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RELEASE AND ACTIONS OF PROSTAGLANDIN E_2 FROM CANINE AIRWAY EPITHELIUM

By

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A Thesis

Submitted to the School of Graduate Studies

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Abstract

Asthma is a condition of the airway characterized by 1) a reversible increase in airway resistance; 2) airway hyperresponsiveness; and 3) airway inflammation (Juniper *et al.*, 1981; O'Byrne, 1986; Boushey and Fahy, 1995). Defects of the airway epithelium have been suggested to play a role in the pathogenesis of asthma (Goldie *et al.*, 1986; Knight *et al.*, 1994), and loss of the epithelium is associated with increased reactivity of the underlying smooth muscle (Jongejen *et al.*, 1991; Candenas *et al.*, 1992). It has been proposed that the airway epithelium releases one or more factors which inhibit smooth muscle contraction, termed the epithelial derived inhibitory factor (Tschirhart *et al.*, 1987; Fernandes *et al.*, 1989; Ullman *et al.*, 1991). The inhibitory prostaglandin PGE_2 has been demonstrated to be released from the epithelium (Barrel and Bigby, 1995) and to modulate airway smooth muscle contraction (Braunstein *et al.*, 1988; Abela and Daniel, 1995).

In patients with asthma, inhalation of an allergen may result in a biphasic response consisting of an early asthmatic response and a late asthmatic response (Dolovich *et al.*, 1989; Sterk *et al.*, 1993). The late asthmatic response is an indirect measure of allergen-induced airway inflammation (Dolovich *et al.*, 1989; Sterk *et al.*, 1993), and an animal model of the late asthmatic response is produced by inhalation of the allergen *Ascaris suum* by dogs (Sasaki *et al.*, 1987).

In the studies presented in this thesis, the release and actions of PGE_2 from canine airway epithelium, both under unstimulated conditions and following inhalation of the *Ascaris suum* antigen, were examined. Tracheal and bronchial tissues were excised and studied in the organ bath where contractile responses to agonists and electrical field stimulation, as well as PGE_2 release were measured. Finally, a potential mechanism by which PGE_2 may effect smooth muscle relaxation was examined.

In unstimulated animals, PGE_2 was released from tracheal epithelium and inhibited smooth muscle contraction. This release of PGE_2 was dependent upon electrical field stimulation, and was not blocked by the addition of neurotoxins.

In the antigen model, tracheal PGE_2 release was increased from animals that inhaled antigen but did not develop late airway hyperresponsiveness compared to animals that inhaled vehicle or inhaled allergen and did develop a late response. The release of PGE_2 was not dependent on field stimulation. Demonstration of *in vitro* hyperresponsiveness of the tracheal smooth muscle was dependent on removal of the epithelium.

Bronchial smooth muscle from the antigen model did not demonstrate *in vitro* hyperresponsiveness, even when hyperresponsiveness was observed *in vivo*. There was an increase in the basal release of PGE_2 from the bronchi of animals that were hyperresponsive *in vivo*.

PGE_2 increases intracellular cAMP concentrations (Madison *et al.*, 1989; Coleman *et al.*, 1994). Our investigations in tracheal smooth muscle demonstrated that cAMP does not lower intracellular Ca^{2+} and cause relaxation of airway smooth muscle by stimulation of the sarcoplasmic reticulum Ca^{2+} pump.

The results indicate that PGE_2 was released from airway epithelium and modulated smooth muscle contraction. Alterations in the release of PGE_2 were demonstrated in an animal model of asthma, as PGE_2 played a protective role in the development of airway hyperresponsiveness. Modulation of PGE_2 may be a possible therapy for the treatment of asthma and prevention of the late asthmatic response.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
Ach	acetylcholine
AH	airway hyperresponsiveness
ANOVA	analysis of variance
ASM	airway smooth muscle
ATP	adenosine 5'-triphosphate
BAL	bronchioalveolar lavage fluid
BB	bronchial biopsies
8-BrcAMP	8-bromo-adenosine 3',5'-cyclic monophosphate
8-BrcGMP	8-bromo-guanosine 3',5'-cyclic monophosphate
BSM	bronchial smooth muscle
Ca^{2+}	calcium
$[Ca^{2+}]_i$	internal calcium concentration
cAMP	adenosine 3',5'-cyclic monophophate
Cch	carbachol
cGMP	guanosine 3'5'-cyclic monophosphate
CPA	cyclopiazonic acid
CR	concentration-response
ω-CTX	ω-conotoxin GVIA

COX	cyclooxygenase
DbcAMP	dibutyryl adenosine 3',5'-cyclic monophosphate
EpDIF	epithelium-derived inhibitory factor
EpDRF	epithelium-derived relaxing factor
EAR	early asthmatic response
EC_{50}	median effective concentration
EFS	electrical field stimulation
EJP	excitatory junction potential
FOR	forskolin
G protein	guanosine-binding protein
G_i	guanosine 5'-triphosphate inhibitory protein
G_s	guanosine 5'-triphosphate stimulatory protein
HETE	hydroxyeicosatetraenoic acid
HPETE	hydroperoxy-eicosatetraenoic acid
5-HT	serotonin
IDM	indomethacin
ISO	isoproterenol
KCl	potassium chloride
KRB	Krebs'Ringer's buffer
LAR	late asthmatic response

L-655,240	3-[1-(4-chlorobenzyl)-5-fluoro-3-methyl-indol-2-yl]2,2-dimethylpropanoic acid
L-NNA	N-ω-nitro-L-arginine
LTA_4	leukotriene A_4
LTB_4	leukotriene B_4
LTC_4	leukotriene C_4
LTD_4	leukotriene D_4
LTE_4	leukotriene E_4
MBP	major basic protein
MLCK	myosin light chain kinase
Mn^{2+}	magnesium
mRNA	messenger ribonucleic acid
Na^+	sodium
NaNP	sodium nitroprusside
NKA	neurokinin A
NON-RESP	animals which inhaled antigen and did not develop hyperresponsiveness
$PC_5/PC_{5.0}$	provocative concentration
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
PGG_2	prostaglandin G_2

PGH_2	prostaglandin H_2
PGI_2	prostaglandin I_2
PK/PrK	protein kinase
RESP/HYP-RESP	animals which inhaled antigen and developed hyperresponsiveness
RIA	radioimmunoassay
R_L	peripheral resistance
SEM	standard error of the mean
SHAM	animals which inhaled vehicle (saline)
SR	sarcoplasmic reticulum
TSM	tracheal smooth muscle
TTX	tetrodotoxin
TXA_2	thromboxane A_2

CHAPTER 1

INTRODUCTION

Chapter 1:Introduction

1.1 Asthma

Asthma is a condition of the airway which is characterized by 1) a reversible increase in airway resistance; 2) airway hyperresponsiveness; and 3) airway inflammation (Juniper *et al.*, 1981; O'Byrne, 1986; Boushey and Fahy, 1995). Although there is no one accepted definition of asthma, according to McFadden and Gilbert (1992), the following symptoms are required for the diagnosis of asthma to be made: episodes of coughing, wheezing, and chest tightness, usually occurring at night or early in the morning; variable severity of symptoms; a history of increased airway responsiveness, exacerbated by exposure to stimuli such as cold air, allergens, stress, and air pollutants, and reversible airway obstruction or demonstration of increased airway responsiveness upon challenge with histamine, methacholine, cold air, or exercise.

The increase in airway resistance seen in asthma is due to an increased constriction of smooth muscle accompanied by airway wall thickening (Juniper *et al.*, 1981). Contraction of the airway is largely responsible for bronchospasm, which is a characteristic feature of an acute asthma attack (Rodger, 1991).

Airway hyperresponsiveness is defined as a lower than normal concentration

of bronchoconstrictor agonists, such as histamine or methacholine, required to elicit the same amount of bronchoconstriction as in non-asthmatics (hypersensitivity) (O'Byrne, 1986; Boushey and Fahy, 1995), a steeper slope of the concentration-response curve (hyperreactivity), and an increase in the maximal response to an agonist (increased airway narrowing; Sterk *et al.*, 1993). Bronchial hyperresponsiveness is regarded as a fundamental feature of asthma as it is ubiquitous in patients with asthma, and the degree of bronchial hyperreactivity is well correlated with the severity of asthma (Bates, 1995; Boushey and Fahy, 1995).

More recently, asthma has been recognized as an inflammatory disorder. Bronchial biopsies of mild asthmatics, and *postmortem* studies on patients who died from asthma, have shown an infiltration of mast cells, neutrophils, and eosinophils, as well as edema and damage to the respiratory epithelium (Bates, 1995; Boushey and Fahy, 1995; Calverly, 1996). Moreover, there exists a correlation between serum IgE levels and prevalence of asthma (Bourney, 1991; Bates, 1995).

Asthma is a common disease: it is estimated that 10 million people in the United States of America are affected (McFadden and Gilbert, 1992). A study of 310 9-year old Canadian children found that 8% had mild asthma, 11% had moderate asthma, and 3% had severe asthma (Bates, 1995). Furthermore, there is evidence that the incidence of asthma is increasing worldwide (Burney, 1992; McFadden and Gilbert, 1992; Bates, 1995). An improved understanding of the pathology of asthma should give insight into improved diagnostic criteria and potential therapeutics.

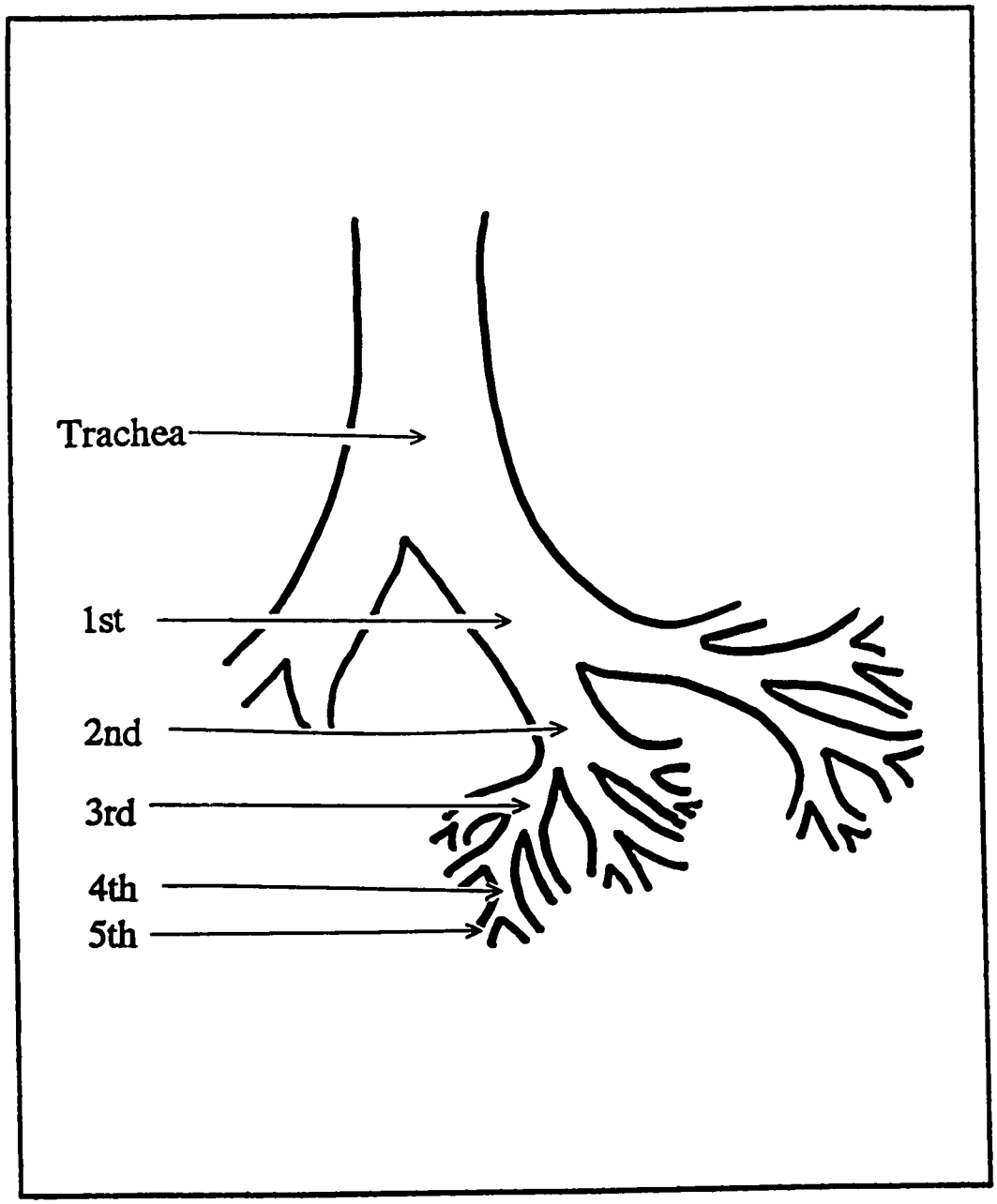
1.2 General Structure of the Airway

The airway can be divided into two major functional components. The conducting airway carries gases into the respiratory airway where gas exchange takes place. The conducting airway is made up of the nose, pharynx, larynx, trachea, bronchi, and bronchioles (Harkema *et al.*, 1991; Figure 1). Air is carried through the nasal passages, where it is warmed and humidified. Air passes through the pharynx and larynx into the trachea. The trachea divides into two branches, the mainstem bronchi. The mainstem bronchi branch into the intrathoracic bronchi which further branch into progressively smaller and smaller airways (Harkema *et al.*, 1991). Finally, in humans and dogs, the terminal bronchioles divide into respiratory bronchioles which join alveolar ducts. It as at the level of the alveolar ducts that gas exchange occurs (Harkema *et al.*, 1991).

1.2.1 Structure of the Airway Epithelium

The epithelium of the conducting airways is made up of several different cell types which line the luminal surface of the airways. The density of the different types of cells varies depending on the species and the level of airway (Harkema *et al.*, 1991). The different types of cells found in the airway and their putative functions will be discussed below.

Figure 1. Structure of the airway. Diagrammatic representation of bifurcation of the trachea and branching of bronchi. For the purpose of the experiments described in this thesis, the trachea and 3rd-5th order bronchi were used. Adapted from (Abela, 1993).


Trachea
1st
2nd
3rd
4th
5th

Basal cells are small flattened cells which attach to the basal lamina of the epithelium. Basal cells contain little cytoplasm, few mitochondria, and rough endoplasmic reticulum. They are characterized by many keratin filaments as well as desmosomes (Spina and Page, 1992). It has been suggested that basal cells are the primary progenitor cells for the epithelium (Harkema *et al.*, 1991; Spina and Page, 1992). Basal cells also function to metabolize many neuropeptides via endopeptidases (Spina and Page, 1992). Ciliated cells are attached to the basal lamina and extend to the luminal surface. These cells contain a nucleus close to the basal surface and many mitochondria close to the apical surface (Spina and Page, 1992). 200-300 cilia per cell are found on the apical surface of these cells (Harkema *et al.*, 1991). The major function of ciliated cells is to propel airway mucous towards the larynx (Harkema *et al.*, 1991; Spina and Page, 1992).

There are three major types of secretory cells in the airway epithelium; mucous goblet cells, serous cells, and Clara cells. Mucous goblet cells are characterized by electron lucent, extensive Golgi apparatuses and granular endoplasmic reticulum. Serous cells contain electron dense granules and granular endoplasmic reticulum, and Clara cells have electron dense granules and granular as well as agranular endoplasmic reticulum (Harkema *et al.*, 1991; Spina and Page, 1992). Mucous cells are the sources of airway mucous which traps, and clears from the airways, inhaled particles: dust, bacterial organisms, and pollutant gases (Harkema *et al.*, 1991; Spina and Page, 1992). Small mucous granule cells have

been identified in the midepithelial layer. The function of these cells is unknown, and it has been postulated that they may be precursors to mucous goblet cells (Harkema *et al.*, 1991).

Two less frequently occurring types of cells are the neuroendocrine cells and brush cells. Neuroendocrine cells lie on the basal lamina but may have cytoplasmic processes which extend to the luminal surface (Harkema *et al.*, 1991). These cells appear individually or in clusters, which are termed epithelial bodies. The characteristic feature of neuroendocrine cells are many round neurosecretory-like granules which degranulate under hypoxia. For this reason, it has been suggested that these cells play a role in chemoreception (Harkema *et al.*, 1991). Brush cells contain a brush border of close packed microvilli, approximately 2 μm in length, on their luminal surface. Little is presently known about the function of these cells (Harkema *et al.*, 1991).

1.3 The Function of the Airway Epithelium in Asthma

It has been suggested that epithelial defects of the airway play a role in the pathogenesis of asthma (Boushey *et al.*, 1980; Laitenen *et al.*, 1985; Goldie *et al.*, 1986; Knight *et al.*, 1994). Structural abnormalities of the epithelium which are associated with asthma include abnormal and damaged epithelial cells as well as a weak attachment between basal and ciliated cells which may lead to areas of

exfoliation (Knight *et al.*, 1994). Moreover, exposure to ozone, which is associated with damage to the epithelium, has been demonstrated to result in airway hyperresponsiveness (Boushey *et al.*, 1980; Murlas *et al.*, 1990; Janssen *et al.*, 1991).

Several studies have identified an association between loss or removal of the airway epithelium and increased reactivity of the underlying smooth muscle. For example, Holroyde (1986) demonstrated that removal of guinea pig tracheal epithelium enhanced sensitivity to 5-HT, histamine, and ACh. Similarly, Goldie *et al.* (1986) found that the potency of histamine was increased four-fold in epithelial-denuded guinea pig tracheal rings. Barnes *et al.* (1985) reported that, in bovine tracheal smooth muscle, the presence of the epithelium reduced both the sensitivity and the maximum contractile force of the smooth muscle to 5-HT, histamine, and ACh. Xie *et al.* (1992) showed that application of isolated epithelial cells to the perfusate surrounding either tracheal or bronchial smooth muscle hyperpolarized cell membranes and suppressed EJP amplitudes. Moreover, Jongejen *et al.* (1991) and Candenas *et al.* (1992) found that removal of the epithelium from human bronchi increased sensitivity to methacholine and endothelins. Thus, it can be seen that the epithelium modulates airway smooth muscle sensitivity in a variety of species and at the level of both the trachea and the bronchi.

There have been several mechanisms proposed through which the epithelium may inhibit airway smooth muscle (ASM) contraction. These mechanisms include:

1) acting as a physical barrier which limits access of agonists to the underlying smooth muscle; 2) acting as an enzymatic barrier which degrades spasmogens before they reach the smooth muscle, and; 3) releasing one or more smooth muscle inhibitory factors (EpDIF).

1.3.1 Physical Barrier to Diffusion

The airway epithelium has been demonstrated to attenuate smooth muscle responses by restricting access of contractile agonists. Several studies have found a difference in ASM responses to spasmogens (histamine, 5-HT, ACh, Cch) dependent upon the side of the epithelium to which the agent is added. An increased sensitivity of the ASM to the agonists was observed when they were added to the serosal side of the tissue compared to agonists added to the epithelial side. Further, the differences in sensitivity were abolished upon removal of the epithelium (Pavlovic *et al.* 1989; Munakata *et al.*, 1989; Iriarte *et al.*, 1990; Yong *et al.*, 1991; Mitchell *et al.*, 1993).

1.3.2 Enzymatic Barrier

Other studies have suggested that the epithelium modulates ASM via degradation of contractile agonists. Koga *et al.* (1992) reported that removal of

guinea pig tracheal epithelium potentiated the response of tracheal smooth muscle to ACh approximately 26-fold. This difference was abolished in the presence of physostigmine, an acetylcholinesterase inhibitor. The authors concluded that the tracheal epithelium inhibited contractions to exogenous ACh by metabolism of the agonist. Similarly, vasoactive intestinal polypeptide, substance P, and endothelins have all been demonstrated to undergo metabolism by the airway epithelium (Takubo *et al.*, 1991; Candenas *et al.*, 1992; Inoue *et al.*, 1992).

1.3.3 Epithelium Derived Relaxing Factor (EpDIF).

There have been several observations, however, that suggest that removal of both a physical and enzymatic barrier cannot fully explain the function of the epithelium in the inhibitory modulation of ASM. There is little acetylcholinesterase activity in airway epithelium, and responses to muscarinic agonists that are not degraded by acetylcholinesterase are also increased by removal of the epithelium. Also, a diffusional barrier would affect the rate, not the absolute magnitude of responses to drugs which are not degraded before they reach the smooth muscle. In rabbit bronchi, removal of the epithelium increased the magnitude of the response to methacholine (Raeburn *et al.*, 1986). Moreover, responses to electrical field stimulation (EFS), which induces the release of ACh from nerves near the muscle inside the epithelial barrier, are increased when the epithelium is removed from ASM

(Flavahan *et al.*, 1985; Stuart-Smith and Vanhoutte, 1988).

It has been proposed that the epithelium releases one or more factors which inhibit ASM. Many experiments have been performed which support this proposal. Tschirhart *et al.* (1987) used a "sandwich protocol" wherein they demonstrated that the presence of an epithelum-intact guinea pig tracheal strip resulted in a decreased response to histamine in adjacent epithelial-denuded tissue. Yu *et al.* (1992) observed that pre-incubation of canine tracheal smooth muscle with cultured epithelial cells resulted in a right-ward shift of a histamine concentration response curve. Fernandes *et al.* (1989) used a co-axial bioassay , in which epithelium-intact guinea-pig trachea was suspended around endothelium-denuded rat aorta, to show that a relaxing factor released from tracheal epithelium could inhibit vascular smooth muscle. Moreover, Ullman *et al.* (1991) demonstrated that the epithelium-dependent relaxation of cholinergic contraction in ferret trachea was transferable between organ baths.

It should be understood that none of the mechanisms mentioned above are mutually exclusive, and the epithelium may modulate ASM by acting as a barrier to diffusion, an enzymatic barrier, and by releasing relaxing factors.

1.4 Nature of EpDIF

Although there is strong evidence to support the existence of an EpDIF in many airway tissues, the chemical nature of EpDIF remains unknown. Several potential candidates have been examined, though most have been ruled out. Hemoglobin, methylene blue, phenidone, and L-NAME, all inhibitors by various mechanisms of actions of nitric oxide, have been demonstrated to have no effect on the relaxation of rat aorta in a co-axial bioassay with rabbit bronchi (Martin *et al.*, 1985; Fernandes *et al.*, 1989; Fernandes and Goldie, 1990; Spina and Page, 1991). These findings suggest that nitric oxide, which is the endothelium derived relaxing factor of vascular smooth muscle, is not EpDIF. Papain and α-chymotrypsin similarly did not alter the epithelial-dependent relaxation in a co-axial bioassay, which suggests that EpDIF is not a peptide (Spina and Page, 1991). Furthermore, EpDIF has been shown not to stimulate β-adrenoceptors, as EpDIF-dependent relaxations were not affected by the β-antagonist propranolol (Fernandes and Goldie, 1990), and epithelium-dependent relaxation was not associated with an increase in adenosine 3':5' cyclic monophosphate (cAMP) levels in the underlying smooth muscle (Hay *et al.*, 1992). Other potential candidates for EpDIF, the inhibitory prostaglandins, will be discussed below.

1.5 The Arachidonic Acid Cascade.

Prostaglandins are cyclooxygenase metabolites of arachidonic acid (AA). AA,

and other related fatty acids, are released from the membrane when phospholipases A_2, C, or D are activated. The activation of phospholipases may be due to pharmacological stimuli, physiological stimuli, or pathological stimuli (Kalant and Roschlau, 1989; Holtzmann 1992). There are three major enzymatic pathways for metabolism of AA; the cyclooxygenase, lipoxygenase, and cytochrome P_{450} monooxygenase pathways (See Figure 2).

1.5.1 Cycloxygenase Pathway

Cyclooxygenase (COX) converts AA to prostaglandin endoperoxides (PGG_2 and PGH_2). PGH_2 is converted to thromboxane A_2 (TxA_2) by thromboxane synthase. TxA_2 is hydrolyzed spontaneously to TxB_2. PGH_2 may also be converted to prostacyclin (prostagandin I_2 or PGI_2; Figure 2) by the enzyme prostacyclin synthase. Finally, prostaglandin endoperoxide isomerase catalyzes the conversion of PGH_2 to PGD_2, PGE_2 (Figure 3), and $PGF_{2\alpha}$ (Holtzman, 1992). TxA_2 stimulates contraction of airway smooth muscle, as do the prostaglandins PGH_2, PGD_2, and $PGF_{2\alpha}$ (Kalant and Roschlau, 1989; Holtzman, 1992). PGI_2 and PGE_2 relax airway smooth muscle, though PGE_2 is more potent (Holtzman, 1992)

Two different isoenzymes of COX exist, COX-1 and COX-2. COX-1 is constitutively expressed in most tissues (Meade *et al.*, 1993; Wu, 1996). COX-2 is

Figure 2. The arachidonic acid cascade. The enzymes involved in metabolism of arachidonic acid and their major metabolites. Adapted from (Kalant and Roschlau, 1989; Holtzman, 1992).

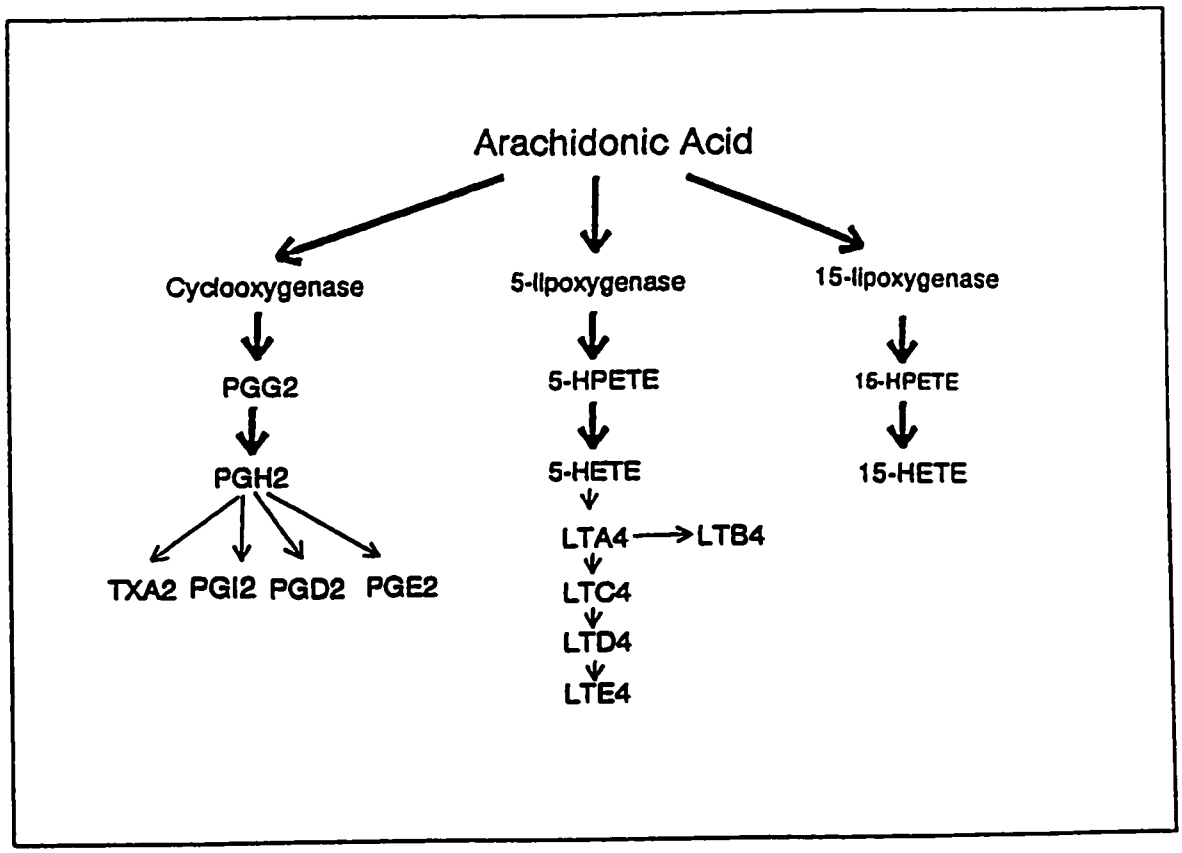
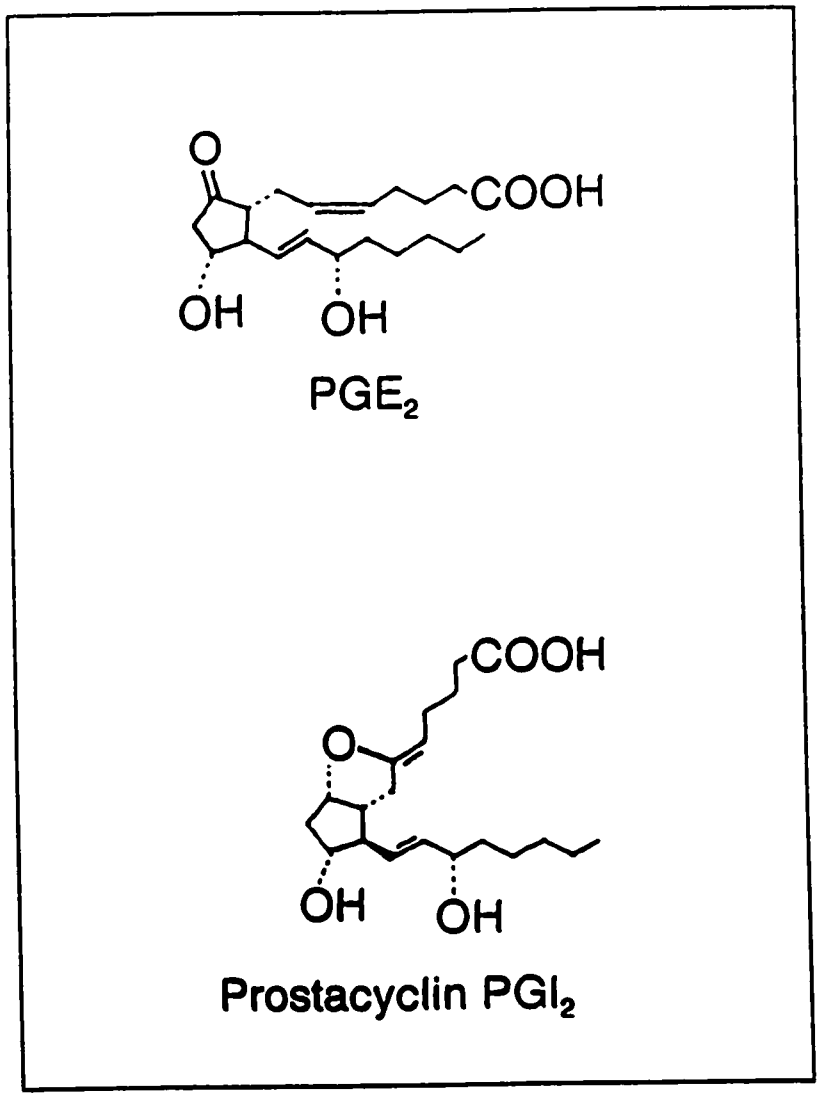

Arachidonic Acid
Cyclooxygenase
5-lipoxygenase
15-lipoxygenase
PGG2
PGH2
TXA2 PGI2 PGD2 PGE2
5-HPETE
5-HETE
LTA4 → LTB4
LTC4
LTD4
LTE4
15-HPETE
15-HETE

Figure 3. PGE2 and PGI2. Chemical structure of prostaglandin E_2 and prostaglandin I_2 (Kalant and Roschlau, 1989).


O
COOH
OH
OH
PGE2
COOH
O
OH
OH
Prostacyclin PGI2

only expressed following cell activation. Synthesis of COX-2 can be stimulated by lipopolysaccahride, serum, growth factors, and cytokines (Meade *et al.*, 1993; Wu, 1996). The pharmacological profiles of the two isomers of COX differ, and COX-1 is more susceptible to inhibition by the non-steroidal anti-inflammatory drugs aspirin and indomethacin than is COX-2. COX-2 is selectively inhibited by 6-methoxy-2-naphthylacetic acid, while ibuprofen, flurbiprofen, and meclofenate inhibit both enzymes equally (Meade et al., *1993*).

1.5.2 Lipoxygenase Pathway

The lipoxygenase pathway involves at least six enzymes; 3-, 5-, 9-, 11-, 12-, and 15-lipoxygenases. The primary product of each is a hydroperoxide (HPETE), which is rapidly reduced to an alcohol (HETE). The alcohols are then converted to biologically active products. Products of 12-HETE are hepoxylins, however the physiological actions and relevance of these products are not yet fully understood (Kalant and Roschlau, 1989; Holtzman, 1992). 15-lipoxygenase converts arachidonic acid to 15-HPETE and, to a lesser degree, 12-HPETE. 15-HPETE or 15-HETE can be reduced by 5-lipoxygenase to lipoxins A and B. Lipoxins are involved in host cellular immune functions, and may modify cell membrane composition and function (Holtzman, 1992). 5-lipoxygenase is of special interest in the study of the airway, as it catalyzes the formation of

leukotriene A_4 (LTA_4). LTA_4 is converted to LTB_4 or LTC_4. LTC_4 is further metabolised to LTD_4 and LTE_4 (Holtzman, 1992). LTB_4 is a potent chemoattractant of neutrophils. LTC_4, LTD_4, and to a lesser extent, LTE_4, are contractile agents in airway smooth muscle (Kalant and Roschlau, 1989; Holtzman, 1992; Abela and Daniel, 1995). LTC_4, LTD_4, and LTE_4 also increase vascular permeability, induce mucous secretion, and decrease mucous transport, all of which lead to pulmonary edema (Sampson, 1996).

1.5.3 Monoxygenase Pathway

The cytochrome P_{450} monooxygenase pathway catalyzes the conversion of arachidonic acid to mono-epoxide derivatives; however the specific enzymes involved are not well understood, and the biological actions of their products have not been described (Kalant and Roschlau, 1989).

1.6 Prostaglandins as candidates for EpDIF

Studies have demonstrated the presence of cyclooxygenase in airway epithelium, (Stuart-Smith *et al.*, 1988; Butler *et al.*, 1992; Yu *et al.*, 1992), and have shown that PGE_2 is the predominant metabolite of cyclooxygenase in the airway epithelium (Barrett and Bigby 1995). Further, it has been found that prostaglandins

are released from the airway epithelium in sufficient concentrations to modulate smooth muscle function, (Barnett *et al.*, 1990; Prie *et al.*, 1991; Holtzman, 1992).

PGE_2 has been demonstrated to play a role in the modulation of airway smooth muscle contraction. Exogenous PGE_2 relaxed histamine-induced constriction in isolated guinea-pig trachea, and trachea pre-treated with PGE_2 displayed a decrease in both the maximal response and the sensitivity to histamine (Braunstein *et al.* 1988). PGE_2, but not PGI_2, decreased the response of canine bronchi to Ach and electrical field stimulation (EFS; Abela and Daniel, 1995). PGE_2 also relaxed canine tracheal smooth muscle precontracted with serotonin or methacholine, and PGI_2 had a synergistic effect on this PGE_2-induced relaxation (Yu *et al.*, 1992). Furthermore, indomethacin, an inhibitor of cyclooxygenase, increased the responses of guinea-pig trachea to histamine, serotonin, ACh, and high potassium (Adcock and Garland, 1980; Tschirhart *et al.*, 1987; Braunstein *et al.*, 1988).

Absence of PGE_2 may also mediate asthmatic attacks. Studies have indicated that inhibitors of cyclooxygenase, such as acetylsalicylic acid and indomethacin, can precipitate asthmatic attacks (Szezekil *et al.*, 1975; Ito and Tajima, 1981). Aspirin-induced bronchoconstriction can be prevented with prior inhalation of PGE_2 (Sestini *et al.*, 1981). Moreover, PGE_2 has been demonstrated to attenuate exercise-induced and allergen-induced bronchoconstriction in asthmatic subjects (Pavord *et al.*, 1992; Melillo *et al.*, 1994).

Based on these results it is possible that prostaglandins are candidates for the EpDIF. Many experiments have been performed to test the ability of the cyclooxygenase products to act in this role; however, results have been contradictory.

Yu *et al.* (1992), treated canine tracheal smooth muscle strips with a buffer containing cultured epithelial cells. A dose-related relaxation of pre-contracted trachea was observed to be markedly reduced following the pre-treatment of cultured epithelial cells with sodium meclofenamate; a cyclooxygenase inhibitor. Analysis of the buffer showed presence of 6-keto $PGF_{1\alpha}$, the stable metabolite of PGI_2, as well as PGE_2. Farmer *et al.* (1987), observed that the qualitative nature of guinea-pig tracheal responses to arachidonic acid was altered in the same manner whether in the presence of indomethacin or following removal of the epithelium. These findings suggest that the epithelial-dependent relaxation of airway smooth muscle is mediated by a cyclooxygenase product, the most likely candidates being PGE_2 and PGI_2, (Tschirhart *et al.*, 1987; Butler *et al.*, 1992; Adcock and Garland, 1980; Braunstein *et al.*, 1988; Orehek *et al.*, 1975).

However, another body of evidence exists which suggests that the EpDIF cannot be a prostanoid. Hay *et al.* (1986), demonstrated that indomethacin did not affect guinea pig tracheal smooth muscle response to histamine in either the presence or the absence of the epithelium. Moreover, Barnes *et al.* (1985) displayed findings which indicated that the effect of the epithelium on bovine tracheal

contractility was not affected by pre-treatment with indomethacin. These findings, and others, (Fernandes and Goldie, 1990), indicated that the EpDIF is not a cyclooxygenase product in these cases.

A complication to the search for the nature of EpDIF is that more than one factor may be involved. It is possible that several factors may act cooperatively to produce epithelium-dependent relaxation. As a result, the stimulation or inhibition of a single substance not having an effect on epithelial-mediated relaxation may not be a true indication that it is not a candidate for an EpDIF. Ullman *et al.* (1990) found that both a prostanoid factor and a non prostanoid factor were involved in the epithelium-dependent relaxation of ferret trachea.

1.7 Prostanoid Receptors

Several different receptors with nanomolar affinities for prostaglandins have been identified. These receptors are classified as DP, EP, FP, IP, and TP receptors on the basis that each is selective for one of the different prostaglandins D_2, E_2, $F_{2\alpha}$, I_2 and TxA_2 (Coleman *et al.*, 1994). The EP and IP receptors are discussed below.

1.7.1 PGI_2 Receptors

mediated via multiple receptor subtypes. PGE_2 receptors are characterized, based on pharmacological profiles, as EP_1, EP_2, EP_3, and EP_4 receptors (Coleman *et al.*, 1994; Negishi *et al.*, 1995).

EP_1 receptors have been identified in guinea pig trachea, gastrointestinal tract, uterus, and bladder (Coleman *et al.*, 1994). Although EP_1 receptors are sparse in non-rodent species, expressions of these receptors has been noted in dog stomach and human uterus (Coleman *et al.*, 1994). Activation of EP_1 receptors is associated with an increase in $[Ca^{2+}]_i$, and EP_1-mediated contraction of guinea-pig trachea is dependent on the extracellular Ca^{2+} concentration. As a result, EP_1 receptor activity is thought to involve the opening of Ca^{2+} channels, but the second messenger system is not yet understood (Coleman *et al.*, 1994; Negishi *et al.*, 1995). The major biological action associated with the EP_1 receptor is contraction of smooth muscle, including that of the gastrointestinal tract, respiratory tract, vas deferens, uterus, and iris sphincter (Negishi *et al.*, 1995).

EP_2 receptors are found in vascular, gastrointestinal, tracheal, and uterine smooth muscle from a variety of species (Coleman *et al.*, 1994; Negishi *et al.*, 1995). These receptors are also found on inflammatory cells where they mediate inhibition of mediator release and on epithelial cells where they mediate secretion (Coleman *et al.*, 1994). There is a large body of evidence which demonstrates that EP_2 receptors are coupled to a Gs protein , and that their activation increases intracellular concentrations of cAMP ($[cAMP]_i$; Hassid *et al.*, 1986; Madison *et al.*,

1989; Coleman *et al.*, 1994; Negishi *et al.*, 1995). EP_2 receptors are thus postulated to mediate smooth muscle relaxation by raising $[cAMP]_i$.

EP_3 receptors are present in gastrointestinal, uterine, and gastric smooth muscle, autonomic nerves, gastric mucosal cells, and the renal medulla (Narumiya *et al.*, 1993; Coleman *et al.*, 1994; Negishi *et al.*, 1995). Several isoforms of the EP_3 receptors have been identified. Alternative RNA splicing leads to changes in the carboxy-terminal tails of these receptors, and several different isoforms exist in various species. Different isoforms are associated with different signal transduction mechanisms. For example, hamster EP_{3A} and $_C$ receptors are coupled to Gi and inhibit adenylate cyclase, EP_{3B} and EP_{3C} are coupled to Gs and activate adenylate cyclase, and EP_{3D} receptors are coupled to Gq, Gi, and Gs, and increase IP turnover and calcium mobilization (Coleman *et al.*, 1994; Negishi *et al.*, 1995). Physiological actions of PGE_2 at EP_3 receptors include inhibition of neurotransmitter release, lipolysis, gastric acid secretion, contraction of uterine smooth muscle, and sodium and water reabsorption from kidney tubules (Coleman *et al.*, 1994; Negishi *et al.*, 1995).

The mRNA of the EP_4 receptor is widely distributed in the body, and these receptors are found in the ileum, thymus, lungs, spleen, heart, uterus, and mast cells. The EP_4 receptor, like the EP_2 receptor is coupled to a Gs protein, and stimulation of this receptor also increases $[cAMP]_i$. Biological actions of the EP_4 receptor include smooth muscle relaxation and inhibition of histamine release

(Coleman *et al.*, 1994; Negishi *et al.*, 1995).

1.8 PGE_2 in the Airway

It has been demonstrated that, in the airway, the most important inhibitory effect of PGE_2 is to inhibit neurotransmitter release, in particular Ach release (Walters *et al.*, 1984; Daniel et al, 1987: Serio and Daniel, 1989; Johansson-Rydberg *et al.*, 1992; Abela and Daniel, 1995). This is presumably via the actions of PGE_2 at presynaptic EP_3 receptors. PGE_2, at higher concentrations than required for inhibition of mediator release, also inhibits airway smooth muscle contraction induced by exogenous Ach, which suggests that PGE_2 acts directly on the smooth muscle as well (Daniel *et al.*, 1987; Abela and Daniel 1995). The actions of PGE_2 on airway smooth muscle are likely due to an increase in $[cAMP]_i$ via EP_2 and EP_4 receptors, both of which have been localized to the lung (Madison *et al.*, 1989; Coleman *et al.*, 1994). It has also been suggested that PGE_2 may increase cGMP in some smooth muscles (Hassid *et al.*, 1986).

PGI_2 may also increase cAMP in smooth muscle (Hassid *et al.*, 1986; Coleman *et al.*, 1994), but PGI_2 has been demonstrated to be a much less effective inhibitor of airway smooth muscle, and its actions are more likely to mediate vascular smooth muscle relaxation (Hassid *et al.*, 1986).

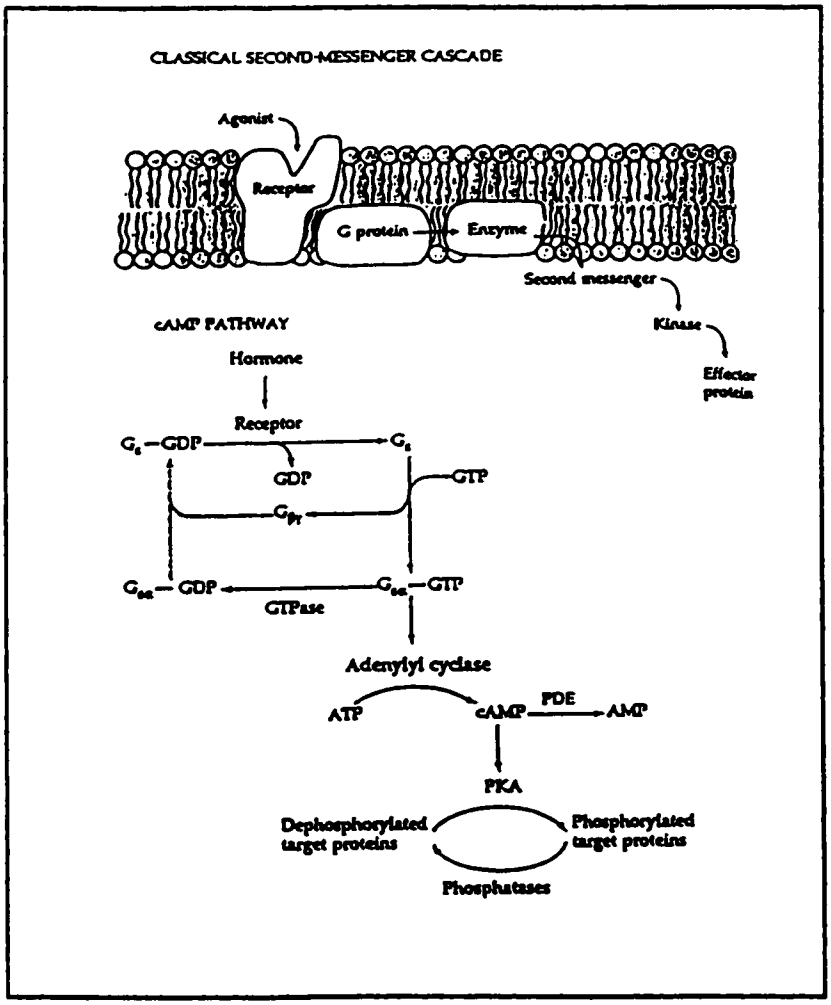
1.9 Cyclic Nucleotides

The cyclic nucleotides cyclic 3',5'-adenosine monophosphate (cyclic AMP; cAMP) and cyclic 3',5'-guanosine monophosphate (cyclic GMP; cGMP) are intracellular second messengers which function in the transduction of a message at the cellular membrane to intracellular effector mechanisms.

cAMP is formed from ATP via adenylate cyclase, which is a protein found on the cell membrane (Stryer, 1988). Adenylate cyclase is activated when a ligand binds to a cell surface receptor and activates a guanyl-nucleotide binding protein (G protein; Stryer, 1988). G proteins are made up of 3 subunits; α, β, and γ. The α subunit contains the guanine nucleotide binding region and has GTPase activity. When a ligand binds to the receptor, bound GDP is released from the α subunit and GTP is bound. Gα-GTP then activates adenylate cyclase and cAMP is produced (Stryer 1988; Benham, 1991). cGMP is formed in a similar manner via activation of guanylate cyclase (Benham, 1991; Figure 4).

There are many different forms of G proteins, and activation of different G proteins produces different effects. For example, Gs stimulates adenylate cyclase and increases $[cAMP]_i$, whereas Gi inhibits the enzyme, and inhibits the production of cAMP (Benham, 1991). Once formed, cAMP activates its target enzyme, cAMP-

Figure 4. Formation and action of cAMP. A schematic representation of the formation of cAMP following activation of a receptor by an agonist. Production of cAMP results in activation of PKA and phosphorylation of target proteins (Cornell and Lincoln, 1988).


CLASSICAL SECOND-MESSENGER CASCADE
Agonist
Receptor
G protein
Enzyme
Second messenger
Kinase
Effector protein
cAMP PATHWAY
Hormone
Receptor
GDP
GTP
GTPase
Adenylyl cyclase
ATP
cAMP
PDE
AMP
PKA
Dephosphorylated target proteins
Phosphorylated target proteins
Phosphatases

dependent protein kinase (protein kinase A; PKA; Stryer, 1988). In its inactivated state, this enzyme is made up of four subunits, two catalytic subunits and two regulatory subunits. When two cAMP molecules bind to the regulatory subunits, the two catalytic subunits are released and activated (Stryer, 1988; Benham 1991). PKA is then able to phosphorylate many different proteins which leads to a multitude of different effects within the cell (Benham,1991).

cGMP also activates a protein kinase, the cGMP-dependent protein kinase, which also phophorylates a number of different proteins and thus has many different actions (Benham, 1991).

1.10 Cyclic Nucleotide Induced Relaxation via Lowering Internal Calcium

A mechanism which has been proposed to explain the method via which cAMP and cGMP elicit smooth muscle relaxation is by lowering the $[Ca^{2+}]_i$ of the smooth muscle cells.

McDaniel *et al.* (1990) measured forskolin-induced changes in aequorin-estimated $[Ca^{2+}]_i$, [cAMP], myosin phosphorylation, and stress in swine arterial smooth muscle precontracted with phenylephrine or histamine. Forskolin increased [cAMP] and reduced $[Ca^{2+}]_i$, myosin phosphorylation, and stress. Relaxation was not associated with an alteration in the $[Ca^{2+}]_i$ sensitivity of phosphorylation, nor the dependence of stress on phosphorylation. These results suggest that cAMP

induced relaxation in their tissue is primarily due to decreases in $[Ca^{2+}]_i$.

Similarly, Cornell and Lincoln (1988) used the Ca^{2+}-dependent conversion of phosphorylase b to a to measure changes in $[Ca^{2+}]_i$. They demonstrated that ANP II, which raises cGMP and relaxes vascular smooth muscle *in vitro*, affects mainly the removal of Ca^{2+} from the cytoplasm. Based on the evidence presented above, it is likely that both cAMP and cGMP effect smooth muscle relaxation via decreasing $[Ca^{2+}]_i$. This can be accomplished through a variety of mechanisms.

1.11 Mechanisms for Decreasing $[Ca^{2+}]_i$

Several mechanisms exist through which cyclic nucleotides may act to decrease $[Ca^{2+}]_i$ (reviewed by Lincoln; 1989 reviewed by Murray, 1990). Firstly, cAMP and cGMP may act to inhibit Ca^{2+} influx. cGMP relaxation has been demonstrated to be associated with decrease in Ca^{2+} uptake in aortic tissue (Collins *et al.*, 1986; Taylor and Meisheri, 1986). Isoproterenol (ISO) and dibutyryl cAMP have been shown to inhibit potassium-stimulated $^{45}Ca^{2+}$-influx into rabbit aorta (Meisheri and van Breeman, 1982). It is evident, however, that inhibition of Ca^{2+} influx cannot fully account for cyclic nucleotide formation (Lincoln, 1989; Murray, 1990). Hwang and van Breeman (1987) reported that when the external potassium concentration was increased, dibutyryl cAMP and forskolin caused a rightward shift in the force versus Ca^{2+}-influx relationship. The observation that these agents had

a greater effect on inhibiting contraction relative to the $^{45}Ca^{2+}$-influx suggests that other mechanisms for lowering $[Ca^{2+}]_i$ play a more important role.

$[Ca^{2+}]_i$ can also be lowered by stimulation of Ca^{2+} transport. There are three transport systems in smooth muscle cells which are capable of removing cytoplasmic Ca^{2+} (reviewed in Murray, 1990). These are:

1. the sarcoplasmic reticulum Ca^{2+}-ATPase,
2. the plasma membrane Ca^{2+}-ATPase, and
3. the Na^+/Ca^{2+} exchange.

Smith and Smith (1987) reported that the Na^+/Ca^{2+} exchange protein contributes to the rapid decline in $[Ca^{2+}]_i$ which occurs following agonist-activated Ca^{2+} signalling. However, the affinity of the Na^+/Ca^{2+} exchange protein for Ca^{2+} is lower than that of the plasma membrane Ca^{2+}-ATPase; thus it is unable to return Ca^{2+} levels back to resting values by itself (Lincoln, 1989). Furukawa *et al.* (1988) observed that 8-bromo-cAMP caused a stimulation of the plasma membrane Ca^{2+}-ATPase. Forskolin, however was ineffective. Scheid and Fay (1984) found that, in toad stomach smooth muscle, ISO and dibutyryl cAMP stimulate Ca^{2+} efflux. They proposed that this was due to cAMP-PrK activation of Na^+/Ca^{2+} exchange.

Finally, $[Ca^{2+}]_i$ may be lowered by the activation of the sarcoplasmic reticulum Ca^{2+}-ATPase, which enhances the sequestration of Ca^{2+} into the internal stores of

the smooth muscle cell. Studies in cardiac muscle (Toda and Katz, 1982) suggest that phosphorylation by cAMP-PrK of phospholamban in SR vesicles results in increased SR Ca^{2+}-ATPase activity and, consequently, increased Ca^{2+} uptake. Moreover, Raeymaekers *et al.* (1988) and Huggin *et al.* (1989) report that cardiac and smooth muscle phospholamban are substrates for both cAMP-PrK and cGMP-PrK.

1.12 Cyclopiazonic Acid

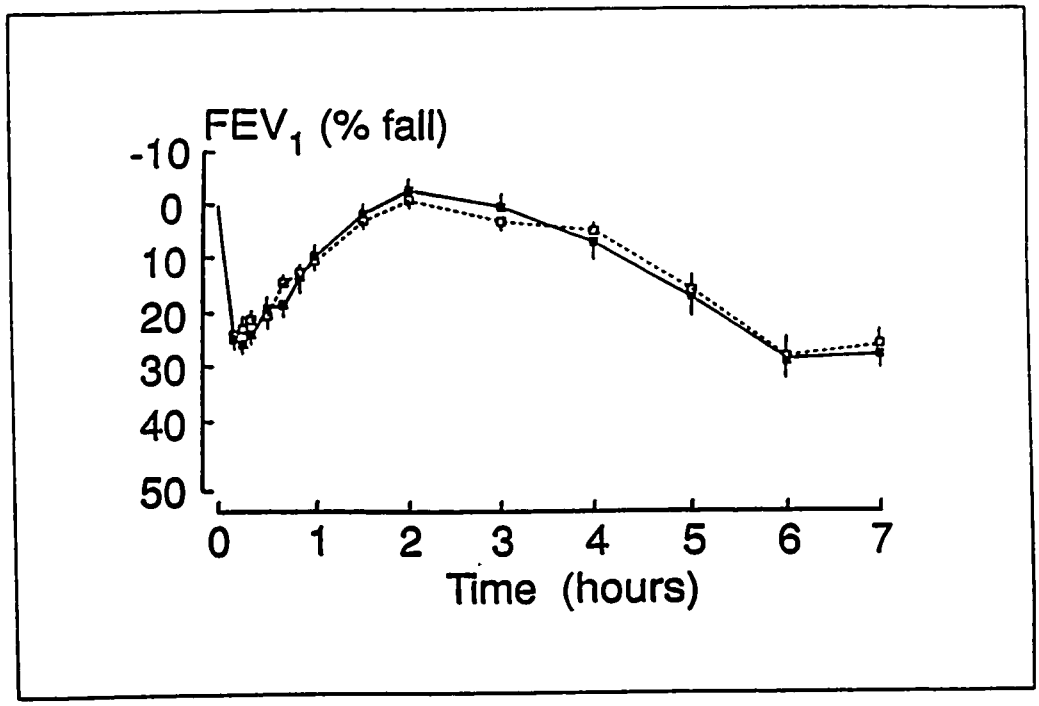
Cyclopiazonic acid (CPA) is a fungal toxin which has been shown to be a selective inhibitor of the SR Ca^{2+}-ATPase pump. Seidler *et al.* (1989) demonstrated that CPA inhibited the Ca^{2+}-stimulated ATP hydrolysis in intact SR and in a purified preparation of Ca^{2+}-ATPase. It did not, however, inhibit kidney and brain Na^+,K^+-ATPase, gastric H^+,K^+-ATPase, mitochondrial F1-ATPase, erythrocyte Ca^{2+}-ATPase, nor the Mg^{2+}-activated ATPase of T-tubules and surface membranes of rat skeletal muscle (Seidler *et al.*, 1989). Deng and Kwan (1991) and Darby *et al.* (1995) showed that CPA was also effective as a SR Ca^{2+} pump inhibitor in smooth muscle cells. It is an objective of mine to use CPA as a tool to determine if cAMP effects smooth muscle relaxation via stimulation of the SR Ca^{2+} ATPase.

1.13 Allergen Inhalation Tests

In patients with asthma, inhalation of an allergen may result in a biphasic reaction consisting of an early asthmatic response (EAR) and a late asthmatic response (LAR; Dolovich *et al.*, 1989; Sterk *et al.*, 1993; Figure 5). The EAR is a period of airflow obstruction which usually begins approximately 10 minutes after inhalation of the allergen. The obstruction is maximal between 10 to 30 minutes following exposure and usually resolves within 1 to 3 hours (Hargreave , 1989; Sterk *et al.*, 1993). The airflow obstruction of the EAR is caused primarily by smooth muscle contraction, likely due to the combination of antigens with IgE antibodies and a resultant release of mediators such as cysteinyl leukotrienes from mast cells (Hargreave, 1989).

The LAR is a subsequent period of airflow obstruction and airway inflammation that occurs in approximately 50% of asthmatic patients (Hargreave, 1989; Sterk *et al.*, 1993), which is associated with an increase in inflammatory cells such as eosinophils and neutrophils (Padalier, 1993; Asano *et al.*, 1994). The LAR begins 3 to 4 four hours following the allergen inhalation, and may continue for up to 24 hours (Sterk, 1993). The LAR is usually associated with an increase in airway responsiveness to bronchoconstrictors such as methacholine or histamine. This hyperresponsiveness may last for several days or even weeks following exposure to the antigen, and is likely an indirect measure of allergen-induced inflammation (Dolovich *et al.*, 1989; Sterk *et al.*, 1993).

Figure 5. Early and late phase response. Time-response curve of forced expiratory volume (FEV_1) from asthmatics following an allergen challenge. As determined from the decrease in FEV_1, the early response resolves approximately 2 hours following inhalation of the allergen, and the onset of the late response occurs approximately 4 hours following challenge. Subjects were challenged with house-dust mite extract on two days separated by a two week interval. Each curve represents the mean and SEM of 10 subjects of each day (Sterk *et al.*, 1993).


FEV1 (% fall)
-10
0
10
20
30
40
50
0
1
2
3
4
5
6
7
Time (hours)

1.14 The Canine Model of Allergen Inhalation

In 1970, Booth *et al.* investigated the potential usefulness of a canine model of antigen-induced airway obstruction. These investigators reported that some dogs demonstrated both skin reactivity and airway obstruction following exposure to the aerosolized antigen *Ascaris suum*. They suggested that the respiratory response was due to bronchoconstrictor mediator release due to cross reactivity of the *Ascaris suum* antigen with a parasitic nematode, *Toxacara canis*, which is commonly found in dogs (Booth, 1970). Sasaki *et al.* (1987) reported that inhalation of *Ascaris suum* resulted in a LAR in dogs that were treated with metapyrone, and suggested that inhalation of this antigen might be a useful tool for the study of the late response of bronchial asthma.

One of my objectives is to examine the canine airway hyperresponsiveness that occurs as a result of inhalation of the *Ascaris suum* antigen. I hope to elucidate the role of PGE_2 in mediating this hyperresponsiveness. In dogs, exposure to ozone is used as another model of asthma. Exposure to ozone resulted in bronchial hyperresponsiveness that was determined to be due to a decreased prejunctional and postjunctional inhibition, potentially mediated by PGE_2 (Janssen *et al.*, 1991). I hope to determine if PGE_2 plays a similar role in the canine allergen inhalation model of asthma.

CHAPTER II

OBJECTIVES AND HYPOTHESES

Chapter II: Objectives and Hypotheses

2 General Hypothesis:

The production and release of PGE_2 from the epithelium regulates ASM contraction, can account for part of the putative EpDIF function, and plays a role in antigen-induced airway hyper-responsiveness.

2.1 Objective 1:

To determine if inhibitory prostanoids are released from canine tracheal epithelium and to establish whether or not they are the EpDIF.

Hypothesis:

The actions of PGE_2 and PGI_2 can account for epithelium-derived inhibition of canine tracheal smooth muscle.

Results:

PGE_2 was released from the epithelium of tracheal smooth muscle in response to EFS. PGI_2 was released both from the epithelium as well as from a non-epithelial source, and its release was not dependent on EFS. Removal of the epithelium was not mimicked completely by the prevention of prostaglandin production, and although PGE_2 was released from the epithelium and inhibited ASM contraction, there also exists a non-prostanoid EpDIF.

2.2 Objective 2:

To determine the role of the epithelium, with a focus on epithelium-derived PGE_2, on mediating antigen-induced airway hyper-responsiveness in canine tracheal smooth muscle.

Hypothesis:

Animals who became hyper-responsive *in vivo* following antigen exposure will demonstrate hyper-responsiveness *in vitro*. There will be a decrease in the release of epithelium-derived PGE_2 from animals who become hyper-responsive *in vivo* following allergen inhalation. This may possibly be due to damage of the epithelium as a result of antigen exposure.

Results:

Tissues from animals who were hyper-responsive *in vivo* only demonstrated hyper-responsiveness *in vitro* when the epithelium was removed. There was not a decrease in the release of epithelium-derived PGE_2 from animals who became hyper-responsive *in vivo* following allergen inhalation. There was an increase, however, in epithelium-derived PGE_2 from animals who were not hyper-responsive following allergen exposure, compared to either sham-exposed animals or animals who became hyper-responsive. The epithelium did not appear to be damaged in animals which became hyper-responsive.

2.3 Objective 3:

To determine the role of the epithelium, and of epithelium-derived PGE_2, on mediating antigen-induced airway hyper-responsiveness in canine bronchial smooth muscle.

Hypothesis:

Animals who became hyper-responsive *in vivo* following antigen exposure will demonstrate hyper-responsiveness *in vitro*. There will be a decrease in the release

of epithelium-derived PGE_2 from animals who become hyper-responsive *in vivo* following allergen inhalation. This may possibly be due to damage of the epithelium as a result of antigen exposure.

Results:

Tissues from animals who were hyper-responsive *in vivo* following antigen exposure were not hyper-responsive *in vitro*. These tissues were hypo-responsive *in vitro*, except in the presence of indomethacin. There was no decrease in PGE_2 from tissues from hyper-responsive animals, in fact there was an increase in the basal release of PGE_2 from these tissues.

2.4 Objective 4:

To use CPA to determine if one mechanism through which PGE_2 effects relaxation of canine tracheal smooth muscle is through a cAMP or cGMP-mediated increase in SR Ca^{2+}-ATPase activity, and consequently, an increased Ca^{2+} uptake.

Hypothesis:

cAMP and cGMP increase the SR Ca^{2+}-ATPase activity, which results in an

increased uptake of Ca^{2+}, and inhibition of contraction.

Results:

cAMP induced-relaxation was not altered in the presence of CPA, thus increased SR Ca^{2+}-ATPase activity is not a likely mechanism of action for cAMP in canine tracheal smooth muscle. CPA only slightly inhibited cGMP-induced relaxation, thus increased SR Ca^{2+}-ATPase activity is not the major mechanism of action of cGMP in canine tracheal smooth muscle.

CHAPTER III

MANUSCRIPT # 1

MANUSCRIPT No. 1

RELEASE AND ACTIONS OF INHIBITORY PROSTAGLANDINS FROM CANINE TRACHEAL EPITHELIUM

Published in the *Canadian Journal of Physiology and Pharmacology* in 1996

Ita McGrogan's contribution:

(i) organ bath studies

(ii) radioimmunoassay of PGE_2 and 6-keto $PGF_{1\alpha}$

(iii) analysis of data

(iv) preparation of first draft of manuscript

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June 20, 1997

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Release and actions of inhibitory prostaglandins from canine tracheal epithelium

I. McGrogan and E.E. Daniel

Abstract: This study evaluated the sources and actions of inhibitory prostanoids on canine tracheal smooth muscle to determine if these accounted for epithelium derived relaxing factor activity. Concentration–response curves of canine tracheal smooth muscle were generated to carbachol in the presence and absence of the epithelium and (or) indomethacin, a cyclooxygenase inhibitor. Removal of the epithelium or addition of indomethacin (10^{-5} M) in the presence of the epithelium shifted the curve significantly leftward compared with epithelial-intact tissue. Furthermore, addition of indomethacin to epithelial-denuded tissue produced the greatest shift in the curve. Removal of the epithelium increased contractility in response to electrical-field stimulation at intermediate frequencies compared with epithelium-intact tissues. The addition of indomethacin to epithelium-intact tissue also increased the contractile responses. Removal of the epithelium in the presence of indomethacin further increased responses. Radioimmunoassay of muscle bath fluid indicated that the inhibitory prostanoids prostaglandin I_2 (PGI_2) and prostaglandin E_2 (PGE_2) were released. PGI_2 was released from the epithelium as well as from a nonepithelial source. PGE_2 was released from the epithelium in response to electrical-field stimulation. The release of PGE_2 was greatly reduced by the addition of indomethacin (10^{-6} M), but not by the addition of ω-conotoxin (GVIA), an N-type Ca^{2+} channel antagonist, nor by the addition of tetrodotoxin, a Na^+ channel blocker. Both toxins abolished contractions to electrical-field stimulation. We conclude that the inhibitory prostanoids PGI_2 and PGE_2 are released, along with a nonprostanoid factor from epithelium, and modulate airway smooth muscle contractility to stimulation of cholinergic nerves and muscarinic agonists. PGE_2 is released from epithelium by electrical-field stimulation independent of nerve function.

Key words: epithelium-derived inhibitory factor, electrical-field stimulation, radioimmunoassay, tetrodotoxin, ω-conotoxin (GVIA).

Résumé : La présente étude a évalué les sources et les actions des prostanoïdes inhibiteurs sur le muscle lisse trachéal canin, afin de déterminer s'ils jouent un rôle dans l'activité du facteur de relaxation dérivé de l'épithélium. Des courbes concentration–réponse du muscle lisse trachéal canin ont été établies pour le carbachol, en présence et en l'absence d'épithélium et (ou) d'indométhacine, un inhibiteur de cyclooxygénase. L'enlèvement de l'épithélium, ou l'addition d'indométhacine (10^{-6} M) en présence de l'épithélium, a nettement déplacé la courbe vers la gauche comparativement à ce qui a été observé pour le tissu avec épithélium intact. L'addition d'indométhacine au tissu sans épithélium a provoqué le plus important déplacement de la courbe. L'enlèvement de l'épithélium a augmenté la contractilité en réponse à une stimulation de champ électrique à fréquences moyennes comparativement à ce qui s'est produit dans les tissus avec épithélium intact. L'addition d'indométhacine au tissu avec épithélium intact a aussi augmenté les réponses contractiles, réponses qui ont augmenté davantage avec l'enlèvement de l'épithélium. Le radio-immunodosage de la préparation musculaire a indiqué la libération des prostanoïdes inhibiteurs, prostaglandine I_2 (PGI_2) et prostaglandine E_2 (PGE_2). Les PGI_2 ont été libérées de l'épithélium ainsi que d'une source non épithéliale. La stimulation électrique (SÉ) a provoqué la libération de PGE_2 de l'épithélium, libération qui a été considérablement réduite par l'addition d'indométhacine (10^{-6} M), mais non par l'addition de ω-conotoxine (GVIA), un antagoniste des canaux Ca^{2+} de type N, ni par l'addition de tétrodotoxine, un bloqueur des canaux Na^+. Les deux toxines ont supprimé les contractions induites par la stimulation électrique. Nous concluons que les prostanoïdes inhibiteurs, PGI_2 et PGE_2, ainsi qu'un facteur non prostanoïque, sont libérés de l'épithélium et qu'ils modulent la contractilité du muscle lisse des voies aériennes en réponse à la stimulation des nerfs cholinergiques et des agonistes muscariniques. La stimulation électrique provoque la libération des PGE_2 de l'épithélium sans mettre en jeu la fonction nerveuse.

Mots clés : facteur d'inhibition dérivé de l'épithélium, stimulation électrique, radio-immunodosage, tétrodotoxine, ω-conotoxine (GVIA).
[Traduit par la Rédaction]

Introduction

The airway epithelium releases one or more factors that have inhibitory effects on airway smooth muscle (ASM) contraction (Tschirhart et al. 1987; Ullman et al. 1988; Fernandes et al. 1990; Yu et al. 1992). Prostaglandin E_2 (PGE_2) and prostaglandin I_2 (PGI_2) may contribute to this epithelium-derived relaxation. Studies have demonstrated that cyclooxygenase, an

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enzyme required for PGE_2 production, is present in airway epithelium (Butler et al. 1992; Barnett et al. 1988). PGE_2 has been shown to have an inhibitory effect on canine ASM tissue as well as on the release of acetylcholine from nerves innervating ASM (Daniel et al. 1987; Abela and Daniel 1995; Serio and Daniel 1989). Moreover, recent studies have demonstrated that PGE_2 is released from airway tissue in other species in response to electrical-field stimulation (EFS) (Ullman et al. 1991; Fernandes et al. 1994).

Ullman et al. (1991), using ferret trachea, and Fernandes et al. (1994), using guinea pig trachea, demonstrated that the level of PGE_2 released from airway tissue in response to EFS was not reduced in the presence of tetrodotoxin (TTX). Ullman et al. (1991) proposed that PGE_2 release was due to TTX-resistant nerves. However, in this study we demonstrated that the PGE_2 release is not affected by TTX or the addition of ω-conotoxin (ω-CTX) (GVIA), an N-type Ca^{2+} channel blocker. Our findings indicate that the epithelium released PGE_2 upon EFS. This release was blocked upon addition of indomethacin but was unchanged by the addition of TTX or ω-CTX (GVIA), suggesting that this release does not require neural activation. We further demonstrated that 6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2, is released from the epithelium and from a nonepithelial source, and modulates airway smooth muscle contraction.

Methods

Animals

Healthy adult mongrel dogs of either sex were euthanized using pentobarbital administration (100 mg/kg body weight). This procedure was approved by the McMaster University Animal Care Committee. Segments of trachea were removed and placed in Krebs solution, constantly bubbled with 95% O_2 – 5% CO_2 to achieve a pH of 7.3–7.4. The composition of the Krebs solution was (in mM) NaCl, 115.5; KCl, 4.6; $CaCl_2$, 2.5; NaH_2PO_4, 1.6; $MgSO_4$, 1.16; $NaHCO_3$, 21.9; glucose, 11.1.

Organ bath studies

Each segment of trachea was cleaned, and the smooth muscle was removed from the cartilaginous tracheal rings. In both epithelium-intact and -denuded tissues, the serosal side was cleaned of connective tissue. In epithelium-intact tissue the epithelium was left unaltered. For epithelium-denuded tissues the epithelium was cut away from the smooth muscle and underlying connective tissue was removed. Dissection was performed under a dissecting microscope to prevent damage to the underlying smooth muscle fibres.

The tracheal muscle was cut into strips 1–2 mm wide and approximately 1 cm long, parallel to the direction of the smooth muscle fibres. The strips were tied with 4/0 silk thread and mounted in 10-mL organ baths containing the same Krebs solution and bubbled with the same gas mixture as mentioned above. The lower ends of the strips were attached to a hook on the bottom of a plastic holder, and the top ends of the tissue were connected to a Grass FT-03C mechanotransducer (Grass Instruments, Quincy, Mass.). Isometric tension was recorded continuously on a Gould 2800 chart recorder (Gould Inc., Cleveland, Ohio). A resting tension of 1.5 g (previously shown to produce maximum active tension) was applied to each strip. The tissues were equilibrated for 1 h in the organ baths before the beginning of the experiments. They were submerged in the Krebs solution and bubbled at 37°C throughout the experiment. KCl (60 mM) was added to the organ bath to contract the tissue. Fifteen minutes later, the KCl was washed out. This procedure was repeated three times, or until consistent, reproducible contractions were generated in each tissue. Half of the tissue strips were preincubated for 1 h in normal Krebs Ringer solution, the other half in Krebs Ringer containing the cyclooxygenase inhibitor indomethacin (10^{-5} M). A concentration–response curve was generated to carbachol (Cch; 10^{-9}–10^{-5} M), using both epithelium-intact and -denuded tissues. For EFS, the tissues were stimulated at 40 V, 0.5 ms duration, at frequencies of 0.1, 0.3, 1.0, 3.0, 10.0, and 30.0 pulses/s. The electrodes used for EFS were two platinum rings, placed 1 cm apart, at the bottom of a holder placed inside the organ bath. The tissue was suspended through the center of the rings.

Tissues to which the nerve toxins were added had the epithelium intact and no indomethacin present. Following equilibration in 60 mM KCl, tissues were incubated with TTX (10^{-6} M) or ω-CTX (GVIA) (3×10^{-7} M) for 20 min. The same stimulus protocol described above was employed. Both toxins abolished EFS-induced contractions.

Following each experiment, tissues were removed from the organ bath, and the epithelium was removed from those tissues that were epithelium intact. The tissues were air dried for at least 48 h and then weighed to obtain the dry weight of the smooth muscle.

Radioimmunoassays

Samples (2×200 μL) of organ bath fluid were collected immediately after the tissues were washed of KCl and as soon as the EFS protocol (or appropriate time controls) was carried out. The samples were collected using plastic syringes, immediately frozen, stored at –70°C, and thawed on the benchtop immediately before the assay was performed.

Samples were analyzed for PGE_2 and 6-keto-$PGF_{1\alpha}$ content, using commercially prepared kits from Advanced Magnetics (Cambridge, Mass.). The radioimmunoassay was based upon competition of the prostaglandin with radioactively labelled prostaglandin for a number of sites on the specific antibody. Antibody-bound prostaglandin was separated from unbound with magnetic dextran-coated charcoal through magnetic separation. The counting rate was correlated with concentration via a standard curve.

Drugs used

Carbachol, indomethacin, ω-CTX (GVIA), and TTX were obtained from Sigma (St. Louis, Mo.). Indomethacin was dissolved in a solution of $NaHCO_3$ (1%), which was then incorporated into the Krebs solution. All other drugs were dissolved in distilled water.

Data analysis

All results are expressed as means ± SE. Significant differences were reported if $p < 0.05$, using the Bonferroni correction for multiple comparisons, performed following analysis of variance. The maximum responses to Cch are reported as gram tension per milligram of dry tissue. Cch responses were expressed as a percentage of the response to 10^{-4} M Cch.

Radioimmunoassay results are expressed as the increment from pre-EFS measurements subtracted from the post-EFS (or time control values). This was done to normalize for any difference in basal release of the prostaglandins between tissues.

Results

Figure 1 displays the effects of removing the epithelium and (or) adding indomethacin on the responses to Cch. The EC_{50} values derived from these curves are given in Table 1. The removal of the epithelium resulted in a significant shift of the curve to the left. This is consistent with the epithelium releasing a factor or factors that inhibit the contraction of the smooth muscle, in support of previous findings. Adding indomethacin, a cyclooxygenase inhibitor, in the presence of epithelium also produced a leftward shift in the concentration–response curve.

Fig. 1. Contractile responses of canine trachea to Cch concentration ($n = 9$). Removal of the epithelium shifted the curve to the left by a half-log step, both in the presence and absence of indomethacin. Adding indomethacin in the presence of epithelium also resulted in a leftward shift. Error bars have been omitted for clarity. For EC_{50} values, see Table 1. Max, maximum.

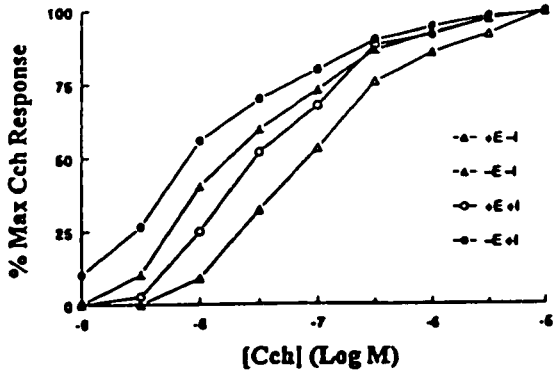


Table 1. EC_{50} values from Cch concentration–response curves (Fig. 1).

	EC_{50} (log M)	SEM	Significantly different from:
A (+E –I)	–7.11	0.13	B, C, D
B (–E –I)	–7.75	0.12	A, D
C (+E +I)	–7.60	0.11	A, D
D (–E +I)	–8.10	0.09	A, B, C

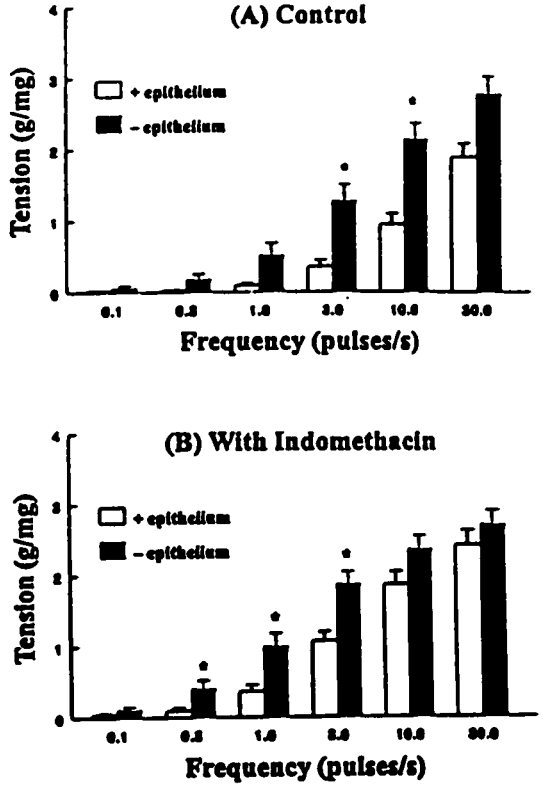
Note: Significant differences are reported if $p < 0.05$, using the Bonferroni correction for multiple comparisons. E, epithelium; I, indomethacin. All data are means ± SEM.

This suggests that prostaglandins inhibit ASM. However, the fact that removing the epithelium as well as adding indomethacin resulted in a further leftward shift may indicate that there is another, nonprostanoid, inhibitory factor released by the epithelium. Putative candidates for this factor are addressed in the discussion.

There were no significant differences in the maximum contractions produced by Cch in any of the four experimental conditions. Removal of the epithelium (E), but not addition of indomethacin (I), consistently but insignificantly was associated with greater maximum response (+E,–I, 3.52 ± 0.41; –E,–I, 4.13 ± 0.59; +E,+I, 3.27 ± 0.46; –E,+I, 4.56 ± 0.77). These findings indicate that (*i*) removal of the epithelium did not damage the underlying smooth muscle and (*ii*) the sensitivity of the smooth muscle to carbachol was altered, without altering the maximal response of the tissue.

In the canine trachea, cholinergic excitatory nerves predominate (Daniel et al. 1987), although adrenergic nerves are also present. Figures 2A and 2B display the results of electrical field stimulated contractions performed in the presence and absence of epithelium and in the presence and absence of indomethacin. Effects on these responses were consistent with the findings of Fig. 1. Removal of the epithelium caused an

Fig. 2. Contractile responses of canine trachea to electrical field stimulation (0.5-ms pulse duration, 40 V per pulse), at 0.1, 0.3, 1.0, 3.0, 10.0, and 30.0 pulses/s. (A) Control, in normal Krebs, in the presence and absence of epithelium. (B) In Krebs containing 10^{-5} M indomethacin. Removal of the epithelium enhanced the contractile response at intermediate frequencies under both conditions. *Significant, $p < 0.05$, using the Bonferroni correction for multiple comparisons ($n = 9$).



increased contractile response to EFS, which was significant at 3 and 10 pulses/s. The addition of indomethacin also increased the contractile response. Moreover, removal of the epithelium in the presence of indomethacin also resulted in a greater contraction compared with that after indomethacin alone, indicating the involvement of a nonprostanoid.

The concentration of 6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2, released into the muscle bath is displayed in Fig. 3. Statistical analysis of Fig. 3 can be found in Table 2. When the epithelium was removed, there was a decrease in the concentration of 6-keto-$PGF_{1\alpha}$ released. However, significantly more of the metabolite was released into tissue baths containing tissue without epithelium compared with the same tissues in the presence of indomethacin. Thus, PGI_2 is released from nonepithelial tissue as well as from epithelium.

Figure 4 displays the concentrations of PGE_2 generated. The data demonstrate that PGE_2 is released from the epithelium in response to EFS. EFS significantly increased the

Fig. 3. Concentration of 6-keto-$PGF_{1\alpha}$ generated in the muscle bath. The data suggest that there are epithelial and nonepithelial sources of PGI_2. *Significant, $p < 0.05$, using the Bonferroni correction for multiple comparisons ($n = 5$).

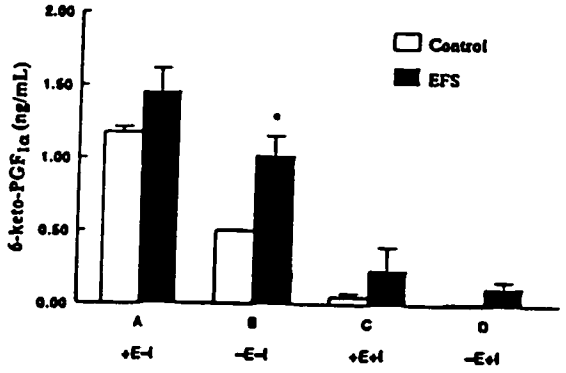
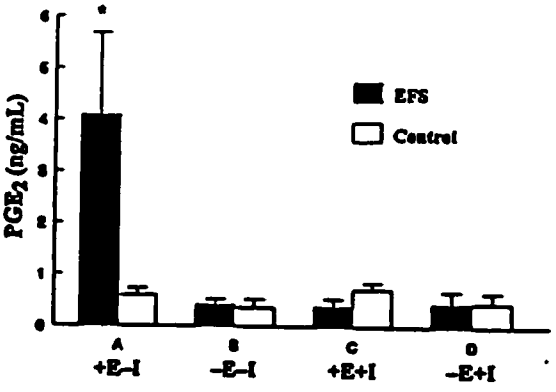


Fig. 4. Concentration of PGE_2 generated in the muscle bath. The data show that PGE_2 is released from the epithelium in response to electrical field stimulation. The data also suggest that the epithelium is the sole source for PGE_2. *Significant, $p < 0.05$, using the Bonferroni correction for multiple comparisons ($n = 5$–8).



amount of PGE_2 release compared with unstimulated tissues. Removal of the epithelium reduced the level of PGE_2.

In Fig. 5, the amount of PGE_2 released in response to EFS in the presence of ω-CTX (GVIA) or TTX is displayed. The level of PGE_2 released is not significantly different in the presence of ω-CTX (GVIA) or TTX than in the absence. Contractile activity in response to EFS was abolished by addition of either toxin, indicating that the toxin was present at effective concentrations in the tissue.

Discussion

The main findings of this study are (*I*) PGI_2 (assessed from its stable metabolite, 6-keto-$PGF_{1\alpha}$) is released from canine tracheal epithelium and from a nonepithelial source, (*II*) PGE_2 is released from canine tracheal epithelium in response to EFS

Fig. 5. Concentration of PGE_2 generated in the muscle bath in the presence of ω-CTX (GVIA) (10^{-6} M) or TTX (10^{-6} M). No significant difference was seen in the presence or absence of the toxins. All tissues had epithelium intact and all tissues were electrical field stimulated. No indomethacin was present. Contractile activity was abolished by the addition of the toxin ($n = 5$).

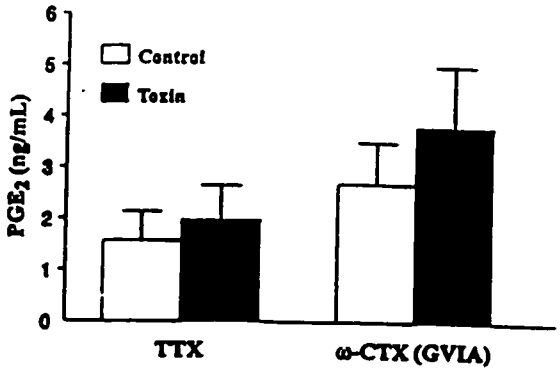


Table 2. Statistical analysis of 6-keto-$PGF_{1\alpha}$ release.

Control	EFS
A vs. B, $p<0.001$	A vs. B, $p>0.05$
A vs. C, $p<0.001$	A vs. C, $p<0.001$
A vs. D, $p<0.001$	A vs. D, $p<0.001$
B vs. C, $p<0.001$	B vs. C, $p<0.01$
B vs. D, $p<0.001$	B vs. D, $p<0.01$
C vs. D, $p<0.05$	C vs. D, $p>0.05$

Note: A, +epithelium, −indomethacin; B, −epithelium, −indomethacin; C, +epithelium, +indomethacin; D, −epithelium, +indomethacin.

and inhibits smooth muscle contractility, and (*III*) the EFS-dependent release of PGE_2 is not blocked by the addition of TTX or ω-CTX (GVIA). Removal of the tracheal epithelium increased smooth muscle contraction in response to EFS and also increased sensitivity to direct muscarinic stimulation. This is in agreement with a large body of evidence (Flavahan et al. 1985; Raeburn et al. 1986; Tschirhart et al. 1987; Yu et al. 1992) and suggests that one or more factor(s) are released from the epithelium to modulate ASM and (or) release of acetylcholine from nerves (Serio and Daniel 1989). The existence of a released epithelium-derived relaxation factor (EpDRF) was proven by demonstrations that inhibition could be transferred with muscle bath fluid (Ullman et al. 1988) and that inhibition could also be transferred between tissues from guinea pig trachea to rat aorta in a coaxial bioassay (Fernandes et al. 1990).

Several authors have suggested that the increase in contractility in response to spasmogens that follows the removal of the epithelium is primarily due to the removal of a barrier. This barrier may include a physical barrier to diffusion and (or) an enzymatic barrier, which degrades spasmogens before they reach the smooth muscle (reviewed in Barnes, 1994, and Sparrow et al., 1995). The removal of the barrier function of

the epithelium does not, however, explain our results. Firstly, the experiments were performed on tracheal strips, not tracheal tubes, such that all surfaces were exposed to carbachol simultaneously, and contractions were assessed after the response reached a plateau. These considerations limit the influence of the epithelium serving as a physical barrier to diffusion. Epithelial-denuded tissues did not exhibit significantly greater contractile responses to Cch than did epithelial-intact tissues. As Cch is a nonhydrolyzable muscarinic agonist, differences cannot be accounted for by an enzymatic barrier that decreased the amount of spasmogen reaching the tissues. Secondly, an increase in contractile response to EFS was observed in epithelium-denuded tissues compared with epithelium-intact tissues. EFS induced the release of endogenous acetylcholine from nerves near the muscle; thus this difference cannot be explained by the absence of a diffusional barrier. Finally, the differences in contractile response between epithelial-intact and epithelial-denuded tissues were reduced in the presence of indomethacin. This suggested that prostanoids are released from the epithelium and modulate airway smooth muscle contractility.

In our present study, adding indomethacin resulted in an increase in sensitivity to Cch in tissue that was epithelium intact. However, neither removal of epithelium, nor addition of indomethacin, nor both increased the maximum response. These findings indicated that (*i*) removal of the epithelium did not damage the underlying smooth muscle and (*ii*) the sensitivity of the smooth muscle to carbachol was altered, without altering the maximum response of the tissue. Thus, a cyclooxygenase product is a candidate for the EpDRF that affects sensitivity to Cch. Levels of PGE_2, the major inhibitory prostaglandin in canine trachea, released into the muscle bath fluid were measured and indicated that the largest source for this prostanoid was the epithelium. Studies measuring 6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2, demonstrated that a significant amount of this metabolite was released following removal of the epithelium. This indicated that the epithelium was not the only source of this prostaglandin, but that the smooth muscle itself may have also released significant levels of PGI_2. Thus, we have shown that PGI_2, like PGE_2, is released from canine tracheal epithelium and may have an inhibitory effect on sensitivity of the underlying smooth muscle. However, removal of the epithelium would diminish but not abolish this effect, an effect modest compared with that of PGE_2 (Serio and Daniel 1989; Abela and Daniel 1995).

Addition of indomethacin to epithelial-denuded tissue resulted in a leftward shift of the Cch concentration–response curve compared with the absence of indomethacin. This may reflect an inability of epithelium removal to stop all PGE_2 release. Alternatively, it may reflect inhibition of PGI_2 production in the smooth muscle. Studies in canine ASM have indicated that PGE_2 reduced EFS-induced contraction primarily through inhibition of the release of acetylcholine from postganglionic nerve endings and secondarily by reducing the smooth muscle response to acetylcholine (Abela and Daniel 1995; Serio and Daniel 1989). Furthermore, the fact that removal of the epithelium in the presence of indomethacin also resulted in increased contractility in the muscle compared with epithelial-intact tissues suggests that a nonprostanoid inhibitory factor is also released from the epithelium. Nitric oxide (NO), the epithelium-derived relaxing factor of vascular smooth muscle, has been proposed to play a role in the epithelial modulation of ASM. However, inhibitors of NO did not effect the concentration–response curve to acetylcholine in canine bronchi (Lorenz et al. 1988), indicating that endogenous NO was not exerting an inhibitory effect. Further, NO has been demonstrated to be a poor relaxant of ASM (Stuart-Smith and Vanhoutte 1990).

In 1991, Ullman et al. reported that EFS-induced release of PGE_2 from ferret trachea was not blocked by the addition of TTX. Fernandes et al. (1994) reported similar findings in guinea pig. Our present study confirms these findings in canine tracheal smooth muscle, and demonstrates that the N-type Ca^{2+} channel antagonist, ω-CTX (GVIA), which blocked contractions to EFS, also did not inhibit epithelial release of PGE_2 due to EFS. Presumably TTX and ω-CTX (GVIA) blocked acetylcholine release from EFS. The mechanism for EFS-induced release of the prostanoid is not known. Ullman et al. (1991) demonstrated that its release was not due to the formation of oxygen free radicals, as EFS-induced PGE_2 release was maintained in the presence of the free radical scavenger ascorbic acid. Ullman et al. (1991) suggested that PGE_2 release may be due to TTX-resistant nerves potentially innervating PGE_2-producing cells in the epithelium, or that the contraction of the smooth muscle itself was responsible for production of PGE_2. In canine trachea, ω-CTX or TTX abolished contractile responses to EFS, eliminating that possibility as a PGE_2 source. Fernandes et al. (1994) demonstrated that addition of capsaicin, substance P, and neurokinin A (NKA) did not induce release of PGE_2, indicating no role for tachykinins or capsaicin-sensitive sensory neurons. The canine trachea has not been demonstrated to possess nerves containing substance P or NKA, and the application of substance P or NKA had no effect on tracheal smooth muscle tone (Salonen et al. 1988). This and our finding that the EFS-induced release of PGE_2 is not blocked by ω-CTX (GVIA) makes the involvement of TTX-insensitive sensory nerves unlikely.

Fernandes et al. (1994) further demonstrated that direct stimulation of vagus nerves resulted in contraction of the smooth muscle but did not effect the release of PGE_2, thereby providing further evidence against the possibility that contraction of the smooth muscle induced the PGE_2 release. These authors suggested that the mobilization of arachidonic acid and the resultant production of prostanoids in response to EFS were an artifact rather than a response to stimulation of intrinsic nerve fibres (Fernandes et al. 1994). This is not our interpretation however, as EFS resulted in an increased release of PGE_2 into the muscle bath without significantly increasing the level of PGI_2 released from tissues with intact epithelium. If the production of prostanoids by EFS was an artifact, it was unexpectedly selective for PGE_2 production.

We propose that the mechanism of release of PGE_2 in response to EFS involves an epithelial non-neural tissue. It is possible that there exist, in the epithelium, cells that release PGE_2 in direct response to EFS. Potential candidates for these cells include mast cells or neuroendocrine cells that exist in the epithelial layer (Daniel 1988, Daniel et al. 1988). The candidate cells produce PGE_2 in direct or indirect response to EFS. This is in contrast with Ullman et al. (1991), who proposed that cells in the epithelium that release PGE_2 are innervated by TTX-insensitive nerves. There are few nerves if any in the canine tracheal epithelium (Daniel and O'Byrne 1991), and no

evidence exists of TTX-insensitive nerves in any region. The nature of the cellular source(s) of PGE_2 is not known and requires further investigation.

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CHAPTER IV

MANUSCRIPT # 2

MANUSCRIPT No. 2

RELEASE OF EPITHELIAL-DERIVED PGE_2 FROM CANINE TRACHEA FOLLOWING ANTIGEN INHALATION

Submitted to the *American Journal of Physiology*

Ita McGrogan's contribution:

(i) organ bath studies

(ii) radioimmunoassay of PGE_2

(iii) analysis of data

(iv) preparation of first draft of manuscript

Release of epithelial-derived PGE_2 from canine trachea following antigen inhalation.

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Abstract

In order to investigate the role of PGE_2 in allergen-induced hyper-responsiveness, dogs inhaled either the allergen, *Ascaris suum,* or vehicle (SHAM). 24 hours following inhalation, some animals exposed to allergen demonstrated an increased responsiveness to acetylcholine challenge in vivo (HYP-RESP), others did not (NON-RESP). Strips of tracheal smooth muscle, either epithelium-intact or epithelium-denuded were suspended on stimulating electrodes and a concentration response curve to carbachol (Cch; 10^{-9}M to 10^{-5}M) was generated. Tissues received electrically field stimulation (EFS) and organ bath fluid was collected to determine PGE_2 content. Epithelia-denuded tissues from animals which had inhaled allergen contracted more to 10^{-5} M Cch than did tissue from SHAM dogs. With the epithelium present, all three groups contracted similarly to 10^{-5} M Cch. In response to EFS, HYP-RESP tissues contracted less than SHAM tissues in the presence of the epithelium, and more than SHAM tissues in the absence of the epithelium. PGE_2 release in the muscle bath was greater in NON-RESP tissues than in SHAM or HYP-RESP tissues when the epithelium was present. Removal of the epithelium greatly inhibited PGE_2 release. We conclude tracheal smooth muscle is hyperresponsive *in vitro* after *in vivo* allergen exposure only when the modulatory effect of the epithelium, largely through PGE_2 release, is removed.

Key words: allergen; smooth muscle; prostaglandin

Introduction

Several studies have suggested a role for the inhibitory prostaglandin, prostaglandin E_2(PGE_2), in regulation of airway hyper-responsiveness and asthma. Responses of canine tracheal and bronchial tissues to stimulation of cholinergic nerves or agonists are reduced by PGE_2 (1,6,21) In 1991, Janssen *et al.* (12) reported that exposure to ozone produced airway hyper-responsiveness *in vitro* and that this was due to decreased prejunctional and postjunctional inhibition, potentially mediated by PGE_2. In 1993, Pavord *et al.* (18) found that PGE_2 inhibited the early and late response to allergen and the allergen-induced bronchial reactivity in human allergen-induced asthma. Moreover, Mellilo *et al.* (15) has shown that inhaled PGE_2 decreased exercise-induced bronchoconstriction in asthmatic subjects. Thus PGE_2 plays a protective role in the airway.

In the present study we determined the role of PGE_2 in an animal model of antigen-induced hyperresponsiveness. This is the first study investigating the release of PGE_2 *in vitro* from the trachea following *in vivo* allergen exposure.

In patients with asthma, inhalation of an allergen may result in a biphasic reaction consisting of an early asthmatic response (EAR) and a late asthmatic response (LAR; 23). The EAR is a period of airflow obstruction which usually begins

approximately 10 minutes after inhalation of the allergen. The obstruction is maximal between 10 to 30 minutes following exposure and usually resolves within 1 to 3 hours (23). The airflow obstruction of the EAR is caused primarily by smooth muscle contraction, likely due to the combination of antigens with IgE antibodies and a resultant release of mediators such as histamine and leukotrienes from mast cells (23).

The LAR is a subsequent period of airflow obstruction and airway inflammation that occurs in approximately 50% of asthmatic patients (23). The LAR begins 3 to 4 four hours following the allergen inhalation, and may continue for up to 8 hours. The LAR is usually associated with an increase in airway responsiveness to bronchoconstrictors such as methacholine or histamine. This hyperresponsiveness may last for several days or even weeks following exposure to the antigen, and is likely a consequence of allergen-induced inflammation (23).

The model of antigen exposure used in the current study was developed and characterized by Wooley (25). In this model, dogs inhaled the antigen *Ascaris suum*, which has previously been demonstrated to evoke a LAR (3,16). We examined *in vitro* the contractile responses of the trachea and the release of PGE_2 in antigen-induced airway hyperresponsiveness in animals which demonstrated a LAR compared to animals which did not demonstrate a LAR.

Methods

Antigen Exposure

The methods of antigen exposure have been described previously (26, 27, 28). Briefly, 15 healthy adult dogs of either sex were anaesthetized using pentobarbitol (10mg/kg body weight). The animals were intubated and attached to a ventilator. Animals inhaled acetylcholine (ACh) and a control concentration response curve was generated (0.7 - 80 mg/ml, doubling concentrations; 27). The concentration of ACh which raised the pulmonary resistance 5 cm H_20/l/s above baseline was termed the provocative concentration (PC5). Ten animals subsequently inhaled either the antigen dissolved in saline, and five dogs inhaled saline. The concentration of allergen was increased until pulmonary resistance was raised to 10 cmH_2O/l/s above baseline, after which the animals were ventilated until pulmonary resistance returned to baseline. The animals were then allowed to recover from the anaesthetic.

Twenty-four hours following the exposure to antigen the dogs were once again anaesthetized and a second ACh concentration-response curve was generated. Five animals demonstrated at least a two-fold decrease in the PC5 following exposure to the antigen, and were labelled hyper-responders (HYP-RESP). Those 5 animals which exhibited less than a two-fold decrease in the PC5 following

exposure to the antigen were labelled non-responders (NON-RESP). Those animals which received only saline and no antigen were labelled sham (SHAM). The animals were then euthanised using pentobarbitol administration (100mg/kg body weight). These procedures were approved by the University Animal Care Committee following the guidelines of The Canadian Council for Animal Care..

Segments of trachea were removed and placed in Krebs solution, constantly bubbled with 95% O_2 and 5% CO_2 to achieve a pH of 7.3-7.4. The composition of the Krebs solution was (in mM): NaCl, 115.5; KCl, 4.6; $CaCl_2$, 2.5; NaH_2PO_4, 1.6; $MgSO_4$ 1.16; $NaHCO_3$, 21.9; glucose, 11.1. A total of eight segments of trachea per animal were mounted in the organ baths, two per experimental group (plus/minus epithelium and/or plus/minus EFS). Dissection was performed under a dissecting microscope in order to prevent damage to the underlying smooth muscle fibres.

Organ Bath Studies

The trachea was immediately removed, segments were cleaned, and the smooth muscle was removed from the cartilaginous tracheal rings. In both epithelium-intact and -denuded tissues, the serosal side was cleaned of connective tissue. In epithelium-intact tissue the epithelium was left unaltered. For epithelium-denuded tissues, the epithelium was cut away from the smooth muscle and underlying connective tissue was removed.

The tracheal muscle was cut into strips 1-2 mm wide and approximately 1 cm long, parallel to the direction of the smooth muscle fibres. The strips were tied with 4/0 silk thread and mounted for electrical field stimulation (EFS) and recording of contraction in 10 mL organ baths containing the same Krebs solution and bubbled with the same gas mixture as mentioned above. The lower ends of the strips were attached to a hook on the bottom of a plastic holder which also held the electrodes for EFS and the top ends of the tissue were connected to a Grass FT-03C mechanotransducer. Isometric tension was recorded continuously on a Gould 2800 chart recorder. A preload tension of 1.5 grams (previously shown to allow maximum active tension) was applied to each strip. For EFS of tracheal strips two platinum rings were placed 1 cm apart and the tissue was suspended through the center of the rings. The tissues were equilibrated for 1 h in the organ baths before the beginning of the experiments and kept submerged in the Krebs solution and bubbled at 37°C throughout the experiment.

In a total of 8 organ baths, 4 strips of trachea were epithelium-intact and 4 were epithelium-denuded. Within each of the two groups of 4, two tissues were electrically field stimulated, and two served as equivalent time controls.

In order to evaluate the equilibration of the tissues, KCl (60 mM) was added to the organ bath to contract the tissues. Fifteen minutes later, the KCl was washed out. This procedure was repeated three times, or until consistent, reproducible contractions were generated in each tissue. A cumulative concentration response

(CR) curve was generated to carbachol, (10^{-8} - 10^{-5}M, half-log steps; 14). For EFS, the tissues were stimulated at 40 V/cm, 0.5 ms duration pulses for 10 s, at frequencies of 1.0, 3.0, 10.0, and 30.0 pps.

Following each experiment, tissues were removed from the organ bath, and the epithelium was removed from those tracheal strips that were epithelium-intact. All tissues were air dried for at least 48 hours and then weighed to obtain the dry weight of the smooth muscle.

Radioimmunoassays

Samples of organ bath fluid were collected immediately after the tissues were washed of KCl and again as soon as the EFS protocol (or equivalent time controls in parallel tissues from the same animal) was carried out. Two samples from each tissue sample were collected for analysis of PGE_2 content by radioimmunoassay (RIA), and the average of the two measurements is reported. The samples were collected using plastic syringes, immediately frozen, stored at -70 C, and thawed on the bench top immediately before the assay was performed.

Samples were analysed for PGE_2 content using a commercially prepared RIA kit from Advanced Magnetics (Cambridge, Massachusetts).

Drugs Used

Acetylcholine and carbachol were obtained from Sigma (St.Louis, MO), and were dissolved in distilled water.

Data Analysis

All results are expressed as mean ± SE. Significant differences were reported if $p < 0.05$, using the Bonferroni correction for multiple comparisons, performed following analysis of variance. The maximum responses to Cch are reported as gram tension per milligram of dry tissue. Cch responses were expressed as a percentage of the response to 10^{-5} M Cch.

Radioimmunoassay results are expressed as the increment when pre-EFS measurements were subtracted from the post-EFS (or time control values). This was done to normalize for any difference in basal release of the prostaglandins between tissues.

Results

Figure 1 shows the degree of airway responsiveness attained following exposure to either vehicle or allergen, demonstrated by the PC_5 24 hours following allergen or saline inhalation as a percentage of the PC_5 prior to inhalation. There was no change in the responsiveness following exposure to saline. Exposure to allergen caused a decrease in airway responsiveness, as determined by the PC5, in those animals labelled NON-RESP. This was consistently less than a two-fold decrease, however, and was not significantly different from the PC5 of animals who received only vehicle. There was, however, an increase in airway responsiveness following exposure to allergen, as determined by a two-fold or greater decrease in the PC5, in those animals labelled HYP-RESP, compared to either SHAM animals or NON-RESP animals.

In Figure 2, the mean tension generated in tracheal strips by 10^{-5}M Cch is displayed. In epithelium intact tissues, there was no significant difference between the three groups. Removal of the epithelium resulted in significantly greater contraction in all three tissue types. In epithelium-denuded tissues, a greater contraction was seen in tissues from HYP-RESP animals than in those from SHAM animals. Thus, the presence of the epithelium attenuated the contraction to the maximum dose of Cch in all tissues more in tissues from HYP-RESP animals..

The concentration-response (CR) curves to Cch (10^{-9} mM to 10^{-5} mM) appear

in Figure 3, where the responses are normalized to the maximum (100%) in each tissue. The EC_{50}s to these curves appear in Table 1. Figure 3A displays the CR response curves generated in response to Cch in tissues with intact epithelium; EC_{50} values from HYP-RESP tissues were significantly more than those in SHAM tissues. Figure 3B displays the curves generated by tissues in which the epithelium had been removed. Removal of the epithelium resulted in a significant leftward shift in the CR curves in all three tissue groups. With the epithelium removed, no significant differences were seen between EC_{50} values from SHAM, HYP-RESP, and NON-RESP animals. Table 1 shows that the presence of the epithelium decreased the responsiveness of all the tissue to Cch, and that this effect was more pronounced in tissues from Sham animals than in those from HYP-RESP animals.

The mean contractile responses to EFS are shown in Figures 4. Figure 4A displays those responses from epithelium-intact tissues, and Figure 4B displays epithelium-denuded tissues. Removal of the epithelium resulted in an increased contraction at 10 pps for SHAM tissues, 10 and 30 pps for HYP-RESP tissues and only at 30 pps for NON-RESP tissues. Moreover, at 30 pps, HYP-RESP epithelium-denuded tissues contracted more than SHAM epithelium-denuded tissues. Once again, the presence of the epithelium attenuated the responses and masked a difference between tissues from SHAM animals and from HYP-RESP animals.

To determine the amount of PGE_2 released into the organ bath during the period when EFS was applied, we performed RIA analysis of organ bath fluid

following EFS and a corresponding time control. The concentrations of PGE_2 collected from baths containing tissues with epithelium are shown in Figure 5A, and those from epithelium-denuded tissues are in Figure 5B. EFS did not increase PGE_2 accumulation. There was no significant difference in the concentration of PGE_2 measured in the organ baths containing tissues from either SHAM or HYP-RESP animals. However, a greater concentration of PGE_2 was found in baths from NON-RESP animals than from SHAM or HYP-RESP animals following EFS or a corresponding time control. When the epithelium was removed, PGE_2 release from all tissues was virtually abolished, with the exception of tissues from HYP-RESP dogs, which, when electrically field stimulated, released small, but significant amounts of the prostanoid.

Discussion

The major findings of the present study are: 1) the demonstration of tracheal smooth muscle hyper-responsiveness *in vitro* (correlated with that determined *in vivo* by airway resistance), depends on the absence of the airway epithelium, 2) the tracheal epithelium release more PGE_2 from NON-RESP animals than from HYP-RESP or SHAM animals and 3) there may be a minor non-epithelial source of PGE_2 in HYP-RESP animals.

In epithelium-intact tissues, there was no difference between any of the groups in contractile responses to 10^{-5}M Cch. However, when the epithelium was removed, the maximum contractile response of tracheal smooth muscle strips to 10^{-5} M Cch was significantly greater in tissues from HYP-RESP animals, compared to tissues from SHAM animals. The response to EFS in the absence of the epithelium was likewise greater in HYP-RESP tissues compared to SHAM tissues. The EC_{50} to Cch of HYP-RESP tissues was, however, greater than that of SHAM tissues in the presence of the epithelium (*i.e.* they were less sensitive to Cch). Similarly, epithelium-intact HYP-RESP tissues contracted less in response to EFS than did epithelium-intact SHAM tissues. Thus, it appears that the epithelium is a major determinant of allergen-induced changes in tracheal smooth muscle responsiveness *in vitro.*

Removal of the epithelium is associated with the removal of a barrier. This barrier may be either a physical barrier to diffusion or an enzymatic barrier which degrades contractile agents before they reach the underlying smooth muscle (4,22). The removal of the barrier properties of the epithelium is not sufficient to explain the data presented in this study, however, for the following reasons. First, these experiments were performed with smooth muscle strips, not tubes, which allowed for simultaneous access of Cch to all exposed surfaces. Second, contractions were assessed after the response to each concentration of Cch reached a plateau. Furthermore, Cch is a non-hydrolyzable muscarinic agonist which is not degraded by enzymes found in the epithelium. Moreover, differences were also seen in response to EFS following the removal of the epithelium. EFS induces the release of endogenous ACh from nerves near the muscle inside the epithelial barrier such that differences could not be attributed to the removal of a diffusional barrier. Consistent with our evidence that removal of the barrier function cannot account for all the inhibitory properties of the epithelium, there have been many studies which give evidence that the epithelium releases one or more inhibitory factors which can modulate the contraction of the underlying smooth muscle (1,8,9,24).

We have previously demonstrated that the inhibitory prostanoids PGI_2 and PGE_2 are released from canine tracheal and bronchial epithelium and relax airway smooth muscle *in vitro* (14). Further, it has been shown that concentrations of PGE_2 as low as 1 nM can cause inhibition of airway smooth muscle contraction, and at 10

nM PGE_2 this effect is significantly increased (1,6,14). As PGI_2 is much less effective in inhibiting canine tracheal smooth muscle, we did not analyze for PGI_2 release.

Measurements of PGE_2 released into the muscle bath fluid in the presence of the epithelium demonstrated that there was no significant difference between tissues from SHAM or HYP-RESP dogs. In contrast, a significantly greater amount of PGE_2 was released from tissues from NON-RESP animals. When the epithelium was removed, release of PGE_2 was virtually abolished. Thus, PGE_2, released from the tracheal epithelium, in sufficient concentrations to inhibit smooth muscle contraction (1,6), appears to have played a role in the prevention of airway hyper-responsiveness in animals exposed to antigen.

There have recently been several studies which demonstrated that PGE_2 may play a protective role in airway disease. Gray *et al.* (10) reported that bronchial PGE_2 was reduced in horses with heaves. Further, inhalation of PGE_2 inhibited the allergen-induced increase in airway hyperresponsiveness seen in asthmatics (17) and inhibited exercised-induced bronchial constriction in mild asthmatics (15). The results in the present study confirm the protective role of PGE_2 and suggest that the PGE_2 is primarily epithelium-derived.

The mechanism through which PGE_2 exerts its protective role may be multi-faceted. PGE_2 has been demonstrated to directly relax canine airway smooth muscle and to inhibit acetylcholine release from nerve endings (1,6). PGE_2 may also

act indirectly through effects on inflammatory cells. PGE_2 has been reported to inhibit mediator release from lung mast cells (18) as well as inhibit eosinophil chemotaxis and survival (2,18).

A question that arises from this study is why were tissues from NON-RESP animals capable of producing and releasing more PGE_2 than tissues from HYP-RESP animals. Another model of asthma, ozone exposure, appears to produce airway hyper-responsiveness partly through destruction of the epithelium. Following ozone exposure, desquamation of the epithelium occurs and a subsequent decrease in PGE_2 is predicted (8,11). Thus, we expected to see a decrease in PGE_2 released from the tissues from HYP-RESP animals. Surprisingly, this was not the case. Instead a large increase in the amount of PGE_2 released from tissues from NON-RESP animals compared to those from SHAM or HYP-RESP animals. The amount of PGE_2 released from SHAM and HYP-RESP tissue was similar to the amount we reported in an earlier study (14), in which animals did not inhale any allergen or vehicle. Thus it appears that, in NON-RESP animals, the protective mechanism of increased epithelial PGE_2 release is turned on. This is in direct contrast with the ozone model of asthma in which the protective mechanism is damaged.

Preliminary ultrastructural studies suggest that tracheal and bronchial tissues from NON-RESP animals contain large numbers of inflammatory cells (eosinophils and neutrophils) located just under the epithelium. This is being investigated further. It is possible that there exists a difference in the extent to which inflammatory cells

are recruited in NON-RESP versus HYP-RESP animals. It has been shown that the numbers of eosinophils and neutrophils are elevated in the bronchio-alveolar lavage fluid (BAL) from asthmatics following allergen challenge (19). Wooley *et al.* (28) reported a significant increase in eosinophils in BAL and biopsy samples in asthmatics following allergen challenge. Using the model described in this study, Wooley *et al.* (27) demonstrated that neutrophils are found in BAL from HYP-RESP animals but are not found in BAL from NON-RESP animals. Thus differences may arise in the extent to which recruited cells pass through the epithelium. Different arachidonic metabolites are produced in different inflammatory cells. In the airway, eosinophils have been shown to release leukotrienes C_4, D_4, and E_4, whereas neutrophils release primarily leukotriene B_4 (19,24). Moreover, PGE_2 may be released from macrophages (13).

It is important to note, however, that the increase in PGE_2 release from tissues from NON-RESP animals may be due to a difference in epithelial PGE_2 synthesis following allergen inhalation, and this synthesis may be independent of inflammatory cell recruitment. Further studies need to be conducted to determine if different inflammatory cells are recruited in different animals and if this can account for differences seen in PGE_2 production following allergen exposure.

Tissues from NON-RESP and SHAM animals were equally sensitive to Cch, even though there was an increased release of PGE_2 from NON-RESP tissues when the epithelium was present. This may have been due to the release of an excitatory

substance from NON-RESP tissues that was not present in either SHAM or HYP-RESP tissues, potentially TXA_2. This may also explain why NON-RESP tissues released more PGE_2 than did SHAM tissues during EFS, yet the NON-RESP tissues contracted more in response to EFS.

Also of interest in the current study are the differences seen in PGE_2 production and hyper- responsiveness between tracheal and bronchial tissues. A similar study investigating allergen-induced hyper-responsiveness in canine bronchi reported that bronchial tissues from HYP-RESP animals exposed to *Ascaris suum* were less responsive to Cch administration or EFS than were tissues from SHAM or NON-RESP animals (12). Further, a greater amount of basal PGE_2 release was reported from HYP-RESP tissues. However, these experiments were all performed on epithelium-intact bronchial tissues. It is not known if removal of the epithelium would have uncovered hyper-responsiveness of the underlying smooth muscle. Thus, the current study suggests that studies which examine responsiveness of airway smooth muscle following exposure to antigen should establish whether effects seen are epithelium-dependent or intrinsic to the smooth muscle itself.

In epithelial-denuded tissues from HYP-RESP animals, a small, but significant increase in PGE_2 was observed following EFS. This concentration was significantly less than that in epithelial-intact field stimulated tissues from HYP-RESP animals. Thus there appears to be a non-epithelial source of PGE_2 in HYP-RESP animals that is stimulated to release the prostanoid during EFS. Further studies should explore

if this could possibly be due to differences in inflammatory cell recruitment, or if PGE_2 is released from the smooth muscle itself under these conditions.

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Figure Legends

Figure 1. Changes in Ach-PC_5

Mean provocative concentration (PC_5) of ACh 24 hours following exposure to allergen as a percentage of baseline PC_5. Airway responsiveness to Ach was measured *in vivo* immediately before inhalation of allergen or saline (baseline), and 24 hours following exposure. Hyperresponsiveness was indicated by a decrease in the PC_5 of two-fold or more.

Figure 2. Contractile responses to Cch (10^{-5}M)

Mean contractile responses of *in vitro* tracheal strips from SHAM, NON-RESP, and HYP-RESP animals to Cch (10^{-5} M) in the presence and absence of the epithelium. Removal of the epithelium significantly increased contraction of strips from animals in all three groups (**$p<0.01$; ***$p<0.001$; $n=5$). Tissues from HYP-RESP animals contracted more than did tissues from SHAM animals in epithelium-denuded tissues ($p<0.05$).

Figure 3. Cch concentration response curves

Cumulative CR curves generated in the muscle bath to Cch (10^{-9} - 10^{-5} M),in tissues

with intact epithelium (A) and in epithelium-denuded tissues (B). In A, the EC50 to Cch of tissues from HYP-RESP animals was significantly greater than those from SHAM animals ($p<0.05$; n=5).

Figure 4. Response to EFS

EFS-invoked contractions of tracheal strips in the muscle bath at 0.1, 0.3, 1.0, 3.0, 10, and 30 pps (40 V/cm, 0.5 ms duration), in epithelium-intact (A) and epithelium-denuded tissues (B). In A, the contractile response to 30 pps was greater in SHAM tissues than in HYP-RESP tissues ($p<0.05$). In B, the response to 30 pps was greater in HYP-RESP tissues than in SHAM tissues ($p<0.05$; n=5).

Figure 5. PGE_2 Measurements

Concentration of PGE_2 measured in samples of muscle bath fluid from epithelial-intact tissues (A) and epithelial-denuded tissues (B). Results are expressed as the difference between the concentration of PGE2 in the bath immediately prior to EFS or a time control, and the concentration immediately following. In A, a greater concentration of PGE2 was measured in the muscle baths containing tissues from NON-RESP animals compared to baths with either SHAM or HYP-RESP tissues (**$p<0.01$). For B, a significantly greater concentration of PGE2 was measured in

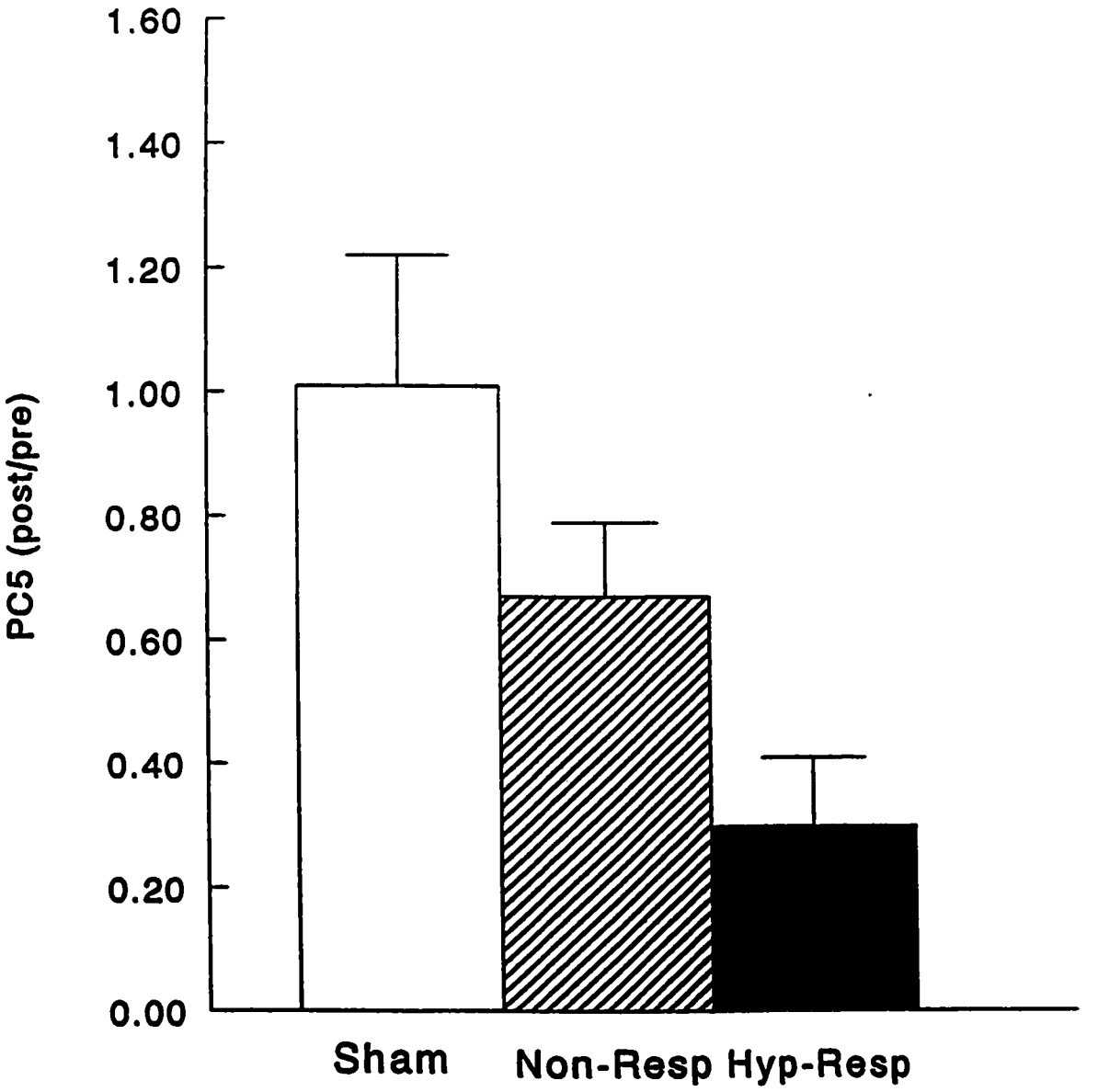
baths containing EFS HYP-RESP tissues compared to time control tissues and EFS SHAM and NON-RESP tissues (**$p<0.01$; $n=5$).

Table 1. Cch EC_{50} Values

EC_{50}s

		Mean (10^{-7})	n	p
Sham	+ epithelium	17.7 ± 0.36	5	<0.01
	- epithelium	5.26 ± 0.78	5	
Non-Responsive	+ epithelium	20.7 ± 0.48	5	<0.001
	- epithelium	9.52 ± 1.8	5	
Hyper-Responsive	+ epithelium	11.2 ± 7.2	5	<0.01
	- epithelium	15.3 ± 2.0	5	

EC_{50}s derived from Cch concentration response curves in the muscle bath (10^{-9}M - 10^{-5}M).


1.60
1.40
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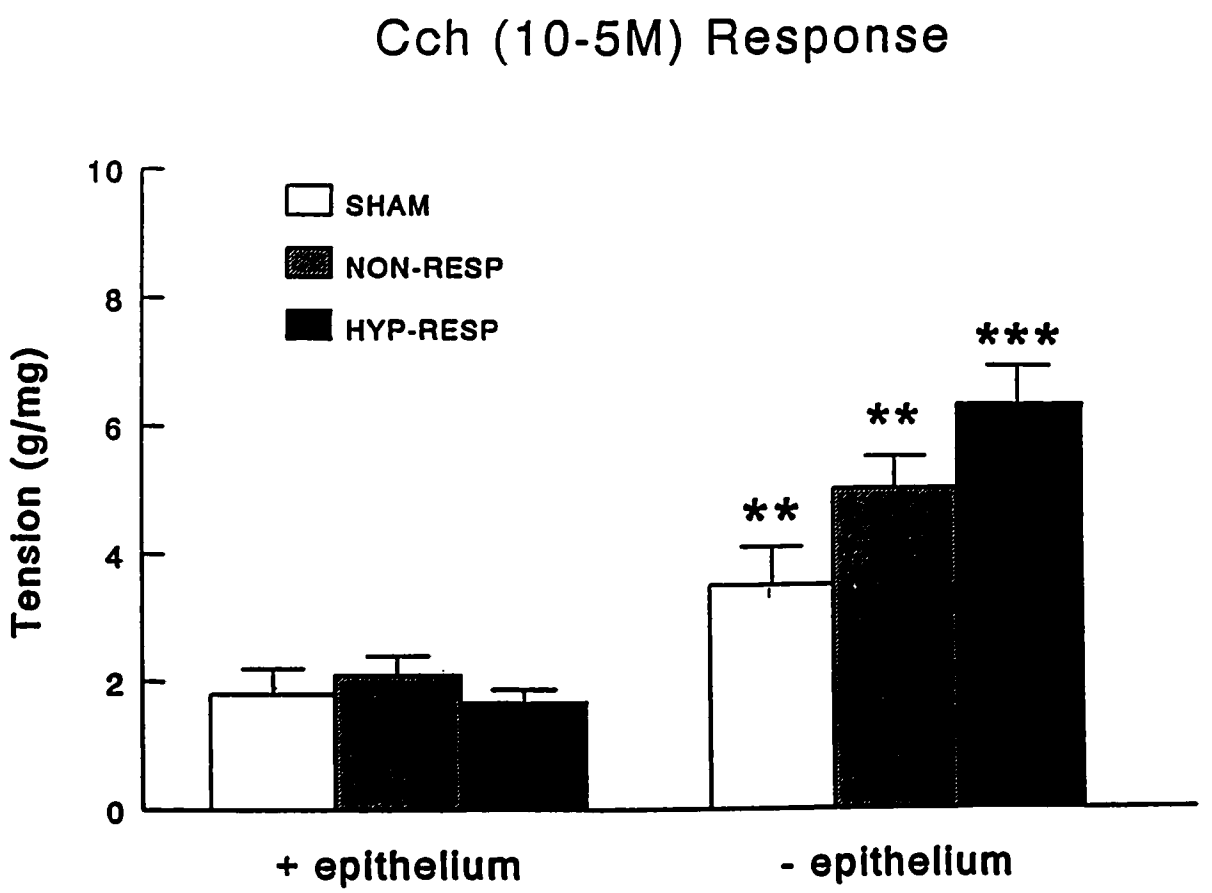
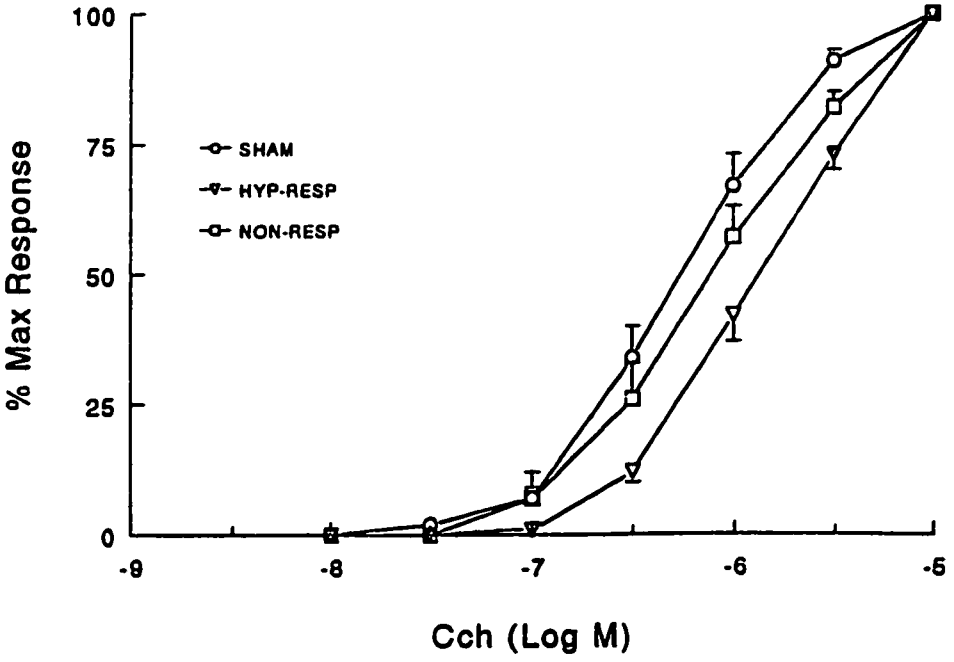
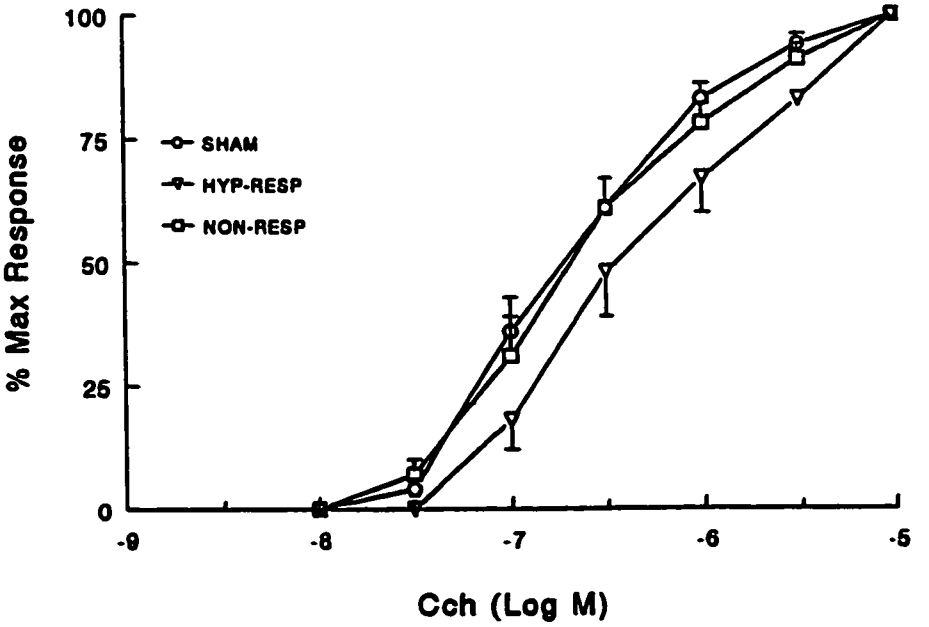
PC5 (post/pre)
Sham
Non-Resp
Hyp-Resp


Cch (10-5M) Response
Tension (g/mg)
10
8
6
4
2
0
SHAM
NON-RESP
HYP-RESP
**
**
***
+ epithelium
- epithelium

A) Epithelium Intact



B) Epithelium Denuded



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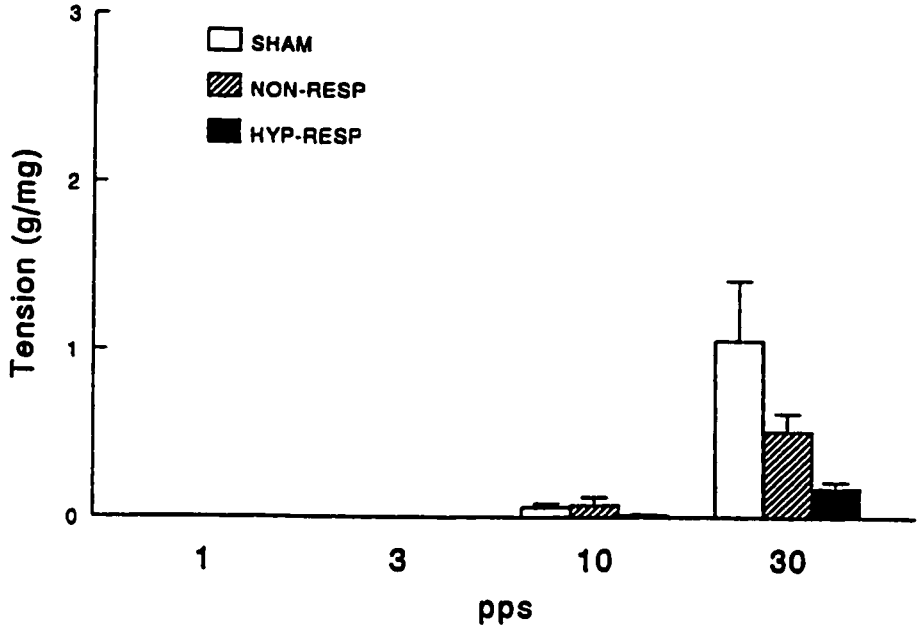

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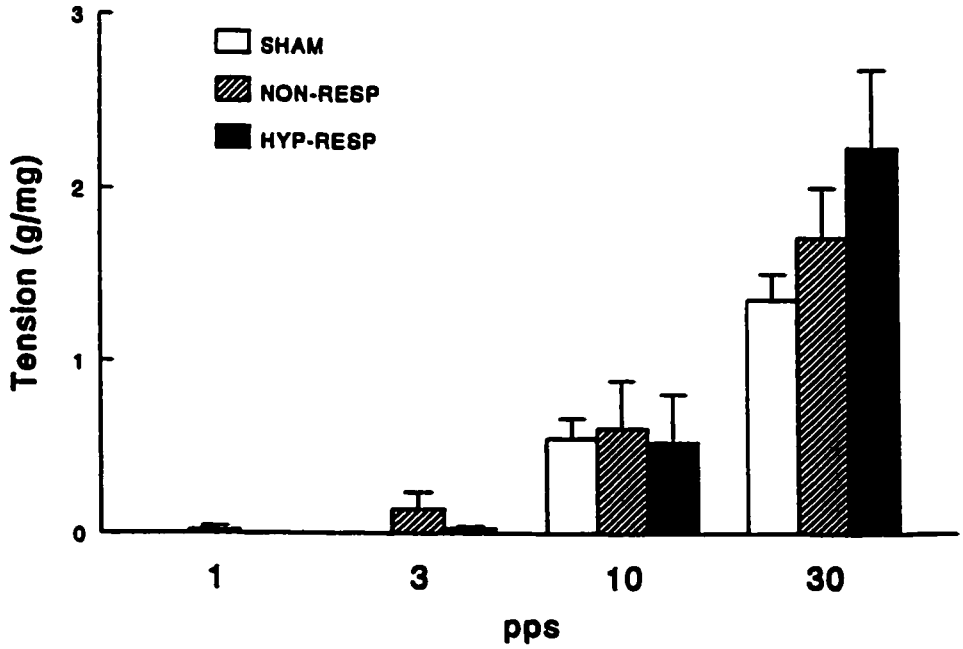

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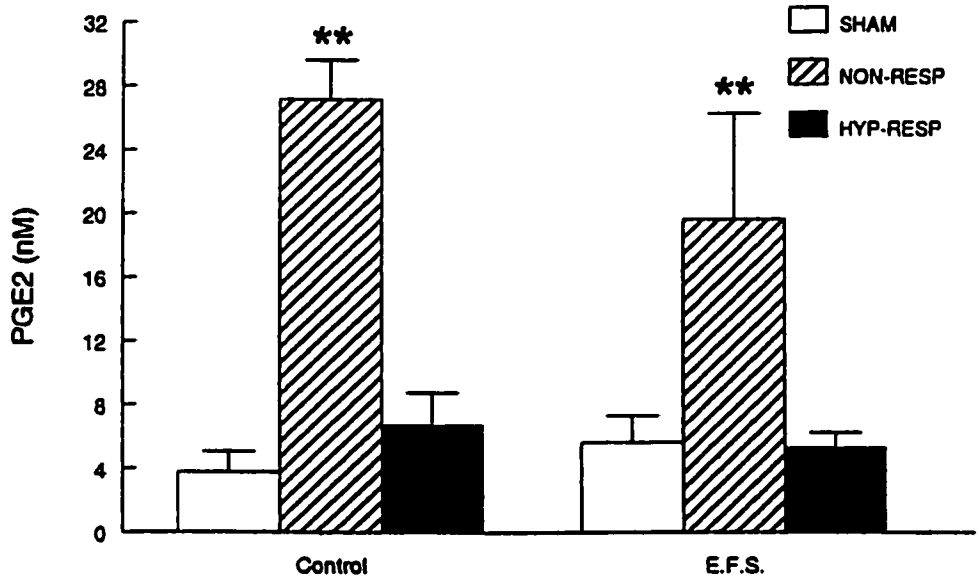

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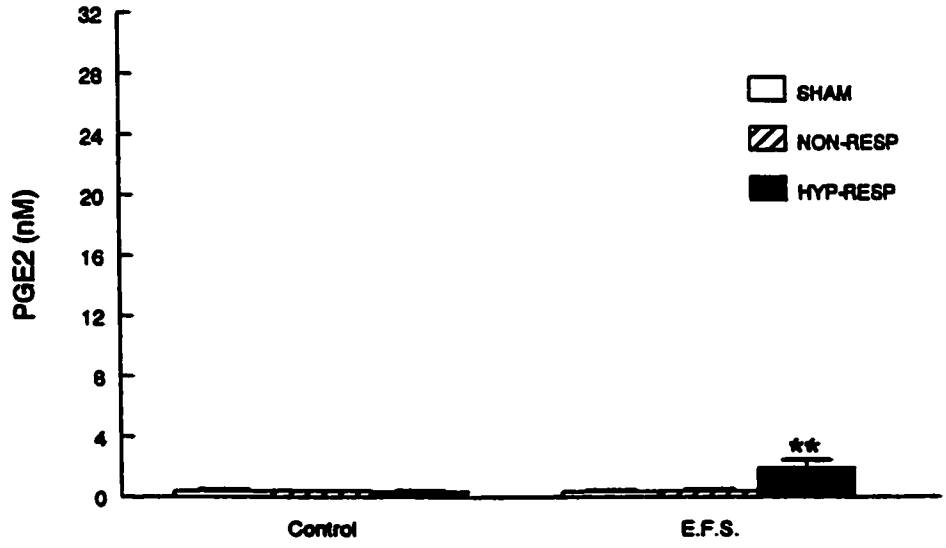

CHAPTER V

MANUSCRIPT # 3

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MYOGENIC AND NEUROGENIC MECHANISMS AND ARACHIDONATE METABOLITES IN BRONCHIAL MUSCLE RESPONSE TO ALLERGEN

Submitted to the *American Journal of Physiology*

Ita McGrogan's contribution:

(i) contractile responses to KCl

(ii) contractile responses to Cch

(iii) frequency-response characteristics of contractions evoked by EFS

(iv) radioimmunoassay of PGE_2

MYOGENIC AND NEUROGENIC MECHANISMS AND ARACHIDONATE METABOLITES IN BRONCHIAL MUSCLE RESPONSE TO ALLERGEN

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Running title: Cyclo-oxygenase metabolites and airway responsiveness

ABSTRACT

We investigated allergen-induced airway hyperresponsiveness (AH) in bronchial tissues obtained from dogs which inhaled *Ascaris suum* leading to AH (RESP) *in vivo* or no change (NON-RESP), as well as those which inhaled saline (SHAM). RESP tissues were not hyperresponsive to KCl or to carbachol, while contractions to electrical field stimulation (EFS) were reduced. This reduction was reversed partially by indomethacin and completely by replacement of the bathing fluid. Radioimmunoassay revealed marked elevation of PGE_2 generation in RESP tissues compared to SHAM and NON-RESP tissues. EFS-evoked contractions were often followed by a slowly-developing secondary contraction in RESP tissues but not in SHAM or NON-RESP tissues. However, indomethacin unmasked such secondary contractions in many SHAM and NON-RESP tissues and markedly enhanced those in RESP tissues, while L-655,240 (TXA_2/PGD_2-receptor antagonist) abolished such contractions in all groups. We were unable to detect thromboxane using radioimmunoassay. We conclude that allergen-induced AH involves altered generation of cyclo-oxygenase metabolites of arachidonic acid (particularly PGE_2) as well as of a non-prostanoid inhibitory factor; as such, the responsiveness of the tissue *in vitro* is dependent on the relative levels of inhibitory and excitatory metabolites.

Key words: prostaglandin E_2; thromboxane A_2; cholinergic innervation;

INTRODUCTION

Asthma is characterized by bronchoconstriction and hyperresponsiveness to various spasmogens. In early attempts to elucidate the pathophysiological mechanisms underlying asthma, attention was initially focussed on the regulation of the airway smooth muscle (ASM) *per se*. It is now recognized that asthma is secondary to airway inflammation, accompanied by influx of inflammatory cells and elevated levels of proinflammatory mediators and cytokines in the airways. Changes in other components of the airway wall participate in reversible airway narrowing. A number of models of asthma have been developed which involve induction of an inflammatory response in the airways. For example, dogs exhibit airway bronchoconstriction, airway inflammation, and hyperresponsiveness to inhaled spasmogens following exposure to the aerosolized allergen *Ascaris suum* (4,10,14,20). The respiratory response was thought to be due to release of bronchoconstrictor inflammatory mediators following cross reactivity of the *Ascaris suum* antigen with a parasitic nematode, *Toxocara canis*, commonly found in dogs. This response is believed to be IgE-mediated, as indicated by positive skin test (*i.e.*, wheal), although IgE levels have not yet been measured directly. Since then, many studies have been carried out using allergen-inhalation to elucidate the mechanisms underlying airway hyperreactivity (5,6,8,15,16,19). Nonetheless, the mechanisms underlying allergen-induced AH are still not completely understood. Many studies indicate the involvement of several arachidonic acid metabolites in the responses to

inhalation of allergen. For example, allergen-induced AH is accompanied by elevation of PGD_2 levels (11) and is antagonized by blockade of TXA_2 receptors (6,11) or activation of PGE_2 receptors (21).

Another model of asthma involves induction of an inflammatory response by exposure to ozone (12). Like asthma and allergen-induced AH, the ozone-induced inflammatory response is associated with AH to spasmogens *in vivo* and, in this case, also *in vitro*; however this model differs from asthma and allergen-induced AH in that it is not IgE-mediated. Ozone-induced AH seems to involve decreased levels of an inhibitory cyclo-oxygenase metabolite(s) (likely PGE_2) and possibly also increased levels of an excitatory cyclo-oxygenase metabolite(s) (possibly TXA_2) (12). There are a variety of *in vitro* data which show that PGE_2 in dogs can affect airway responsiveness either by inhibiting release of acetylcholine from airway nerves or inhibiting the responsiveness of the airway muscle (2,17).

In the present study, we investigated whether allergen-induced AH developed *in vivo* could also be demonstrated in isolated tissues *in vitro*, and examined the roles of various arachidonic acid metabolites in the changes in airway responsiveness. Bronchial airway tissues were used, since these are primarily responsible for determining peripheral resistance to airflow. The data suggest that metabolism of arachidonic acid is markedly altered during allergen-induced inflammation. This manifests primarily as increased generation of PGE_2 (as well as a non-prostanoid inhibitory factor) accompanied by reduced mechanical responses;

however, there is also evidence that production of an excitatory autacoid is simultaneously increased, leading to a contraction which develops and resolves slowly. These data have been presented in abstract form (13).

MATERIALS AND METHODS

In vivo measurements and inhalation of allergen Dogs (15-30 kg; either sex) were anaesthetized using intravenous pentobarbital sodium (30 mg/kg) to induce surgical anaesthesia; this level of anaesthetization was maintained during the course of the *in vivo* study by additional injections as required. An endotracheal tube and an esophageal balloon catheter were inserted. The endotracheal tube was connected to a constant volume ventilator set at a tidal volume of 10 ml/kg and frequency of 30 min^{-1}. The esophageal balloon catheter and a port at the equipment end of the endotracheal tube were connected to a differential pressure transducer (Hewlett-Packard 267B, Waltham, MA) and pressure amplifier (Hewlett-Packard 8805C) to monitor transpulmonary pressure. Measurements of peripheral resistance (R_L) were obtained at constant volume using techniques described previously, and airway responsiveness to aerosolized acetylcholine was assessed 30 minutes after induction of anaesthesia, using methods which have been described previously (10,12,25). Dose-response curves to ACh were constructed by plotting the baseline and peak values of R_L after each concentration of ACh aerosol delivered. From each curve, an ACh-provocative concentration ($PC_{5.0}$, which is the concentration of

ACh that increased R_L by 5.0 $cmH_2O/l/s$ above the baseline value) was calculated by interpolation as previously described (10,12,25).

Dogs were then exposed to allergen (*Ascaris suum* in 0.9% saline) or saline alone. During this one-time challenge, the concentration of allergen was increased from 10^{-5} M in 10-fold increments until R_L was elevated 10 $cmH_2O/l/s$ above baseline, after which the dogs were ventilated with air until R_L returned to baseline. 24 hours later, dogs were anaesthetized as described above and the airway responsiveness to ACh was again assessed, after which they were euthanized with pentobarbital sodium (100 mg/kg). Dogs were defined as being hyperresponsive when there was a decrease in the ACh-$PC_{5.0}$ of 2-fold or more (10,12,25).

Tissue dissection and organ bath studies Following euthanization, pulmonary lobes were excised, pinned out in physiological solution, and the overlying parenchymal tissue and vasculature were dissected away, thereby exposing the bronchiolar tree from which ring segments were excised (5-10 mm wide; 2-10 mm outer diameter), as described previously (12). Ring segments were mounted vertically in 3 ml organ baths using platinum hooks inserted through the lumen (taking care to not damage the epithelium); one of the platinum hooks was fastened to a force displacement transducer, while the other served as an anchor. Throughout the studies, tissues were bathed in Krebs-Ringer's buffer (KRB) bubbled with 95% O_2 / 5% CO_2; in some cases, KRB also included either indomethacin (10^{-5} M) or 3-[1-(4-chlorobenzyl)-5-fluoro-3-methyl-indol-2-yl]2,2-dimethlypropanoic acid

(L-655,240; 10^{-6} M; see ref. 9). Preload tension was maintained at ≈1.25 grams (determined previously to allow maximal responses). Electrical stimulation (60 V; 0.5 ms pulse duration) was supplied via the platinum hook which served as the anchor and another platinum pole not in direct contact with the tissue (the two poles were 2 cm apart). Isometric changes in tension in response to various agonists and conditions were recorded on an ink-writing polygraph.

Experimental protocol Tissues from each dog were studied using one of two protocols. One set of 8 tissues were mounted in the muscle baths containing standard KRB or KRB plus indomethacin (10^{-5} M) or L-655,240 (10^{-6} M). Tissues were then electrically stimulated (20 pulses at 20 Hz) at 20 minute intervals for 140 minutes, each time preceded by a wash and preload adjustment. Immediately after the final stimulation at 140 minutes, tissues were washed with standard KRB or KRB containing indomethacin (10^{-5} M) or L-655,240 (10^{-6} M) and the frequency-response relationship of the tissues was examined: 20 pulses were delivered at 5 minute intervals at frequencies of 0.5-20 pps, without any wash or preload adjustment between stimulations. In some cases, the twitch contraction evoked by EFS (which is known to be mediated by release of acetylcholine [12]) was followed by a slowly-developing secondary contraction which did not always resolve fully before the subsequent response was elicited. Next, the response to KCl (100 mM added hypertonically) was assessed. Finally, the tissues which had been exposed to indomethacin were washed and allowed to equilibrate for ≈60 minutes, after which

cumulative concentration-response curves for carbachol (CCh) were generated in the presence or absence of nifedipine (10^{-7} M; added ≈20 minutes before CCh).

A second set of 8 tissues from each dog was set aside at room temperature in standard KRB (bubbled with 95% O_2 / 5% CO_2) until the conclusion of the experiments described above (≈5 hours after euthanization). Tissues were mounted in the organ baths, washed with standard KRB or KRB containing IDM (10^{-5} M) at 37°C, then allowed to equilibrate for ≈45 minutes before obtaining a contractile response to KCl (60 mM). Next, the bath fluid surrounding the tissues was collected before and after three series of EFS stimulations for quantitation of PGE_2 released by the tissues (see below). Each series of EFS involved trains of pulses (10 second train duration) with frequencies ranging from 0.1 to 30 pps, without any wash or preload adjustment between stimulations; some tissues were not electrically stimulated during this 40 minute period in order to serve as time controls. Finally, after the second collection of bath fluid for radioimmunoassay, cumulative concentration-response curves for CCh were generated.

In all cases, tissues were saved and dried for standardization of contractile responses.

Radioimmunoassay Samples of organ bath fluid were collected immediately after the tissues were washed of KCl as well as following the third series of EFS trains. Samples were collected using plastic syringes, then immediately frozen and stored at -70°C. On the day of RIA, samples were thawed on the bench top, then

immediately analyzed for PGE_2 or TXB_2 content using a commercially prepared kit from Advanced Magnetics (Cambridge, Mass). The RIA is based upon competition of the prostaglandin with radioactively labelled prostaglandin for a number of sites on the specific antibody. Antibody-bound prostaglandin was separated from unbound with magnetic dextran-coated charcoal through magnetic separation. The counting rate was correlated with concentration via a standard curve. The sensitivity of this assay is 2.5 pg/0.1 ml.

Solutions and chemicals Experiments were conducted using standard KRB containing (in mM): NaCl, 116; KCl, 4.2; $CaCl_2$, 2.5; NaH_2PO_4, 1.6; $MgSO_4$, 1.2; $NaHCO_3$, 22; D-glucose, 11. Chemicals were obtained from Sigma Chemical Company. All agents were prepared as neutral aqueous solutions, with the exceptions of indomethacin and L-655,240 (both in 22 mM HCO_3^-) and nifedipine (absolute EtOH).

Statistical analysis The concentration of CCh required to evoke a half-maximal response (EC_{50}) was derived from the concentration-response curve of each tissue. Contractile responses were standardized using tissue dry weight. Data are reported as mean ± SEM. Statistical comparisons were made using ANOVA with corrections for multiple comparisons using the Bonferroni technique. The sources of significant differences were determined using a Student's t-test, with P values <0.05 being considered significant.

RESULTS

In vivo measure of airway responsiveness

Inhalation of allergen (*Ascaris suum*) caused an immediate bronchoconstrictor response in all 20 dogs assayed, while inhalation of vehicle did not. When assayed 24 hours later, 10 of the allergen-exposed animals were found to be hyperresponsive to ACh (*i.e.*, exhibited more than a 2-fold decrease in ACh $PC_{5.0}$; RESP), while the other 10 did not (NON-RESP). The mean magnitudes of the change in $PC_{5.0}$ (*i.e.*, PRE/POST) were 0.84±0.17 in SHAM dogs, 1.29±0.17 in NON-RESP dogs, and 3.56±0.38 in RESP dogs. Figure 1 indicates the range of changes in sensitivity to ACh in these animals. None of the 8 dogs which were exposed to vehicle ("SHAM") exhibited AH to inhaled ACh.

In vitro responses to exogenously-added KCl or cholinergic agonist

To determine if hyperresponsiveness measured *in vivo* could also be detected *in vitro*, we examined the contractile responses to KCl (60 mM) or the cholinergic agonist carbachol (CCh). While the former acts exclusively through electromechanical coupling mechanisms, the latter acts through both electromechanical and pharmacomechanical coupling mechanisms.

In the absence of IDM, there were no significant differences between the 3 groups with respect to the contractile response to KCl (Fig. 2), the maximal response to CCh (*i.e.*, 10^{-4} M) or the CCh EC_{50} (Table 1). IDM had no effect on the KCl-

responses in any of the 3 groups (Fig. 2), but displaced the CCh concentration-response curves ≈½ log unit to the left (Table 1); there were no significant differences between the maximal responses nor the EC_{50} values for CCh in SHAM, NON-RESP, and RESP tissues exposed to IDM (Table 1). Preincubation of the tissues with nifedipine (10^{-7} M; in the maintained presence of IDM) to eliminate the electromechanical component of the CCh-contraction caused the concentration-response curves to be shifted slightly to the right (Table 1) and caused a small but statistically insignificant reduction in the maximal response to CCh; the CCh-responses (EC_{50} values and maxima) in the presence of nifedipine were not significantly different between the 3 groups.

In vitro responses to nerve-released cholinergic agonist

The frequency-response characteristics of the tissues were examined using trains of pulses with frequencies ranging from 0.1 to 30 pps delivered at 5 minute intervals (Fig. 3); tissues were electrically stimulated over prolonged periods without replacement of bath fluid between stimulations. In tissues stimulated in this way in the absence of IDM, there were no statistically significant differences between SHAM and NON-RESP while those in RESP tissues were significantly reduced (Fig. 3A). IDM potentiated the responses in all 3 groups, particularly at low EFS frequencies, consistent with its prevention of the inhibitory effect of one or more cyclo-oxygenase metabolites on neurotransmission in this tissue (2). Nonetheless, the responses in

RESP tissues exposed to IDM were still significantly smaller than those in the other 2 groups (Fig. 3B), suggesting that an inhibitory factor unrelated to cyclo-oxygenase metabolism is generated in RESP tissues.

We obtained somewhat different results when the tissues were washed immediately before electrical stimulation to wash out any accumulated autacoids. In this case, we used trains of pulses (20 pulses at 20 Hz) delivered at 20 minute intervals in standard KRB or KRB containing IDM (10^{-5} M) or L-655,240 (10^{-6} M; TXA_2/PGD_2 receptor antagonist), and the bathing fluid was replaced ≈5 minutes before each response. In the absence of pharmacological agents, EFS-responses within each group increased during the first 60 minutes, then decreased over the next 80 minutes (Fig. 4A). There was no significant difference in the responses at each time point between the 3 groups. In the presence of IDM (10^{-5} M), the EFS-responses at 20 minutes were not significantly different from those obtained in its absence (compare points at t=20 minutes in Fig. 4A and 4B), but all subsequent responses within each group showed a progressive increase in magnitude (Fig. 4B). Again, there were no significant differences between the groups at each time point in the presence of IDM. In tissues pretreated with L-655,240, the EFS-responses obtained at 20 minutes in each group were not significantly different from each other (Fig. 4C) nor from those obtained in the same group in the absence of L-655,240 (compare Fig. 4A and 4C). In SHAM and NON-RESP tissues, the magnitudes of responses to successive electrical stimulations remained relatively constant (as

opposed to the progressive changes in control and IDM-treated tissues described above), while RESP tissues showed a progressive and marked decrease, such that the response at 140 minutes was almost negligible (Fig. 4C).

In some cases, primarily among tissues from responders, the EFS-evoked twitch contraction was followed by a smaller contraction which developed relatively slowly, reaching a peak approximately 1-2 minutes after EFS, then relaxing partially during the period between stimulations (Fig. 5A). These were more prevalent and notably larger in RESP than in NON-RESP or SHAM tissues (Fig. 5B). Indomethacin (10^{-5} M) unmasked small secondary contractions in some SHAM and NON-RESP tissues, and markedly enhanced the prevalence, magnitude, and duration of such secondary contractions in RESP tissues (Fig. 5B). L-655,240 (10^{-6} M) did not significantly alter the initial twitch contractions, but eliminated the secondary contractions in all three groups (Fig. 5B).

Radioimmunoassay for PGE_2 and TXB_2

Levels of PGE_2 in the bathing medium before EFS were less than the detection limit (*i.e.*, 2.5 pg/0.1ml) in SHAM and NON-RESP groups, but were markedly elevated to ≈1 ng/ml in RESP (Fig. 6A). The concentration of PGE_2 in the latter case (*i.e.*, $\approx 10^{-9}$ M) has previously been shown to be sufficient to mediate prejunctional inhibition of cholinergic neurotransmission (2); the concentration of PGE_2 within the tissue is likely to be even higher. PGE_2 levels were increased by

2-3 ng/ml in all 3 groups over the subsequent 90 minutes (Fig. 6B; right panel). This accumulation was not significantly increased in tissues which were electrically stimulated (Fig. 6B; left panel compared to right panel). PGE_2 accumulation in the electrically-stimulated tissues as well as the time-controls was greatly reduced, if not abolished, by IDM (data not shown). Levels of TXB_2 never exceeded the detection limit of the assay.

DISCUSSION

Allergen-induced AH has been used in many studies as a model to elucidate the mechanisms underlying asthma. However, these mechanisms remain poorly understood, in part because the majority of the studies were carried out using *in vivo* preparations in which the various effects of allergens on the nervous, immune, and vascular systems in the airways confound interpretation of the data. In the present study, we used isolated airway tissues, in which these effects are absent or controlled. We also used CCh as a cholinergic agonist rather than ACh, as was done in earlier studies (11), since ACh is susceptible to degradation by cholinesterases, and it is possible that cholinesterase activity is altered during AH: for example, in a previous study of a canine model of asthma (11), ACh had no effect over the concentration range 10^{-7}-10^{-6} M, while these concentrations are effective in non-hyperresponsive dogs. CCh, on the other hand, is not susceptible to cholinesterase activity. In contrast to our expectations, we found that bronchial

tissues isolated from hyperresponsive dogs demonstrated no change in responses to exogenously-added spasmogens and reduced responses to electrical stimulation (this reduction was reversed partially by indomethacin or by replacement of the bathing medium to remove any inhibitory factors in the bathing medium). The data are consistent with allergen-exposure having enhanced generation of inhibitory arachidonic acid metabolites from cyclo-oxygenase, as well as of a possible non-prostanoid inhibitory factor. Therefore, these data suggest that the responsiveness of the airway tissues *in vitro* is determined by the relative levels of inhibitory and excitatory factors elicited by exposure to allergen, as discussed below.

Changes in myogenic mechanisms? Contraction of ASM is a Ca^{2+}-dependent event, primarily involving activation of myosin light chain kinase in response to an elevation of cytosolic $[Ca^{2+}]$. The latter is a result of agonist-induced release of internally sequestered Ca^{2+} and/or depolarization-induced opening of voltage-dependent Ca^{2+} channels. A number of Ca^{2+}-homeostatic mechanisms contribute to restoring $[Ca^{2+}]_i$ to basal levels and thereby mediate recovery from excitation, including uptake into the internal store and extrusion from the cell. It has been suggested that AH may involve changes in the function of the ASM *per se*, such as changes in Ca^{2+} handling or increased sensitivity to Ca^{2+} (14,15,24). We did not obtain any evidence for such changes under the present experimental conditions. For example, there seems to be no change in electromechanical coupling mechanisms (*i.e.*, voltage-dependent Ca^{2+}-influx), since we found no difference

between SHAM and RESP tissues with respect to the sensitivities or magnitudes of responses to CCh in the absence of nifedipine (Table 1), nor to KCl (Fig. 2). The lack of a significant difference between the CCh responses in the presence or absence of nifedipine, as well as the KCl responses, also suggests that there is no change in the sensitivity of the contractile apparatus to Ca^{2+} following exposure to allergen. Likewise, there seems to be no change in agonist-induced release of internally sequestered Ca^{2+}, since there was no change in the maximal contractile response nor in the EC_{50} for CCh (Table 1). Measurements of ionic currents in single cells also did not reveal any difference between SHAM and RESP tissues with respect to membrane currents at rest nor during cholinergic stimulation (Janssen, unpublished communication), consistent with our claim that basal $[Ca^{2+}]_i$ is not elevated in these tissues and agonist-induced release of internal Ca^{2+} is not altered.

Airway inflammation may involve a change in airway tissue elastance (via lymphocyte-mediated destruction of the matrix), which would result in a change in the optimal preload for these tissues. If this occurred in the present experiments, our use of the same preload tension (≈1.25 g) for all tissues could account for the observed decrease in responses in allergen-exposed tissues. However, there was no difference in the magnitude of responses between the three groups when tissues were washed before stimulation (to remove any inhibitory substances; Figure 4A), which contraindicates an allergen-induced change in the optimal preload. It is worth re-iterating that when this type of experiment is repeated without a wash before each

stimulation, there is a marked difference between the three groups (Fig. 3A).

Changes in neurogenic mechanisms? In canine ASM the excitatory innervation is almost exclusively cholinergic in nature. We have demonstrated above that the myogenic response to cholinergic stimulation is unaltered. However, it is possible that allergen-induced airway inflammation may result in changes in the release of ACh from the nerve endings, leading to exaggerated neurogenic excitation. For example, Elbon *et al.* (8) found that allergen-induced AH in guinea-pigs involves release of eosinophil-derived mediators which down-regulate prejunctional inhibitory muscarinic receptors, leading to increased cholinergic neurotransmission. Similarly, Mitchel *et al.* (19) have shown that basal and histamine-induced release of ACh is increased in ragweed-sensitized dogs, suggesting immune sensitization facilitates the release of the neurotransmitter from postganglionic parasympathetic nerves. In a different canine model of AH — that induced by inhalation of ozone (12) — we have found that AH seems to involve decreased prejunctional inhibition (likely due to decreased generation of PGE_2) and possibly also increased prejunctional excitatory input (perhaps due to increased generation of TXA_2).

Surprisingly, we found that tissues from dogs which had demonstrated AH *in vivo* after allergen exposure were not more excitable to EFS than control tissues. In fact, there was a significant decrease in the neurogenic responses in RESP tissues compared to SHAM and NON-RESP tissues. This reduction of the neurogenic responses was reversed partially by indomethacin (Fig. 3) and

completely by replacement of the bathing medium to remove any inhibitory factors (Fig. 4). Thus, allergen-induced inflammation may be accompanied by generation of inhibitory factors for mediator release. Radioimmunoassay and the effect of indomethacin on mechanical responses indicate that one of these factors is PGE_2 (discussed in more detail below); however, the inability of indomethacin to completely reverse the hyporesponsiveness suggests a non-prostanoid inhibitory factor is also produced. Studies of epithelium-dependent inhibition of canine *trachealis* are also consistent with involvement of both PGE_2 and a non-prostanoid factor (McGrogan and Daniel, unpublished communication).

Changes in cyclo-oxygenase metabolites? Our observations and those of others suggest that allergen-induced airway inflammation is accompanied by marked changes in the metabolism of arachidonic acid.

First, we found basal levels of PGE_2 were markedly increased in RESP tissues (Fig. 6A). The overall bath concentration of PGE_2 reached $\approx 10^{-9}$ M (the effective concentration within the tissue is likely to be higher); this concentration of PGE_2 has been shown to mediate marked inhibition of neurogenic responses, but not those to exogenously-added ACh, in canine bronchial smooth muscle (2). Thus, PGE_2 accumulation could explain the sensitivity of responses in allergen-exposed animals to neurally-released cholinergic agonist, but not those to cholinergic agonist added exogenously. The accumulation of $[PGE_2]$ was sensitive to the cyclo-oxygenase antagonist indomethacin. Similarly, Itabashi *et al.* (11) have shown that inhalation

of allergen by dogs is accompanied by significant accumulation of another prostanoid (PGD_2), although this was not prevented by indomethacin.

Second, we found that the responses to EFS in SHAM and NON-RESP tissues were not significantly altered by L-655,240, while those in RESP tissues were markedly reduced in the presence of this TX/PG receptor antagonist (Fig. 4). Itabashi *et al.* (11) also found that blockade of thromboxane receptors (using an antagonist distinct from the one used in our study) had no effect on the cholinergic responses in control and non-responder groups, but markedly antagonized those in the hyperresponder group. Similarly, Chung *et al.* (6) have shown that the late phase of allergen-induced AH in dogs is antagonized by yet another TXA_2 receptor antagonist.

Third, the slowly-developing secondary contractions which followed the initial EFS-evoked twitch contractions were larger and much more prevalent in RESP tissues than in SHAM or NON-RESP tissues, and these were enhanced in all three groups by indomethacin but eliminated by L-655,240 (Fig. 5). Similar secondary contractions have been shown to be induced by leukotrienes and to be sensitive to inhibition by PGE_2, although the mechanism involved is as yet unclear (1).

These observations are consistent with allergen-exposure initiating changes in the generation of both excitatory and inhibitory arachidonic acid metabolites via cyclo-oxygenase. As a result, the effect of allergen-induced inflammation on airway responsiveness depends on the relative levels of these metabolites as well as the

sensitivity of the tissue to them (*e.g.* density of receptors for the metabolites within the particular tissue). For example, allergen causes airway responsiveness to be increased in the small airways (5,11), decreased in the trachealis (5,17), and unchanged in the larger bronchi (this study; 11), perhaps reflecting the relative levels of arachidonic acid metabolites and their receptors throughout the airways.

Distinct mechanisms underlying different models of airway inflammation

The epithelium is the major source of PGE_2 and of a non-prostanoid inhibitory factor (17,18,23). Ozone-induced inflammation in canine ASM is accompanied by destruction of the epithelium and increased responsiveness of isolated bronchial tissues, the latter of which seems to be secondary to decreased generation of an inhibitory cyclo-oxygenase metabolite, likely PGE_2 (12). In the present study, tissues excised from dogs exhibiting allergen-induced hyperresponsiveness demonstrated reduced *in vitro* mechanical responses (Fig. 3) accompanied by increased levels of PGE_2 (Fig. 6); electronmicroscopic examination of the tissues showed no appreciable damage of the epithelium in tissues from hyperresponsive dogs compared to their control counterparts (Daniel, unpublished communication). Thus, the mechanisms underlying ozone-induced and allergen-induced AH seem to differ markedly, and this could account for the contrasting observations (*i.e.*, hyperresponsiveness *versus* hyporesponsiveness) made in excised tissues from these two models. Decreased airway smooth muscle responsiveness is also seen in the mouse following Toxocara-induced eosinophilic inflammation (5) as well in the

Basenji-Greyhound model of asthma (even though the tissues were excised from dogs which demonstrated hyperresponsiveness *in vivo*) (7). In the rat, induction of an airway inflammatory response causes increased generation/release of nitric oxide from the airway epithelium (22) and hyporesponsiveness of the smooth muscle which is secondary to the effects of nitric oxide on the smooth muscle (16). In the present study, L-NNA (a blocker of nitric oxide synthesis) had no effect on the magnitudes of contractions evoked by EFS (Janssen, unpublished communication).

The mechanisms underlying other models of asthma may also differ from those described above. For example, in canine airway tissues which have been sensitized to allergen but which are not actually inflamed at the time of study, there are changes in shortening velocity which were interpreted to reflect increased myosin light chain kinase levels within the smooth muscle, although maximum force generation is not increased (14). Moreover, studies of the changes in airway function following acute exposures to allergen or ozone do not take into account the structural changes which accompany repeated/prolonged exposures (which are also seen in the airways of asthmatics). Thus, the relationship between these various animal models of asthma and the clinical (*i.e.*, human) condition is equivocal. Studies in humans vary with respect to whether or not smooth muscle force generation is increased; we are not aware of any reports of decreased responsiveness of human airway smooth muscle following inflammation nor in asthma.

Conclusion We conclude that allergen-induced changes in airway responsiveness studied *in vitro* reveal altered metabolism of arachidonic acid by cyclo-oxygenase, leading to increased generation of the inhibitory metabolite PGE_2 and possibly other excitatory prostanoids. In addition, a non-prostanoid inhibitory factor may also be produced. As such, the responsiveness of the tissue is dependent on the relative levels of inhibitory and excitatory autacoids.

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Figure 1. Changes in ACh-$PC_{5.0}$ values in sham and allergen-exposed animals Airway responsiveness to ACh (reflected in $PC_{5.0}$ value; see Methods) was measured immediately before (PRE) and 24 hours after (POST) exposure to diluent (n=8) or allergen (n=20). Airway hyperresponsiveness, indicated by a decrease in the $PC_{5.0}$ of 2-fold or more, was noted only in 10 allergen-exposed dogs (dotted lines).

Figure 2. Contractile responses to KCl Tissues were challenged with KCl (60 mM, added hypertonically) in the presence or absence of indomethacin. There were no significant changes in the response to KCl in RESP tissues (solid bars) compared to SHAM (open bars) or NON-RESP tissues (shaded bars). Furthermore, indomethacin did not significantly alter the response to KCl, nor unmask any significant differences between the 3 groups. n = 4-5 in each case.

Figure 3. Frequency-response characteristics of contractions evoked by EFS Twitch contractions evoked by electrical stimulation (0.5 ms pulse duration; maximal voltage) at various pulse frequencies in SHAM, NON-RESP, and RESP tissues (open, shaded, and filled bars, respectively); responses were evoked in the absence and presence of indomethacin (**A** and **B**, respectively). In this experiment, bath fluid was not replaced at the beginning, nor in between, each series of electrical stimulations.

Figure 4. EFS-evoked responses following wash-out of accumulated endogenous autacoids Tissues were mounted in organ baths in the absence (**A**) or presence of indomethacin (10^{-5} M; **B**) or L-655,240 (10^{-6} M; **C**). Contractions were evoked by electrical stimulation (20 pulses at 20 Hz; 0.5 ms pulse duration; maximal voltage) delivered at 20 minute intervals (each time preceded by a wash) for a total of 140 minutes. There were no significant differences between SHAM (——○——), NON-RESP (----▽----), or RESP (----●----) groups in the response at any given time point in standard or indomethacin-containing KRB. L-655,240, however, reduced the responses in RESP tissues, this effect becoming significant only after 80 minutes.

Figure 5. EFS evokes a twitch contraction and a slowly developing secondary contraction Twitch contractions and secondary contractions evoked by electrical stimulation; in this experiment, tissues were washed before evoking the mechanical responses. **A** Original tracing showing twitch contraction evoked by EFS (20 pulses at 0.5 Hz [■] or 20 Hz [○]) in SHAM (left) and RESP (right) tissues: in the latter case, the twitch contraction was followed by a secondary contraction which developed slowly and relaxed slowly. Dotted lines indicate zero tone. **B** Mean magnitudes of contractile responses evoked by EFS (20 pulses at 20 Hz) in SHAM (open bars), NON-RESP (shaded bars), and RESP (filled bars) tissues in the absence or presence of indomethacin or L-655,240, as indicated. The twitch contraction is indicated by the first bar in each pair of bars; the secondary

contraction, when present, is indicated by the second bar in each pair of bars.

Figure 6. Radioimmunoassay for PGE_2 PGE_2 levels in bath fluid prior to electrical stimulation ("BASAL") were not significantly different between SHAM and NON-RESP tissues (open and shaded bars, respectively), but were markedly elevated in fluid from RESP tissues (filled bars). Next, the tissues were washed and the accumulation of PGE_2 over the course of 90 minutes was quantified. During this 90 minute period, half the tissues in each group were electrically stimulated ("FS"; see Methods), while the other half served as time controls: electrical stimulation did not increase the accumulation of PGE_2.

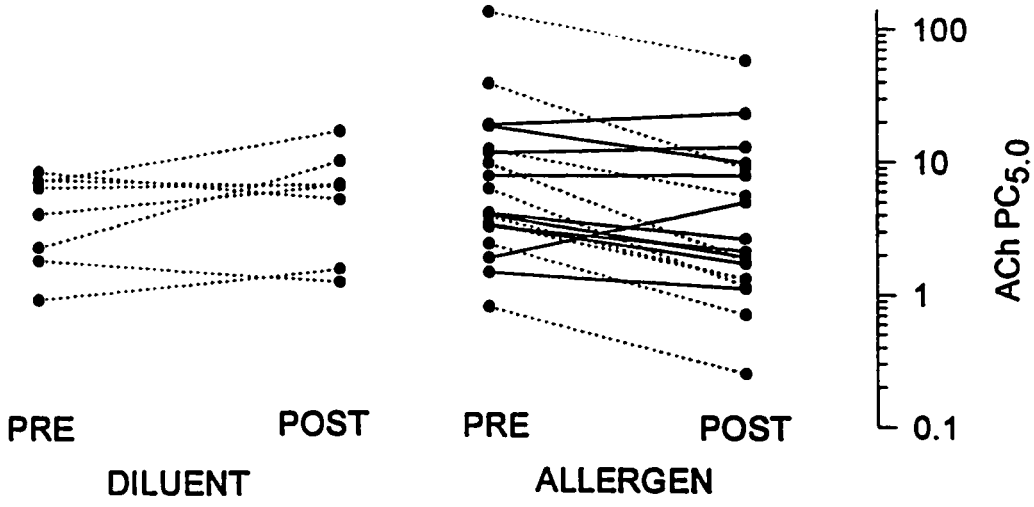
FOOTNOTES

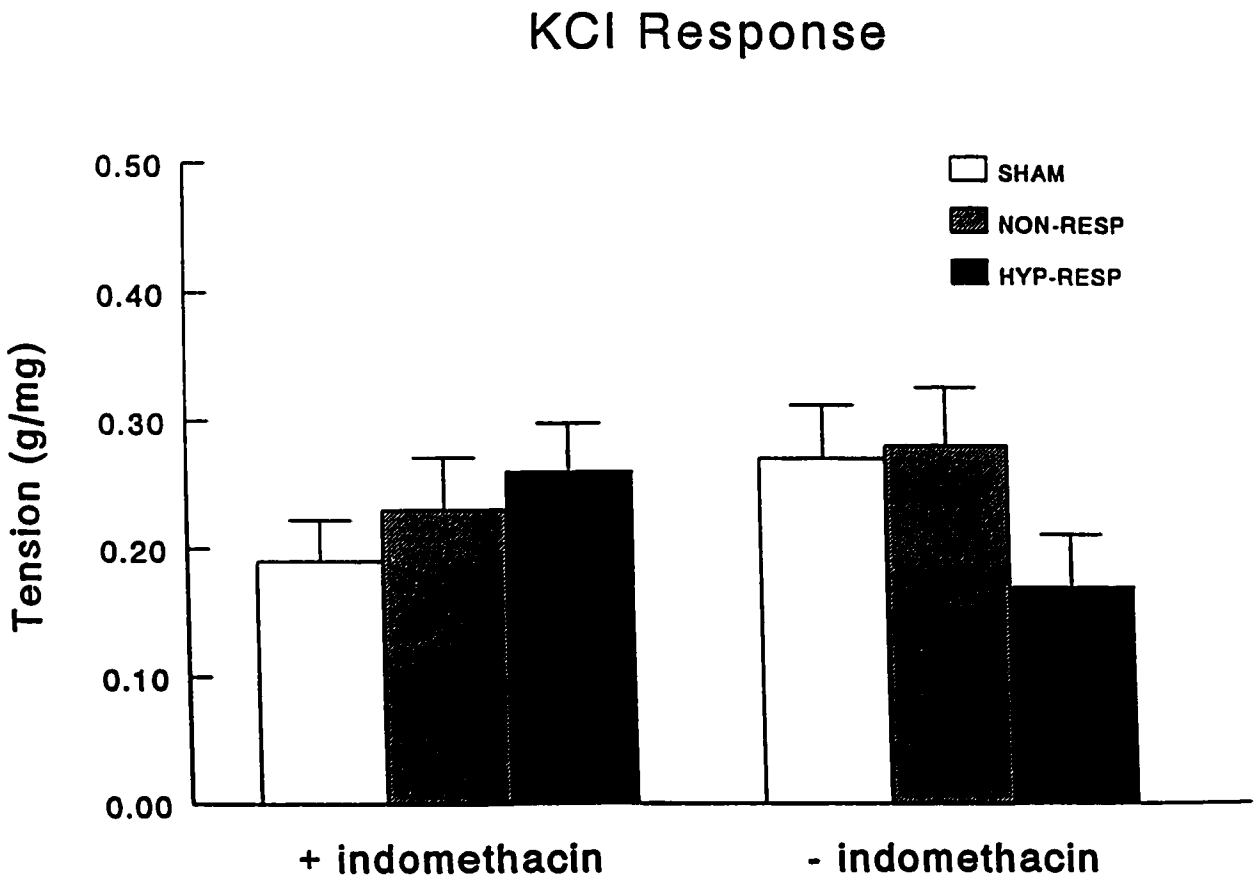
Abbreviations used:

ACh	- acetylcholine
AH	- airway hyperresponsiveness
ASM	- airway smooth muscle
CCh	- carbachol
EC_{50}	- concentration eliciting 50% of maximal excitatory response
EFS	- electrical field stimulation
IDM	- indomethacin
KRB	- Krebs-Ringers buffer
L-655,240	- thromboxane/prostaglandin endoperoxide receptor antagonist
NON-RESP	- airway tissues exposed to allergen but which did not become hyperresponsive
$PC_{5.0}$	- concentration of ACh that increased R_L by 5.0 $cmH_2O/l/s$ above baseline
RESP	- airway tissues exposed to allergen and which became hyperresponsive
RIA	- radioimmunoassay
R_L	- total lung resistance
SHAM	- airway tissues exposed to vehicle

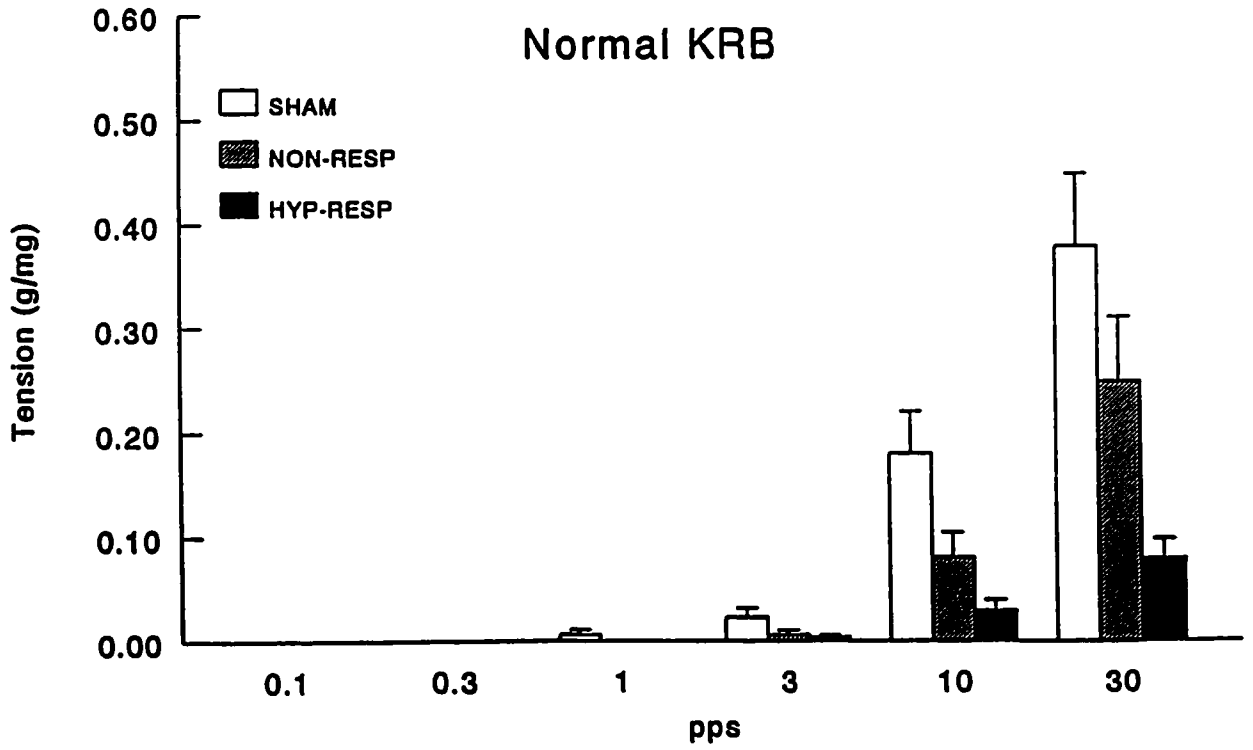
Table 1 Mean values (±S.E.) for the carbachol EC_{50} (log[M]) in the presence or absence of IDM (10^{-5} M) and/or nifedipine (10^{-7} M).

		EC_{50} log(M)
SHAM	-IDM	5.8±0.6
	+IDM	6.4±0.7
	+IDM + NIFED	6.1±0.2
NON-RESP	-IDM	5.6±0.6
	+IDM	6.1±0.7
	+IDM +NIFED	5.9±0.2
RESP	-IDM	5.8±0.7
	+IDM	6.2±0.7
	+IDM +NIFED	6.0±0.2

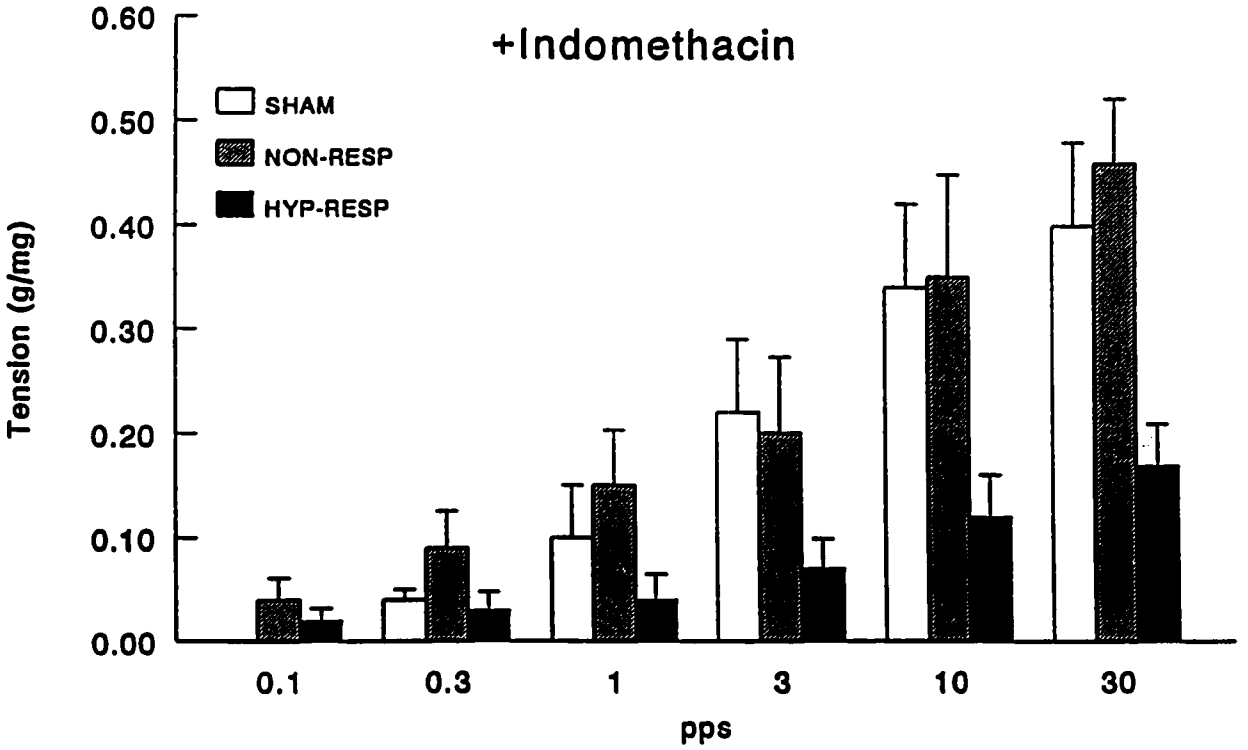

100
10
1
0.1
ACh PC5.0
PRE
POST
PRE
POST
DILUENT
ALLERGEN

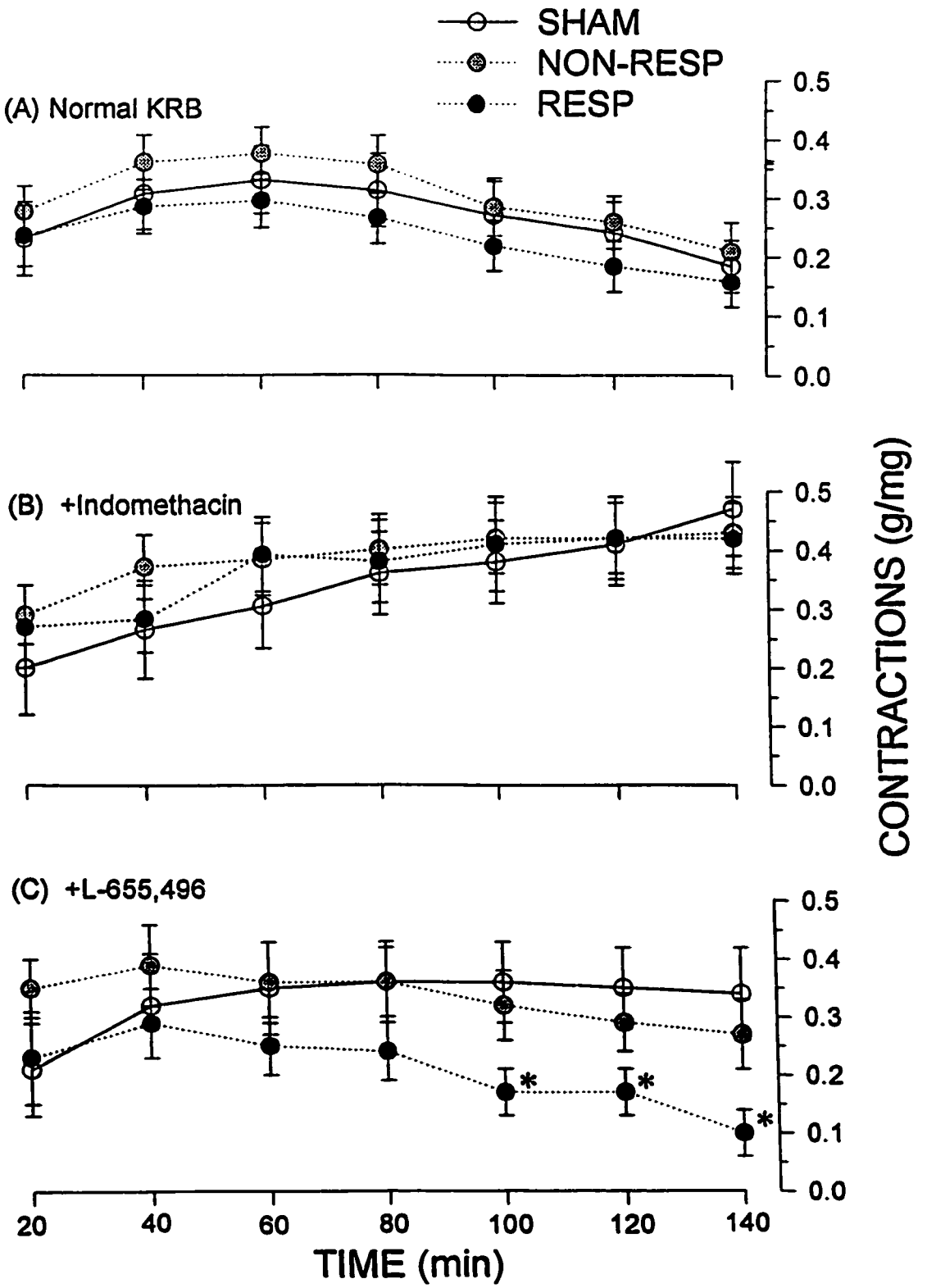

KCl Response
SHAM
NON-RESP
HYP-RESP
Tension (g/mg)
0.50
0.40
0.30
0.20
0.10
0.00
+ indomethacin
- indomethacin

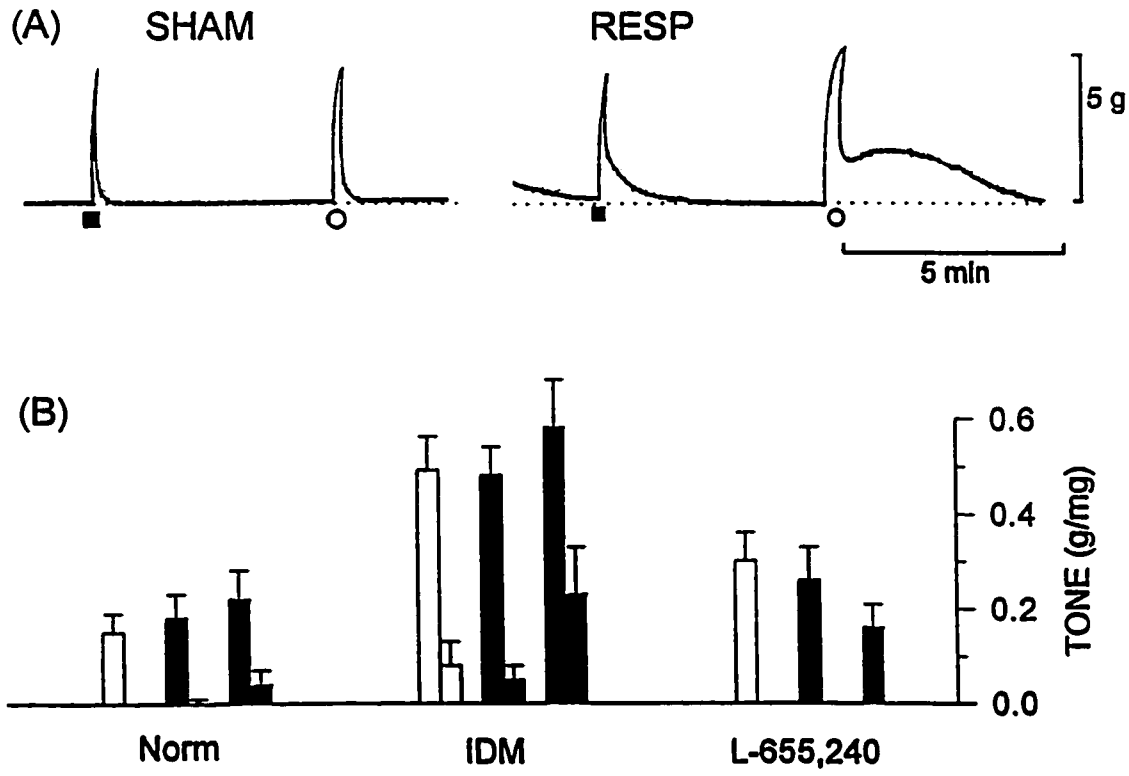
(A)

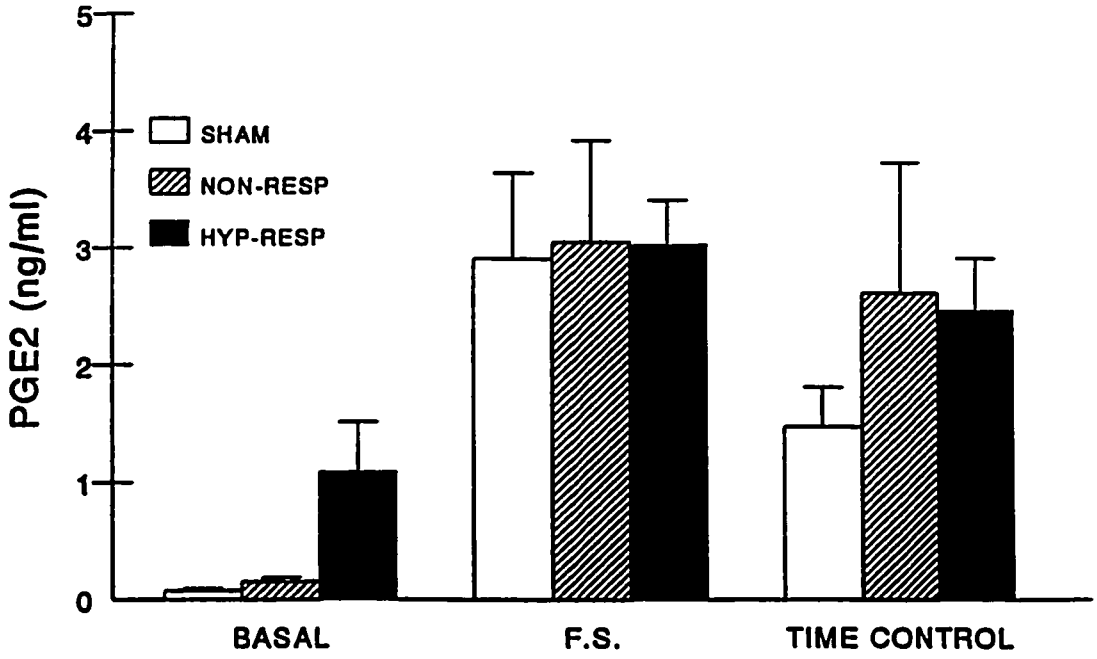

Normal KRB
SHAM
NON-RESP
HYP-RESP
Tension (g/mg)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.1
0.3
1
3
10
30
pps

(B)


+Indomethacin
SHAM
NON-RESP
HYP-RESP
Tension (g/mg)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.1
0.3
1
3
10
30
pps


SHAM
NON-RESP
RESP
(A) Normal KRB
(B) +Indomethacin
(C) +L-655,496
0.5
0.4
0.3
0.2
0.1
0.0
CONTRACTIONS (g/mg)
*
20
40
60
80
100
120
140
TIME (min)


(A)
SHAM
RESP
5 g
5 min
(B)
0.6
0.4
0.2
0.0
TONE (g/mg)
Norm
IDM
L-655,240


PGE2 (ng/ml)
5
4
3
2
1
0
SHAM
NON-RESP
HYP-RESP
BASAL
F.S.
TIME CONTROL

CHAPTER VI

MANUSCRIPT # 4

MANUSCRIPT No. 4

MECHANISMS OF CYCLIC NUCLEOTIDE-INDUCED RELAXATION IN CANINE TRACHEAL SMOOTH MUSCLE

Published in the *American Journal of Physiology* in 1995

Ita McGrogan's contributions:

(i) coordination and supervision of experimental protocols

(ii) preparation of first draft of manuscript

Mechanisms of cyclic nucleotide-induced relaxation in canine tracheal smooth muscle

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McGrogan, I., S. Lu, S. Hipworth, L. Sormaz, R. Eng, D. Preocanin, and E. E. Daniel. Mechanisms of cyclic nucleotide-induced relaxation in canine tracheal smooth muscle. *Am. J. Physiol.* 268 (*Lung Cell. Mol. Physiol.* 12): L407–L413, 1995.—The effects of exogenous cyclopiazonic acid (CPA, 10 μM), a selective inhibitor of the sarcoplasmic reticulum (SR) Ca^{2+} adenosinetriphosphatase, on cyclic nucleotide-induced relaxations of canine airway smooth muscle were examined. Strips of tracheal muscle were precontracted with carbachol (50% median effective concentration, 0.1 μM) or with 60 mM KCl. The β-agonist isoproterenol (ISO, 10 μM) relaxed the tissue by ~50%. The relaxation was reduced in the presence of CPA when L-type Ca^{2+} channels were available but not when these were blocked by 0.1 μM nifedipine. Forskolin (1.0 μM), an adenylate cyclase activator, was less effective at inhibiting the contraction than ISO, and addition of CPA did not block its inhibitory effect as effectively as when ISO was used. Radioimmunoassay indicated that both these agents raised adenosine 3',5'-cyclic monophosphate (cAMP) levels to the same degree. Very little relaxation of the precontracted smooth muscle was elicited by 3 mM 8-bromo-adenosine 3',5'-cyclic monophosphate (8-BrcAMP), and addition of CPA had no effect. Sodium nitroprusside (100 μM) and 8-bromo-guanosine 3',5'-cyclic monophosphate (10 mM) inhibited contraction to a greater degree than any agent that raised cAMP. These inhibitions were greatly reduced in the presence of CPA when L-type Ca^{2+} channels were available. We conclude that pumping of Ca^{2+} into SR plays a major role in guanosine 3',5'-cyclic monophosphate-produced but not cAMP-induced relaxation; L-type Ca^{2+} channels must be available for the relaxant role of Ca^{2+} pumping into the SR to be expressed; and ISO-induced relaxation may not involve primarily elevation of cAMP.

canine trachea; cyclopiazonic acid; isoproterenol; forskolin; adenosine 3',5'-cyclic monophosphate; sodium nitroprusside; guanosine 3',5'-cyclic monophosphate; sarcoplasmic reticulum; calcium; adenosinetriphosphatase

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ELEVATION OF INTRACELLULAR cAMP levels and guanosine 3',5'-cyclic monophosphate (cGMP) levels is followed by relaxation in airway smooth muscle. One mechanism that has been proposed to explain how these cyclic nucleotides elicit smooth muscle relaxation is lowering of the internal Ca^{2+} concentration ($[Ca^{2+}]_i$) of the smooth muscle cells.

McDaniel et al. (23) measured forskolin (FOR)-induced changes in aequorin-estimated $[Ca^{2+}]_i$, cAMP concentration ([cAMP]), myosin phosphorylation, and stress in swine arterial smooth muscle precontracted with phenylephrine or histamine. Forskolin increased [cAMP] and reduced $[Ca^{2+}]_i$, myosin phosphorylation, and stress. Relaxation was not associated with an alteration in the $[Ca^{2+}]_i$ sensitivity of phosphorylation nor with the dependence of stress on phosphorylation. In 1991, Ohta et al. (27) reported that the rise in $[Ca^{2+}]_i$ evoked by carbachol (Cch) was reduced by dibutyryl cAMP (DBcAMP).

Similarly, Cornwell and Lincoln (9) used the Ca^{2+}-dependent conversion of phosphorylase *b* to *a* to measure changes in $[Ca^{2+}]_i$. They demonstrated that atrial naturietic peptide II, which raises cGMP and relaxes vascular smooth muscle in vitro, affects mainly the removal of Ca^{2+} from the cytoplasm. The ability of cGMP to decrease $[Ca^{2+}]_i$ was reported by Chen and Rembold (8), who demonstrated that nitroglycerin attenuated histamine-induced increases in Mn^{2+} influx, $[Ca^{2+}]_i$, and force in swine arterial smooth muscle.

Based on the evidence presented above, it is likely that both cAMP and cGMP effect smooth muscle relaxation, at least in part, by decreasing $[Ca^{2+}]_i$. An important mechanism by which $[Ca^{2+}]_i$ can be lowered is by Ca^{2+} removal. There are three transport systems in smooth muscle cells that are capable of removing cytoplasmic Ca^{2+}. These are *1*) the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase, *2*) the plasma membrane Ca^{2+}-ATPase, and *3*) the Na^+/Ca^{2+} exchange (25).

Smith and Smith (34) reported that the Na^+/Ca^{2+} exchange protein contributes to the rapid decline in $[Ca^{2+}]_i$ that occurs after agonist-activated Ca^{2+} signaling. However, the affinity of the Na^+/Ca^{2+} exchange protein for Ca^{2+} is lower than that of the plasma membrane Ca^{2+}-ATPase; thus it is unable to return Ca^{2+} levels back to resting values by itself (19). Furukawa et al. (13) observed that 8-bromo-adenosine 3',5'-cyclic monophosphate (8-BrcAMP) caused a stimulation of the plasma membrane Ca^{2+}-ATPase. FOR, however, was ineffective. Scheid and Fay (29) found that in toad stomach smooth muscle, ISO and DBcAMP stimulate Ca^{2+} efflux. They proposed that this was due to cAMP-protein kinase (PK) activation of Na^+/Ca^{2+} exchange.

Finally, $[Ca^{2+}]_i$ may be lowered by the activation of the SR Ca^{2+}-ATPase, which enhances the sequestration of Ca^{2+} into the internal stores of the smooth muscle cell. Studies in cardiac muscle (35) suggested that phosphorylation by cAMP-PK of phospholamban in SR vesicles resulted in increased SR Ca^{2+}-ATPase activity and, consequently, increased Ca^{2+} uptake. Moreover, Raeymaekers et al. (28) and Huggins et al. (17) report that cardiac and smooth muscle phospholamban are substrates for both cAMP-PK and cGMP-PK.

Cyclopiazonic acid (CPA) is a fungal toxin that is a selective inhibitor of the SR Ca^{2+}-ATPase pump. Seidler et al. (31) demonstrated that CPA inhibited the Ca^{2+}-stimulated ATP hydrolysis in intact SR and in a purified

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preparation of Ca^{2+}-ATPase. It did not, however, inhibit kidney and brain Na^+-K^+-ATPase, gastric H^+-K^+-ATPase, mitochondrial F1-ATPase, erythrocyte Ca^{2+}-ATPase, nor the Mg^{2+}-activated ATPase of T-tubules and surface membranes of rat skeletal muscle. Deng and Kwan (12) showed that CPA was also effective as a SR Ca^{2+} pump inhibitor in vascular smooth muscle cells. In our laboratory, Darby et al. (11, and personal communication) demonstrated that CPA selectively inhibited oxalate-stimulated Ca^{2+} transport, an exclusive property of SR-derived microsomes (14), in rat vas deferens and dog mesenteric artery. A variety of other studies in smooth muscle, including airway (3, 4), show that CPA prevents refilling of Ca^{2+} stores from the cytoplasm. In this study we used CPA to delineate the role of the SR Ca^{2+} ATPase in effecting cyclic nucleotide-induced relaxation of canine airway smooth muscle. Various cyclic-nucleotide generating agents were used to induce relaxation in canine tracheal smooth muscle. By blocking the SR Ca^{2+} ATPase, we were able to determine to what extent these agents utilize this pump to effect relaxation.

METHODS

Animals. Healthy adult mongrel dogs of either sex were killed with the use of pentobarbital administration (100 mg/kg body wt). This procedure was approved by the University Animal Care Committee. Segments of trachea were removed, placed in Krebs solution, and constantly bubbled with 95% O_2-5% CO_2. The composition of the Krebs solution was (in mM) 115.5 NaCl, 4.6 KCl, 2.5 $CaCl_2$, 1.6 NaH_2PO_4, 1.16 $MgSO_4$, 21.9 $NaHCO_3$, and 11.1 glucose.

Organ bath studies. Each segment of trachea was cleaned and the smooth muscle was removed from the cartilaginous tracheal rings. The epithelium and all connective tissue were removed under a dissecting microscope to prevent damage to the underlying smooth muscle fibers.

The tracheal muscle was cut into strips 1- to 2-mm wide and 1- to 2-cm long, parallel to the direction of the smooth muscle fibers. The strips were tied with 4-0 silk thread and mounted in 10 ml organ baths containing the same Krebs solution and bubbled with the same gas mixture as mentioned above. The lower ends of the strips were attached to a hook on the bottom of a plastic holder, and the top ends of the tissue were connected to a Grass FT-03C mechanotransducer. Isometric tension was recorded continuously on a Gould 2,800 chart recorder. A resting tension of 1.5 grams (previously shown to produce maximum active tension) was applied to each strip. The tissues were equilibrated for 1 h in the organ baths before beginning the experiments. They were submerged in the Krebs solution and bubbled at 37°C throughout the experiment. KCl (60 mM) was added to the organ bath to contract the tissue. Fifteen minutes later, the KCl was washed out. This procedure was repeated three times or until consistent, reproducible contractions were generated in each tissue. The tissues were then contracted with the previously determined 50% median effective concentration (EC_{50}) of carbachol (Cch; 0.1 μM) in the presence and absence of nifedipine or with 60 mM KCl. The two different agents were used to elicit contraction by two different mechanisms. Cch acts on muscarinic receptors to induce inositol 1,4,5-trisphosphate production that releases Ca^{2+} from internal stores (26). KCl depolarizes the smooth muscle membrane, which opens L-type Ca^{2+} channels and allows Ca^{2+} to enter the cytoplasm and elicits contraction. Once a stable level of tension was reached, various cyclic nucleotide inducing agents were added to effect relaxation. These were added cumulatively in increasing concentrations. For the purpose of this manuscript, relaxations to the maximal concentrations are reported. The tissues were then washed and incubated in Krebs solution containing 10 μM CPA for 20 min, and the procedure was repeated.

Radioimmunoassays. For cAMP measurements, tissue strips were equilibrated and precontracted with either Cch or KCl, as previously described. The maximum concentrations of either ISO or FOR used for relaxation were added to tissues precontracted with either Cch in the presence of nifedipine or with KCl. The tissue was then immediately fast-frozen in liquid nitrogen and placed in preweighed plastic Eppendorf tubes. The tubes were placed on dry ice until they were reweighed to determine the wet weight of the tissue. All samples were then immediately placed in a freezer set at −70°C until the time when the assay could be performed. Radioimmunoassays were carried out using a commercial kit from Amersham (Oakville, Ontario, Canada), following the instructions of the makers.

Drugs used. ISO, FOR, 8-BrcAMP, sodium nitroprusside (NaNP), 8-BrcGMP, Cch, and CPA were all obtained from Sigma (St. Louis, MO).

Data analysis. Although concentration-response curves were generated for each agent used to induce relaxation in the tissues (ISO, FOR, 8-BrcAMP, NaNP, and 8-BrcGMP), only the effects of maximum concentrations are given. This was done because a plateau was not always reached at the maximum concentration used, thus EC_{50} values obtained would not be accurate. To prevent noncyclic nucleotide-mediated effects, higher concentrations of the agents were not used. Results shown are expressed as the active tension in the presence of contraction inhibitor as a percent of active tension in the absence of contraction inhibitor and is denoted as percent of maximal contraction maintained after the addition of each relaxant agent. Under each condition, control contractions were elicited to demonstrate that, in the absence of the relaxant agent, contraction was maintained throughout the experimental procedure.

All results are expressed as means ± SE. Paired *t*-tests were used to analyze the data before and after the addition of CPA. Significant change is reported if $P < 0.05$.

RESULTS

ISO (10 μM) relaxed the tissue to 27.5 ± 4.5% of the contraction elicited by 0.1 μM Cch in the presence of 0.1 μM nifedipine. The nifedipine was added to block entry of Ca^{2+} through L-type channels and this reduced the amplitude of the response to Cch by ~50%. Despite inhibition of Ca^{2+} entry through L-type Ca^{2+} channels, contractions were well sustained for up to 60 min in the absence of relaxants. CPA did not significantly reduce the amount of relaxation brought about by ISO (residual contraction = 41.5 ± 9.0% of control, $n = 7$). In the absence of nifedipine, CPA inhibited the relaxations induced by ISO, when either Cch or KCl was used as contractile agent. The residual contraction after the addition of ISO was increased significantly ($P < 0.05$) from 43.6 ± 3.4 and 44.2 ± 2.5 to 62.1 ± 3.4 and 65.1 ± 3.6% ($n = 3, 6$), respectively. Residual contraction, as opposed to relaxation, is reported in order to facilitate interpretation of the figures. These results may indicate that L-type Ca^{2+} channels need to be available if inhibition of the SR Ca^{2+} ATPase is to affect ISO-induced relaxation. Figure 1 summarizes these results.

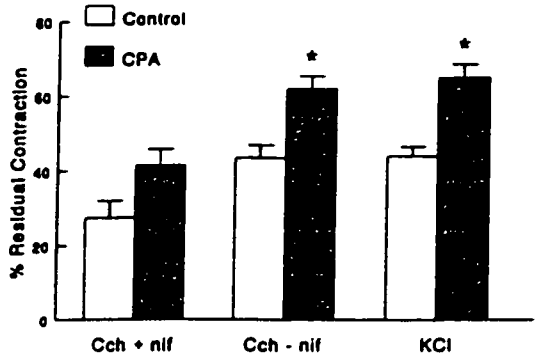

Fig. 1. Effect of 10 μM cyclopiazonic acid (CPA) on relaxation generated by addition of maximal concn of 10 μM isoproterenol (ISO), expressed as %inhibition of maximal contraction elicited by each contractile agent. Carbachol (Cch; 0.1 μM) in presence of 0.1 μM nifedipine (Cch+nif), 0.1 μM Cch with no nifedipine (Cch−nif), and 60 mM KCl were used as contractile agents. Results are shown as means ± SE. *Significantly different ($P < 0.05$) change from value before addition of CPA; $n = 3–7$.

FOR (1.0 μM) relaxed the tissue to 64.8 ± 6.7% of the level of contraction produced by Cch in the presence of nifedipine, and no significant change in this effect occurred after the addition of CPA (60.2 ± 6.7, $n = 8$, Fig. 2). When the tissue was contracted with Cch in the absence of nifedipine, similar results were observed with FOR (54.3 ± 5.0% of control compared with 58.0 ± 2.5% in the presence of CPA, $n = 10$). Only when KCl was used as the contractile agent did CPA significantly block the FOR-induced inhibition of contraction (residual contraction; 64.2 ± 4.1% of control vs. 79.9 ± 3.4%, $n = 8$, see Fig. 2). Since high external potassium levels preclude hyperpolarization through K^+ channels, these results suggest that when hyperpolarization can occur, the action of the SR Ca^{2+} ATPase in relaxation is masked. The degree of inhibition induced by FOR when KCl was the stimulant was less than when Cch was used, consistent with this suggestion.

FOR was not as effective as ISO at inhibiting the contraction produced by either Cch or KCl. If both agents acted by elevation of cAMP levels, this result implies that FOR was less able to raise cAMP. Radioimmunoassay studies were done to determine the amount of elevation of cAMP produced by each. Both ISO and FOR raised cAMP to a similar degree, 201 ± 35% compared with 204 ± 35% of control ($n = 3$) when Cch was used to stimulate the tissue, and 150 ± 18% vs. 159 ± 48% of control ($n = 4$) when KCl was used as the contractile agent (Fig. 3).

8-BrcAMP was then used to determine what portion of the relaxation induced by either ISO or FOR was a result of elevations in cAMP levels and what portion was due to non-cAMP-related mechanisms. The cyclic nucleotide derivative at 3 mM relaxed the tissue to 48.3 ± 6.2% of the control contraction elicited by Cch in the presence of nifedipine (Fig. 4). This value was not changed significantly by the addition of CPA (57.2 ± 7.1%, $n = 5$). It was also greater than the residual contraction after ISO ($P < 0.05$). The ability of 8-BrcAMP to relax the tissue was less in the absence of L-type Ca^{2+} channel blockade. Thus, in the absence of nifedipine, the Cch-stimulated tissue relaxed only to 81.2 ± 2.5 and 81.8 ± 3.6% ($n = 3$) of its original contraction before and after the addition of CPA, respectively. Similarly, the tissue contracted with KCl relaxed minimally to 94.2 ± 3.9 and 93.0 ± 3.8% ($n = 5$) with or without CPA. CPA had no effect under any contractile condition, suggesting that the sequestration of Ca^{2+} into intracellular stores is not a major mechanism by which cAMP per se elicits relation of the smooth muscle.

NaNP, at a maximal concentration of 100 μM, relaxed the trachea smooth muscle strips to 28.2 ± 3.1% of their original contraction produced by the addition of Cch in the presence of nifedipine. This was not affected by the addition of CPA, and the tissue contraction was inhibited to 37.2 ± 5.3% ($n = 9$) in its presence. Thus CPA had no effect once again when L-type Ca^{2+} channels

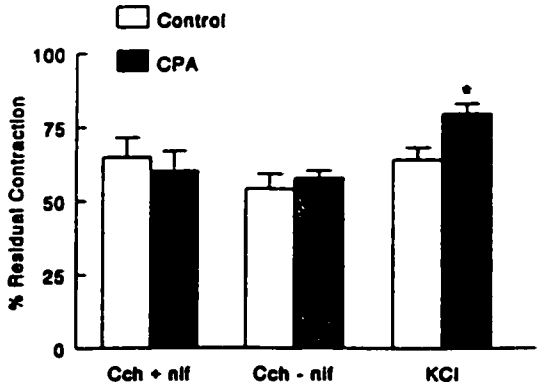

Fig. 2. Effect of 10 μM CPA on relaxation generated by addition of maximal concn 1 μM forskolin (FOR), expressed as %inhibition of maximal contraction elicited by each contractile agent. Cch (0.1 μM) in presence of 0.1 μM nifedipine (Cch+nif), 0.1 μM Cch with no nifedipine (Cch−nif), and 60 mM KCl were used as contractile agents. Results are shown as means ± SE. *Significantly different ($P < 0.05$) change from value before addition of CPA; $n = 8–10$.

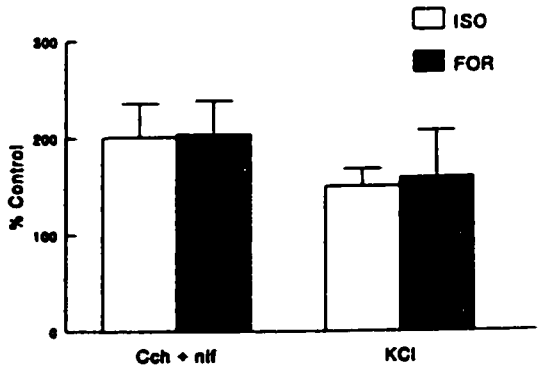

Fig. 3. Elevation of cAMP production after addition of 10 μM ISO and 1 μM FOR, expressed as %control cAMP production. Cch (0.1 μM) in presence of 0.1 μM nifedipine (Cch+nif) and 60 mM KCl were used as contractile agents. Results are means ± SE; $n = 3–4$.

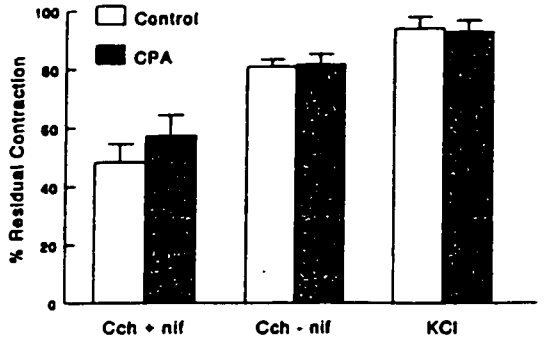

Fig. 4. Effect of 10 μM CPA on relaxation generated by addition of maximal concn of 3 mM 8-bromoadenosine 3',5'-cyclic monophosphate (8-BrcAMP), expressed as %inhibition of maximal contraction elicited by each contractile agent. Cch (0.1 μM) in presence of 0.1 μM nifedipine (Cch+nif), 0.1 μM Cch with no nifedipine (Cch−nif), and 60 mM KCl were used as contractile agents. Results are shown as means ± SE.

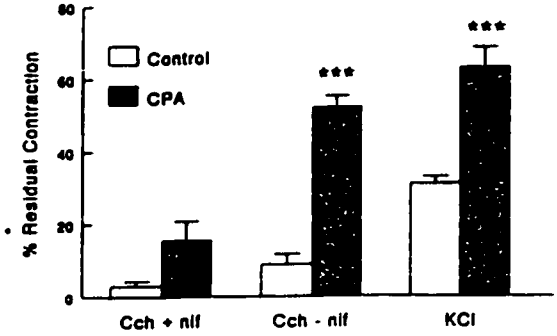

Fig. 6. Effect of 10 μM CPA on relaxation generated by addition of maximal concentration of 3 mM 8-BrcGMP, expressed as %inhibition of maximal contraction elicited by each contractile agent. Cch (0.1 μM) in presence of 0.1 μM nifedipine (Cch+nif), 0.1 μM Cch with no nifedipine (Cch−nif), and 60 mM KCl were used as contractile agents. Results are shown as means ± SE. ***Significantly different (P < 0.001) change from value before addition of CPA; n = 3–7.

were blocked. When the Ca^{2+} channels were available, however, CPA significantly reduced the relaxation induced by NaNP. When Cch was used to contract the tissue in the absence of nifedipine, the tissue was relaxed by NaNP to 26.1 ± 5.2% of control contraction in the absence of CPA and to 49.3 ± 5.3% (n = 7, P < 0.05) in the presence of CPA. When KCl was used, the results were 60.1 ± 2.6% of control contraction before and 72.7 ± 2.8% (n = 6, P < 0.05) after addition of CPA (Fig. 5).

The results elicited by addition of the membrane permeant analogue 8-BrcGMP were similar to those elicited by NaNP. After contraction with Cch in the presence of nifedipine, the tissue was relaxed by 8-BrcGMP almost to baseline (3.0 ± 1.3%; Fig. 6). This effect was not significantly inhibited by the addition of CPA (15.5 ± 5.3%; n = 7). In the absence of nifedipine the tissue was relaxed to 8.8 ± 2.9% of the control contraction induced by Cch. This was greatly inhibited after the addition of CPA (51.0 ± 3.1% of control contraction, n = 3, P < 0.001). Similarly, when KCl was used to stimulate the tissue, the cyclic-nucleotide derivative relaxed the tissue to 30.9 ± 1.9% of the control response without CPA, and this was reduced to 62.7 ± 5.5% (n = 5, P < 0.001) with CPA present. These results indicate that when L-type Ca^{2+} channels are available, cGMP elicits relaxation in part by increasing the sequestration of Ca^{2+} into internal stores.

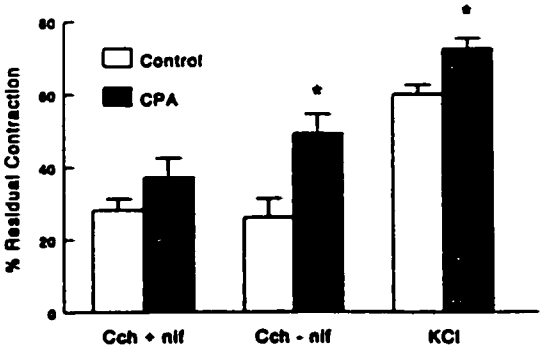

Fig. 5. Effect of 10 μM CPA on relaxation generated by addition of maximal concn of 100 μM sodium nitroprusside (NaNP), expressed as %inhibition of maximal contraction elicited by each contractile agent. Cch (0.1 μM) in presence of 0.1 μM nifedipine (Cch+nif), 0.1 μM Cch with no nifedipine (Cch−nif), and 60 mM KCl were used as contractile agents. Results are shown as means ± SE. *Significantly different (P < 0.05) change from value before addition of CPA; n = 6–9.

DISCUSSION

The main findings of this study are *1*) pumping of Ca^{2+} into SR plays a major role in cGMP-produced but not cAMP-induced relaxation; *2*) L-type Ca^{2+} channels must be available for the relaxant role of Ca^{2+} pumping into the SR to be expressed; and *3*) ISO-induced relaxation may not involve primarily elevation of cAMP.

Effects of ISO and FOR. ISO is usually considered to act by binding to β-adrenoceptors and activating a G_s stimulatory protein that in turn activates adenylate cyclase and results in increased production of cAMP (see Ref. 25 for review) FOR is a direct activator of adenylate cyclase (see Ref. 30 for review). Our studies showed that addition of either ISO or FOR resulted in similar elevations of intracellular cAMP in canine trachea. However, in the present experiments ISO was more effective at relaxing the canine airway smooth muscle than was FOR when either KCl or Cch was used as the contractile agent.

This difference was not related to differential abilities to raise cAMP; both agents raised cAMP by the same amount, to ~200% of control levels in the presence of Cch and ~150% of control in the presence of KCl. These results suggest that total elevation of cAMP does not determine the extent of relaxation and are consistent with those of Shafiq et al. (33), who reported that cAMP was increased more by NKH-477, a water-soluble FOR derivative, than by ISO but that both agents inhibited high K^+- or acetylcholine-induced contraction to the

same extent. Similarly, Marshall and Fain (22) reported that, in rat myometrium, FOR had to elevate cAMP levels to a greater extent than did ISO to produce a similar functional response. Thus a given elevation of cAMP by ISO is more effective than elevation by FOR at causing relaxation; alternatively, cAMP elevation is not the sole or major determinant of the relaxant response.

One possible explanation of the disparity between relaxation induced by each of the agents is that various components of the cAMP/cAMP-PK cascade may exist in distinct compartments. If so, not all the cAMP generated in response to FOR might have access to cAMP-PK. Zhou et al. (38) demonstrated that, compared with ISO, FOR needed to induce greater increases in cAMP accumulation in order to achieve the same activation levels of cAMP-PK and the same degree of relaxation. Another putative explanation is that ISO also mediates relaxation through a cAMP-independent mechanism. Lohmann et al. (20) reported that ISO could relax bovine tracheal smooth muscle by 85–90% without significantly elevating cAMP levels. Moreover, Lau and Lum (18) provided evidence that the selective β_2-blockers butoxamide and $H_{35/25}$ antagonized the increase in cAMP but not the relaxation produced by ISO in the same tissue (see Ref. 6 for review).

Effects of 8-BrcAMP. To eliminate any contributions from non-cAMP-related effects, the membrane-permeable analogue 8-BrcAMP was used to relax the tissue. No significant change in the ability of the cyclic nucleotide to relax the tissue was noted after the addition of CPA. This suggests that stimulation of the SR Ca^{2+} ATPase and subsequent enhancement of Ca^{2+} uptake into stores are not mechanisms by which cAMP elevation per se evokes relaxation in canine tracheal smooth muscle. It is possible that the cyclic nucleotide produces relaxation by decreasing the sensitivity of the contractile apparatus to the internal Ca^{2+} concentration. In other words, cAMP-dependent phosphorylation of myosin light-chain kinase (MLCK) can decrease its affinity for calmodulin. This results in decreased myosin phosphorylation and, consequently, relaxation. Sellers and Adelstein (32) demonstrated that gizzard MLCK could be phosphorylated at two sites and that this phosphorylation reduced the affinity of MLCK for the Ca^{2+}-calmodulin complex. This evidence was supported by Bhalla et al. (1) and Hathaway et al. (15), who demonstrated similar results in vascular smooth muscle.

The above explanation, however, does not account for the cAMP-induced decrease in $[Ca^{2+}]_i$ reported by McDaniel et al. (23). Alternatively, Scheid and Fay (29) reported that cAMP stimulated Ca^{2+} extrusion across the plasma membrane.

There is growing evidence that cAMP may induce relaxation through modulation of ion channels in the membrane. Our observation that the ability of 8-BrcAMP to relax the tissue was severely inhibited when high K^+ was used to stimulate the tissue may indicate that K^+ channel opening is necessary for cAMP to evoke maximal relaxation. Haynes et al. (16) reported that 8-BrcAMP-induced relaxation in rat pulmonary artery was decreased by the addition of TEA, a nonselective K^+ channel blocker. Moreover, Carl et al. (7) demonstrated an increase in the open probability of Ca^{2+}-activated K^+ channels after the addition of the catalytic subunit of porcine cAMP-PK in single canine colonic smooth muscle cells. Ca^{2+} channels may also be involved. Lory and Nargeot (21) have shown that cAMP-dependent modulation plays a role in maintaining the basal activity of cardiac L-type Ca^{2+} channels expressed in *Xenopus* oocytes. They reported a two- to threefold increase in Ba^{2+} current amplitude after microinjection of cAMP and a decrease in the Ba^{2+} current amplitude after inhibition of cAMP-PK. The relaxant effect of 8-BrcAMP was inhibited when L-type Ca^{2+} channels were available and Cch was the stimulant. However, it is possible that Cch interferes with cAMP actions on ion channels.

CPA inhibited the relaxation induced by ISO when L-type Ca^{2+} channels were available. In view of the fact that CPA had no effect on the relaxation elicited by 8-BrcAMP, these results suggest that ISO may stimulate the SR Ca^{2+} ATPase through a cAMP-independent mechanism.

Effects of NaNP and 8-BrcGMP. When L-type Ca^{2+} channels were available, CPA inhibited the relaxation produced by NaNP by ~30%. Addition of CPA also inhibited the relaxation generated by 8-BrcGMP, once again, only when L-type Ca^{2+} channels were available. The amount of relaxation elicited by NaNP was less than that produced by 8-BrcGMP (30 compared with 50%). This may indicate that the level of cGMP was not elevated by NaNP to the same concentration as was added directly with the cyclic nucleotide analogue. The present data indicate that, when L-type Ca^{2+} channels are available, pumping of Ca^{2+} into the SR store is an important mechanism by which cGMP lowers $[Ca^{2+}]_i$ and produces relaxation of canine tracheal smooth muscle. These results are consistent with the findings of Twort and Van Breemen (36), who reported that cGMP increased Ca^{2+} uptake into skinned rat aortic smooth muscle cell SR stores. Moreover, Cornwell et al. (10) demonstrated that the SR Ca^{2+} ATPase regulatory protein, phospholamban, was phosphorylated in the presence of cGMP.

However, the relaxation elicited by NaNP and 8-BrcGMP was not fully inhibited in the presence of CPA. Either CPA was not present in sufficient concentrations to fully inhibit the SR Ca^{2+} pump, or cGMP also acts through SR Ca^{2+} ATPase-independent mechanisms to produce relaxation. Most studies indicate that 10 μM produces maximal effects on the SR Ca^{2+} pump (12, 31). The second explanation is supported by the present data that demonstrate that NaNP and 8-BrcGMP induced relaxation was inhibited when KCl was used as the contractile agent and the K^+ gradient across the cell membrane was reduced. This suggests that, as when cAMP was the relaxant agent, K^+-channel opening is necessary for cGMP to exert its maximal effect. Vrolix et al. (37) demonstrated that cGMP-PK stimulated the plasma membrane Ca^{2+} ATPase purified from the smooth muscle of pig stomach and rat aorta. This suggests that cGMP may also relax smooth muscle by enhancement of Ca^{2+} extrusion. Moreover, cGMP may

also act by decreasing the sensitivity of the contractile apparatus to $[Ca^{2+}]_i$ as reported by McDaniel et al. (23).

When L-type Ca^{2+} channels were blocked by the presence of nifedipine, neither NaNP- nor 8-BrcGMP-induced relaxation was significantly changed by the addition of CPA. However, with both relaxation agents, as with 8-BrcAMP, the amount of relaxation elicited was reduced. This might suggest a trend that could possibly become significant if experimental numbers were increased. This is unlikely, however, as the data are quite consistent and error is fairly small. If further experiments did prove a significant difference, this might give insight into the role of Ca^{2+} channels in cyclic nucleotide-induced relaxation. The role of the SR pump, however, would remain unchanged.

Role of Ca^{2+} channels. The ability of CPA to inhibit relaxation was enhanced when L-type Ca^{2+} channels were available. When Ca^{2+} channels are available, the entry of Ca^{2+} into the cell is likely to be increased, so that pumping of Ca^{2+} into the SR was required to effect relaxation. Emptying of Ca^{2+} stores by Cch or CPA may have made available an entry route into the SR via an L-type Ca^{2+} channel that did not require the SR Ca^{2+} pump. Bourreau et al. (5) have demonstrated that a dihydropyridine-sensitive Ca^{2+} channel exists that was controlled by the status of internal Ca^{2+} stores. When this Ca^{2+} entry route is available (in the absence of nifedipine), it allows Ca^{2+} to move from the extracellular space, through the SR and into the cytoplasm, contributing to contraction. When it is not available (in the presence of nifedipine), Ca^{2+} entry during tonic contraction may be minimal and involve recycling of internal Ca^{2+} across the plasma membrane (2; L. M. Montano, C. Barajas-Lopez, and E. E. Daniel, unpublished observations).

The ability of ISO and 8-BrcAMP to relax the tissue was inhibited when L-type Ca^{2+} channels were available. This may result from the ability of additional Ca^{2+} entry through L-type Ca^{2+} channels into the SR with subsequent leakage into the cytoplasm. CPA would not inhibit this extra Ca^{2+} influx (4).

Tonic vs. phasic contractions. Bourreau et al. (4) have presented evidence that Ca^{2+} entrance plays a more important role in sustaining a tonic compared with the initial phasic contraction to Cch. Our study involved only tonic contractions. Thus these data may not address the mechanisms of relaxation (or inhibition of contraction) modulating phasic contraction of airway smooth muscle. For example, the importance of Ca^{2+} entry through L-type Ca^{2+} channels may be less in affecting relaxation of phasic contractions.

In conclusion, cAMP appears not to activate the SR Ca^{2+} ATPase. As a result, the cyclic nucleotide does not cause relaxation by stimulation of Ca^{2+} uptake into the internal stores. cGMP, on the other hand, does stimulate the pump and the uptake of Ca^{2+} into the SR is an important mechanism by which cGMP causes relaxation. The relaxation induced by both cyclic nucleotides was decreased when the muscle is stimulated by high external K^+, indicating that K^+ channel opening may be another mechanism by which both cAMP and cGMP produce relaxation. Other mechanisms, such as decreased sensitivity to internal Ca^{2+}, may also play a role.

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CHAPTER VII

DISCUSSION

Chapter VII: Discussion

7.1 Summary of Findings

In the series of experiments outlined in this thesis, the role of epithelial-derived PGE_2 in the canine airway was investigated. The mechanism of PGE_2 release and its actions were first examined from the trachea of control animals. Subsequently, we determined if PGE_2 release was altered in an animal model of asthma, antigen inhalation. We also studied the bronchi of animals exposed to allergen to note differences between the trachea and the bronchi, and to determine if PGE_2 played the same protective role at both levels of airway smooth muscle. Finally, we examined a potential mechanism for PGE_2 modulation of airway smooth muscle tone.

In determining whether PGE_2 is released from the epithelium, and if this prostanoid can account for the actions of the EpDIF, we discovered that both prostanoids PGI_2 and PGE_2 are released from unchallenged canine tracheal epithelium. Although the epithelium appeared to be the only source of PGE_2, PGI_2 was also released from a non-epithelial source. There was virtually no basal release of PGE_2. The release of PGE_2 from the epithelium occurred only when the tissue was electrically field stimulated (EFS), and this release was not blocked by either

TTX or ω-CTX (GVIA). We postulated that perhaps the release of PGE_2 was from neuroendocrine cells located within the epithelium.

In our subsequent study, changes in tracheal smooth muscle prostanoid release following exposure to allergen were investigated. It was shown that the release of PGE_2 from animals that were exposed to allergen but did not develop airway hyperresponsiveness, as determined *in vivo* (NON-RESP), was increased. Release of PGE_2 from animals that inhaled only vehicle (SHAM), or who inhaled antigen and developed airway hyperresponsiveness (HYP-RESP), was similar to the release of PGE_2 from Chapter 3. However, the release of PGE_2 was not dependent on EFS from any of the tissues from animals that inhaled either vehicle or allergen. It was determined that demonstration of hyperresponsiveness *in vitro* depended on the absence of the epithelium, and therefore the removal of the major source of PGE_2. However, in animals that became hyperresponsive *in vivo* following inhalation of allergen, there was a small but significant release of PGE_2 from a non-epithelial source. This release was dependent on EFS.

When we compared the release of PGE_2 from trachea to that from third to fifth order bronchi, we noted differences from the results in trachea, not only in the release of the prostanoid but also in the responses of the tissues to EFS and the contractile agonist carbachol (Cch). The response of the tissues to Cch was not altered in animals that inhaled either vehicle or allergen, whether the development of airway hyperresponsiveness *in vivo* occurred or not. Tissues from HYP-RESP

animals were less responsive to EFS than were those from SHAM or NON-RESP animals. This decrease was partially reversed by preventing the production of PGE_2 with the cyclooxygenase inhibitor, indomethacin. Furthermore, the potential release of a non-prostanoid inhibitory factor was once again noted. Finally, an increase in basal release of PGE_2 occurred in tissues from animals who developed hyperresponsiveness. The release of PGE_2 from tissues from all three groups of animals was not dependent on EFS.

Upon investigating the potential mechanism of action of PGE_2 on canine tracheal smooth muscle, we examined the PGE_2-dependent increase in cAMP, and a possible mode of action of cAMP in decreasing the intracellular Ca^{2+} concentration, thus effecting relaxation. It was demonstrated that pumping of Ca^{2+} into the SR is not a major mechanism by which cAMP induces relaxation.

7.2 Differences Between Studies

When the results of the four studies are examined together, certain differences in results between the studies become evident. These differences include altered responses to Cch and EFS between the tracheal and bronchial smooth muscles, as well as different patterns of PGE_2 release between the two different sites. Moreover, the release of PGE_2 was dependent on EFS in our first study in trachea, however the release was never EFS-dependent on any of the following studies. A summary

of the differences between the studies is displayed in Table 1.

7.2.1 Responses to Cch

Tracheal responses to Cch and EFS in the antigen study (Chapter 4) were measured in epithelium-intact and epithelium-denuded tissues. As a result, the results from tracheal smooth muscle (TSM) strips demonstrate the effect of the epithelium as a whole. There is no delineation between potential prostanoid and non-prostanoid influences. The epithelium of the bronchi is much more difficult to remove; thus the responses of bronchial smooth muscle (BSM) strips were measured in the presence and absence of the cyclooxyygenase inhibitor indomethacin (Chapter 5). This has the advantage of allowing the examination of the influence of prostaglandins on the BSM. However, the epithelium releases both prostanoid and non-prostanoid factors which are capable of modulating smooth muscle (Ullman *et al.*, 1991; Chapter 3), and this technique did not allow investigation of the full effect of the epithelium. It is important to keep these different protocols in mind when comparing the results of the antigen studies on TSM and BSM.

In Chapter 4, removal of the epithelium increased the TSM maximum response and the EC_{50} to Cch. The addition of indomethacin did not, however, increase the

		Cch (max)		EFS (30pps)		PGE_2 EFS		PGE_2 Control	
Source:	**TSM**	+e	-e	+e	-e	+e	-e	+e	-e
Chapter 3	Control	>	=	>	=	>	=	<	=
Chapter 4	SHAM	-	-	-	-	-	-	-	-
	NON-RESP	=	=	<	=	>	=	>	=
	HYP-RESP	=	>	<	>	=	>	=	=
	BSM	-i	+i	-i	+i	-i	+i	-i	+i
Chapter 5	SHAM	-	-	-	-	-	-	-	-
	NON-RESP	<	=	=	=	=	-	=	-
	HYP-RESP	<	=	<	<	=	-	>	-

Table 1: Summary of Results

Summary of differences in contractile responses to Cch (10^{-7}M) and EFS, and in concentration of PGE_2 measured during EFS or time control from manuscripts 1, 2, and 3. All results are compared to SHAM tissues of either trachea or bronchi. Trachea results from Chapter 3 are compared to SHAM results from Chapter 4. In chapter 5, no PGE2 was measured in the presence of indomethacin (-). TSM, tracheal smooth muscle, BSM, bronchial smooth muscle; >, significantly greater than SHAM; <, significantly less than SHAM; =, not significantly different than SHAM; +e, with epithelium; -e, without epithelium; -i, without indomethacin; +i, with indomethacin.

BSM responses to Cch (Chapter 5). This may be interpreted several ways. It may suggest that the bronchial epithelium does not release any inhibitory prostanoids, thus BSM contractions are not affected by the presence of indomethacin. Alternatively, it may be an indication that it is a non-prostanoid which is released from the epithelium which decreases the response to the contractile agonist, or that Cch contraction occurs in BSM by a PGE_2 and cAMP resistant mechanism. The differences seen between BSM and TSM may be due to altered recruitment of immune cells in the TSM and BSM. Furthermore, in the allergen studies, HYP-RESP tissues from TSM contracted more in response to Cch than did SHAM or NON-RESP tissues once the epithelium was removed. In BSM, however, there were no significant changes between the three groups of BSM tissues, either in the presence or absence of indomethacin.

It is also interesting to note that in Chapter 3, when animals did not inhale any vehicle or allergen, no differences were seen in the maximal contraction to Cch in either epithelial-intact or epithelial-denuded tracheal tissues, or in the presence or absence of indomethacin. Thus the inhalation protocol itself is sufficient to alter the effect of the epithelium on TSM. This may be due to an inflammatory response occurring following inhalation of saline or *Ascaris suum*. Alternatively, it may be a result of damage to the TSM following intubation. However, upon removal of the epithelium, responses from HYP-RESP tissues were greater than those from tracheal tissues which received no allergen and responses from trachea of SHAM

or NON-RESP animals were no different than those from trachea from animals that did not undergo any inhalation protocol. These results did not correlate with possible damage to smooth muscle by intubation suggesting that other factors determined alterations in responsiveness.

7.2.2 Responses to EFS

EFS-evoked responses were greatly reduced in HYP-RESP tissues compared to SHAM or NON-RESP tissues in epithelial-intact TSM and in BSM in both the presence and absence of indomethacin. Removal of the epithelium from TSM however, resulted in a greater response of HYP-RESP tissues to EFS compared to SHAM tissues. Once again this suggests that either release of a non-prostanoid EpDIF or altered recruitment of immune cells and their mediators occurred.

Clearly, to facilitate direct comparisons of contractions from TSM and BSM, it is highly desirable that the experiments be repeated in which TSM experiments are performed in the presence of indomethacin, and BSM experiments are carried out on epithelial-denuded tissues.

7.2.3 PGE_2 Measurements

Measurements of PGE_2 release from TSM and BSM (Chapters 4 and 5,

respectively) were all performed with no indomethacin present. Removal of the epithelium from TSM abolished the release of PGE_2 except from HYP-RESP tissues that were field stimulated. As a result, it is possible to make comparisons of PGE_2 release between TSM and BSM. Although there were no significant differences in the release of PGE_2 between SHAM, NON-RESP, and HYP-RESP tissues from bronchi, in tracheal tissues there was a significant increase in the PGE_2 released from NON-RESP tissues. This release of PGE_2 from NON-RESP tissues was independent of field stimulation. We suggest that it was this large increase of the inhibitory prostanoid concentration that prevented NON-RESP animals from developing airway hyperresponsiveness. It is possible that inhalation of the allergen resulted in an inflammatory response which resulted in excitatory mediators being released in both NON-RESP and HYP-RESP tissues. In the NON-RESP, however, the ratio of inhibitory to excitatory mediators was greater than in the HYP-RESP and thus the NON-RESP did not develop hyperresponsiveness either *in vivo* or *in vitro*. The mechanisms underlying this difference remain to be elucidated.

In BSM, when PGE_2 production was eliminated, there may have been other inhibitory factors present in sufficient concentrations to prevent hyperresponsiveness, and in the case of HYP-RESP tissues, to have caused *in vitro* hyporesponsiveness of the BSM.

A similar phenomenon may explain why the presence of the epithelium masked HYP-RESP in TSM. In TSM the HYP-RESP tissues only displayed increased

responses to Cch and EFS once the epithelium was removed. Removal of the epithelium eliminated the influence of any EpDIF, and may have altered the ratio of inhibitory to excitatory mediators as suggested above.

In TSM there was a small, but significant, release of PGE_2 from epithelium-denuded HYP-RESP tissues. Similarly, in BSM, there was a small, but significant, increase in basal PGE_2 release from HYP-RESP tissues. Thus, in tissues from HYP-RESP animals, there may have been an induction of an epithelium-independent PGE_2 source. The nature of this PGE_2 source will be discussed in section 7.3.2.

Interestingly, the TSM of all three groups of animals that inhaled either saline or allergen (Chapter 4) released PGE_2 independently of EFS. In our original study (Chapter 3), we found that the release of PGE_2 was dependent on EFS and that tracheal tissues that were not field stimulated released almost no PGE_2. This adds support to our theory that the inhalation protocol itself was sufficient to alter TSM responses.

In BSM, PGE_2 release was significantly increased in SHAM tissues that were field stimulated compared to the SHAM time controls. However, in animals that received allergen, the release of PGE_2 from EFS tissues was not significantly different from that of time controls.

It is possible that intubation of the trachea physically stimulated the tissue and resulted in the induction of cyclooxygenase, and thus PGE_2 production. Furthermore, inhalation of the allergen might also have been sufficient to induce

PGE_2 production. Possible mechanisms of induction of cyclooxygenase will be discussed in section 7.3.3.

It appears that the development of airway hyperresponsiveness may be due to an alteration in the relative concentrations of inhibitory and excitatory mediators. For this reason, the remainder of the discussion will focus on sources and actions of various mediators in the airway. The main focus will be on PGE_2, but other mediators and their putative inflammatory cell sources will also be examined.

7.3 Potential Sources of PGE_2

7.3.1 Epithelial Source of PGE_2

A large body of evidence exists which suggests that the epithelium is the major source of PGE_2 in the airway. The enzymes necessary for the production of PGE_2 have been demonstrated in airway epithelium (Stuart-Smith *et al.*, 1988; Butler *et al.*, 1992; Yu *et al.*, 1992). PGE_2 is released from the epithelium in sufficient concentrations to modulate ASM (Chapter 3; Barnett *et al.*, 1990; Prie *et al.*, 1991; Holtzman, 1992; Matsumoto *et al.*, 1996). Moreover, removal of the epithelium greatly reduces PGE_2 release from airway tissues (Undem *et al.*, 1988; Gray *et al.*, 1992).

There have been few studies, however, which have investigated which specific

cells from the epithelium release PGE_2. This is likely due to the difficulty in isolating individual cell types from the epithelium. In Chapter 3, we proposed that the neuroendocrine cell (NEC) of the epithelium was responsible for EFS-induced production of epithelium-derived PGE_2. This was based on the observations that the release of PGE_2 that was induced by EFS was not blocked by the Na^+ channel blocker TTX nor the N-type Ca^{2+} channel blocker ω-CTX. Thus, release was from an excitable cell within the epithelium which was not dependent on neural activity for mediator release.

NEC, often found in clusters termed neuroepithelial bodies, are small granule-containing cells located in the epithelium at all levels of the airway (Sorokin *et al.*, 1983; Youngson *et al.*, 1993; Boers *et al.*, 1996). Once believed to be of little importance in the adult airway, these cells have been demonstrated to play a role in oxygen sensing. Youngson *et al.* (1993) found pO_2-sensitive K^+ channels in the NEC. They proposed that hypoxia caused K^+ channels to close, which depolarized the membrane and thus caused voltage-dependent Ca^{2+} channels to open which led to Ca^{2+} influx and mediator release. Mediators which have been demonstrated to be contained within NEC include serotonin, calcitonin, substance P, enkephalin, somatostatin, and cholecystokinin (Sorokin *et al.*, 1983; Adriaensen and Scheuermann, 1993). It has not yet been demonstrated that NEC cells are capable of producing prostaglandins; however, with the technique that Youngson *et al.* (1993), developed for isolating NEC cells this should be possible to determine.

7.3.2 Non-Epithelial Sources of PGE_2

The epithelium does not appear to be the sole source of PGE_2 in the airway. In chapter 4, we found that epithelial-denuded tissues from HYP-RESP animals released PGE_2 in response to EFS. Moreover, Gao and Vanhoutte (1992) demonstrated that canine bronchial release of PGE_2 was not altered following removal of the epithelium. Two possible non-epithelial sources of PGE_2 include the smooth muscle itself and inflammatory cells.

7.3.2.1 Smooth Muscle as a Source of PGE_2

Delamere *et al.* (1994) reported that TSM itself is capable of producing PGE_2. This group demonstrated that bovine cultured TSM cells released PGE_2 when stimulated by arachidonic acid or bradykinin. They later reported that the release of PGE_2 from bovine cultured TSM cells was increased by incubation with albuterol, forskolin, or dibutyryl cAMP (Barry *et al.*, 1995). Vigano *et al.* (1997) found that human BSM cells, when challenged with interleukin 1β, produced PGE_2 through the COX2 enzyme. It is possible that, in our tissue, induction of COX2 may occur within the muscle cells, and PGE2 may be produced and released from the smooth muscle.

7.3.2.2 Inflammatory Cell Sources of PGE_2

Several investigators have studied the release of PGE_2 from inflammatory cells. It has been demonstrated that, in mice, peritoneal macrophages release a basal level of PGE_2 (Adarem *et al.*, 1986; Dollob, 1996). This release of PGE_2 was increased by stimulation of the macrophages with lipopolysaccharide (LPS) from *E. coli* (Aderem et al, 1986).

Eosinophils are also capable of production and release of PGE_2. In 1975, Hubscher reported that an eosinophil-derived inhibitor, released following sonication or stimulation of eosinophils with antigen, was a mixture of prostaglandins E_1 and E_2. Foegh *et al.* (1986) demonstrated that the Ca^{2+} ionophore A23187 induced release of PGE_2 from human peritoneal eosinophils. Moreover, it has been shown that platelet activating factor (PAF) induced a greater than basal release of PGE_2 from guinea pig (Giembycz *et al.*, 1990) and human (Kroegel and Matthys, 1993) eosinophils.

7.3.3 COX1 and COX2

The existence of two different enzymes responsible for the production of prostaglandins may help explain the differences in PGE_2 production between SHAM, NON-RESP, and HYP-RESP animals, and between TSM and BSM.

As mentioned previously (Chapter 1) there exist two different isomers of cyclooxygenase, COX1 and COX2. COX1 is constitutively expressed, but

expression of COX2 requires stimulation (Meade *et al.*, 1993; Wu, 1996). Induction of COX2 expression has been demonstrated in many different cells by cytokines, growth factors, hormones, and LPS (Herschman, 1996; Wu, 1996). Cells in which COX2 induction has been reported that may be relevant to this thesis include lung fibroblasts, bronchial epithelial cells, alveolar macrophages, and mast cells (Herschman, 1996). Moreover, induction of COX2 mRNA has been demonstrated in vascular smooth muscle (Pritchard *et al.*, 1994; Rimarachin *et al.*, 1994) and it is possible that airway smooth muscle may be capable of expression of COX2 as well.

In our studies, it is possible that an allergen-induced increase in COX2 expression in the epithelium of NON-RESP led to the large increase in PGE_2 released from these tissues. The question that remains, however, is why an increase in COX2 expression would occur in only some of the animals studied. Perhaps it is a difference in the ability of an animal to induce COX2 expression in the airway which determines whether it will develop hyper- or hypo-responsiveness. Similarly, the non-epithelial PGE_2 which was released from denuded HYP-RESP TSM may have been produced as a result of induction of COX2 in the smooth muscle. Once again, however, it is unclear why this increase in expression of the enzyme would be limited to a subset of animals. Studies with selective COX2 inhibitors may illustrate the role of COX2 expression in prostaglandin production following allergen exposure.

7.3.4 Isoprostanes

Isoprostanes are prostaglandin-like compounds which are formed independently of cyclooxygenase (Morrow and Roberts, 1996). Isoprostanes are formed by free radical-catalyzed peroxidation of AA, and the level of measured isoprostanes has been demonstrated to be a reliable indicator of oxidant injury (Morrow and Roberts, 1996).

The existence of isoprostanes may confound interpretation of the results contained within this thesis. If the antibody raised against PGE_2 in the RIA reacts specifically with the cyclopentane ring, then the RIA would also measure E_2 isoprostanes, as this region is conserved. Thus, production and release of PGE_2 measured may not be an indicator of cyclooxygenase activity but rather an indicator of free radical-induced damage in the tissue. However, in a study by Ullman *et al.* (1991), PGE_2 release was measured from ferret trachea epithelium, in a similar manner as the release of PGE_2 seen in Chapter 3. Its release was determined to be dependent upon EFS and not inhibited by addition of TTX. These experiments were performed in presence of the free radical inhibitor ascorbic acid (Ullman *et al.*, 1991). The similarity of results between our experiments and those of Ullman suggest that EFS induced oxidative damage did not occur in our tissue. Furthermore, no change in PGE_2 release was seen from SHAM exposed EFS tissue, which suggests that the inhalation protocol itself did not induce the production of

isoprostanes. If inhalation of the allergen did cause production of E_2 isoprostanes, we would predict an increase in RIA PGE_2 measurements, from both NON-RESP and HYP-RESP tissues, above that seen in Chapter 3 and in Chapter 4 SHAM animals. However, an increase was only seen in PGE_2 release from NON-RESP tissues and not from HYP-RESP tissues.

The results of the present studies may be further confounded by the fact that isoprostanes have been demonstrated to have biological effects. While PGE_2 is a potent relaxer of vascular smooth muscle, the isoprostane 8-isoPGE$_2$ is a potent constrictor of vascular smooth muscle (Morrow and Roberts, 1996). If the same is true of ASM, then the contractile responses that were measured may have been modulated not only by PGE_2, but also by any isomers of PGE_2 that may have been formed.

7.4 Inflammatory Cells

As discussed previously, many of the differences in results between the manuscripts presented in this thesis may have been a result of alterations in which inflammatory cells were present. Different inflammatory cells release different profiles of mediators with distinct actions. Which cells are present in the lungs of asthmatic subjects and our allergen model, as well as which mediators they release and their biological actions, will be described below.

Inflammatory cells believed to be involved in the pathogenesis of asthma are usually measured in the bronchoalveolar fluid (BAL) or in bronchial biopsies (BB). In 1987, Kirby *et al.* demonstrated an increase in the number of eosinophils found in the BAL from asthmatics compared to non-asthmatics. Similarly, Beasley *et al.* (1989) reported a mucosal infiltration of eosinophils in the BAL from asthmatic subjects compared to non-asthmatic controls. During bronchoscopy, they also noted mast cell degranulation, and active recruitment of eosinophils, neutrophils, and monocytes in subjects with asthma (Beasley *et al.*, 1989). Foresi *et al.* (1990) also demonstrated an increase in eosinophils and mast cells in the BAL of asthmatic subjects compared to healthy subjects. Bousquet *et al.* (1990) found a significant increase in eosinophils in patients with asthma, and demonstrated that the number of peripheral blood eosinophils in asthmatics was positively correlated with the severity of asthma. Pradalier (1993) reported that the number of eosinophils and neutrophils are elevated in the BAL from asthmatics following allergen challenge. Moreover, Howarth *et al.* (1995) noted that biopsy and BAL studies have shown the presence of mast cells, eosinophils, T-lymphocytes, as well as activated macrophages in patients with asthma.

In studies with animal models of asthma, Itoh *et al.* (1996) reported a correlation between airway eosinophilia and airway hyperresponsiveness in guinea pigs which inhaled allergen. In the model used in this thesis, canine inhalation of *Ascaris suum*, Wooley *et al.* (1993) showed that dogs which developed antigen-

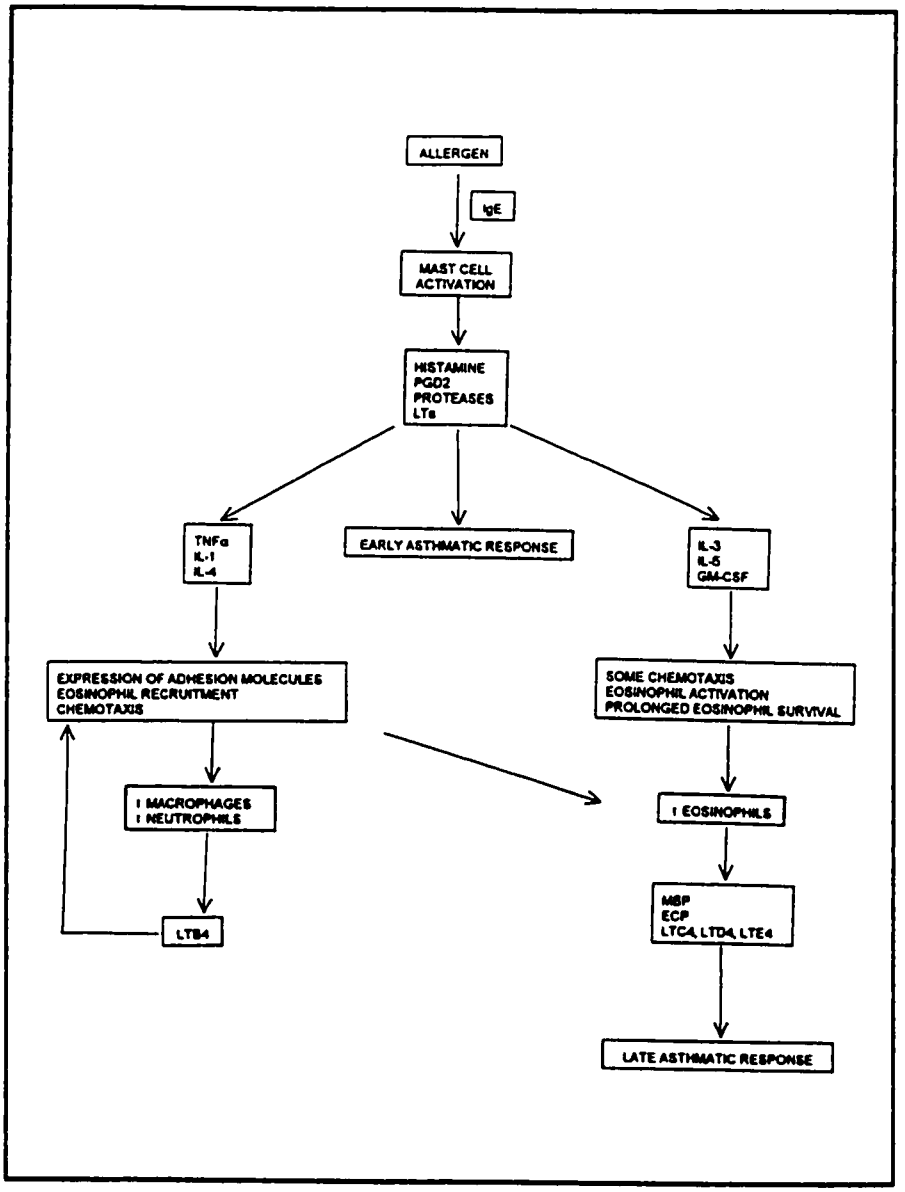
induced hyperresponsiveness contained an increased number and increased activation of eosinophils compared to those animals that did not develop hyperresponsiveness.

It can be seen that many inflammatory cells play a role in asthma, including mast cells, eosinophils, neutrophils, and macrophages (Figure 1). The mast cell is thought to be mainly responsible for the EAR (Hargreave , 1991; Sterk *et al.*, 1993; Abbas, 1994). Interaction of an antigen with IgE bound to mast cells results in activation of the mast cells. Activation results in three responses: 1) release of histamine and proteases from pre-formed granules; 2) synthesis of lipid mediators including platelet activating factor, PGD_2, LTC_4, LTD_4, and LTE_4; and 3) production and secretion of cytokines, namely tumor necrosis factor (TNFα), interleukin (IL)-1, IL-3, IL-4, IL-5, IL-6, and granulocyte monocyte-colony stimulating factor (GM-CSF) (Abbas, 1994; Howarth, 1995).

Interaction of the cytokines released from mast cells with selectins located on endothelial cells and leukocytes results in the expression of integrins and the adhesion molecules ICAM-1 and VCAM-1 (Howarth, 1995). These adhesion molecules enable inflammatory cells to migrate from the vasculature into the tissue (Howarth, 1995). The cytokine IL-5 further acts as an activator of eosinophils and prolongs the survival rate of these cells (Abbas, 1994; Howarth, 1995).

Eosinophils, once activated, release eosinophil cation protein and major basic protein (MBP) (Flavahan, 1988; Abbas, 1994; Howarth, 1995). MBP is cytotoxic to

Figure 1 Potential pathways of the development of 1) early asthmatic responses, and 2) late asthmatic responses.


ALLERGEN
IgE
MAST CELL ACTIVATION
HISTAMINE
PGD2
PROTEASES
LTs
TNFα
IL-1
IL-4
EARLY ASTHMATIC RESPONSE
IL-3
IL-5
GM-CSF
EXPRESSION OF ADHESION MOLECULES
EOSINOPHIL RECRUITMENT
CHEMOTAXIS
SOME CHEMOTAXIS
EOSINOPHIL ACTIVATION
PROLONGED EOSINOPHIL SURVIVAL
↑ MACROPHAGES
↑ NEUTROPHILS
↑ EOSINOPHILS
LTB4
MBP
ECP
LTC4, LTD4, LTE4
LATE ASTHMATIC RESPONSE

the respiratory epithelium as well as other tissues (Flavahan, 1988; Frigas *et al.*, 1991; Abbas, 1994). Eosinophils have also been demonstrated to release LTC_4 (Owen *et al.*, 1987) and prostaglandins E_2 and I_2 (Hubscher, 1975; Giembycz *et al.*, 1990; Kroegal and Matthys, 1993; White *et al.*, 1993).

Neutrophils, as well as macrophages, release LTB_4, which is a potent chemoattractant (Kalant and Roschlau, 1989; Holtzman, 1992; Howarth, 1995). Macrophages may also generate IL-1, IL-6, GM-CSF, and TNFα (Howarth, 1995).

The leukotrienes derived from these inflammatory cells have been demonstrated to be directly or indirectly responsible for the LAR, as a leukotriene antagonist, zafirlukast, greatly inhibited the appearance of the LAR (Sampson, 1996; Larsen and Jackson, 1996). As described in Chapter 1, LTC_4, LTD_4 and LTE_4 have potent bronchoconstrictor activity, increase vascular permeability, induce mucous secretion, and decrease mucous transport (Kalant and Roschlau, 1989; Henderson, 1994; Sampson, 1996). Further, LTB_4 is a potent chemoattractant of neutrophils (Holtzman, 1992). All these actions of the leukotrienes contribute to the LAR.

7.5 Potential Actions of PGE_2

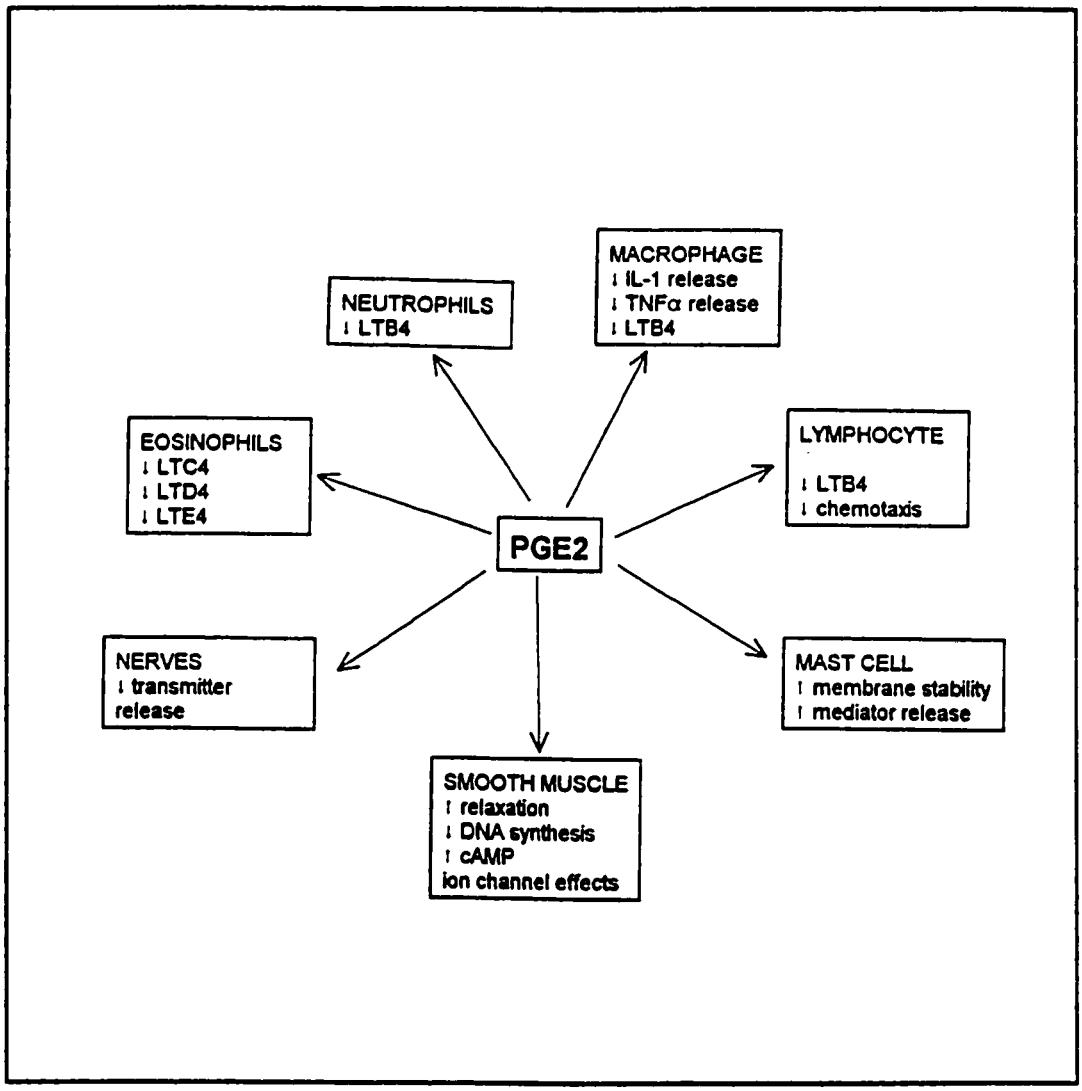
It was demonstrated that animals that did not develop airway hyperresponsiveness following the inhalation of allergen (NON-RESP) released significantly more PGE_2 from TSM than did SHAM or HYP-RESP animals (Chapter

4). Also, BSM strips which were hyperresponsive (HYP-RESP) *in vivo*, were hyporesponsive in the muscle bath and also displayed an increased basal release of PGE_2 *in vitro* (Chapter 5).

Several other studies have also demonstrated a potential protective role of PGE_2 in the development of airway hyperresponsiveness. Pavord *et al.* (1991) found that nebulized PGE_2 protected against bronchoconstriction induced by sodium metabisulphite in asthmatic subjects. PGE_2 also decreased exercised-induced bronchoconstriction in asthmatics (Pavord *et al.*, 1992; Mellilo *et al.*, 1994). O'Byrne *et al.* (1997) reported that indomethacin blocked the refractory period that follows exercise-induced bronchoconstriction, and that pretreatment with PGE_2 prevented exercise-induced bronchoconstriction. Moreover, inhaled PGE_2 also greatly inhibited both the early and late responses to allergen-induced increases in bronchial reactivity seen in asthmatics (Pavord *et al.*, 1993).

The development of hyperresponsiveness following exposure to an allergen is a complex process, as modeled in Figure 1. Given this model, there are many potential sites of action for PGE_2 in the inhibition of the development of a LAR (Figure 2). In 1993, Hogaboam *et al.* demonstrated that PGE_2 or misoprostol, a PGE agonist, inhibited the release of PAF, TNFα, and histamine from rat peritoneal and intestinal mucosal mast cells. Leal-Berumen et al (1995) found that PGE_2 inhibited production of TNFα in rat peritoneal mast cells. Furthermore, PGE_2 inhibited the release of histamine from hamster cheek pouch mast cells (Raud *et al.*,

Figure 2 Potential mechanisms of action of PGE_2.


MACROPHAGE
↓ IL-1 release
↓ TNFα release
↓ LTB4
NEUTROPHILS
↓ LTB4
EOSINOPHILS
↓ LTC4
↓ LTD4
↓ LTE4
LYMPHOCYTE
↓ LTB4
↓ chemotaxis
PGE2
NERVES
↓ transmitter release
MAST CELL
↑ membrane stability
↑ mediator release
SMOOTH MUSCLE
↑ relaxation
↓ DNA synthesis
↑ cAMP
ion channel effects

1987). It is possible that PGE_2 also prevents the release of histamine and cytokines from airway mast cells. This theory is supported by the observation that PGE_2 inhibited the EAR, which is mast cell dependent, from asthmatic subjects (Pavord, 1993).

PGE_2 has also been demonstrated to have inhibitory effects on the release of mediators from other inflammatory cells. Christman *et al.* (1993) reported that PGE_2 inhibited the synthesis and release of LTB_4 from rat alveolar macrophages. PGE_2 also suppressed the release of IL-1 from murine peritoneal macrophages (Kunkel and Chensue, 1985). In neutrophils, PGE_2 and PGI_2 also inhibited the production of LTB_4 (Ham *et al.*, 1983; Haurand and Flohe, 1989). Furthermore, PGE_2 and other EP receptor agonists inhibited chemotaxis of human neutrophils (Armstrong, 1995). PGE_2, as well as forskolin, inhibited the production of IL-2 from human T cells (Chouaib *et al.*, 1987; Minakuchi *et al.*, 1990; Paliogianni *et al.*, 1993). Finally, PGE_2 inhibited, and indomethacin stimulated, the synthesis of LTC_4 from eosinophils (Tenor, 1996).

PGE_2 does not act only on inflammatory cells, however, as it has direct effects on both nerves and smooth muscle of the airway. PGE_2 inhibits neurotransmitter release, in particular Ach release (Walters *et al.*, 1987; Serio and Daniel, 1989; Johansson-Rydberg *et al.*, 1992; Abela and Daniel, 1995). PGE_2 also acts directly on the smooth muscle to inhibit contraction (Daniel *et al.*, 1987; Abela and Daniel, 1995), likely due to an increase in cAMP (Madison *et al.*; 1989; Coleman *et al.*,

1994). Moreover, PGE_2 inhibited DNA synthesis in human BSM cells, and this has been suggested to inhibit hypertrophy and/or hyperplasia of ASM (Johnson *et al.*, 1995).

7.6 Allergen Inhalation as a Model of Asthma

The model of asthma we used in the present investigations was inhalation of the allergen *Ascaris suum* by dogs. In humans with atopic asthma, allergens are a cause of chronic airway inflammation, and reduction of allergen exposure results in a reduction of asthmatic symptoms (Hendeles and Harman, 1997). Inhalation of allergens by asthmatics produces a physiological response similar to natural exposure of allergen including an EAR and a LAR (Hendeles and Harman, 1997). The EAR is a result of the combination of antigens with IgE antibodies and the resultant release of chemical mediators from mast cells (Hargreave, 1989). The LAR is a result of the interaction of various inflammatory cells, mediators, and cytokines (Pradalier, 1993; Charlesworth, 1996). The LAR is of particular interest, as it has been noted the LAR can be an indication of the severity of asthma (Charlesworth, 1996). Further, it has been suggested that the recent increase in morbidity due to asthma may be a result of clinicians paying inadequate attention to the inflammatory aspects of asthma (Charlesworth, 1996).

Inhalation of the *Ascaris* antigen by dogs also resulted in an EAR in almost all

animals (Booth *et al.*, 1970), and a LAR in approximately fifty percent of animals treated with metapyrone (Sasaki *et al.*, 1987). This percentage is similar to the percentage of persons who develop an EAR that also develop a LAR (O'Byrne *et al.*, 1987; Hargreave, 1989). Further, Itabashi *et al.* (1993) demonstrated that the LAR induced by *Ascaris suum* inhalation in dogs was associated with hyperresponsiveness of ASM. Thus, it was proposed that inhalation of *Ascaris suum* is a useful tool for the study of the LAR (Sasaki *et al.*, 1987).

Using our animal model, we were able to investigate not only the *in vivo* effects of allergen inhalation on airway resistance, but also alterations in smooth muscle contractions *in vitro*. Through the use of pharmacological tools, e.g. indomethacin, we determined the role of different mediators on *in vitro* responses. Further, we were able to measure the amount of mediator released from the tissue. Use of this model will also allow investigation by electron microscopy into which inflammatory cells are recruited to the airway. Moreover, this model could be used to screen potential anti-asthmatic medications.

As with any model, however, certain caveats as to its relevance to human disease are warranted. The relative importance of different mediators may not be the same in the animal model as in humans. For example, PGD_2 and the cysteinyl leukotrienes, contractile agents in the human airway, have little effect of dog ASM (Stevens *et al.*, 1994; Abela and Daniel, 1995). Secondly, it cannot be assumed that what is happening in the muscle bath is also happening in the entire organism.

Damage to tissues during dissection and preparation for muscle bath studies may lead to altered responses. Moreover, dilution of mediators, both inhibitory and excitatory, may occur in the muscle bath.

In the model, we found that demonstration of *in vivo* hyperresponsiveness was not always followed by *in vitro* hyperresponsiveness. In fact, BSM tissues removed from HYP-RESP animals were hyporesponsive to Cch and EFS in the muscle bath.

In the tracheal tissues, removal of the epithelium unmasked hyperresponsiveness. This was presumably due to the removal of inhibitory mediators, including PGE_2, which were released from the epithelium. Removal of the epithelium may not have only removed inhibitory factors released by the epithelium itself, but might have also resulted in removal of inflammatory cells, located in the interstitial tissue between the epithelium and the smooth muscle. These cells may have also been a source of inhibitory mediators, and loss of these cells may have played a role in unmasking hyperresponsiveness.

In bronchial tissues, the addition of indomethacin reduced but did not prevent hyporesponsiveness of tissues from HYP-RESP dogs in the muscle bath. It is possible that other, non-prostanoid, inhibitory mediators were released from the bronchial epithelium, and that removal of the epithelium may have also unmasked hyperresponsiveness of bronchial smooth muscle.

The observation that *in vivo* responses and *in vitro* responses differ is not novel (Vincenc et al., 1983; Roberts et al., 1984; Cerrina et al., 1986). These investigators

suggested that differences in the responsiveness of ASM *in vivo* compared to *in vitro* may be attributed to the site of airway under study. Accordingly, the peripheral airways may contribute more to the increase in pulmonary resistance measured *in vivo* than larger airways. Thus, as we investigated only trachea and third to fifth order bronchi, we may have missed any increased responsiveness present in the lower bronchi and bronchioles.

The fact that exposure to ozone caused airway hyperresponsiveness in healthy humans and animals has led some investigators to study ozone inhalation as a model of asthma (O'Byrne *et al.*, 1984). It has been demonstrated in dogs that the ozone-induced bronchial responsiveness was due to a decreased prejunctional and postjunctional inhibition that was likely mediated by PGE_2 (Janssen *et al.*, 1991). It was therefore suggested that ozone inhalation may cause decreased PGE_2 synthesis, possibly as a result of damage to the epithelium (Janssen *et al.*, 1991).

Heaves is a condition of the airway in horses in which hay-borne antigens lead to airway obstruction, hyperresponsiveness, and inflammation (Gray *et al.*, 1992; Yu *et al.*, 1994). Thus, heaves is also used as an animal model of asthma. In horses with heaves it has been shown that there was a decrease in epithelial-derived PGE_2 compared to horses without heaves (Gray *et al.*, 1992).

The results from the ozone studies and heaves studies suggest that a decrease in PGE_2 results in hyperresponsiveness. In our allergen model, however, we found that an increase in PGE_2 prevented the development of airway hyperresponsiveness

in vitro (NON-RESP). All three animal models clearly indicate that PGE_2 plays a protective role in antigen-induced airway hyperresponsiveness.

7.7 Significance of Findings

The general hypothesis at the onset of our studies was "The production and release of PGE_2 from the epithelium regulates ASM contraction, can account for part of the putative EpDIF function, and plays a role in antigen-induced airway hyperreactivity" (Chapter 1). We believe that our results have confirmed this hypothesis. The findings presented in this thesis aid in the further delineation of the role of PGE_2 in airway inflammation and the modulation of ASM.

We have demonstrated that PGE_2 is derived primarily from an epithelial source. This epithelial source is not of neural origin, but is dependent on EFS and may involve neuroendocrine cells. PGE_2 release was EFS-independent following inhalation of saline or allergen. Also, in animals that developed airway hyperresponsiveness following antigen inhalation, production of PGE_2 was stimulated from a non-epithelial source. Thus, the release of PGE_2 may be induced, following appropriate stimulation, from both the epithelium and from a non-epithelial source, as summarized in Figure 3.

Upon examination of airway hyperresponsiveness in animals exposed to allergen, we found that, in NON-RESP, a greater than normal release of PGE_2 was

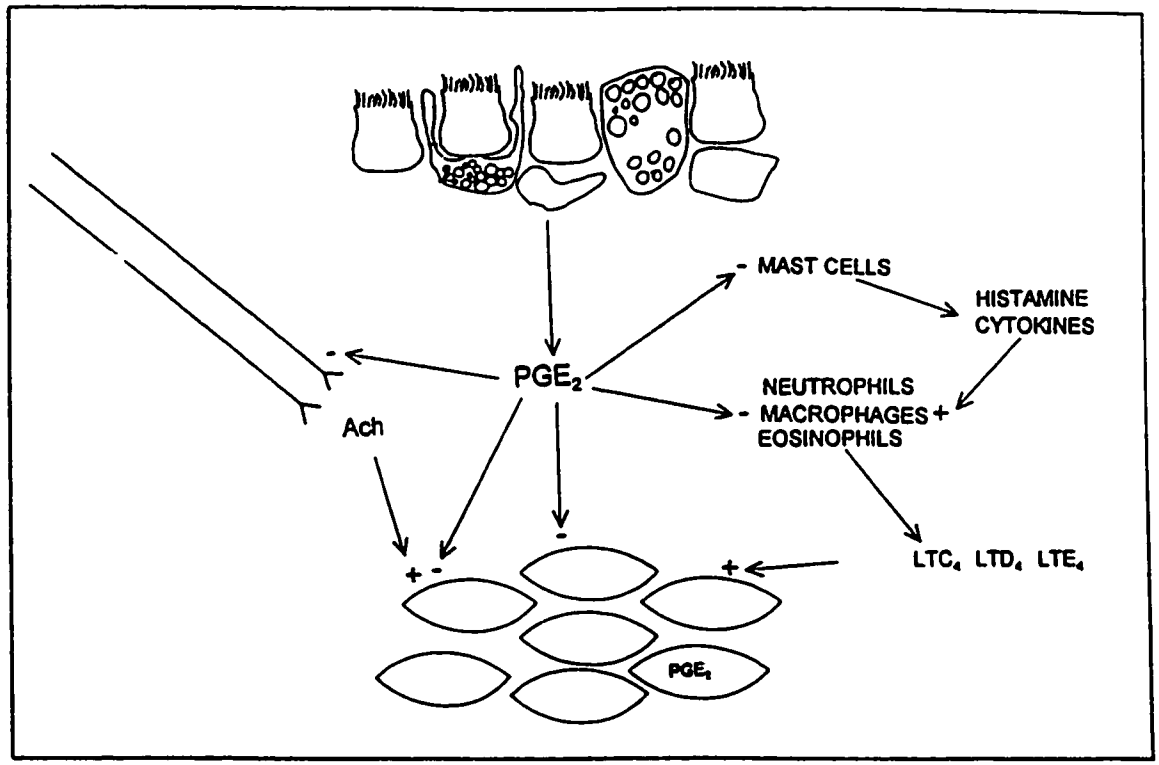
accompanied by a failure to produce hyperrepsonsiveness in TSM, either *in vivo* or *in vitro*. Moreover, removal of the primary source of PGE_2, the epithelium, unmasked an *in vitro* hyperresponsiveness from HYP-RESP animals that was not observed with the epithelium intact. Thus, in the trachea, PGE_2 is a major determinant of *in vitro* HYP-RESP.

Hyperresponsiveness of BSM was not seen *in vitro* from animals that displayed increased responsiveness to Ach *in vivo*. In fact, there was a reduction in the responses to Cch and EFS in these tissues when the epithelium was intact. Inhibition of PGE_2 production inhibited, but did not prevent, this reduction in responses. This reduction was prevented by washing the tissue and thus removing any other inhibitory mediators which may have been present. Thus, in BSM, PGE_2 as well as non-prostanoid inhibitory mediators modulate *in vitro* smooth muscle contractions following *in vivo* exposure to allergen. Therefore, although PGE_2 is important in the modulation of airway responsiveness, it may play a more significant role in TSM than in BSM.

Finally, we investigated a potential mechanism of action for PGE_2 in the relaxation of TSM, an increase in intracellular cAMP resulting in stimulation of the SR-Ca^{2+}ATPase. We demonstrated that this is not a major mechanism by which PGE_2 evokes relaxation of TSM.

Together, the findings presented in this thesis demonstrate the importance of PGE_2, in particular epithelial-derived PGE_2, in the modulation of ASM. Further, they

Figure 3. Summary Diagram. PGE_2, released from the epithelium, inhibits release of Ach, chemotaxis of inflammatory cells, mediator release, and smooth muscle contraction. PGE_2 also may be released from the smooth muscle itself.


MAST CELLS
HISTAMINE
CYTOKINES
PGE2
NEUTROPHILS
MACROPHAGES
EOSINOPHILS
Ach
LTC4 LTD4 LTE4
PGE1

suggest a role for PGE_2 in the prevention of the LAR both *in vivo* and *in vitro*.

It is possible that PGE_2, through many distinct sites of action, is capable of regulating the inflammatory response of the airway to an inhaled antigen. Regulation of which mediators and cytokines are expressed and released from mast cells may determine which inflammatory cells are recruited to the airway. Further, PGE_2 may regulate the release of mediators from inflammatory cells, in particular leukotrienes. Thus, whether or not airway hyperresponsiveness occurs in response to allergen inhalation, may be dependent on the relative level of inhibitory prostaglandins to leukotrienes that are present in the airway.

7.8 Future Directions

Although these studies have expanded our knowledge of the protective roles of PGE_2, there remain many unanswered questions and many avenues for future study.

The question, "what is the nature of non-prostanoid inhibitory factors of epithelial origin?", has yet to be answered. Though this question has been extensively researched, adequate answers have not been found, and it remains to be addressed.

The inflammatory cells which are recruited into the airway during allergen-induced airway hyperresponsiveness in dogs have been studied in BAL (Woolley,

1993; Inman *et al.*, 1996). Currently, this is also being studied in our laboratory through electron microscopy of biopsies from SHAM, NON-RESP, and HYP-RESP animals. Once it is known which inflammatory cells are present, it will be important to determine which if any if these cells are capable of producing PGE_2, and under what stimulus.

As mentioned previously, the antigen challenge experiments need to be repeated such that the organ bath studies performed on TSM strips are compared in the presence of indomethacin or epithelium-denuded BSM. This will allow for closer comparison between the tissues from the two different sites in the airway. Removal of the bronchial epithelium is a technically difficult procedure, and its removal would need to be confirmed through electron microscopy.

With regards to the treatment of asthma, or the prevention of allergen-induced airway responses, it will be important to determine if there is potentially a mechanism by which the production of PGE_2 can be induced from cells already present in the airway, of either epithelial or non-epithelial origin. If, for example, BSM cells can be stimulated pharmacologically to produce PGE_2 during an EAR, the occurrence of a LAR may be prevented.

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APPENDIX 1

METHODS

Methods

Animals

Random source, healthy adult mongrel dogs of either sex were euthanised using pentobarbital administration (100mg/kg body weight). This procedure was approved by the University Animal Care Committee.

Organ Bath Studies

Each segment of trachea was cleaned and the smooth muscle was removed from the cartilaginous tracheal rings. In both epithelium-intact and -denuded tissue the serosal side was cleaned of connective tissue. In epithelium-intact tissue the epithelium was left unaltered. For epithelium-denuded tissues the epithelium was cut away from the smooth muscle and underlying connective tissue was removed. Dissection was performed under a dissecting microscope in order to prevent damage to the underlying smooth muscle fibres.

Segments of tissue, once removed were placed in Krebs solution, constantly bubbled with 95% O_2 and 5% CO_2. The composition of the Krebs solution was (in mM): NaCl, 115.5; KCl, 4.6; $CaCl_2$, 2.5; NaH_2PO_4, 1.6; $MgSO_4$ 1.16; $NaHCO_3$, 21.9;

glucose, 11.1.

The tracheal muscle was cut into strips 1-2 mm wide and 1-2 cm long, parallel to the direction of the smooth muscle fibres. The strips were tied with 4/0 silk thread and mounted in 10 mL organ baths containing the same Krebs solution and bubbled with the same gas mixture as mentioned above. The lower ends of the strips were attached to a hook on the bottom of a plastic holder and the top ends of the tissue were connected to a Grass FT-03C mechanotransducer. Isometric tension was recorded continuously on a Gould 2800 chart recorder. A resting tension of 1.5 grams (previously shown to produce maximum active tension) was applied to each strip. The tissues were equilibrated for 1 h in the organ baths before the beginning of the experiments. They were submerged in the Krebs solution and bubbled at 37°C throughout the experiment. KCl (60 mM) was added to the organ bath to contract the tissue. Fifteen minutes later the KCl was washed out. This procedure was repeated three times, or until consistent, reproducible contractions were generated in each tissue.

For dissection of bronchial tissues, pulmonary lobes were excised, pinned out in physiological solution, and the overlying parenchymal tissue and vasculature were dissected away, thereby exposing the bronchiolar tree from which ring segments were excised (5-10 mm wide; 2-10 mm outer diameter). Ring segments were mounted vertically in 3 ml organ baths using platinum hooks inserted through the lumen (taking care to not damage the epithelium); one of the platinum hooks was

fastened to a force displacement transducer, while the other served as an anchor.

Half of the tissues (tracheal strips or bronchial rings) were pre-incubated for an hour in normal Krebs Ringer solution, the other half in Krebs Ringer containing indomethacin (10^{-5}M). A concentration response curve was generated to carbachol, (10^{-9} - 10^{-5}M), using both epithelium-intact and -denuded tissues. For electrical field stimulation, the tissues were stimulated at 40 V, 0.5 ms duration, at frequencies of 0.1, 0.3, 1.0, 3.0, 10.0, and 30.0 pps.

For cyclic nucleotide studies no indomethacin was present, and all strips were epithelium-denuded. KCl (60 mM) was added to the organ bath to contract the tissue. Fifteen minutes later, or once the muscle had reached a steady level of contraction, the KCl was washed out. This procedure was repeated three times. Cch (10^{-7}M) or KCl (60mM) was then added as a contractile agent until a plateau was reached. At this point the substance used to induce cyclic nucleotide production (INA, forskolin, or NaNP), or the cyclic nucleotide derivatives (8-bromo-cAMP or 8-bromo-cGMP) were added in increasing concentrations (half-log steps) until maximum relaxation was reached. The tissues were then washed and CPA (10^{-5}M) was added to the organ baths. Following a twenty minute wait to ensure complete emptying of the SR, the procedure was repeated in the presence of CPA.

Radioimmunoassays

Samples were collected from organ bath fluid prior to and following electrical field stimulation. The samples were collected using plastic syringes, stored at -70 °C, and thawed immediately before the assay was performed.

Samples were analysed for 6-keto $PGF_{1\alpha}$, the stable metabolite of PGI_2, and PGE_2 content using a commercially prepared kit from Advanced Magnetics. The RIA was based upon competition of the prostaglandin with radioactively labelled prostaglandin for a number of sites on the specific antibody. Antibody-bound prostaglandin was separated from unbound with magnetic dextran-coated charcoal through magnetic separation. The counting rate was correlated with concentration via a standard curve.

In the case of cAMP measurements, tissue strips were equilibrated and precontracted with KCl or Cch. The maximum concentrations of each relaxant agent were added. The tissue was then fast-frozen in liquid nitrogen, and placed in pre-weighed eppendorf tubes. The tubes were placed on dry ice until they were re-weighed to determine the wet weight of the tissue. All samples were then immediately placed in a freezer set at -70 ℃ until the time when the assay could be performed. Radioimmunoassays were carried out using a commercial kit from Cedarlane laboratories.

Antigen Exposure

The method of antigen exposure has been described previously (Wooley, 1993). Dogs were anaesthetized using intravenous pentobarbital sodium (30 mg/kg) to induce surgical anaesthesia; this level of anaesthetization was maintained during the course of the *in vivo* study by additional injections as required. An endotracheal tube and an esophageal balloon catheter were inserted. The endotracheal tube was connected to a constant volume ventilator set at a tidal volume of 10 ml/kg and frequency of 30 breaths per minute. The esophageal balloon catheter and a port at the equipment end of the endotracheal tube were connected to a differential pressure transducer (Hewlett-Packard 267B, Waltham, MA) and pressure amplifier (Hewlett-Packard 8805C) to monitor transpulmonary pressure. Measurements of peripheral resistance (R_L) were obtained at constant volume, and airway responsiveness to aerosolized acetylcholine was assessed 30 minutes after induction of anaesthesia. Animals inhaled acetylcholine (ACh) and a control concentration response curve was generated (0.7 - 80 mg/mL, doubling concentrations). The concentration of ACh which raised the pulmonary resistance 5 cm H_20/l/s above baseline was termed the provocative concentration (PC_5).

Dogs were then exposed to allergen (*Ascaris suum* in 0.9% saline) or saline alone. During this one-time challenge, the concentration of allergen was increased from 10^{-5} M in 10-fold increments until R_L was elevated 10 cmH_2O/l/s above baseline. Each concentration of the allergen was administered for 50 inhalations of 3 seconds duration, with 10 minute intervals between concentrations. Following antigen

inhalation, the dogs were ventilated with air until R_L returned to baseline. The animals were then allowed to recover from the anaesthetic. Twenty-four hours later, dogs were anaesthetized as described above and the airway responsiveness to ACh was again assessed, after which they were euthanised with pentobarbital sodium (100 mg/kg). Dogs were defined as being hyperresponsive when there was a decrease in the ACh-PC_5 of two-fold or more; non-responsive If less than a two-fold decrease in the ACh-PC_5 was observed, and sham if the dogs inhaled vehicle alone (Wooley, 1993). The use of a two-fold difference as the threshold for determining responsiveness was based on previous findings from repeated ACh challenges. These studies demonstrated that the probability of a two-fold difference in the ACh-PC_5 occurring as a result of repeated ACh inhalation was less than five percent likely to be due to chance alone (Inman, personal correspondence).

Drugs Used

Carbachol, indomethacin, nifedipine, isoproterenol, forskolin, sodium nitroprusside, cyclopiazonic acid, 8-Br-cAMP, 8-Br-cGMP, L-655,240, ω-CTX(GVIA), and TTX were obtained from Sigma (St. Louis, Mo.). Indomethacin and L-655,240 were dissolved in a solution of $NaHCO_3$ (1%), which was then incorporated into the Kreb's solution. Nifedipine was dissolved in absolute ethanol. All other agents mentioned above were dissolved in distilled water. *Ascaris suum* extract (0.4 %

phenol) was obtained from Greer laboratories, Lenoir, NC., and was diluted with saline (Wooley, 1993).