliminary or pilot experiments in which other techniques were employed in order to test the above hypothesis. The techniques aimed to demonstrate, and if possible to quantify, the direct release of the neurotransmitter(s) mediating NANCᵢ nerve responses in the guinea-pig trachea by EFS (and possibly the voltage-sensitive Na⁺ channel activators, veratridine and aconitine). Ultimately it was hoped to determine whether the neurotransmitter was released by the PKC activator, 4β-PDBu. This approach of direct measurement of the NANCᵢ neurotransmitter is obviously severely limited by information available on the identity of the NANCᵢ species.

The first approach employed was to measure VIP-like immunoreactivity (VIP-IR) by an ELISA technique, with which the ability of 4β-PDBu to cause accumulation of VIP-IR in the bathing medium of isolated guinea-pig tracheal strips was tested. However, quantification of VIP-IR by ELISA revealed no change in 4β-PDBu-stimulated and control tracheal tissues, when compared within each of the two experiments performed. There was considerable variation (approx. 30 fold) in VIP-IR in samples between the two experiments. The reason for this anomaly was not clear, but problems in the methodology of the assay were encountered, and in order to validate the apparent negative result to phorbol ester stimulation the necessity for the inclusion of a positive control such as EFS, known to cause accumulation of VIP-IR into the bathing medium (Matsuzaki et al., 1980), should have been foreseen. It is unlikely that the VIP-IR measured, particularly in the second experiment, could be attributed solely to spontaneous accumulation since the "no tissue blank" (KH solution only) produced a change in optical density not dissimilar from one of the controls (no 4β-PDBu) which was incubated with tracheal tissues. The reason for the disparity between these two experiments may lie in the antigen (VIP)-antibody binding stage, known to be affected by various ion concentrations present in the KH solution.
The antibody which was used to VIP in ELISA is known to exhibit little or no cross-reactivity with other peptides such as substance P or PHI. If the negative result is to be believed, the possibility of 4β-PDBu evoking the release of another relaxant peptide (or for that matter a non-peptide) which is not recognised by the antibody must still be considered. However, this particular approach stimulated other experiments which resulted in the reported finding that 4β-PDBu was able to facilitate release of VIP-IR from enteric nerves in the rat ileum (Belai et al., 1990; see Chapter 5).

In another approach an attempt was made to collect and concentrate the NANC\textsubscript{i} neurotransmitter using Sep-Pak \textsubscript{C\textsubscript{18}} cartridges. These are often employed to "clean" and concentrate peptide-containing samples for chromatographic analysis. However, since the identity of the NANC\textsubscript{i} neurotransmitter(s) in guinea-pig airways is unknown, this approach had to make two fundamental assumptions: (i) that the NANC\textsubscript{i} neurotransmitter(s) was peptidergic in nature and (ii) that it would bind to the packing in the \textsubscript{C\textsubscript{18}} cartridge. A preliminary experiment in which a sample was "spiked" with VIP, a putative NANC\textsubscript{i} peptide neurotransmitter, demonstrated that as far as this particular peptide was concerned, the technique was suitable.

Initially, EFS was used to activate NANC\textsubscript{i} neurones prior to collection of the samples. Matsuzaki et al. (1980) had demonstrated that EFS caused a frequency-dependent and TTX-sensitive accumulation of VIP-IR in the bathing medium of guinea-pig trachea. Further when a pulse duration of 2ms was used the accumulation of VIP-IR, measured by radio-immunoassay, was doubled when compared to a pulse duration of 1ms. With this in mind an initial experiment was performed using a pulse duration of 2ms, in which a weak transient relaxation was observed to the "stimulus" sample when added to the assay tissue, but which was not seen in the case of the "prestimulus" sample. However, it was considered that a pulse duration of 2ms could have a direct effect on the smooth muscle and/or cause the release of substances not necessarily derived from neuronal structures, or relevant to the situation in the organ bath, where pulse durations of 0.1-0.5ms are more commonly used to produce responses which are largely abolished by the
neurotoxin, TTX. Further experiments employing this pulse duration (2ms) were not continued. However, when EFS parameters similar to those used in organ bath studies were employed, no difference could be detected between "prestimulus" and "stimulus" samples, both of which produced concentration-dependent relaxation which was reproducible. The nature of this "relaxant" was not investigated further, but did appear to be a real effect since relaxation was not obtained to the "no tissue blank" (see section 8.3.2) suggesting that impurities in the methanol, or substances carried over from the KH solution used to "clean and wet" the Sep-Pak C_{18} cartridges, did not appear to be responsible for the relaxation which was observed with other samples, in which tracheal tissues had been incubated. In addition, the off-white powder obtained on drying the samples, was not obtained in the "no tissue blank" suggesting that the relaxation observed in the assay tissues may well be non-specific, but dependent on exposure of the KH solution to tracheal tissues during the sample collection stage of the procedure.

In another experiment it was decided to stimulate tracheal strips with the voltage-sensitive Na\(^+\) channel activator, aconitine (AC), which it was hypothesised causes the release of a NANC\(_1\) relaxant (see Chapter 6). This "stimulus" sample produced marked relaxation in the assay tissue (which was less obvious with the "prestimulus" sample). The response however, was indistinguishable from that produced by AC, when both were compared in the absence and presence of eserine. The possibility that AC had bound to, and had been eluted-off, the cartridge during the concentration procedure and thereby causing contamination of the sample, was one which would require careful and extensive investigation before pharmacological stimuli such as aconitine, veratridine or 4\(\beta\)-PDBu (emphasising the advantage of the non-invasive nature of EFS in activating neuronal mechanisms) could be investigated further to test the working hypothesis. This situation was not aided by the large numbers of animals required and financial cost involved for this technique.
The final method employed to detect the NANC\textsubscript{i} neurotransmitter released by EFS, was that of cascade-superfusion — a technique developed by Vane (see Vane, 1983), and instrumental in advancing the research and identity of cyclo-oxygenase products, the proposed anti-inflammatory mechanism of the glucocorticoids and more recently, the pharmacology of endothelium-derived relaxing factors. However, at this time there are few or no reports in the literature, of which we are aware, where this technique has been used (successfully or otherwise) to investigate the NANC\textsubscript{i} neurotransmitter in guinea-pig airways.

The inability to detect a relaxant in the effluent of the syringe after EFS suggests that the concentration of biologically-active material, which presumably includes the NANC\textsubscript{i} neurotransmitter(s), is below the threshold for detection by the assay tissue (guinea-pig trachea). This may be due to rapid enzymic degradation of the NANC\textsubscript{i} species or due to some intrinsic labile property of the relaxant. The former possibility is unlikely to be due to bacitracin- or aprotinin-sensitive proteases since these inhibitors were present throughout. Further, the assay tissue was shown to be responsive to exogenous VIP. Addressing the problem of labileness or very short active biological half-life, no activity was observed even when the flow rate was doubled to reduce the latency between the syringe and assay tissue to less than five seconds. The possibility that the half-life of the putative NANC\textsubscript{i} neurotransmitter is much shorter or that nothing was released under the experimental conditions, must also be considered. However, the "monitoring signal" on the oscilloscope was very similar to that seen in organ bath studies (see section 2.2.3) where relaxant responses to EFS were readily observed. Recently, reports have appeared in the literature to suggest that in mouse and rat anococcygeus muscle (Gibson et al., 1989; Gillespie et al., 1989) and in canine gastrointestinal tract (Bult et al., 1990) the elusive NANC\textsubscript{i} neurotransmitter is nitric oxide.

It is of interest to note then when the assay tissue was treated with ouabain, which in organ bath studies converted phorbol ester-induced tracheal relaxation into contraction (Huang et al., 1987; see figure 4.4), the phorbol ester, 4\beta-\textsubscript{PDBu} (after having first passed through the syringe) produced a rapid and sustained relaxation. It may also have
been useful to test the effect of 4β-PDBu directly on the assay tissue after treatment with ouabain. However, this observation must beg the question of whether the relaxant action of 4β-PDBu under these conditions involved a direct or an indirect mechanism – the subject of this thesis.

In summary, no firm conclusions can be drawn from these preliminary experiments. However, they draw attention to the difficulty experienced in demonstrating NANC$_i$ nerve-mediated responses in isolated tissues outside the organ bath. It is intriguing to ask whether these responses are indeed peptidergic in view of the negative results obtained when protease inhibitors, antioxidants, and experimental conditions under which most peptide responses can readily be demonstrated, are carefully employed using methods considered most likely to activate NANC$_i$ nerves and presumably cause release of their sequestered neurotransmitters. This is especially worthy of consideration when concentration procedures and large numbers of animals are employed apparently to little avail.
CHAPTER NINE

GENERAL DISCUSSION
It is not the intention of this final chapter to discuss in detail the results of individual chapters since this has already been done. However, it is important to summarise and consolidate the main findings.

In October 1986, the project set out to examine some of the possible interactions which might occur between preparations of isolated airway smooth muscle and inflammatory cells (e.g., platelets, eosinophils and polymorphonuclear cells) when added to the organ bath. In addition, it was decided to examine the extent to which protein kinase C (PKC) activation (by phorbol esters) might be involved in this interplay, using preparations of guinea-pig lung parenchyma as a model of airway smooth muscle.

Chapter three highlighted some of the controversy which exists as to the extent to which vascular smooth muscle contributes to the response of the guinea-pig lung parenchyma, challenging its status as a model of peripheral airway pharmacology. The results of this chapter did not resolve adequately this controversy and so it was decided to study the response of both tracheal and lung parenchymal preparations in parallel.

Phorbol esters, considered to activate PKC directly, caused qualitatively different responses in these two respiratory tract preparations from guinea-pig (Chapter 4). Contraction in the lung parenchyma (Dale and Obianime, 1985) and relaxation in the trachea (Menkes et al., 1986). At the time it seemed unlikely that one preparation of guinea-pig respiratory tract smooth muscle should respond to phorbol esters by an intrinsically different mechanism from that of another taken from further down the bronchial tree, but which has an identical embryological origin. Chapter four addressed the question: Was the unexpected relaxation due to the tracheal muscle itself responding directly to phorbol esters in an intrinsically different manner from that of the lung parenchyma, or was it due to an indirect effect involving release of mediators which in turn might cause relaxation either directly or indirectly? Menkes et al. (1986) considered that this relaxation was indeed direct, involving PKC-mediated inhibition of the signal transduction pathways to contractile agonists such as histamine and carbachol. The results of this chapter implied that phorbol esters were able to inhibit relaxation to agonists such as isoprenaline (but not salbutamol), but had no effect on the contraction to either histamine or carbachol - presumed to act
predominantly at receptors located on the airway smooth muscle. Consideration of these results, together with evidence from the literature implicating PKC in the proposed "latch-bridge" state of smooth muscle tension maintenance (see Chapter 1), suggested that the explanation put forward by Menkes et al. (1986) was incorrect, and that an indirect mechanism could be responsible for relaxation of the guinea-pig trachea to phorbol esters. However relaxation to the phorbol ester, 4β-PDBu, was not prevented by a series of receptor antagonists/ enzyme inhibitors or after removal of the luminal airway epithelium. One possibility not eliminated by the results obtained in this chapter was that relaxation in the guinea-pig trachea to 4β-PDBu could result from PKC-mediated release of a relaxant neurotransmitter(s), possibly from inhibitory non-adrenergic non-cholinergic (NANC_i) nerve fibres. This seemed to be a reasonable hypothesis to test since there was evidence accumulating in the literature that phorbol esters were indeed able to cause the release of sequestered neurotransmitters. This possibility then became the main focus of this thesis.

The remaining chapters (Chapter 5 onwards) set out to test this hypothesis by comparing relaxation produced by 4β-PDBu with relaxation caused by NANC_i nerve stimulation by EFS (in the presence of atropine and propranolol) and a putative NANC_i peptide neurotransmitter, VIP. Experiments were designed in which the current state of knowledge of the distribution of functional NANC_i nerve innervation and VIP-like immunoreactivity in respiratory tissues was applied to the relaxant response which occurred with the phorbol ester. Similarities were also found in a possible role for cyclic nucleotides in mediating VIP (and NANC_i nerve)-induced relaxation of guinea-pig trachea, and in the mechanisms reported to be responsible for termination of the biological actions of the putative neurotransmitter(s) involved in these responses.

There is now a considerable number of reports in the literature, most of which have appeared during the last four years, implicating a role for PKC in the control of neuronal function, particularly in neurotransmitter release (see Discussion, Chapter 5). Moreover, NANC_i nerves occur in the tissues in which phorbol esters produce relaxation (eg. guinea-pig ileum, rat uterus and guinea-pig trachea). In guinea-pig isolated respiratory tract preparations, relaxation to 4β-PDBu seemed to be associated with the previously reported NANC_i neurone distribution,
assessed by EFS and PHI/VIP-like immunoreactivity. Further, the results in Chapter 5 were consistent with relaxation of the trachea to 4β-PDBu being due to release of a cyclic GMP-independent relaxant, possibly a peptide from NANC$_i$ neurones.

If relaxation in the guinea-pig trachea to 4β-PDBu does involve release of a NANC$_i$ neurotransmitter, then a role for PKC, the endogenous PKC activator (DAG) and phosphoinositide (PI) hydrolysis is implied in the release process of sequestered neurotransmitter from NANC$_i$ nerves. This was considered in Chapter 6. A number of reports in the literature demonstrated that agents such as veratridine (VT), aconitine (AC) and the toxin from the venom of the scorpion, Leiurus quinquestriatus, which cause Na$^+$ influx through voltage-sensitive Na$^+$ channels in cerebral cortical slices from rat, and in preparations of nerve endings (cerebral cortical synaptoneurosomes) from guinea-pig, also stimulate molecular changes consistent with PI hydrolysis (Batty et al., 1985; Gusovsky et al., 1986, 1987). This would be expected to cause generation of DAG and activation of PKC - an effect which leads to neurotransmitter release in several tissues. Moreover, VT has been reported to release the putative NANC$_i$ peptide neurotransmitter, VIP, from rat ileum (Besson et al., 1983), which also occurs in this tissue with 4β-PDBu (Belai et al., 1990). Examination of the alkaloids, VT and AC, in the trachea and other respiratory tract smooth muscle preparations suggested that the relaxant response of the trachea to the alkaloids closely reflected the component of EFS which is attributed to NANC$_i$ nerve stimulation.

Investigation of inhibitors of DAG metabolism (see Introduction, Chapter 6), with the assumption that they were specific for the DAG metabolising enzymes, suggested that the generation of DAG and its subsequent metabolism by DAG lipase might well be involved in NANC$_i$ nerve-mediated relaxation.

Finally, in chapter seven, various techniques were employed in attempts to differentiate neuronal and non-neuronal relaxant responses of the guinea-pig trachea caused by NANC$_i$ nerve stimulation and the PKC activator, 4β-PDBu. The main finding was that relaxation of guinea-pig
trachea induced by NANC\textsubscript{i} nerve stimulation, veratridine and 4\beta-PDBu was inhibited by methods most likely to cause neurotransmitter depletion, in particular from NANC\textsubscript{i} nerves.

Taken together, these results could imply that the transduction events for the release mechanism of the sequestered NANC\textsubscript{i} neurotransmitter from nerve terminals may be as follows: plasma membrane depolarisation (Na\textsuperscript{+} influx), followed by PI hydrolysis occurring via a Ca\textsuperscript{2+}-dependent phospholipase C, generation of DAG and the subsequent activation of PKC, and finally neurotransmitter release (see figure 9.1). Data in favour of this suggestion has already been discussed in detail. However, an additional and interesting report by Diamant et al. (1987) has shown that neomycin, reported to selectively bind phosphatidylinositol 4,5-bisphosphate and thereby acting as a phospholipase C inhibitor (Lipsky and Leitman, 1982), inhibited VT-induced neurotransmitter release from rat brain slices and synaptoneurosomes. Further, in a recent report by Ari et al. (1989) polymyxin B, reported to selectively inhibit PKC (Allgaier et al., 1986), blocked VT-induced neurotransmitter release from rat brain cortical slices.

This hypothesis, that 4\beta-PDBu relaxes guinea-pig trachea by releasing a potent NANC\textsubscript{i} neurotransmitter, could explain the main results reported by Menkes et al. (1986) and Huang et al. (1987). They had discarded the possibility of a neuronal effect on the basis of the lack of effect of both propranolol and TTX. This was also shown in Chapter four (figure 4.8a and c), but was to be expected according to the data of Gusovsky et al. (1986, 1987) on the transduction events in synaptoneurosomes, and in the scheme presented in figure 9.1 showing a post ganglionic nerve ending in guinea-pig trachea, since PI hydrolysis (and thus the role of PKC) would be expected to occur further along the train of the transduction events than Na\textsuperscript{+} influx (see figure 9.1).

Chideckel et al. (1986) reported that K\textsuperscript{+}-induced depolarisation of methacholine precontracted guinea-pig trachea in the presence of the calcium channel antagonist, verapamil, caused relaxation. Verapamil and other organic voltage-sensitive calcium channel antagonists are thought to exhibit greater selectivity for the channels found in smooth muscle cells, rather than in neuronal cells (see Tsien et al., 1988). This effect with K\textsuperscript{+} is analogous to the reversal of carbachol-induced tone by
phorbol esters which was reported by Menkes et al. (1986). Similarly, the relaxations reported by both Menkes et al. and Chideckel et al. were not prevented by the cyclo-oxygenase inhibitor, indomethacin. However, Chideckel et al. (1986) was able to demonstrate that relaxation in the guinea-pig trachea to K⁺ (in the presence of verapamil) was resistant to propranolol, but was abolised by TTX. The authors suggested that the relaxant effect of K⁺ was due to depolarisation of nerve fibres and the subsequent TTX-sensitive release of a relaxant non-adrenergic neurotransmitter (cf. figure 9.1). More recently, Watson et al. (1990) have shown that K⁺ causes inositol phosphate (IP) accumulation (suggestive of PI hydrolysis) in the guinea-pig ileum longitudinal strip. The accumulation of IP was found to be only partially sensitive to nifedipine, an inhibitor of L-type voltage sensitive Ca²⁺ channels which are found on both neurones and smooth muscle cells (Tsien et al., 1988). However, the dihydropyridine-insensitive component of the response to K⁺ was inhibited by ω-conotoxin which blocks N-type Ca²⁺ channels present in neuronal cells, but absent in smooth muscle (Tsien et al., 1988). The authors concluded that the response to K⁺ (ie. IP accumulation) appeared to occur predominantly in neuronal tissue, and went on to suggest the presence of a Ca²⁺-activated phospholipase C mechanism. Watson et al. (1990) also reported similar results (ie. neuronal IP accumulation) with the persistent voltage-sensitive Na⁺ channel activators, veratridine and scorpion toxin, showing remarkable accord in the peripheral nervous system with the findings of Gusovsky et al. (1986, 1987) and in the scheme suggested in figure 9.1. Moreover, phorbol esters also produce relaxation in this tissue (Sasaguri and Watson, 1990).

K⁺-induced relaxation of guinea-pig trachea, first described by Chideckel et al. (1986), has also been explained in terms of enhanced blocking effects of voltage-sensitive Ca²⁺ channel antagonists due to depolarisation-induced increase in their binding to smooth muscle Ca²⁺ channels (Koga et al., 1989). However, although these authors were able to show that the luminal airway epithelium was not involved in the K⁺ response, they failed to test the effect of TTX or propranolol, precluding the elimination of the neural mechanism described by Chideckel et al. (1986) from this response.
Could relaxation of the isolated guinea-pig tracheal strip preparation to 4β-PDBu involve a direct action on the airway smooth muscle?

Although this question has in part been discussed above, it was a consideration which was always in mind throughout the design and interpretation of experiments reported in this thesis, and which received particular attention in chapters 4 and 7. Phorbol esters, through activation of PKC are known to phosphorylate many different cellular proteins, some of which are believed to be involved in the control of tension in smooth muscle (Introduction, section 1.3). Other such proteins might bring about inhibition of Ca\(^{2+}\) release, augmentation of the efficiency of processes responsible for lowering [Ca\(^{2+}\)]\(_{j}\) (eg. uptake into non-mitochondrial Ca\(^{2+}\) sequestration sites, Ca\(^{2+}\)-efflux or Na\(^{+}\)/Ca\(^{2+}\) exchange), inhibition of calmodulin or inhibition of the biochemical sequences leading to myosin phosphorylation and eventually contraction, or even prevent influx of Ca\(^{2+}\) across the plasma membrane (Introduction, section 1.4). Interference with any of these mechanisms could conceivably lead to smooth muscle relaxation. However, such an effect in the guinea-pig trachea was considered unlikely since the contractile response of this preparation to both histamine and carbachol was unaffected by 4β-PDBu (see above).

More recently, however, several reports in the literature suggest that phorbol esters (by activating PKC) produce relaxation of smooth muscle through augmentation of the plasma membrane electricgenic Na\(^{+}\)/K\(^{+}\)-ATPase pump, leading to reduced [Na\(^{+}\)]\(_{j}\) in guinea-pig tracheal strips (Souhrada and Souhrada, 1988, 1989) and guinea-pig ileum longitudinal smooth muscle strips (Sasaguri and Watson, 1989, 1990). Whilst it is accepted that a component of the relaxation of the trachea to phorbol esters might involve this mechanism to a greater or lesser extent, the above authors base much of their conclusion on the ability of ouabain, an inhibitor of the Na\(^{+}\)/K\(^{+}\)-ATPase pump, to convert relaxation of the tissues by phorbol esters to contraction. Inhibition of the Na\(^{+}\)/K\(^{+}\)-ATPase pump in the guinea-pig trachea is known to cause plasma membrane depolarisation (Souhrada et al., 1981). This is associated with Ca\(^{2+}\) entry via voltage-sensitive Ca\(^{2+}\) channels and/or inhibition of Na\(^{+}\)/Ca\(^{2+}\) exchange (Chideckel et al., 1987). The results reported in this thesis and of others (Menkes et al., 1986; Huang et al., 1987) have already clearly demonstrated that agents which increase [Ca\(^{2+}\)]\(_{j}\) in
Figure 9.1  Schematic representation of the proposed events leading to the release of the NANC<sub>i</sub> neurotransmitter from a hypothetical neurone innervating guinea-pig tracheal smooth muscle. Depolarisation of the nerve ending (1) leads to Na<sup>+</sup> influx (2) followed by Ca<sup>2+</sup> influx (3). Activation of a phospholipase C (PLC) by increased intracellular Ca<sup>2+</sup> (4) causes phosphoinositide (PI) turnover and the generation of the endogenous PKC activator, diacylglycerol (DAG, 5) which in turn leads to the release of the NANC<sub>i</sub> neurotransmitter (6). Note that the phorbol ester, 4β-PDBu, is effective much further down the train of events than Na<sup>+</sup> influx and that in addition the toxin from black widow spider venom, α-latrotoxin (αLTx), can also lead to PI turnover. Modified from the data of Gusovsky et al. (1986, 1987) and Vicentini and Meldolesi (1984).
addition to ouabain (e.g. KCl and A23187), also convert phorbol ester-induced relaxation of the trachea to contraction. In addition, the results of Sasaguri and Watson (1989, 1990) show only a small increase (24% above basal) in ouabain-sensitive $^{86}$Rb$^+$ uptake (an index of Na$^+/K^+$-ATPase activity) in guinea-pig ileum longitudinal muscle strip, evoked by a maximally effective concentration of 4β-PDBu (3μM). Further, no data was presented showing the relative extent to which this effect occurred in either neuronal or smooth muscle tissues.

The activity of the Na$^+/K^+$-ATPase pump can be reduced by cooling (Fleming, 1980), which also reduces neuronal function (Day and Vane, 1963). Inhibition of the pump in the isolated guinea-pig tracheal preparation leads to depolarisation of smooth muscle (see above) which may account for the otherwise fortuitous result (shown in figure 4.4) obtained on cooling, where contraction instead of relaxation to 4β-PDBu was observed (see also Discussion, Chapter 7). However, the extent to which either of these two possible explanations account for this phenomenon is not known.

It should be realised that "activation of the electrogenic Na$^+/K^+$-ATPase pump" can be described either by an increase in pumping rate of individual pumps or an increase in the number of ouabain binding sites (ie. pumps). The latter would only be expected to occur over 6-8h (Boardman et al., 1974). However, the possibility that phorbol esters may accelerate this process has not been considered. Finally, the Na$^+/K^+$-ATPase pump theory, in explaining relaxation of the trachea to phorbol esters appears not to account for the graduated loss in relaxation to 4β-PDBu which occurred at different levels down the tracheobronchial tree - an effect more likely to parallel changes in the density of pulmonary innervation, than changes in the density or properties of the Na$^+/K^+$ ATPase pumps for which there does not appear to be any reported evidence in the guinea-pig tracheobronchial tree.

This project has attempted to characterise an unexpected response in the isolated guinea-pig tracheal preparation: relaxation to phorbol esters. The study has employed numerous and varied approaches and paid particular attention to the neural mechanisms innervating this preparation. A proportion of the findings and their interpretations in this project have been based on often rather small numbers of

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replicates, and where appropriate, attention has been drawn to this deficiency. It should be stressed, however, that many different possible mechanisms for the relaxation were under investigation during the time available, and it was not considered necessary to "build up" the number of replicates in a group when many of the results were negative.

Another criticism fundamental to this study might be the use of electrical field stimulation (EFS) in the presence of adrenergic and cholinergic blockade, as a model for NANC\textsubscript{i} nerve activation in the guinea-pig trachea, and the use of VIP as a putative neurotransmitter mediating NANC\textsubscript{i} responses. EFS of isolated tissues will excite all neural elements present. Guinea-pig airways possess an excitatory non-adrenergic non-cholinergic mechanism which, although it is predominant in bronchial smooth muscle, may occasionally be present in the trachea (Grundstrom et al., 1981). Consequently responses to EFS (after adrenergic and cholinergic blockade) represent a balance between excitatory and inhibitory components. Furthermore, speculation exists as to the precise identity of the neurotransmitter(s) mediating NANC\textsubscript{i} responses (Karlsson and Persson, 1984; Ellis and Farmer, 1989a,b), and the proposed role of VIP or a related peptide seems increasingly more precarious.

It may be worth considering whether the neurally-mediated relaxation to EFS \textit{in vitro}, is an experimental artifact caused by the resultant action of a cocktail of largely uncharacterised mediators released by EFS? The ingredients of this cocktail may vary between species, contributing to the puzzle in the literature as to the identity \textit{in vitro} of the NANC\textsubscript{i} neurotransmitter. \textit{In vivo}, the NANC\textsubscript{i} neurotransmitter may indeed be VIP or a related peptide which is released on physiological excitation of a precise population of nerve fibres, and it may be that this does not occur by gross electrical stimulation of the vagus \textit{in vivo}, or EFS of airway smooth muscle preparations \textit{in vitro}. If this were the case, then EFS of isolated preparations possessing multiple innervation, may not be a suitable model with which to examine NANC\textsubscript{i} nerve-mediated relaxation or to develop antagonists to the neurotransmitter mediating NANC\textsubscript{i} nerve responses.

Future approaches aimed to test the working hypothesis of PKC-mediated release of a relaxant from NANC\textsubscript{i} nerves, could involve culture of an homogeneous population of isolated tracheal smooth muscle cells from
guinea-pig, and measuring subsequent changes in length in response to different concentrations of phorbol esters to determine the direct effects of PKC activators on individual cells. In rat, seven subspecies of PKC (α, βI, βII, γ, δ, ε, ζ) have been characterised (see Shearman et al., 1989) which have different tissue expression and subtle differences in their preference for various PKC activators. Exploitation of this accumulating information and the development of subspecies-specific activators, may for example allow differentiation of the effect of PKC activation in nerves (e.g. NANCι nerve fibres) and PKC activation in airway smooth muscle cells, in innervated smooth muscle preparations such as the guinea-pig trachea.

In conclusion, this project has been concerned with characterising relaxation of the isolated guinea-pig tracheal strip in response to phorbol esters - the assumption being that in this preparation the relaxation was representative of PKC activation. During the study, a hypothesis was proposed that the relaxation in the guinea-pig trachea produced by the PKC activator, 4β-PDBu (and the voltage-sensitive Na+ channel activators, veratridine and aconitine), might involve a component of PKC-mediated release of a relaxant neurotransmitter, particularly from NANCι nerves. While the results obtained do not prove this hypothesis, most do appear to support it. However, it is recognised that other actions of phorbol esters (presumably through activation of protein kinase C, discussed above) may contribute to the relaxant response of the isolated guinea-pig tracheal preparation.
REFERENCES


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