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EFFECTS OF ALTERED ARACHIDONIC ACID METABOLISM ON TONE AND NEUROTRANSMITTER RELEASE IN EQUINE AIRWAY SMOOTH MUSCLE

A Thesis

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by

KULBIR K. GILL

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

DEDICATED TO MY FAMILY,

ESPECIALLY

MY PARENTS WHO

ENCOURAGED ME AND STOOD BY ME

EVERY STEP OF THE WAY.

I LOVE YOU.

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ABSTRACT

Equine airway smooth muscle (EASM) is innervated by vagal efferents and, in addition, displays spontaneous mechanical activity. The preparation thus appears to contain at least two discrete excitable components, the cholinergic neural elements and the smooth muscle membrane. Indomethacin (INDO), a cyclooxygenase (CO) inhibitor, exerts a considerable potentiation of function in this preparation. The latter may be effected indirectly, through loss of the inhibitory effect of endogenous prostaglandin E_2 (PGE_2) on neural acetylcholine (ACh) release and through direct effects on smooth muscle of the generally antagonistic CO and lipoxygenase (LO) metabolites. The present studies were designed to assess the relative contributions of altered arachidonic acid metabolism on these respective elements. The utility of the model, in terms of distinguishing neural and myogenic components, was assessed by examining the effects of the muscarinic antagonist atropine (ATR) and the neurotoxin, tetrodotoxin (TTX), on the stimulus-response (S-R) relationship. The substantial rightward (but not downward) shift of the S-R curve effected by ATR (10^{-6}M) is consistent with a selective activation of the muscle by its endogenous neural elements at lower voltages and a direct stimulation of the muscle at higher voltages. This was confirmed with the use of TTX and D-600 (10^{-5}M), a blocker

of potential-dependent calcium channels. TTX (10^{-6}M)) was added to ATR-pretreated muscles to ensure that cholinergic nerves are the only ones relevant to electrical field stimulation (EFS) responses and, thus, the adequacy of the ATR-based model. TTX added to ATR-pretreated muscles had no further effect on the S-R relation. D-600 virtually abolished responses to EFS in ATR- pretreated muscles. Hexamethonium bromide, (HBR, 10^{-5}M) a ganglionic blocker, produced no shift of the S-R curve thus eliminating ganglionic influences from consideration.

INDO (10^{-5}M) potentiated both the neural and myogenic components of the S-R curve, effects which were sensitive to ATR and 5,8,11,14-eicosatetraynoic acid (ETYA, 3.3 x 10^{-5}M)), an inhibitor of LO and PGE_2. The finding that PGE_2 at low doses (10^{-8}M) shifted the S-R curve to the right and that at higher concentration (10^{-7}M) it shifted the S-R curve right- and downward suggested that neurotransmitter release is more sensitive to PGE_2 inhibition than is muscle response. These results suggest that INDO exerts its effects both indirectly, through regulation of cholinergic neurotransmitter release and/or via direct effects relating to decreased levels of cyclooxygenase products and increased lipoxygenase metabolism.

Further studies were designed to assess the effects of a INDO, ETYA, CO and LO metabolites on overflow of acetylcholine from electrically and non electrically -stimulated endogenous cholinergic nerves. These studies utilized EASM

preincubated in radiolabelled choline. INDO enhanced both basal and EFS-stimulated efflux in an ATR-pretreated preparation, an effect which was substantially inhibited by PGE_2 (10^{-6}M). This finding supports the notion that INDO may act through PGE_2-induced inhibition of pre-junctional ACh release. In ATR-pretreated muscles, ETYA inhibited both basal and EFS-induced ACh release. This effect was reversed upon administration of leukotriene D_4 (LTD_4, 6 x 10^{-7}M). These results suggest that lipoxygenase metabolites may also regulate neurotransmitter release.

The present findings suggest that altered arachidonic acid metabolism via the CO and the LO pathway affects at least two components present in EASM, the cholinergic neural elements and the myogenic components. The results show that 1) INDO exerts its effects directly through decreasing levels of CO metabolites and increasing levels of LO metabolites, 2) INDO also exerts its effects indirectly through PGE_2-mediated regulation of cholinergic neurotransmitter (NT) release, and 3) lipoxygenase metabolites enhance pre-junctional cholinergic neurotransmitter release. These results offer direct evidence for a regulatory role of PG's and LT's in NT release at the pre-synaptic membrane.

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Mechanisms affecting tone in smooth muscle.

Smooth muscle is widely distributed throughout the body of vertebrates and is the major constituent of the muscular walls of the alimentary canal, blood vessels, the urogenital tract and the airways. The contractile activity, and consequently the tone, of smooth muscle is under myogenic and neurogenic control, and its proper control is essential for normal functioning of the respective organ systems. In the present context, tone will be defined as the state of activation of muscle strips under specified conditions. It should be noted that under isotonic conditions, an increase in tone will result in shortening while in isometric studies an increase in force is registered.

I) Extrinsic control of smooth muscle tone: Neurogenic

A) Parasympathetic System

The parasympathetic system is the major neural mechanism for the control of airway smooth muscle in humans (Richardson and Beland, 1976), dogs (Brown et al., 1982; Russell, 1978), cats (Olsen et al., 1965), guinea pigs (Coburn and Tomita, 1973), baboons (Middendorf and Russell, 1980) and cows (Kirkpatrick, 1975).

Cholinergic nerve terminals can be identified with electron microscopy on the basis of small agranular vesicles of 50-70 nm diameter (Burnstock, 1970), or by histochemical techniques demonstrating the presence of acetylcholinesterase, diffusely distributed between and on canine tracheal muscle cell membranes. (Suzuki et al., 1976). Richardson and Ferguson (1980) have demonstrated the presence of ganglia receiving preganglionic cholinergic input in airway smooth muscle. Utilizing a ganglion blocker, hexamethonium, they were able to abolish effects of the vagal nerve fibres. This suggested the involvement of ganglia in the parasympathetic pathway.

Vagal stimulation releases acetylcholine (ACh) from small agranular or electron-transparent vesicles. The ACh attaches to muscarinic receptors on the smooth muscle cells and is rapidly hydrolyzed by acetylcholinesterase forming choline and acetate. The acetate is usually lost to the circulation but 50% of the choline is recovered by the nerve for the resynthesis of new transmitter (Burnstock, 1979). Colebatch and Halmagyi (1968) have shown that electrical stimulation of isolated tracheal smooth muscle preparation caused the release of ACh The accompanying contractile response was blocked by atropine, a muscarinic antagonist, but not hexamethonium. This demonstrated that the contraction was mediated by postganglionic cholinergic fibres. Similar results have been shown in the in vitro canine

tracheal preparation (Russell, 1978; Stephens and Kroeger, 1980).

Spontaneous basal release of ACh has been shown in the guinea pig trachea *in vitro* (Carlyle, 1963), in human trachea *in vivo* (Vincent et al., 1970) and in dog trachea *in vivo* (Severinghaus and Stupfel, 1955). Addition of atropine, vagal cooling or vagotomy produced bronchodilation in healthy and asthmatic individuals, as well as dogs. This demonstrated that a low level of cholinergic activity maintains airway smooth muscle tone in these preparations (Severinghaus and Stupfel, 1955).

B) Sympathetic System

Neural control of airway smooth muscle occurs largely through the postganglionic parasympathetic neural release of ACh which stimulates postjunctional muscarinic receptors (Widdicombe, 1963; Russell, 1978) to produce contraction. The presence and functional role of an adrenergic system innervating airway smooth muscle is not clear. Histological evidence indicates the presence of adrenergic nerves in the airways of cats (Silvia and Ross, 1974), guinea pigs (Richardson and Ferguson, 1979) and dogs (Suzuki, 1976). Since many adrenergic fibers accompany blood vessels in the lung (Kadowitz et al., 1976), it is difficult to show a direct control of airway smooth muscle histologically. Bergen and Kroeger (1979) demonstrated a functional adrener-

gic innervation of canine tracheal smooth muscle and the presence of alpha- and beta-adrenoceptors which mediate contractile and relaxant responses respectively.

1) beta-adrenergic control

Propranolol, a beta-adrenoceptor antagonist, relaxes canine airways from the trachea to bronchi of 1.5mm diameter in response to exogenous norepinephrine on a histamine-induced contraction (Russell, 1980). Cabezas et al. (1971), produced bronchodilation in canine trachea by stimulating the thoracic sympathetic nerves in preparations where bronchomotor tone was already present. Further studies have shown that sectioning sympathetic fibers to the airways in dogs resulted in a mild bronchoconstriction. Green and Widdicombe (1966) suggested that the bronchodilator activity served to counterbalance the bronchoconstricting effect of parasympathetic activity and direct measurements have shown a basal rate of sympathetic activity . Goldie et al. (1988) demonstrated a significant beta-adrenoceptor hypofunction in bronchial preparations from severely asthmatic lung. Bergen and Kroeger (1986) showed that a propranolol-sensitive relaxation of canine tracheal smooth muscle could be produced in response to exogenous norepinephrine in the presence of active tone. Thus beta-adrenergic receptors in the canine airways mediate bronchodilation.

2) alpha-adrenergic control

A bronchoconstrictor action of the sympathetic nervous system via alpha-adrenoceptors has been suggested in humans and guinea pigs (Adolphson et al., 1971). Pandya (1977) has demonstrated alpha-adrenoceptor-induced contraction of canine tracheal smooth muscle superimposed on ACh-induced tone without beta-adrenoceptor blockade. He also demonstrated a progressive loss of alpha-adrenoceptor induced response with age. Bergen and Kroeger (1979, 1980) have shown native alpha-adrenoceptor induced contractions (without beta-blockade) to low doses of norepinephrine or to tyramine-induced release of endogenous norepinephrine in canine tracheal muscle with tone elevated by histamine, serotonin, Ach or potassium. They also demonstrated a contraction of tracheal strips from ovalbumin sensitized dogs in response to norepinephrine or tyramine without pre-induced tone. However, littermate controls without pre-induced tone remained quiescent. These results suggested that sensitization may increase alpha-adrenergic sensitivity or responsiveness or that increased endogenous basal tone was present in the sensitized tracheal preparations prior to alpha-adrenergic stimulation.

It should be noted that the expression of alpha-adrenoceptor-modulation of contractile responses depends on pre-existent tone. Bergen and Kroeger (1985) demonstrated that a maximum alpha-adrenoceptor mediated contraction was

produced in canine tracheal smooth muscle when initial tone was developed with potassium, histamine or acetylcholine.

II) Intrinsic control of smooth muscle: Myogenic

A) Metabolic control of smooth muscle tone

1) cyclic nucleotides

In 1960 Sutherland and Rall proposed that the relaxation following stimulation of beta adrenoceptors in smooth muscle by epinephrine was mediated by an increase in cyclic 3', 5'- monophosphate (cAMP) levels. Cyclic nucleotides modulate contraction or relaxation by influencing transport of calcium by cell membranes (Andersson and Nilssen, 1977). Calcium ionophores increase cAMP levels in guinea pig trachea (Creese and Denborough, 1980a).

Bronchomotor tone is prominently regulated by the availability of cytosolic free calcium and the resulting phosphorylation of myosin. Intracellular sequestration or export of calcium results in relaxation of muscle whereas increased intracellular calcium levels enhance muscle contraction.

Whether cAMP modulates contraction or relaxation is still controversial. The relaxant actions of prostaglandins of the E series (Murad and Kimura, 1974), the contractile actions of Ach and histamine (Creese and Denborough, 1980a),

and beta-adrenergic agonists (Katsuki et al., 1977; Marshall and Kroeger, 1973) have all been shown to be mediated by increase cAMP levels. In most cases, however, addition of cAMP to smooth muscle preparations causes a relaxing action (for review see Lundholm et al, 1975).

A role for cGMP in smooth muscle relaxation is a more recent proposal (Schultz et al., 1977; Katsuki et al., 1977). Exogenously added cGMP may relax some smooth muscle preparations with a potency less than cAMP (Lundholm et al., 1975). Thus cGMP was originally speculated to participate in smooth muscle contraction as its levels were shown to increase after acetylcholine administration. It has since been shown that cGMP plays a functional role in smooth muscle relaxation. Kukovetz et al. (1979) reported a cGMP-mediated relaxation in coronary smooth muscle. Subsequent investigations have demonstrated an increase in cGMP levels and in cGMP-dependent protein kinase activation coincident with the mechanical effects of vasodilators (Furchgott et al., 1984; Rapoport et al., 1983).

cGMP appears to be the intracellular messenger involved in smooth muscle relaxant effects of three groups of vasodilators, the atrial naturietic factors, the nitrovasodilators (e.g. nitroglycerin, sodium nitrite) and the endothelium dependent vasodilators (e.g. histamine, acetylcholine). For an excellent review see Fiscus (1988).

2) Protein kinase C

In the late 1940's polyphosphoinositides were isolated from the brain by Folch. Hokin and Hokin (1954) reported that cholinergic stimulation of pancreatic cells increases the turnover of phosphatidylinositol. Hawthorne and coworkers (1977) described the breakdown of phosphatidylinositol 4,5-bisphosphate in iris smooth muscle to yield inositol triphosphate and diacylglycerol (DAG). Nishizuka (1984) then demonstrated that DAG activated protein kinase C (PKC). Recently, Rasmussen et al. (1987) proposed a theory stating that PKC participates in smooth muscle contraction and maintains contraction at low energy costs by phosphorylation of cytoskeletal elements.

The effects of temperature on pathways involving PKC in guinea pig tracheal smooth muscle was examined in Menkes's lab (1987). He and his co-workers discovered that contractions elicited by phorbol esters, activators of PKC, at low temperature were inhibited by 5-lipoxygenase inhibitors, ETYA (5,8,11,14- eicosatetraynoic acid) and NDGA (nordihydroguaiaretic acid). Thus cooling has the potential to modify a major intracellular pathway regulating physiological responses of the airways.

PKC activation by phorbol 12-myristate 13-acetate resulted in no detectable rise in cytosolic calcium but completely blocked release of internal calcium by histamine in cultured airway smooth muscle cells (Kotlikoff et al., 1987).

3) Inositol tri-phosphate

One of the products of the hydrolysis of phosphatidylinositols is inositol triphosphate which release intracellular calcium (Berridge and Irvine, 1984). Rapoport (1987) demonstrated that norepinephrine-induced contraction in rat aorta is associated with increased hydrolysis of phosphatidylinositols. This may occur through the activation of phospholipase C by alpha-adrenoceptor agonists. This was shown to be the case for many agonists that induced contraction in vascular smooth muscle (Griendling et al., 1986), and bovine tracheal smooth muscle (Takuwa et al., 1986).

Inositol triphosphates generated upon receptor activation can induce calcium release in smooth muscle cells (Hashimoto et al., 1986). For a review on inositol triphosphates refer to Berridge and Irvine (1987).

B) Membrane related control of smooth muscle tone

1) Ion transport mechanisms and control of smooth muscle tone

Ion transport mechanisms control smooth muscle tone indirectly through control of ion gradients in both muscle cells and endogenous neural elements.

The principal ion transport mechanism in smooth muscle cells is generally the sodium/potassium (Na/K) pump which has four main functions. It supports ion gradients which are essential for a normal transmembrane potential in the muscle and the nerve, the Na gradient supports sodium/calcium, sodium/hydrogen exchange and its electrogenicity affects the membrane potential (E_m).

Generally, K permeability is greater than Na permeability and the operation of the pump results in normal membrane potential.

The Na/Ca exchange is influenced by Na/K transport. The former involves calcium extrusion driven by the sodium gradient. Scheid et al. (1979) reported that isoproterenol-induced relaxation of isolated cells from Bufo Marinus stomach was ouabain sensitive and proposed a model involving Na/Ca exchange driven by the Na/K transport. At the present moment, the role of Na/Ca exchange in smooth muscle contrac-

tion is very controversial (VanBreemen, 1979; Blaustein et al., 1986).

The Na/H exchange is also influenced by Na/K pump action. Na/H is an electroneutral way of regulating cytoplasmic pH in many vetebrate cells (Bobik et al., 1988). Alterations in cytoplasmic pH profoundly affects biochemical processes within the cell (Busa and Nuccitello, 1984). Many activation processes are associated with increased cytoplasmic pH or increased cellular acidity (Grinstein and Rothstein, 1986). The Na/H exchange is also regulated by cytosolic calcium and these and other factors have been discussed in an excellent review by Grinstein and Rothstein, 1986.

The Na/K transport mechanism may also have a direct electrogenic contribution to the membrane potential (Fleming et al., 1980) and thus affect muscle tone. Na/K pump stabilizes the membrane by hyperpolarizing the membrane potential causing the cell to be less excitable and therefore less responsive to depolarizing agonists. Kolbeck et al. (1982) demonstrated that ouabain, an inhibitor of the Na/K electrogenic pump, caused a contraction in guinea pig trachea that was 40% of the maximum produced by histamine. Souhrada et al. (1981) suggested that the electrogenic Na pump may be involved in allergic airway disease as they reported that the resting membrane potential of the guinea pig trachea was hyperpolarized by 15mV upon active immunological sensitization, a phenomenon that was ouabain-sensi-

tive. Electrogenicity may contribute to hyperpolarization, but its role in the control of tone is controversial.

2) Autacoids

A variety of membrane related receptors have been documented that interact with agonists to control tone in smooth muscle. These include histaminergic-, serotonergic-, vasoactive intestinal peptidergic- and substance P receptors.

The complexity of neural control of airways is increasingly appreciated. In addition to classical cholinergic and adrenergic pathways, neural mechanisms which are neither adrenergic nor cholinergic have been described (Barnes, 1986b). There is increasing evidence that neuropeptides may be the neurotransmitters of these nonadrenergic noncholinergic (NANC) nerves. Both excitatory and inhibitory NANC have been described in airways (Diamond and Altiere, 1989; Barnes, 1987c), but the physiological significance of these pathways will remain uncertain until specific blockers become available.

a) Histamine

Histamine is released from mast cells upon degranulation and causes constriction. Its actions may be effected via a direct pathway on smooth muscle cells (Antonissen et al., 1980) or indirectly on a vagal reflex

(Yanta et al., 1981). It causes constriction of isolated airways through H_1 (mepyramine-sensitive) receptors. H_2 (metiamide sensitive) receptors exist in sheep (Eyre, 1973) and cats (Eyre, 1973; Moengwyn-Davies, 1968) and produce bronchorelaxation. Histamine H_1 affects smooth muscle contraction, increases vascular permeability (Metzger et al., 1985), neural reflexes (Shore et al., 1985) and induces tachycardia (Levi et al., 1982). Histamine H_2 receptors affect mucus secretion (Shelhamer et al., 1980).

Histamine-induced generation of prostaglandins is also of interest in asthma. Platshon and Kaliner (1978) showed that antigen-induced anaphylaxis of human lung resulted in release of histamine as well as PGF_{2a}, PGE_2 and thromboxane-B_2. Antol et al. (1988) recently demonstrated that histamine tachyphylaxis occurs in anesthetized dogs <u>in vivo</u> despite prostaglandin synthesis inhibition with indomethacin. In contrast, Tesarowski (1987) demonstrated that INDO potentiated responses to histamine by apparent reversal of tachyphylaxis. The suggested mechanism involved the rerouting of arachidonic acid substrates to the contractile lipoxygenase pathway as well as diminished prostaglandin production. Shore et al. (1983) suggested that histamine increases ACh release from cholinergic nerves or interacts supra-additively with ACh in smooth muscle. However Antol et al. (1988) showed that histamine tachyphylaxis occurred even after pretreatment with large doses of atropine. Thus the mechanism of histamine tachyphylaxis

remains controversial and may involve receptor uncoupling and/or down regulation of histamine receptors.

b) Serotonin

Serotonin, or 5-hydroxytryptamine (5-HT), causes contraction in a variety of tissues including rat aorta (Cohen et al., 1981), rat portal vein (Lemberger et al., 1984) and guinea-pig tracheal smooth muscle (Cohen et al., 1985). Serotonin receptors have been found in the guinea pig trachea (Cohen and Wittenhauer, 1987) and in the canine trachea (Gunst et al., 1987). Recent work with infant lung shows that 5-HT-immunoreactivity is restricted to non-ciliated bronchial epithelial cells (Plowman et al., 1988).

Serotonin receptor activation in rat aorta results in an increase in phosphoinositide turnover (Nakaki et al., 1985). Recently, however, Cohen and Wittenauer (1987) demonstrated that although serotonin was a potent contractile agonist in guinea pig trachea and rat stomach fundus, it did not increase inositol monophosphate formation. Thus the biochemical mechanisms associated with receptor activation may not always be the same. The reader is referred to an excellent review by Frohlich and Van Zwieten (1987) regarding serotonin, its receptors and its actions.

c) Nonadrenergic, noncholinergic system

The airways of many species, including guinea pig, cat, chicken, sheep, monkey, baboon, and man are innervated by a nonadrenergic, noncholinergic (NANC) inhibitory system (Diamond and Altiere, 1989). Other species, such as dog, pig and rat do not possess an airway NANC inhibitory system (Diamond and Altiere, 1989). Barnes et al. (1986b) demonstrated that electrical field stimulation of intramural nerves in human isolated central airway elicits a biphasic response comprising an initial contraction followed by a prolonged relaxation. The contractile phase is abolished by muscarinic cholinoceptor antagonists, whereas the relaxant phase is only partly attenuated by beta-adrenoceptor blockade. The relaxation response remaining is defined empirically as being the NANC inhibitory response. Of the several neuropeptides identified in airways, only vasoactive intestinal peptide (VIP), the related peptide histidine isoleucine (PHI) and its human equivalent, peptide histidine methionine (PHM), relax airway smooth muscle (Barnes, 1988).

i) Vasoactive Intestinal Peptide (VIP)

VIP, a 28 amino acid peptide originally discovered as a vasoactive substance in lung extracts, potently relaxes airway smooth muscle *in vitro* (Said, 1982). VIP is the neu-

ropeptide which is most abundant in human lung and immunoreactive nerve fibres have been located in large airways in humans (Laitinen et al., 1985). VIP-induced relaxation of human bronchi in vitro is 50-100 times more potent than isoprenaline, making it the most potent endogenous bronchodilator so far discovered (Palmer et al, 1986a). Mapping of VIP-receptors in human lung by autoradiography confirms the presence of receptors on airway glands, epithelium and vascular smooth muscle, and also demonstrates the presence of receptors on smooth muscle of bronchi but not bronchioles (Carstairs and Barnes, 1986a). Binding of VIP to its receptors stimulates cyclic AMP formation, thereby leading to relaxation of airway smooth muscle (Lazarus et al., 1986).

Ultrastructural studies suggest that VIP may be present in cholinergic nerves in the airways, and may therefore function as a co-transmitter with ACh (Laitinen et al, 1985). It is possible that VIP is co-released only under certain patterns of neural activation, such as high frequency firing, acting as a protective mechanism. In bovine tracheal smooth muscle VIP reduces the contractile effect of exogenous ACh only with high frequency firing (Palmer et al., 1986a). This indicates that VIP counteracts cholinergic bronchoconstriction and thus may function as a 'braking' mechanism for airway cholinergic nerves (Barnes, 1987c). Definitive evidence for VIP and its actions awaits the development of specific antagonists. Currently available ones lack specificity and potency (Thompson et al.,

1989). Matsusaki et al. (1980) reported that VIP antibody attenuated NANC relaxant responses in the guinea pig trachea, suggesting a role for VIP as the NANC neurotransmitter. A detailed analysis is beyond the scope of this text (for a excellent review of VIP, PHI and PHM, refer to Barnes, 1988).

ii) Substance P

Substance P (SP) is localized to unmyelinated sensory nerves (C-fibres) in airways and contracts airway smooth muscle (Barnes, 1987c). Autoradiograph studies suggest that SP receptors are localized to smooth muscle of all airways in man and guinea pig (Carstairs and Barnes, 1986b), and may therefore regulate tone in these small airways. Non-cholinergic bronchoconstrictory nerves have been demonstrated in guinea pigs. The effects of nerves stimulation are antagonized by SP antagonists, suggesting that SP may be the excitatory neurotransmitter (Andersson and Grundstrom, 1983). Capsaicin, the "hot" extract of pepper, releases SP from unmyelinated sensory nerve endings and, in rats and guinea pigs, causes acute bronchoconstriction and airway microvascular leakiness (Saria et al., 1983). Chronic treatment with capsaicin leads to depletion of SP-immunoreactivity and is associated with a reduced bronchoconstrictor response to allergen in sensitized animals (Saria et al., 1983). While these effects of SP are

seen in rodents, their relevance to human airways is less certain. Reports of SP innervation of human airways are conflicting. Lunberg et al. (1983) reported that SP caused contraction of human airways in vitro. However rapid decrease of SP with age and smoking explains the difficulty in demonstrating this peptide in some studies (Fuller et al., 1985).

SP is representative of a novel class of neuropeptides, the tachykinins. While SP was isolated over 50 years ago, tachykinins called neurokinase A and B (Nawa et al., 1983), calcitonin gene-related peptide (Lundberg et al., 1985) and neuropeptide Y (Sheppard et al., 1984) have been recently identified. For a detailed review of SP and the neuropeptides, refer to Barnes, 1988.

3) Arachidonic acid metabolites and the control of smooth muscle tone.

Arachidonic acid metabolites constitute potent modulators of smooth muscle tone in the gastrointestinal and urogenital tracts, in vascular and pulmonary systems, and they affect glandular and neuronal function (see review by Bergstrom et al., 1968). Arachidonic acid is a membrane bound phospholipid in cell membranes. Upon stimulation by mechanical deformation of membrane or by ACh, histamine, norepinephrine and a large number of other agents (Burka, 1983), it is metabolized by phospholipase A or phospholipase

C into the free arachidonic acid. The latter is then metabolized via the cyclooxygenase pathway or the lipoxygenase pathway. The cyclooxygenase pathway leads to the formation of prostaglandins (PG) with their characteristic cyclopentane ring structure and the bicyclic thromboxanes (Tx). The lipoxygenase pathway results in the production of hydroperoxy-eicosatetraynoic acids (HETE), leukotrienes (LT) and lipoxins (LX).

a) Inhibition of arachidonic acid release

Corticosteroids inhibit phospholipase A_2 activity, ultimately inhibiting the formation of cyclooxygenase and lipoxygenase metabolites. Their inhibitory action is due to the release of the protein lipocortin (Wallner et al., 1986). There has been some dispute over lipocortin action for it appears to be identical to calpactins. The latter bind calcium and also phospholipid and this property is responsible for the reduction in eicosanoid formation (Davidson et al., 1987).

b) (i) Cyclooxygenase products and pathway

Arachidonic acid is primarily esterified at the 2 position of cellular phospholipids and composes nearly 20% of the membrane lipid of rabbit alveolar macrophages (Mason et al., 1972) and human blood monocytes (Stossel et al,

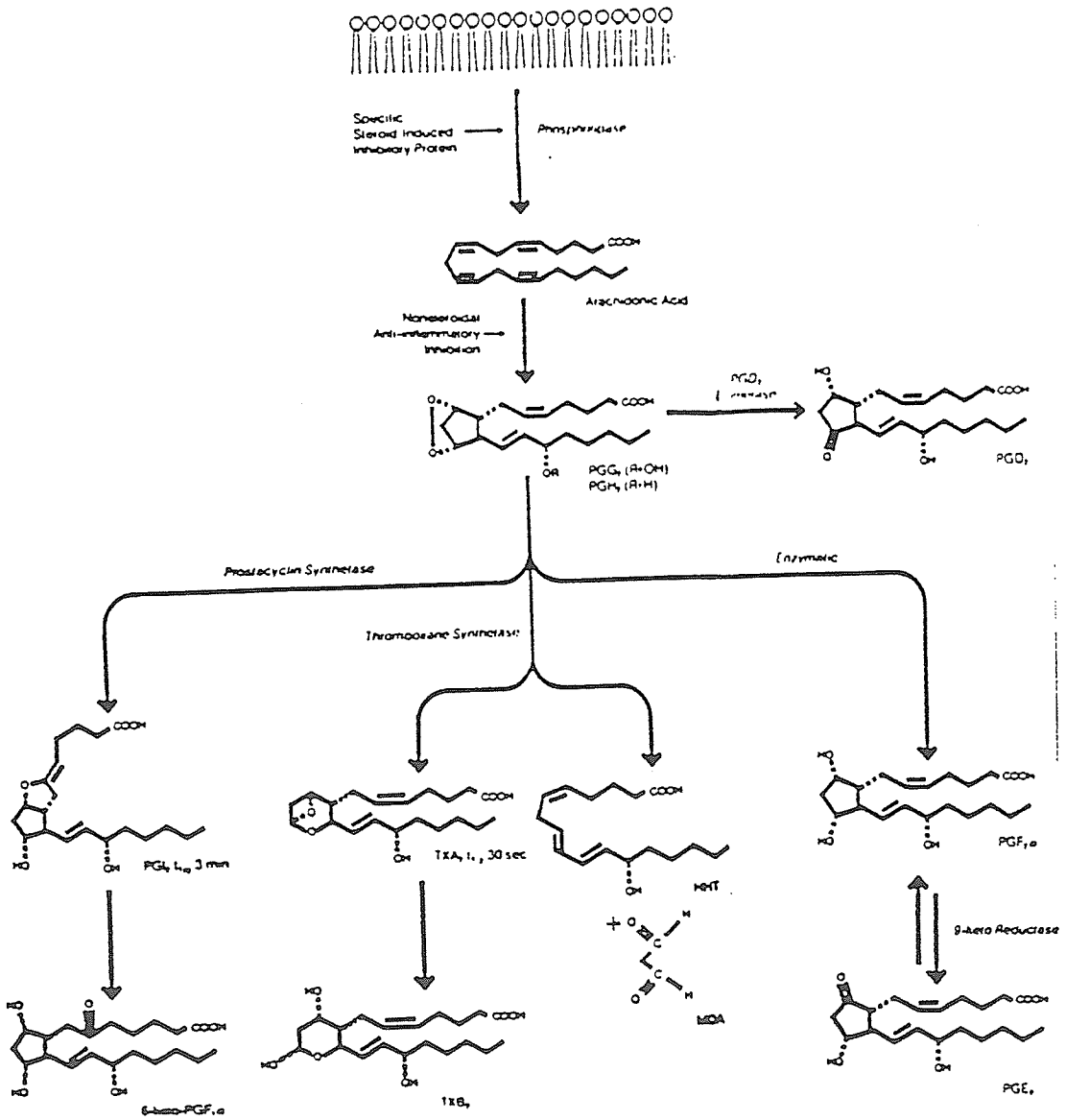
1974). A bis-dioxygenation reaction with arachidonic acid occurs via the cyclooxygenase pathway to form prostaglandin (PG) G_2, which is unstable and rapidly converted by a hydroperoxidase to another unstable endoperoxide PGH_2 (Figure 1, Gorman and Marcus, 1981). The endoperoxides are capable of stimulating contraction in vascular (Kadowitz et al., 1977), gastrointestinal (Moncada et al., 1976) and bronchial smooth muscle (Hamberg et al., 1975). Wasserman (1976) and Spannhake et al. (1978) demonstrated that injections of stable analogs of endoperoxides into dogs resulted in increases of airway resistance, decreases in compliance and an increase in respiratory rate.

The bronchoconstrictor PGD_2 and the bronchodilator PGE_2 are formed from PGH_2, either nonenzymatically or enzymatically, by the action of a PGH-PGD isomerase or a PGH-PGE isomerase; also formed from PGH_2 is PGF_{2a}, a bronchoconstrictor (Hamberg and Samuelsson, 1974).

Thromboxane (Tx) A_2 and the prostacyclin (PGI_2) synthetases act upon the PG endoperoxides to form TxA_2 and PGI_2 respectively (Hamberg and Samuelsson., 1974; Moncada et al., 1976).

TxA_2, a potent platelet aggregator, is very labile and is usually isolated in biological samples as its inactive hydrolysis product TxB_2 (Hamberg et al., 1975). PGI_2, which inhibits platelet aggregation, is also very labile in aqueous solution and forms its inactive hydrolysis product 6-keto-PGF_{1a} (Lands, 1979).

Figure 1. The cyclooxygenase pathway of arachidonic acid metabolism. This diagram is a modification of one presented by Gorman and Marcus (1981).



(ii) Inhibition of cyclooxygenase

Non-steroidal anti-inflammatory drugs are the drugs most widely used to inhibit cyclooxygenase (See Flower, 1974 for review). Vane (1971) described the ability of acetylsalicylic acid (ASA) to inhibit PG production. ASA is an irreversible inhibitor as it acetylates a serine residue on the active site of cyclooxygenase (Roth et al., 1975). Indomethacin (INDO) is also a cyclooxygenase inhibitor. Its action exhibits time and substrate dependency (Gryglewski, 1978). Berend et al. (1986) found that in inflammation-induced airway hyperresponsiveness, not only was PG formation inhibited but so was that of TxA_2.

INDO has varying effects on different tissues from different species. In the canine trachea, which has no discernable basal tone, INDO has no effect but potentiates responses to histamine (Anderson et al., 1980.) In the equine trachea, which has the potential for phasic activity and sharp twitches associated with ACh, ouabain and near threshold potassium depolarization, INDO induces an increase in basal tone (Tesarowski, 1987).

Orehek and co-workers (1975) demonstrated that guinea pig trachea responded to INDO with a relaxation. They suggested that intramural release of contractile PGF_{2a} was responsible for the inherent tone in the preparation. Burka (1988), in contrast, suggested that the major contributor to

intrinsic tone in guinea pig trachea was PGE_2, and that this prostanoid is contractile at low concentrations.

Arachidonic acid-induced relaxation of the guinea-pig trachea was converted to contraction with the addition of INDO (Burka and Saad, 1984). Farmer et al. (1987) demonstrated that this contraction was abolished with NDGA, suggesting that INDO, in addition to inhibiting CO may also be redirecting substrates to the competing LO pathway.

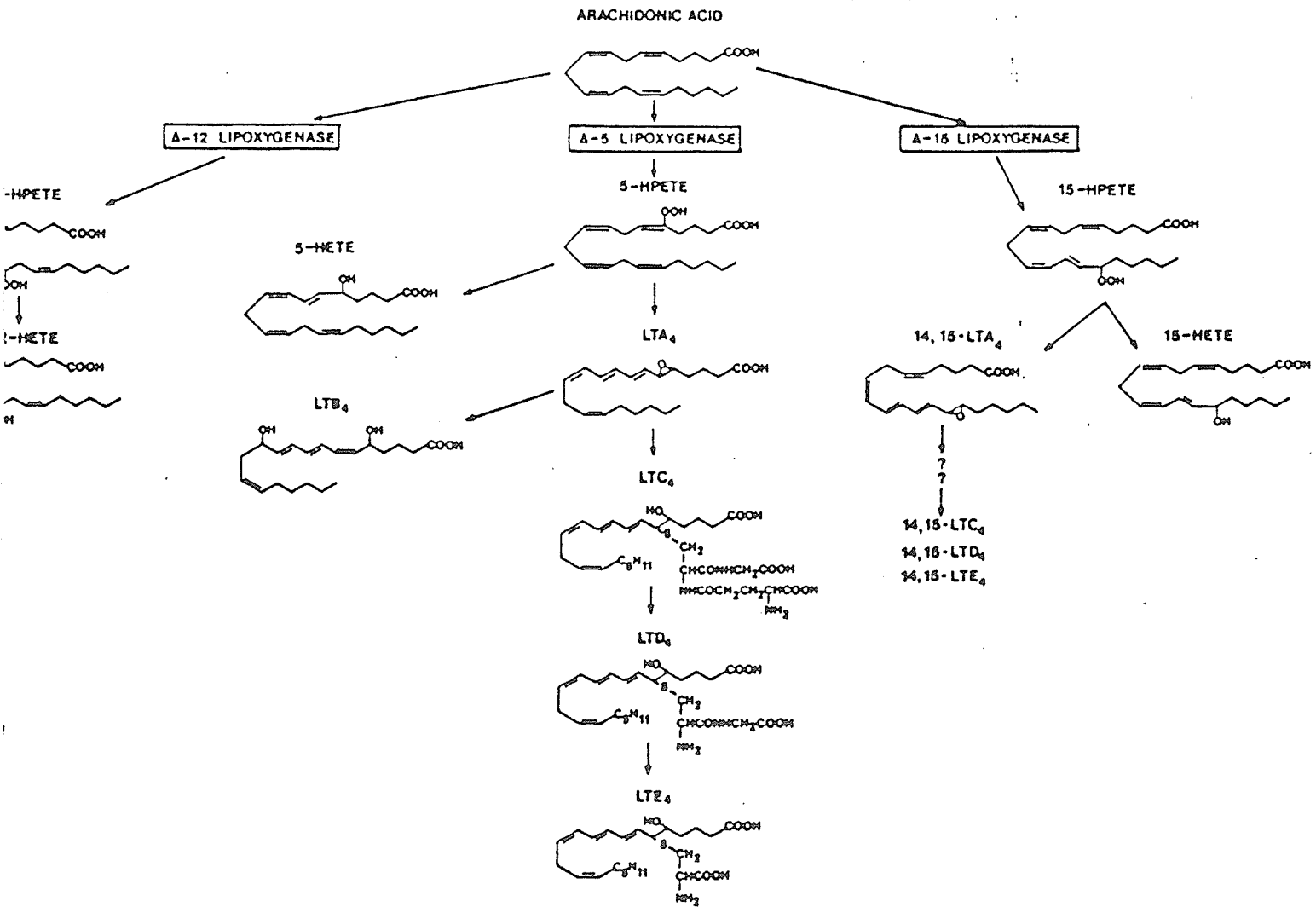
c) (i) Lipoxygenase products and pathway

Initial peroxidation of arachidonic acid is catalyzed by the lipoxygenase enzymes to form hydroperoxy-6,8,11,14-eicosatetraynoic acids (HPETEs) (Samuelsson, 1983). HPETEs are the precursors of hydroxyeicosatetraynoic acids (HETEs) via a reaction that is catalyzed by glutathione peroxidase system. HPETEs are also the key intermediates in the formation of leukotrienes (LT) with their conjugated triene structure (Samuelsson, 1983). Figure 2 illustrates the scheme of events (Anderson, 1985).

LTA_4 is an unstable epoxide derivative of 5-HPETE. The addition of water to LTA_4 by an epoxide hydrolase forms LTB_4 which has potent chemotactic activity (Goetzl et al., 1981).

The addition of glutathione by glutathione transferase to the epoxide ring of LTA_4 at the C-6 position leads to the formation of a group of sulfidopeptide LT, LTC_4, LTD_4 and LTE_4, that can cause increased vascular permeability and

Figure 2. Transformation of arachidonic acid by 5-, 12-, and 15- lipoxygenase. This diagram is taken from Anderson (1985).



smooth muscle contraction. Synthesis of various sterioisomers of the sulfido-leukotrienes has indicated that their hydrophobic region is necessary for contractile activity (Drazen et al., 1981).

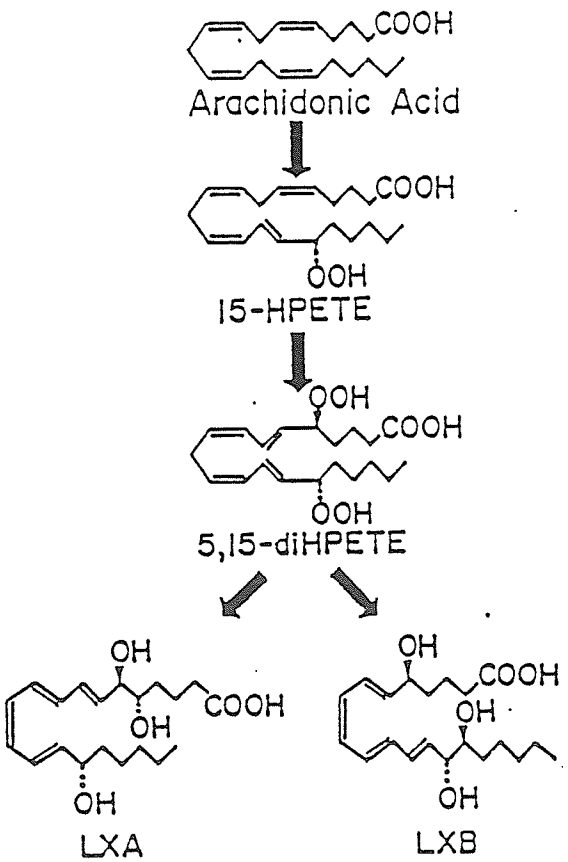
Lipoxins (LX, "lipoxygenation interaction products"), have a unique synthesis pathway (Figure 3, Yamamoto et al., 1988). They are formed by oxygenation of arachidonic acid by 15-lipoxygenase and subsequent oxygenation by a dual function 5-lipoxygenase involving oxidation and dehydration to yield 5,6-epoxytetraene. This epoxide is then opened by electrophilic attack at the C-6 by an epoxide hydrolase to yield LXA_4, or via a conjugate attack of hydrolase at the 14-position to yield LXB_4. (For review see Samuelsson et al., 1987).

(ii) Inhibition of lipoxygenase products

In the past, specific antagonists for 5-lipoxygenase have been difficult to synthesize, although several dual inhibitors of both the cyclooxygenase and lipoxygenase pathways are available. These include BW755C (Higgs et al., 1979) and ETYA (5,8,11,14-eicosatetraynoic acid).

NDGA, nordihydroguaiaretic acid, a catecholic antioxidant, inhibits soyabean lipoxygenase-1 very effectively (Flamberg et al., 1988). However, Burka (1988) recently noted that NDGA not only inhibited an LTD_4 contraction in

Figure 3. Lipoxins A and B derived from 5, 15-diPETE. This diagram is taken from Yamamoto et al. (1988)..



guinea pig trachea, but also enhanced contractions to histamine and carbachol. He proposed that it might also be exerting some effect on the cyclooxygenase pathway.

Recently, a novel series of acetohydroxamic acids have been described, BW A4C, BW797C and BW A137C that selectively inhibits 5-lipoxygenase in anaesthetized, ovalbumin sensitized guinea pigs challenged with antigen (Tateson et al., 1988).

Koshihara et al. (1988) reported that a compound called AC-5-1, isolated from an Indonesian plant, Artocarpus communis, was a highly selective 5-lipoxygenase inhibitor with a half inhibition dose of $5.00 \pm 0.12 \times 10^{-8}$M.

d) Cyclooxygenase products and smooth muscle (an overview)

In most vascular beds, PG's of the E series are potent vasodilators, often resulting in a reflex increase in cardiac output. PGI_2 seems to occupy a role in the maintenance of cardiac homeostasis. The effect of PGD_2 in the vascular bed depends on species and the tone of the vessel. In the guinea pig, it causes a drop in blood pressure, followed by the pressor effect. In the sheep, rabbit and pig, PGD_2 only causes a pressor effect (Jones et al., 1978). PGE_2 and PGD_2 can potentiate the effects of histamine and bradykinin on vascular permeability and cause hyperalgesia to both agents (Williams and Peck, 1977). PG's

also produce labour-like contractions of the uterus (Goldberg and Ramwell, 1975) and stimulate the gastrointestinal musculature causing nausea, vomiting, diarrhea and cramps. PG's of the E series and its analogs inhibit gastric acid secretion and are cytoprotective to the gastric and intestinal mucosa (Roberts, 1979). PGD_2, PGI_2 and PGE_1 all inhibit platelet aggregation (Tateson et al., 1977.) and increase cAMP levels in airway smooth muscle (Murad and Kimura, 1974).

e) Cyclooxygenase products and airway smooth muscle

(i) Prostaglandins of the E series

PGE_2 generally relaxes airway smooth muscle from most species including guinea pig (Schneider et al. 1980), dog (Krell et al., 1978), human (Karim et al., 1980) and horses (Tesarowski, 1987). It has been demonstrated to increase cAMP-dependent protein kinase activity and increase phosphorylation of microsomal proteins and myosin light chain kinase, consistent with a role for cAMP in relaxation (Torphy et al., 1982). The smooth muscle relaxation produced by PGE_2 is thought to be mediated by the phosphorylation of specific cellular proteins through the hormone-receptor-adenylate-cyclase protein kinase cascade. PGE_2 increases cAMP concentrations and cAMP-dependant protein kinase activ-

ity in the canine TSM thus causing relaxation (Torphy et al., 1982).

The relaxations elicited by the PGE series (PGE_1, PGE_2 and PGE_3) are not affected by atropine, mepyramine, methysergide or alpha- and beta-adrenoceptor blocking agents, therefore, these events seem to have a direct action on the smooth muscle cells, possibly through a specific prostaglandin receptor (Mathe, 1976).

Ito and Tajima (1981) noted that low concentrations of PGE_2 (10^{-11} to 10^{-8} M) markedly reduced the amplitude of the twitch contractions and excitatory junction potentials (e.j.ps) evoked by activation of excitatory cholinergic fibres in the canine trachea, with no change in resting membrane potential, input resistance and sensitivity of the muscle membrane to exogenous ACh. Thus, the nerve terminals of the cholinergic fibres were much more sensitive to PG's than are the smooth muscle cells in the dog trachea. SC-19220, a selective antagonist for PGE_2 and PGF_{2a} (Farmer et al., 1974) in the guinea pig trachea and INDO reversed the decremental response of twitch contractions or e.j.ps evoked nerve stimulations and SC-19220 produced a sustained contraction of the dog tracheal tissue which was suppressed by atropine or PGE_2 (Inoue et al., 1984). This evidence indicates that endogenous PG's play a role in inhibiting the release of ACh from vagal nerve terminals in both resting and active states.

Walters et al. (1984) noted that the contractile response of canine tracheal smooth muscle to electrical field stimulation declined over 2 hours while measured PGE_2 increased. The addition of INDO reversed this decline and actually increased electrical field stimulated responses. In contrast, incubation alone, treatment with INDO or addition of PGE_2 had no effect on ACh-induced contractions. They concluded that progressive increase of PG release from canine trachealis occurs and inhibits pre-junctional cholinergic neurotransmitter release.

(ii) Prostaglandin D_2

PGD_2 is the major prostaglandin generated from human lung and rat peritoneal mast cells (Lewis et al., 1982). It not only causes bronchoconstriction in dogs (Wasserman et al., 1980), monkeys (Patterson et al., 1980), and humans (Hardy et al., 1984) but also potentiates the bronchoconstrictor response to various inhaled stimuli in patients with atopic asthma (Fuller et al., 1986) suggesting that it may cause airway hyperresponsiveness.

Tamaoki et al. (1987) showed that PGD_2 induced increases in active tension in canine tracheal smooth muscle, an effect that was attenuated by pretreatment with atropine. This suggests that, in addition to a direct action of PGD_2 on airway smooth muscle, the airway response to PGD_2 is partially mediated by a local cholinergic

muscarinic action. This notion is also supported by their finding that physostigmine, an acetylcholinesterase inhibitor, enhanced the contractile response to PGD_2. Hexamethonium, a ganglionic blocker, did not inhibit the contractile response to PGD_2 therefore ruling out the involvement of ganglia. These observations have implicated PGD_2 as a neuromodulator via pre-synaptic potentiation of vagally induced contraction of airway smooth muscle. Omini et al (1986) reported that PGD_2 potentiated contraction due to post-ganglionic parasympathetic fibre stimulation in guinea pig trachea. In contrast Armour et al. (1988) found that PGD_2 inhibited both pre- and postganglionic stimulation-induced contraction in the rabbit trachea. They proposed that since PGE_2 and PGD_2 are isomeric, the effects of PGD_2 may be mediated through the PGE_2 receptors.

(iii) Prostaglandin F_{2a}

PGF_{2a} constricts guinea pig trachea and parenchyma (Ono, 1979), human bronchioles (Hutas et al., 1981), and horse isolated airway smooth muscle (Mirabahar, 1985). Dog and cat trachea are unresponsive to PGF_{2a} although smaller airways do exhibit some response (Lulich et al., 1976). PGF_{2a} also primes tissues to other agonists. Subthreshold doses of PGF_{2a} causes a leftward shift of the dose response curve to histamine (Walters et al., 1982).

This prostanoid has also been reported to affect neuromuscular transmission (Leff et al., 1985). Armour et al. (1988) demonstrated that PGF_{2a} potentiated contractile responses to electrical field stimulation in rabbit bronchi. They further demonstrated that this potentiation was at a site distal to the parasympathetic ganglion as PGF_{2a} induced a significant increase in both pre- and post- ganglionic stimulation to 140% of the control values.

(iv) Prostacyclin

Prostacyclin was discovered accidentally while Moncada and coworkers (1976) were attempting to study thromboxane generation in blood vessels and microsomes. They uncovered an unstable substance which relaxed blood vessels and inhibited platelet aggregation. These features are the physiologic opposites of thromboxane A_2.

PGI_2 is released from both human bronchi and lung parenchyma and guinea pig lung parenchyma on immunological stimulation. It is a weak bronchodilator of guinea pig airways in vitro (Burka, 1983). Its activity may be predominant in pulmonary vascular bed as PGI_2 receptors linked to adenylate cyclase in the lung are located on vascular tissue and not on airways (MacDermot et al., 1981).

(v) Thromboxane

TxA_2 is a potent bronchoconstrictor, but is released in low concentrations in humans (Schulman et al., 1981). It may contribute to allergic bronchoconstriction, but may also contract pulmonary vessels and induce intravascular platelet aggregation which can lead to the release of other spasmogens, such as serotonin. TxA_2 exerts potent bronchoconstrictor effects in intact animals as well as in isolated tracheal rings and pulmonary parenchymal strips (Darius et al., 1985). Work in Nadel's laboratory has shown that U46619, a TX mimic, at sub-threshold doses causes fourfold increase in airway responsiveness to ACh in dogs (Aizawa et al., 1985). Chung et al. (1986) showed that antigen induced airway hyperresponsiveness in dogs in vivo can be inhibited by a Tx synthetase inhibitor, OKY-046. Recently, Serio and Daniel (1988) demonstrated that U-46619 enhanced electrical field stimulated contractions in canine trachea, an effect that was unaffected by INDO but completely antagonized by SQ 29548, a selective TxA_2 inhibitor. They concluded that TxA_2 may enhance neural transmission by increasing ACh release or another as yet undetermined mechanism.

(vi) Prostaglandin receptors

A classification of prostanoid receptors has been proposed by Kennedy et al. (1982) and Coleman et al. (1984). This classification suggests that the effects of prostaglandins are mediated through six different receptors: the Tx receptor (TP), the PGI_2 receptor (IP), the PGD_2 receptor (DP), two PGE receptors, one mediating contraction (EP_1) and one mediating relaxation (EP_2), and finally a PGF_{2a} receptor (FP). One problem is that most tissues contain more than one PG receptor while many PG and synthetic analogues have affinity and efficacy at more than one PG receptor.

f) Lipoxygenase products and smooth muscle

(an overview)

Lipoxygenase refers to an enzyme that catalyses the hydroperoxidation of fatty acids. Its activity is characterized by positional specificity and by the specific formation of only one enantiomer. At present there are lipoxygenases that can peroxidate arachidonic acid at the 5, 8, 9, 11, 12, or 15 positions. In addition, arachidonic acid can be metabolized by a cytochrome P450-dependent monooxygenase, in the presence of NADPH, to four regioisomeric epoxyeicosatetraynoic acids (5,6; 8,9; 11,12; 14,15) which can be hydrolyzed to form diols, and 19-, 20- hydrox-

yeicosatetraynoic acids (Escalante et al., 1989). Furthermore, lipoxins are also uniquely synthesized as mentioned previously.

The processing of arachidonic acid via the 5-lipoxygenase pathway leads to four classes of biologically active product- HETEs, LTB_4, the "SRS-A" (LTC_4, LTD_4 and LTE_4) and the LXs.

(i) Hydroxyeicosatetraynoic acids

5-HETE is incorporated into membrane lipids, modulating the motility (Goetzyl, 1980) and possibly the glucose transport of neutrophils (Bass et al., 1980). 15-lipoxygenase metabolites, 15-HETE, are present in the human lung (Hamberg et al., 1980), neutrophils (Soberman et al., 1985), eosinophils (Laviolette et al., 1986), macrophages (Pawlowsi et al., 1982) and airway epithelial cells (Hunter et al., 1985). They are immunosuppressive agents (Bailey et al., 1982) and both 5-HETE and 15-HETE are weak stimulants of contraction in human bronchi and guinea pig trachea (Copas et al., 1982; Sirois et al., 1981). Seaman (1983) reported that when HETE production is inhibited by lipoxygenase inhibitors, natural killer cell activity is also reversibly affected. Chemotaxis and chemokinesis of cells have also been attributed in part to HETEs (Burka, 1981).

(ii) Leukotrienes

LTB_4 is a potent chemotactic factor. It enhances vascular permeability and attracts and activates leukocytes (Samuelsson et al., 1987). Peptidoleukotrienes LTC_4, LTD_4 and LTE_4 have vasoactive spasmogenic activities more potent than histamine (Lewis et al., 1980). Their effect on the pulmonary vascular bed is variable. In the monkey, the predominant responses to injection of LTC_4 is a fall in pulmonary arterial pressure; on the other hand, aerosol administration of LTC_4 caused a marked rise in pulmonary arterial pressure (Smedegard et al., 1982). In the rat, injections caused a dose-related fall in pulmonary arterial pressure (Iacopino et al., 1983). In contrast to LTC_4, LTD_4 caused a marked increase in pulmonary vascular resistance in sheep (Kadowitz et al., 1988).

LTs cause prolonged alterations in systemic blood pressure and promote extensive plasma extravasation by increasing the permeability of postcapillary venules (Feuerstein, 1985). Elevated levels of LTs have also been found in the bile of animals exposed to endotoxemia (Denzslinger et al., 1985).

(iii) Lipoxins

Lipoxin (LX) A_4 induces leukocyte lysosomal membrane leakiness to release lysosomal hydrolases and superoxide free radicals (Serhan et al., 1985). LXA_4 also elicits long-lasting contractions of the guinea pig lung strip and relaxes vascular smooth muscle at concentrations less than 1uM (Dahlen et al., 1987). However, unlike LTC_4, LXA_4 does not stimulate contraction of the guinea pig ileum, which suggests tissue selectivity associated with LX-induced responses (Dahlen et al., 1987). Little is known about LXB_4 in these experimental conditions. Immunologically, both LXA_4 and LXB_4 induce arteriolar vasodilation and inhibit natural killer cell activity. Their actions were not due to release of ACh, histamine, norepinephrine, or cyclooxygenase products (Dahlen et al., 1988). It was interesting to note that LXA was more potent than DAG in activating PKC and thus may prove to be an activator of intracelluar events in smooth muscle cells (Hansson et al., 1986). Dahlen et al. (1988) also reported that LXA_4 induces contraction in guinea pig trachea and the human bronchi. The onset of the contraction was slower in the latter and was reversed by L648-015, a LT antagonist. Thus the mechanism of action of LXs could indeed be via a receptor sharing mechanism with the cysteinyl LTs. A detailed review of LXs is beyond the scope

of this discussion. The reader is referred to the text by Wong et al., 1988 for a thorough review.

g) Lipoxygenase products and airway smooth muscle

i) Leukotrienes and airway smooth muscle

LTs were originally known as slow-reacting substances (SRS) released from guinea pig lung perfusates when stimulated by cobra venom (Feldberg and Kellaway, 1938). Kellaway and Trethewie (1940) also demonstrated that SRS was released from lungs following antigen challenge. In 1960, Brocklehurst coined the term slow-reacting substance of anaphylaxis (SRS-A).

Significant levels of LTs have been found in the nasal washes of allergic patients after ragweed challenge (Creticos et al., 1984) and in the plasma, sputum and bronchial lavages of asthmatics (Ishihara et al., 1985).

LTs contract airway smooth muscle from guinea pig (Piper et al., 1981), human (Dahlen et al., 1980), monkey (Smedegard et al., 1982), and horses (Mirabahar, 1985). There is no response to LTs in the cat or dog (Krell et al., 1981).

LTD_4 and LTE_4 have been shown to sensitize or prime tissues inducing them to be hyperresponsive to other agonists. Creese and Bach (1983) demonstrated that at low calcium concentrations (0.1mM) LTD_4, at subthreshold concentrations, significantly enhanced in vitro contractions to

histamine and ACh. Fennessey et al. (1986) demonstrated that aerosolized and intravenously administered LTD_4 dose-dependently enhanced histamine-induced increases in airway resistance, whereas LTC_4 was unable to produce such effects.

LTs contribute to airway hyperresponsiveness, increase mucus secretion and slow mucociliary transport (Russi et al., 1985). Studies have also indicated that LTD_4 generated during both immunologic and non-immunologic airway mast cell degranulation not only contributes to the immediate bronchoconstrictor response but is also important for the late responses or late increases in airflow resistance associated with worsening symptoms in asthma patients (Cartier et al., 1982). It follows, thus, that antagonism of immunologically released LT products would be expected to prevent these pathophysiological developments.

ii) Lipoxins and airway smooth muscle

Dahlen et al. (1988) reported that five minute treatments with 1μM LXA_4 depressed LTC_4-induced contractions in the guinea pig trachea. The nature of this interaction is subject to further investigations. In the human bronchi, LXA_4 elicited a contraction that was antagonized by L-648,051, a competitive and selective antagonist for LTD_4 receptors (Dahlen et al., 1988). LXA_4 also promoted the release of TxA_4 from the guinea pig lung. Dahlen et al. (1988) suggested that this enhancement may reflect a gener

al capacity for this compound to stimulate formation of cyclooxygenase products. Interaction of LXA_4 and LTC_4 at the receptor level suggests that both up- and down- regulation of effector cell responsiveness may be achieved within the lipoxygenase system.

In contrast, Lefer et al. (1988) failed to detect any significant quantities of TxA_2, or leukotrienes in pulmonary parenchymal strips contracted with LXA_4 and LXB_4. Lefer et al. (1988) have recently shown that LXB_4 possesses a very similar biological profile to LXA_4 in that it contracts pulmonary smooth muscle, dilates vascular smooth muscle, fails to aggregate platelets and increases lysosomal membrane permeability.

h) Leukotriene receptors and their antagonists

Peptidoleukotrienes exert their actions on smooth muscle by binding to receptors (Samhoun and Piper, 1983). In recent years, specific radioligand binding sites for peptidoleukotrienes have been identified on numerous tissues and cells: guinea pig lung and heart, human lung, alveolar macrophages, rat glomeruli and smooth muscle and RBL-1 cells in culture (see Robertson, 1986; Sarau et al., 1987 for review). These receptors have been characterized by the use of FPL 55712, an antagonist developed by Fisons (Augstein et al., 1973). Krell et al. (1983) reported that FPL 55712 appeared to be a more effective antagonist of LTD_4 than LTC_4

in some tissues. In contrast, Hand et al. (1981) reported that FPL 55712 was more effective against LTC_4 than LTD_4 in guinea pig pulmonary arteries. Further work has lead to the development of more specific receptor antagonists. LY 171883 (Abraham et al., 1986) is an orally active LTD_4/E_4 antagonist which is 1000-fold more specific than FPL 55712 (Aharony et al., 1987). ICI 198,615, another novel LTD_4/E_4 antagonist in guinea pig lungs is 3000-fold more specific than FPL 55712 (Aharony et al., 1987)

Enzyme inhibitors of LT metabolism have also aided the evaluation of specificity of receptor antagonists. L-Cysteine is an aminopeptidase inhibitor and prevents the formation of LTE_4 from LTD_4 and LTC_4 (Snyder et al., 1984). L-serine borate, an inhibitor of glutamyl transpeptidase prevents the conversion of LTC_4 to LTD_4 (Snyder et al., 1984; Hand and Schwalm, 1987).

The mechanism of receptor action is controversial. Anderson and co-workers (1982) demonstrated that LTC_4 and LTD_4 inhibited adenylate cyclase in guinea pig trachea, lowering cAMP levels, thereby causing contraction. This effect has not been observed by others. Baud et al. (1987) reported ligand-gated, voltage-independent Ca^{2+} channels in differentiated HL-60 cells that appear coupled to LTD_4 receptors. Mong et al. (1987) demonstrated that LTD_4 induced rapid and sustained hydrolysis of phosphoinositides in minced guinea pig lung. They suggested that the LTD_4 receptor in the guinea pig lung was coupled to a phospholi-

pase C, that when activated caused the hydrolysis of phosphoinositides to diacylglycerol and inositol triophosphate. They recently demonstrated that in fura-2 loaded sheep tracheal smooth muscle cells, LTD_4/E_4 induced transient intracellular calcium mobilization. This suggests that DAG or inositol triphosphate may serve as intracellular messengers that contribute to the contractile effect in sheep tracheal smooth muscle.

Lin et al. (1988) have also described a novel series of LTB_4 analogues that block the binding of radioactively-labeled LTB_4 to human neutrophil membranes. These analogues replace the carbons 7-9 of the cis-trans-trans triene unit of LTB_4 with a meta-substituted benzene or pyridine or furan ring. They are potent inhibitors, more potent in isolated membranes suggesting that they are metabolized by intact cells. Such compounds may prove to be useful in treating inflammatory diseases.

i) Pharmacology

It should also be noted that mechanically, smooth muscle at the central and peripheral airways are similar. Pharmacologically, however, major differences exist. Peripheral airways respond with a greater degree of narrowing than central airways when exposed to the same contractile agonist (Shioya et al., 1987).

In canine tracheal smooth muscle, Tesarowski (1987) found that exogenous arachidonic acid induced a relaxation of responses to histamine, 5-HT, and ACh. Canine tracheal smooth muscle requires an increase in tone before the effects of arachidonic acid or INDO can be demonstrated. The necessity for active tone in unmasking INDO's effects suggests that cyclooxygenase pathway dominates in this tissue. In the equine tracheal smooth muscle, however, arachidonic acid induced a contractile response that was inhibited by FPL 55712, a LTD_4 receptor antagonist suggesting that the lipoxygenase pathway of arachidonic acid metabolism predominates. Addition of arachidonic acid or INDO induced a contractile response in unstimulated equine tracheal smooth muscle. In addition, NDGA, a lipoxygenase inhibitor (Morris et al., 1979) also attenuated the response to INDO.

The divergent expressions of INDO-sensitivity in the two species suggests that the potentiation of responses caused by INDO may involve the rerouting of substrate from cyclooxygenase through the lipoxygenase and also an inhibiytion of prostaglandin synthesis. Adcock and Garland (1980), Drazen et al. (1981), Walker (1983) , Burka and Eyre (1977), demonstrated that the inhibitors of cyclooxygenase enhanced SRS-A release. Using high pressure liquid chromatography, Tesarowski (1987) showed the release of large quantities of LTE_4 in the presence of INDO in equine airway smooth muscle. This increased release was not

achieved by arachidonic acid alone. Canine airways, unlike equine, are weakly responsive to exogenous leukotriene administrations.

Exogenous addition of LTs has little or no effect on isolated canine airway smooth muscle (Tesarowski, 1987). However, Kannan et al. (1986) demonstrated a response to LTs in vivo. In contrast, LTC_4 ($10^{-6}M$) elicited maximal responses in equine airway smooth muscle. The basic pharmacology of canine airways, insensitivity of basal tone to relaxants and cooling, absence of spontaneous activity and absence of phasic response to graded increases in extracellular potassium (Beamino et al., 1969), causes it to be described as "inexcitable". Equine airways, on the other hand, shows a potential for spontaneous activity and the depolarizations produced by 10-12 mM K^+, ouabain and ACh are accompanied by sharp twitches or coarse tetani. It is therefore termed "excitable".

STATEMENT OF THE PROBLEM

Metabolites of arachidonic acid are among the diverse group of chemical mediators released from the airway epithelium and have been implicated in the regulation of tone in airway smooth muscle (Lewis and Austen, 1981). Airway smooth muscle is a central component in determining the calibre of the airways. Consequently, metabolites affecting the tone of these conduits could contribute to pulmonary diseases, mucous production and asthma.

The oxidative products of arachidonic acid play a role in the pathogenesis of airflow obstruction in asthma. Arachidonic acid is a unique precursor molecule which is transformed into potent mediators that influence smooth muscle in the gastrointestinal and urogenital tracts as well as in vascular and pulmonary systems (for review see Bergstrom, 1968). They may also affect neuronal and glandular systems.

Arachidonic acid is normally bound to phospholipids in cell membrane and can be metabolized by the action of phospholipase C or phospholipase A (Burka, 1983). Free arachidonic acid is then metabolized via the cyclooxygenase and/or the lipoxygenase enzyme systems. Cyclooxygenase metabolites include prostaglandins, thromboxane and prostacyclin. Lipoxygenase metabolites include leukotrienes and lipoxins. Leukotrienes induce contraction of smooth muscle, whereas prostaglandin of the E series generally induces relaxation (Burka, 1988). Thus the functional balance between the production of bronchoconstrictor and bronchodilator thromboxane

and prostaglandins, as well as contractile lipoxygenase metabolites, would ultimately influence airway smooth muscle tone. The importance of this functional balance is shown in a subpopulation of asthmatics who are sensitive to acetylsalicylic acid, a cyclooxygenase inhibitor (Kowalski et al., 1986). The mechanisms by which the resultant bronchospasms are induced are as yet unclear.

The intent of this study was to examine the effects of indomethacin, a cyclooxygenase inhibitor, on airway smooth muscle tone. Our previous studies suggested that indomethacin may redirect substrate to the competing lipoxygenase pathway and thereby elicit contraction of airway smooth muscle. The present results suggest that the mechanism of indomethacin's actions are more complex and involve cyclooxygenase metabolites, lipoxygenase metabolites and neurotransmitter release.

At a gross and subgross level, the structure of the equine lung most closely resembles that of humans (Hanna and Eyre, 1979). In addition, it should be noted that horses are subject to a chronic obstructive respiratory disease, heaves, which parallels human asthma in many ways (Gerber, 1973; Halliwell et al., 1979). Thus, in many respects equine airways present a useful model in studies of mechanisms of regulation of airway tone as related to allergic diseases. It is also hoped that these studies may perhaps provide new insights into the aspirin-sensitivity of some asthmatics.

METHODS

A. DISSECTION

Lungs and tracheae were collected from horses killed by captive bolt at an abattoir in Edmonton. The tissues were placed in plastic bags, kept on ice and delivered by courier, usually within 7 hours of removal form the animal. The tracheal smooth muscle layer was separated from the cartilage, dissected free from the tunica fibrosa and the tunica mucosa and subsequently dissected into parallel strips according to a modification of the method described by Stephens and al. (1969).

One end of each muscle was fastened with a loop of 000 surgical silk thread to a clamped aerating tube and placed in a double-jacketed 15ml organ bath. The upper end of each muscle was attached by the surgical silk to a Statham UC-3 force/displacement transducer through a lever assembly for isometric tension recording. The mechanograms were recorded on a four-channel Gould-Brush 2400 recorder.

The muscle strips were bathed in physiological salt solution (PSS) of the following composition (mM): NaCl, 115; $NaHCO_3$, 25; NaH_2PO_4, 1.38; KCL, 4.7; $MgCl_2$, 1.00; $CaCl_2$, 1.91; and dextrose, 11.1. The baths were aerated with 95% O_2- 5% CO_2 maintaining a pH of 7.40 at 37°C. The muscles were equilibrated for 90 minutes during which time they were stretched to near their optimal length, L_0, and were washed frequently with fresh, aerated PSS. The equilibration period included a series of three brief (5 min) exposures to

a high-K solution, prepared by substituting KCl for NaCl on an equimolar basis. In experiments utilizing electrical field stimulation (EFS), the equilibration period included three supramaximal stimuli.

B) EXPERIMENTAL PROTOCOL

1) Stimulus-Response (S-R) experiments

EFS was effected through two rectangular platinum electrodes placed parallel to smooth muscle strips in the bath and connected to a 60Hz AC source. Voltage was measured with a digital voltmeter (Beckman Tech 300) in parallel with the bath electrodes. All stimuli were of 40 seconds duration, this being sufficient to produce peak tension in all experimental conditions. Each stimulus was applied at 5-minute intervals to permit recovery of the muscle and nerve. Varying strengths of stimuli were applied until maximal responses were elicited. Responses were stable over the experimental period. Muscle responses to EFS were expressed as a percentage of the maximal response obtained in control untreated muscles. In each paradigm a control S-R curve was performed. The voltage eliciting 50% of the maximal response (MR), V_{50}, was calculated and provided a normalizing device to quantitate mean left/right shifts produced by subsequent drug treatments. Thus a V_{50} smaller than the control value (100%) indicated a leftward shift of the S-R curve.

2) Radiolabelled choline efflux experiments

a) Basal efflux experiments

Tracheal muscle strips were dissected as above. The aerating tubes used in these experiments possessed two hooks: one towards the middle of the tube and the other at the bottom of the tube. One end of each muscle was fastened with 000 surgical silk to the bottom hook of the aerating tube. The muscle was then slightly stretched and the upper end was fastened isometrically to the middle hook with surgical silk thread.

The muscles attached to the aerating tubes (95% O_2 and 5% CO_2) were then placed in a beaker containing PSS and immersed in a water bath of 37^0C. The muscles were equilibrated for 90 minutes.

After the equilibration period, the muscles were transferred to a 10-inch test tube. The latter contained 10^{-6}M concentration of ^{14}C-choline with a specific activity of 2 - 5mCi/mol. Choline rather than acetylcholine was used since nerve endings take up choline rather than acetylcholine to resynthesize transmitter.

A cold carrier, choline chloride (10^{-6}M), was also added as ^{14}C-choline has been found to adsorb to glass walls and thus compromise assay conditions. The muscles were incubated in labeled solution for 60 minutes before the collection period began.

A series of culture tubes containing fresh PSS, cold carrier and the appropriate drugs were placed in a tube rack

and then placed in the water bath. Each muscle was transferred from the labeled solution to their respective culture tubes and the collection period began. The muscles were transferred from one tube to the next every 10 minutes.

The first three samples were discarded since they represented primarily the extracellular label. It was found that by the fourth or fifth sample, a stable rate of transmitter ^{14}C-choline release was attained. It was after this sample that the muscles were exposed to appropriate drugs (present in the culture tubes) as specified in the results.

b) Electrical field stimulated (EFS) efflux experiments

Tracheal smooth muscle strips were mounted in organ baths (as above). Subsequent to the 90 minutes equilibration period, the muscles were incubated for 60 minutes with ^{3}H-choline in PSS to estimate the efflux of acetylcholine. Supramaximal electrical field stimuli (approximately 14 volts, 60Hz, 40 seconds duration) were applied through platinum plate electrodes at 10 minute intervals. This method increases transmitter turnover and therefore promotes neural uptake of ^{3}H-choline into the nerve varicosities. The concentration of ^{3}H-choline used was 3.8×10^{-5}M with a specific activity of 1Ci/mmol. Atropine (10^{-6}M) was also added prior to the incubation period. This ensures that all effects seen are a result of

direct muscle stimulation or an inhibition of pre-junctional release of neurotransmitter.

Eserine (10^{-6}M) was used (added at the end of the incubation period) in these experiments to prevent the breakdown of ^{3}H-acetylcholine released and thus prevent the re-uptake of label released.

After the incubation period, a collection period began in which labeled solution (10ml) was replaced with fresh (PSS) cold solution (10ml) containing the cold carrier and eserine. The first three samples collected every 10 minutes were discarded as they represented the washout of extracelluar (EC) label. They were not used in the calculation of rate coefficients.

After the initial 30 minute washout period, electrical stimuli were given every 15 minutes. Bath samples were then collected every 5 minutes. Thus for each stimulus three efflux samples were collected. The first two stimuli represented the internal control for each muscle as no drugs were added yet. It was found that a stable basal rate of ^{3}H-choline release was attained by the fifth and sixth intervals (i.e. after the second stimulation). Appropriate drugs were subsequently added during the efflux periods and were replenished after each washout. Thus, addition of drugs represented a cumulative procedure.

After the collection period, the experimental procedure for both the basal efflux and the electrical field

stimulated efflux experiments were the same as described in the following paragraphs.

After the sampling period, 2 ml aliquots of the respective samples collected were placed in scintillation vials, filled with 9 ml of scintillation fluid (Beckman, HP scintillation cocktail) and radioactivity was measured on a Beckman LS-350 scintillation spectrometer. After the last sample, the muscles were weighed and solubilized in vials containing 250 ul NCS (Amersham/Searle). NCS, a surface-active organic base dissolved the tissues over a period of 3 days which freed any radiolabel in the tissues. Scintillation fluid was added to the muscles and radioactivity was determined.

c) Calculations

The efflux was normalized and expressed as the rate coefficient (R.C.) in units of % of tissue content of (^{3}H) or (^{14}C) released per minute (Shanes and Bianchi, 1959). The R.C. was obtained as follows:

$$\frac{\text{number of counts per minute (cpm) released per sample}}{\text{number of cpm remaining in the muscle at the mid-point of the collection period of this sample}} \times 100\%$$

All values were normalized after washout of the E.C. space at 30 min (EFS efflux) or 50/70 min (basal efflux).

In the basal efflux experiments, the mean of the R.C. achieved during the 4^{th} and 5^{th} collection was taken as 1. This represented an internal control for each muscle strip

as no drug had been added at that time. All subsequent R.C. were normalized to that value. The data were plotted as efflux fraction vs time to display the effects of various treatments on label release.

The calculation for efflux in electrical field stimulated release is as follows: Two control stimulations, 15 min apart, and 6 samples collected every 5 minutes were counted. For each stimulation, the peak efflux subtract the efflux immediately preceding the onset of each stimulation represents the efflux value for each stimulation. The first two control stimulation values are averaged and then normalized to a value of 1 and all subsequent values are expressed as a fraction of 1. The data were plotted as a ratio of test:control efflux fraction vs collection period time to graphically display the effects of treatment on label release.

C) Drugs

Table 1 contains a complete listing of the drugs used in this study. The drug names, abbreviations, and common actions are included in the table. All drug concentrations expressed in the results are final bath concentrations. All drugs were obtained from Sigma Chemical Company (St. Louis, Mo.) with the exception of the following: LY 171883 was a generous gift of the Eli Lilly laboratories (Indianapolis, Ind.); leukotriene D_4 from Merck Frosst Ltd. (Dorval, Quebec); D-600 from Knoll A.G. (Germany); FPL 55712 from

Fisons Pharmaceuticals (Loughborough, England) and both ^{14}C-choline and ^{3}H-choline from New England Nuclear (Boston, Mass.)

INDO was dissolved in dimethylsulfoxide (DMSO); ETYA, eserine, ^{14}C-choline, ^{3}H-choline and PGE_2 were dissolved in 95% ethanol. ATR was dissolved in 0.01N hydrochloric acid, PHENT, D-600 and LY171883 were dissolved in distilled water. A maximum volume of 2uL/ml of DMSO or ethanol was added to the muscle baths and did not affect resting or active tension.

D) STATISTICAL ANALYSIS

Statistical tests in these studies included analysis of variance and Student's T-test as indicated in the text. Statistical significance was established when p values were less than 0.05. Tests were performed using the I.B.M-compatible Number Cruncher Statistical System software package.

Table 1. List of drugs utilized in this study, their action and their respective abbreviations.

DRUG	ABBREVIATION	ACTION
Atropine	ATR	muscarinic receptor antagonist
^{3}H-choline		acetylcholine precursor
^{14}C-choline		acetylcholine precursor
D-600		potential-dependent calcium channel blocker
Eserine	ESER	acetylcholinesterase inhibitor
5,8,11,14-eicosa-tetraynoic acid	ETYA	cyclooxgenase/lipoxy-genase inhibitor
FPL 55712	FPL	Leukotriene D_4 antagonist
Hexamethonium bromide	HBr	ganglionic blocker
Indomethacin	INDO	cyclooxygenase inhibitor
Leukotriene C_4	LTC_4	bronchoconstrictor
Leukotriene D_4	LTD_4	bronchoconstrictor
LY 171883		Leukotriene D_4 antagonist
Prostaglandin E_2	PGE_2	bronchodilator
Phentolamine	PHENT	alpha-adrenoceptor agonist
Serine-borate	SB	Inhibitor of LTC_4 conversion to LTD_4
Tetrodotoxin	TTX	selective neural sodium channel blocker

RESULTS

A. Effects of INDO on equine airway smooth muscle.

The four panels of mechanograms in figure 4 depict the effects of various agonists on an INDO (10^{-5} M)-induced contraction. ATR (10^{-6}M) and LY171883 ($3x10^{-6}$M), a LTD_4 receptor antagonist, partially antagonized discrete components of this contraction, thus verifying the involvement of both cholinergic- and LT- related moieties. The concentrations of ATR and LY171883 used produced maximum effects. The residual contraction was unaffected by PHENT (10^{-5}M) and therefore is not a function of alpha-adrenoceptor activation. The addition of PGE_2 (10^{-7} M), however, relaxed this component to baseline values. The latter residual contraction might therefore result from decreased PGE_2 production, although this evidence is indirect.

B. The separation of two components in the stimulus response through the use of ATR.

The stimulus response (S-R) relation and the effects of ATR on responses to EFS are shown in figure 5 (panel A and B). Control S-R curves are illustrated in panel A and demonstrate that the S-R relation is reproducible over the experimental time period. These responses are comparable with the peak of contraction to 127mM potassium and carbachol (10^{-5}M). This figure, and all other subsequent S-R graphs, present individual experiments, representative of a series. All relevant mean data are presented in Table 2.

Figure 4. Effect of serial additions of atropine (10^{-6}M), indomethacin (10^{-5}M), LY171883 ($3x10^{-6}$M), phentolamine (10^{-5}M) and PGE_2 (10^{-6}M). These mechanograms are representative of 5 experiments.

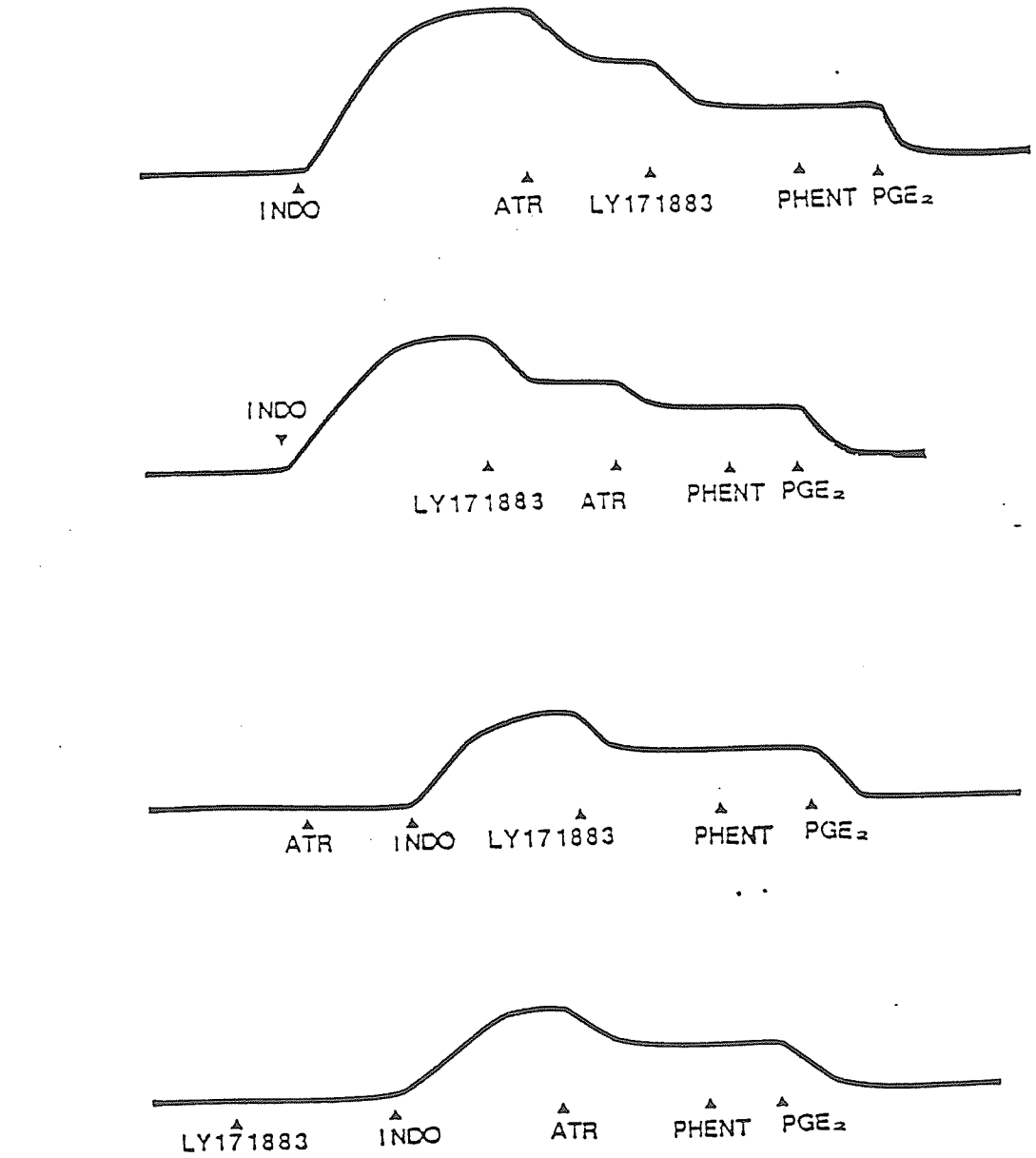


Figure 5. Contraction of untreated equine tracheal smooth muscle induced by electrical field stimulation. In this and subsequent figures, the number next to each curve represents the order in which the stimulus-response relations were conducted. Responses are given as a percentage of maximum tension developed in untreated muscles. This myogram is representative of nine experiments.

Panel A

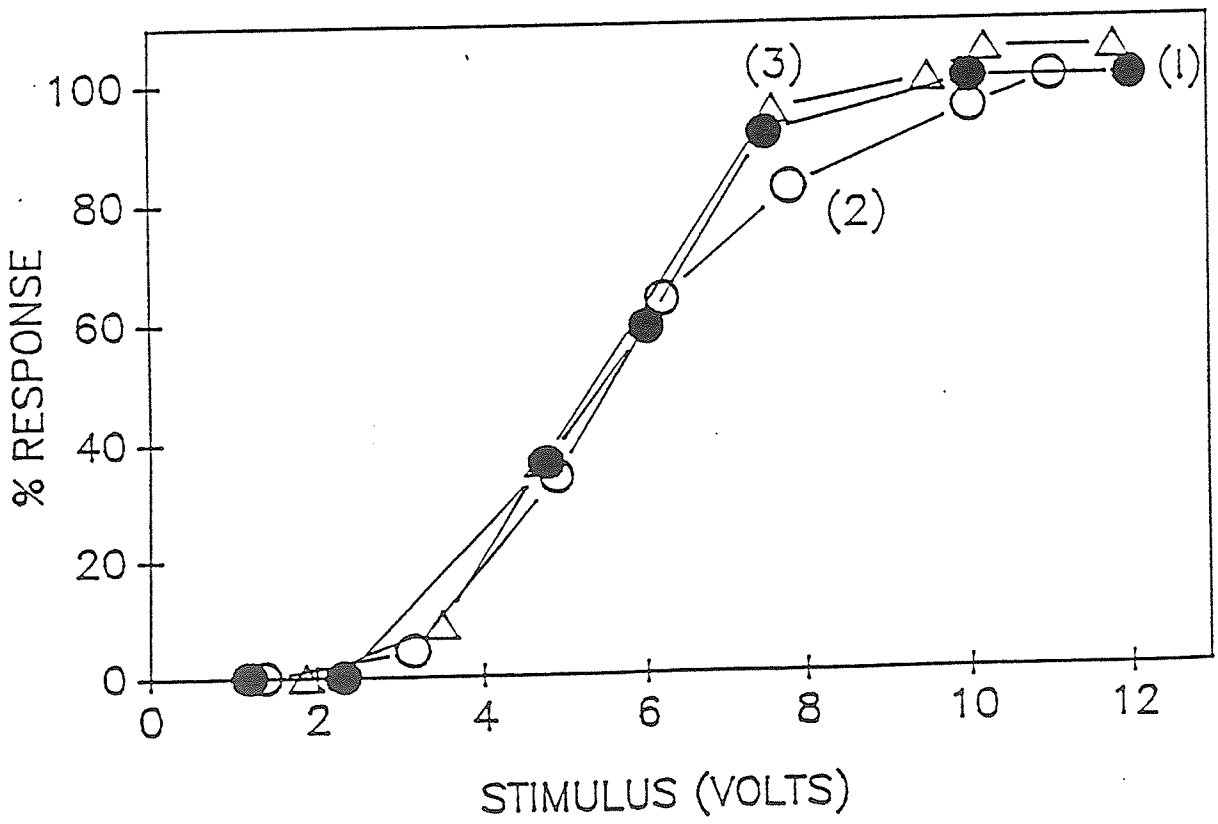


Figure 5.Effect of atropine (10^{-6}M) on contraction of equine tracheal smooth muscle induced by electrical field stimulation. Responses are given as a percent of maximum tension developed in untreated muscles. The number next to each curve in this and subsequent figures represents the order of addition of the drugs. This myogram is representative of seven experiments.

PANEL B

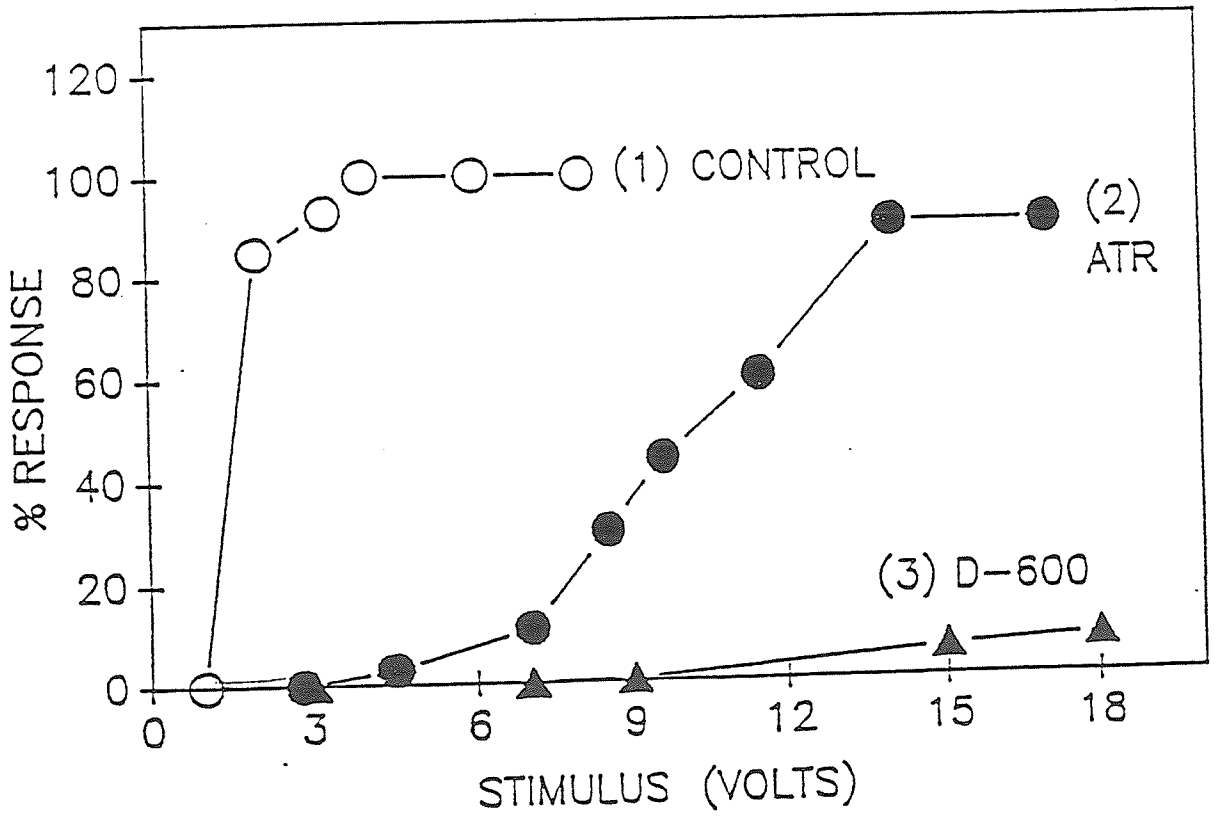


TABLE 2 The effect of various drug treatments on V_{50} and MR in EASM

TREATMENT[a]	N[b]	V_{50}[c]	SEM	MR	SEM
CONTROL	9	100	0	100	0
+INDO, 10^{-5}M	9	74 *	7	139 *	10
+INDO+ATR	9	154 * ++	18	65 * ++	9
CONTROL	7	100	0	100	0
+ATR, 10^{-6}M	7	261 *	20	80	4.5
+ATR+INDO	7	168 * ++	20	85	11
CONTROL	7	100	0	100	0
+ETYA, 3.3×10^{-5}M	7	123 *	2	80 *	2
+INDO, 10^{-5}M	7	83 *	8	94	5
CONTROL	7	100	0	100	0
+INDO	7	68 *	8	141 *	14
+INDO+ETYA	7	115 * ++	5	97 ++	9
CONTROL	7	100	0	100	0
+PGE_2, 10^{-8}M	7	134 *	15	86 *	4
+PGE_2, 10^{-7}M	7	168 *	15	51 * ++	6
CONTROL	4	100	0	100	0
+ATR, 10^{-6}M	4	190	20	80	5
+PGE_2, 10^{-7}M	4	190	19	50 * ++	5

[a] S-R experiments were conducted using muscles treated with agents in the sequence indicated (see Methods). The concentration of the drug was consistent throughout the experiment except where indicated.

[b] The number of experiments performed on tissues from different animals under each protocol is represented by N. Statistical significance was established when $p<0.05$ using analysis of variance and compared with *control or ++previous treatment.

[c] Values indicate the mean V_{50}, (normalized as a percentage of the voltage eliciting 50% of the maximal response, MR).

In order to determine the contribution of cholinergic neurotransmitter release to these responses, the stimulus response (S-R) relation was performed after equilibration with ATR (10^{-6} M). The latter shifted the S-R curve rightward, suggesting that at low stimulus strengths the mechanical response is a function of neurotransmitter release. This is consistent with a role of the autonomic nervous system in electrical field stimulated responses at low stimulus strengths. Stronger stimuli elicited responses which likely resulted from direct stimulation of the muscle component. This was confirmed through the use of D-600 (10^{-5}M), a potential calcium channel blocker. Its addition to ATR-pretreated muscles virtually abolished all responses to EFS.

To ensure that all residual responses were indeed myogenic and not influenced by other neurotransmitter release (e.g. norepinephrine, VIP, SP), TTX (10^{-6}M), a selective neural sodium channel blocker, was added to ATR-pretreated muscles (Figure 6). The S-R relation showed no further decrease in V_{50} and MR. Thus, ATR appeared to produce effective blockade of nerve-related effects and the ATR-insensitive response could be attributed to the myogenic component.

In order to preclude ganglionic modulation of responses, hexamethonium bromide, HBR (10^{-5}M), a ganglion blocker, was added after the control S-R curve was described. Figure 7 demonstrates that HBR had no effect on

Figure 6. The effect of tetrodotoxin (10^{-6}M) on responses to electrical field stimulation in atropine (10^{-6}M) pre-treated muscles. Responses are expressed as a percentage of the maximal tension developed in the muscles before the addition of drugs. This figure is representative of four experiments.

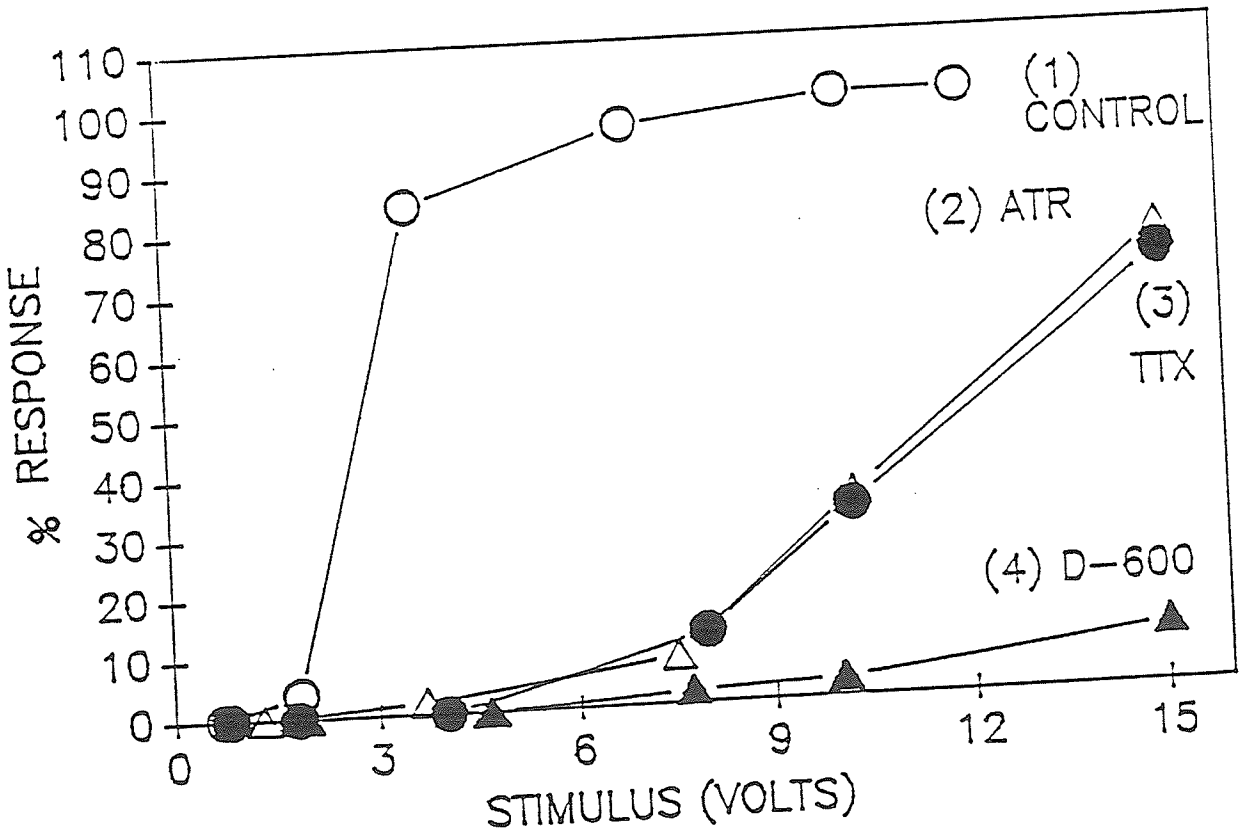
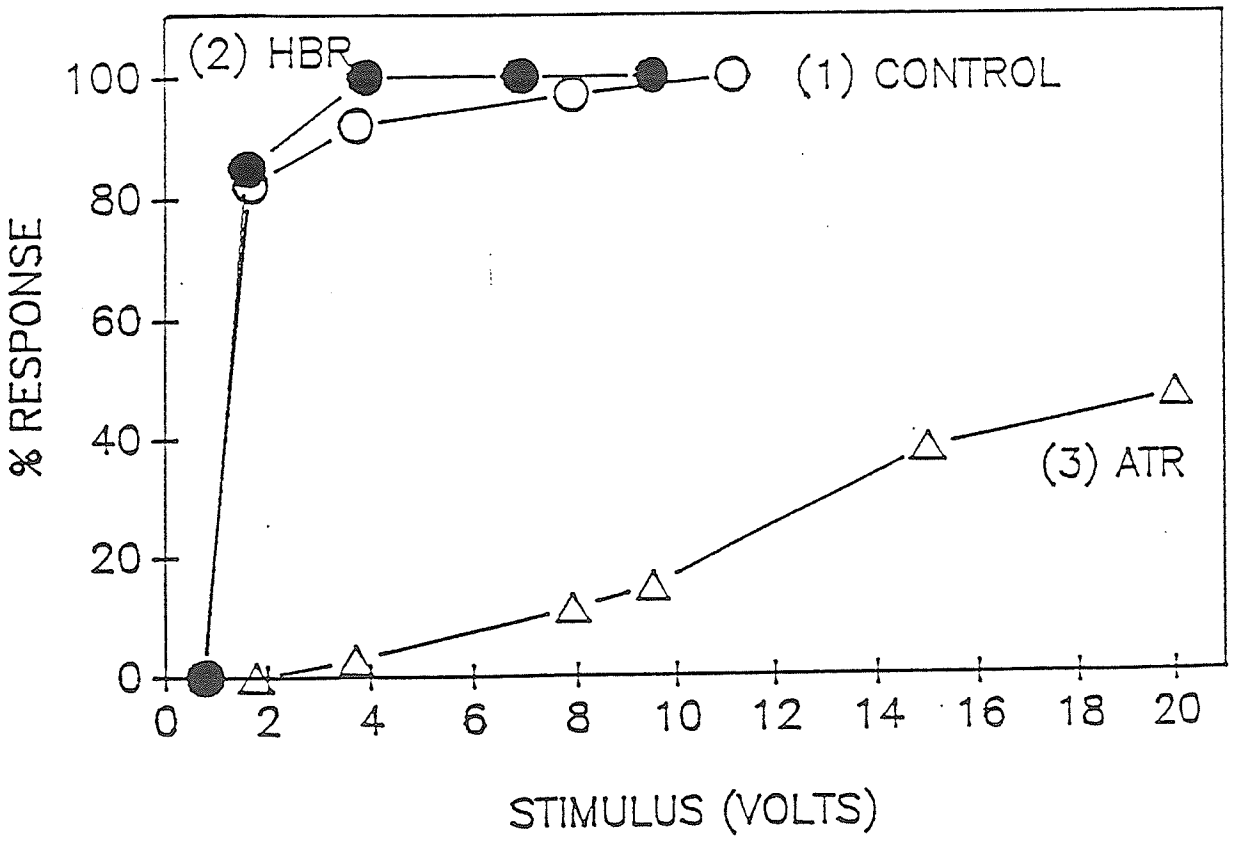


Figure 7. The effect of hexamethonium bromide (10^{-5}M) and atropine (10^{-6}M) on the stimulus-response relationship in equine airway smooth muscle. Responses are given as a percentage of the maximal tension developed in control untreated muscles. This figure is representative of four experiments.



the S-R relation. The subsequent addition of ATR shifted the S-R curve right- and downwards as previously stated. Thus the utility of this model was confirmed: lower stimulus strengths targeted the neural component, while higher stimulus strengths affected the myogenic component directly.

C. Effects of INDO on neural and myogenic components in EASM.

In order to determine whether INDO potentiated responses of equine TSM to EFS, we examined the effects of the drug on the S-R relation. Figure 8 indicates that INDO shifted the S-R curve left- and upward with respect to the control. Thus, INDO (10^{-5}M) potentiated responses to EFS in a component associated with nerve stimulation. The increase in basal tone was 20% (for this experiment) and was substantially ATR sensitive (not shown, but consistent with that shown in figure 4). The V_{50} for INDO was 74% $\pm$ 7% and the MR was 139% $\pm$ 10% (Table 2) with respect to the control. Treatment of INDO-pretreated muscles with ATR shifted the S-R relation rightward with a V_{50} of 154% $\pm$ 18% (Table 2). In this, and all subsequent figures with INDO treatment, the effects noted are those exclusive of the increase in baseline tension.

In order to determine whether INDO affected the excitability of the muscle component, another series of experiments was performed with the exposure of ATR-pretreated muscles to INDO. Figure 9 demonstrates that INDO

Figure 8. Effect of atropine (10^{-6}M) on the stimulus-response relationship in indomethacin(10^{-5}M) pre-treated muscles. Responses are given as a percent of maximum tension developed in untreated control muscles. This figure is representative of nine experiments.

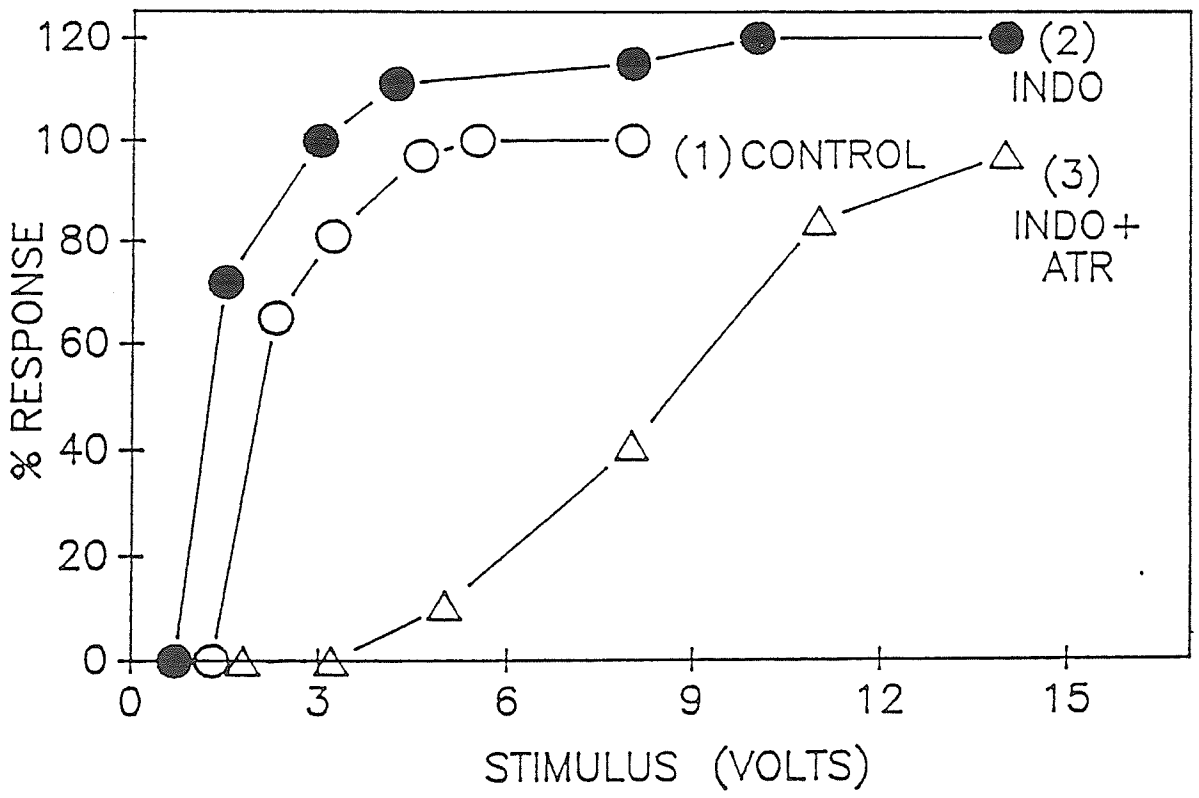
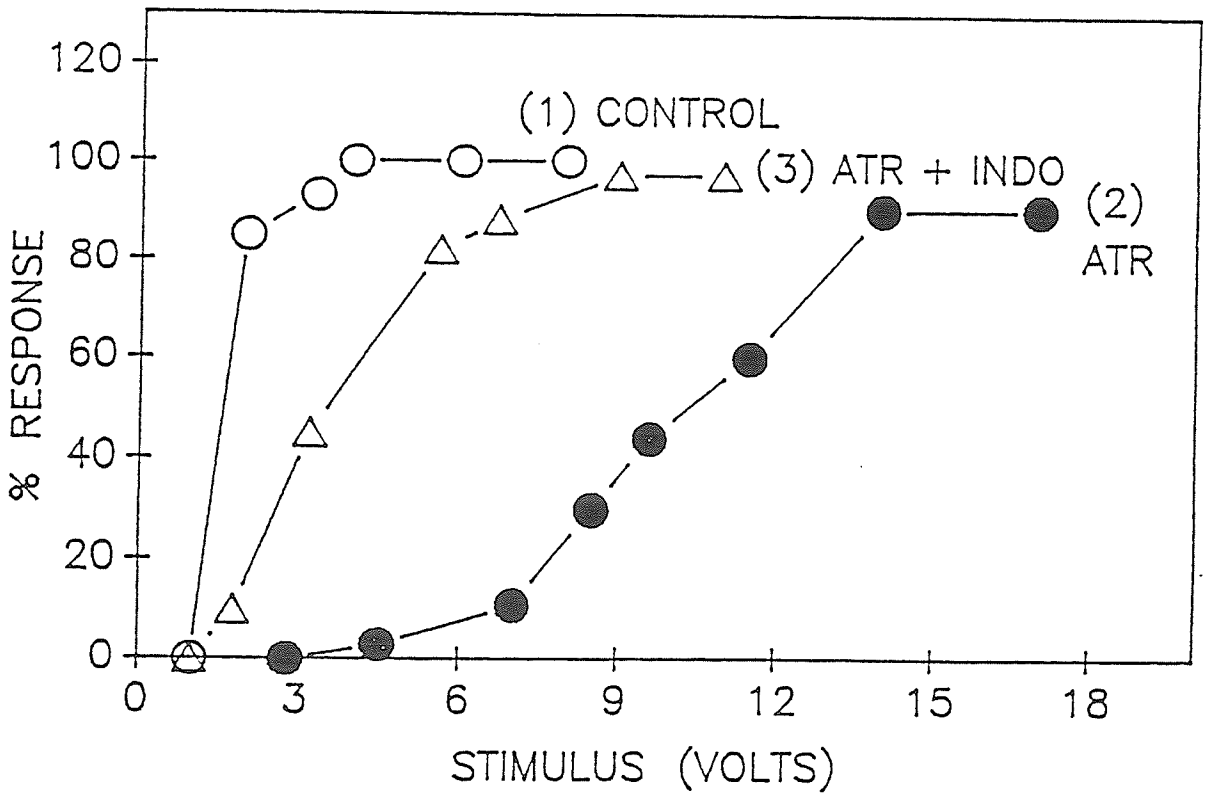


Figure 9. Effects of indomethacin (10^{-5}M) on atropine (10^{-6}M) pretreated muscles. Responses are given as a percentage of the maximum tension developed in untreated muscles. This figure is representative of seven experiments.



shifted the S-R curve of ATR-blocked muscles leftward and upward thus suggesting a non-cholinergic mechanism producing increased muscle excitability and responsiveness. INDO also produced an increase in basal tone of 10% in this experiment (not shown, but consistent with that shown in figure 4). ATR-pretreated muscles yielded a S-R curve with V_{50} of 261% ± 20% and MR of 80% ± 4.5% (Table 2). Exposure to INDO caused a leftward and upward shift of the S-R curve with the V_{50} decreasing from 261% ± 20% to 168% ± 20% and MR increasing from 52% ± 10% to 85% ± 11% (Table 2). It should be noted that the difference in the curves between this and the previous figure simply demonstrates individual variability.

Since the effects of INDO might be produced through inhibition of PGE_2-mediated regulation of Ach release, we examined the effects of PGE_2 on the S-R relation of untreated control muscles. The results (figure 10 panel A) show that PGE_2 at low doses (10^{-8}M) inhibited primarily the cholinergic neural responses. Though this figure does not indicate a significant shift with PGE_2 (10^{-8}M), the mean values are significant as shown in Table 2. At higher doses, PGE_2 (10^{-7}M) inhibited both the cholinergic neural and the myogenic components. The V_{50} and MR compared to control (100%) values were 168 ± 15% and 51 ± 6% respectively. In an ATR-pretreated preparation, PGE_2 (10^{-7}M) still shifted the S-R curve downward from an MR of 80 ± 4.5% in an untreated preparation to 50 ± 5% (Figure 10, panel B). The V_{50} did not differ significantly from the untreated prepara-

Fig 10. Effect of PGE_2 mechanical responses to electrical field stimulation. Responses are quantitated as a percentage of the maximum tension developed in untreated control muscles. This figure is representative of seven experiments.

PANEL A

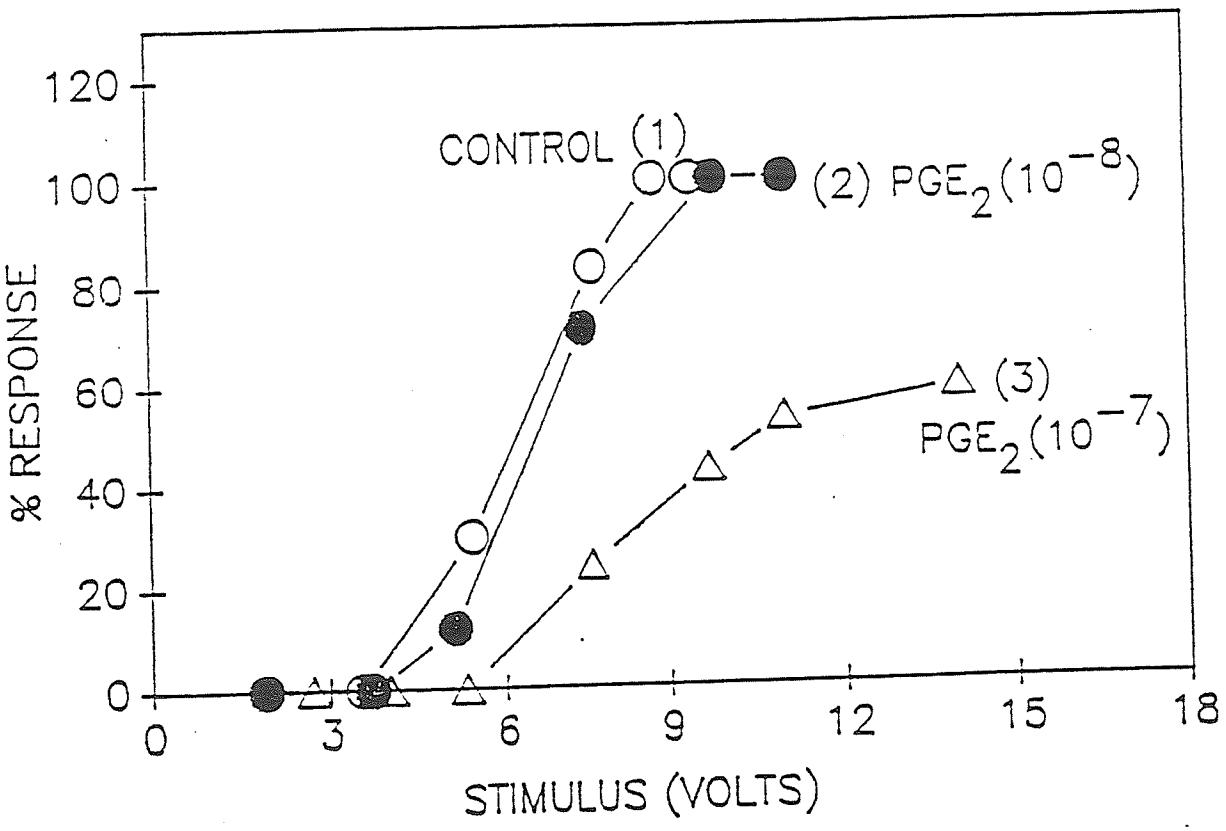
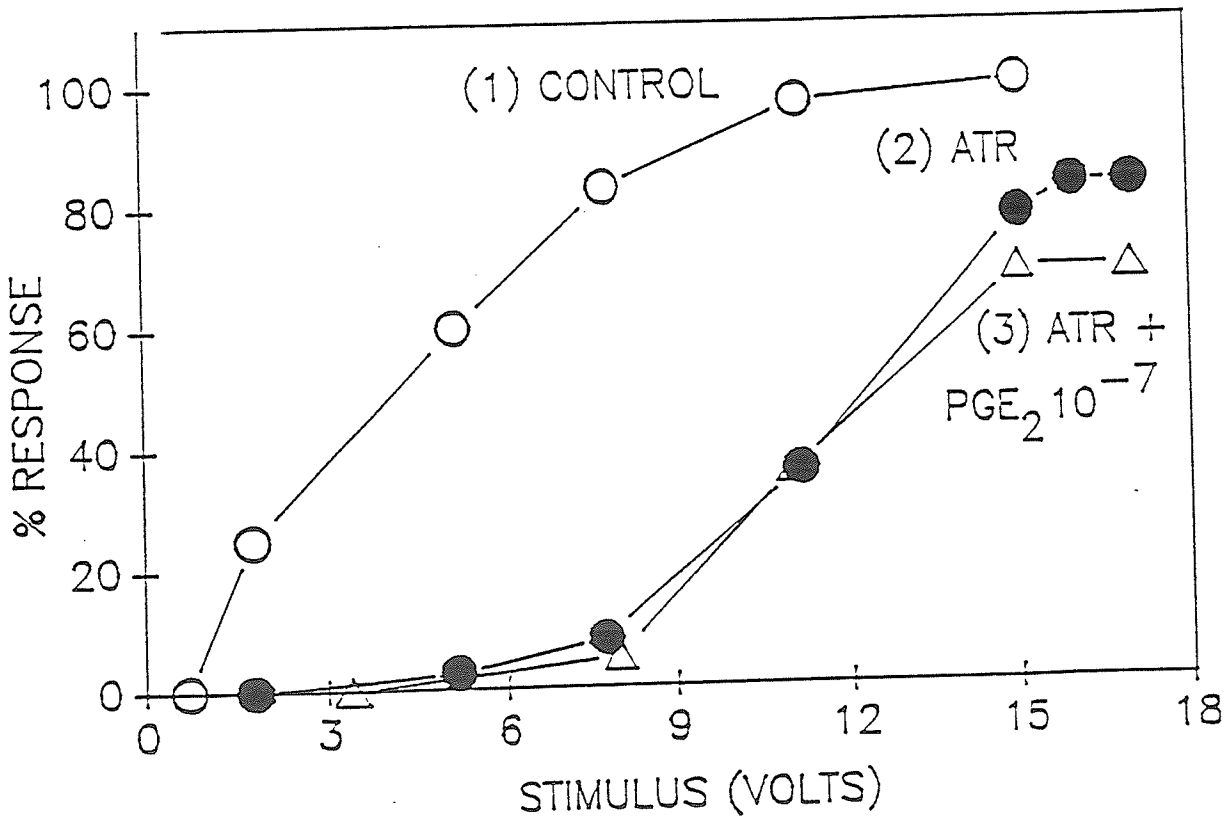


Figure 10. The effect of PGE_2 ($10^{-7}M$) on responses to electrical field stimulation in atropine ($10^{-6}M$) pretreated muscles. Responses are given as a percentage of the maximum tension developed in untreated muscles. This figure is representative of 4 experiments.

Panel B



tion (Table 2). This confirms that higher doses of PGE_2 indeed inhibit both the neurogenic and myogenic components in equine tracheal smooth muscle.

INDO induced a contraction of 76% ± 9% with respect to 127mM potassium contracture 9not shown). PGE_2 inhibited the INDO-induced effects with an IC_{50} of $5x10^{-9}$ M (Figure 11, panel A).

It should be noted that contraction to potassium in EASM is phasic: an initial spike followed by a frequent relaxation to a stable elevated level (Figure 11, panel B). The latter is highly variable, from 60% to 5% of the initial maximum. However, responses to EFS-stimulation are comparable to the initial maximum response. Thus when comparing INDO-induced contraction to 127mM potassium, this comparison is relative to the stable elevated value. Consequently the percentage is larger than when compared to the initial spike.

The effects of INDO on the myogenic component may result not only from decreased production of CO metabolites but also from the rerouting of substrate through the lipoxygenase pathway. In order to investigate the latter possibility, the lipoxygenase inhibitor ETYA was added to untreated control muscles (Figure 12, panel A) and INDO-pretreated muscles (Figure 12, panel B). As is shown in panel A, ETYA shifted the S-R curve rightward and downward with V_{50} being 123% ± 2% and MR being 80% ± 2% (Table 2) affecting both the neural and the myogenic components. ETYA

Fig 11. Dose related effect of PGE_2 on indomethacin ($10^{-5}M$)-induced contraction. Inhibition is expressed as a percentage of maximal contraction induced by indomethacin (76 ± 9% of 127mM potassium contraction). This figure represents the mean of 11 experiments.

PANEL A

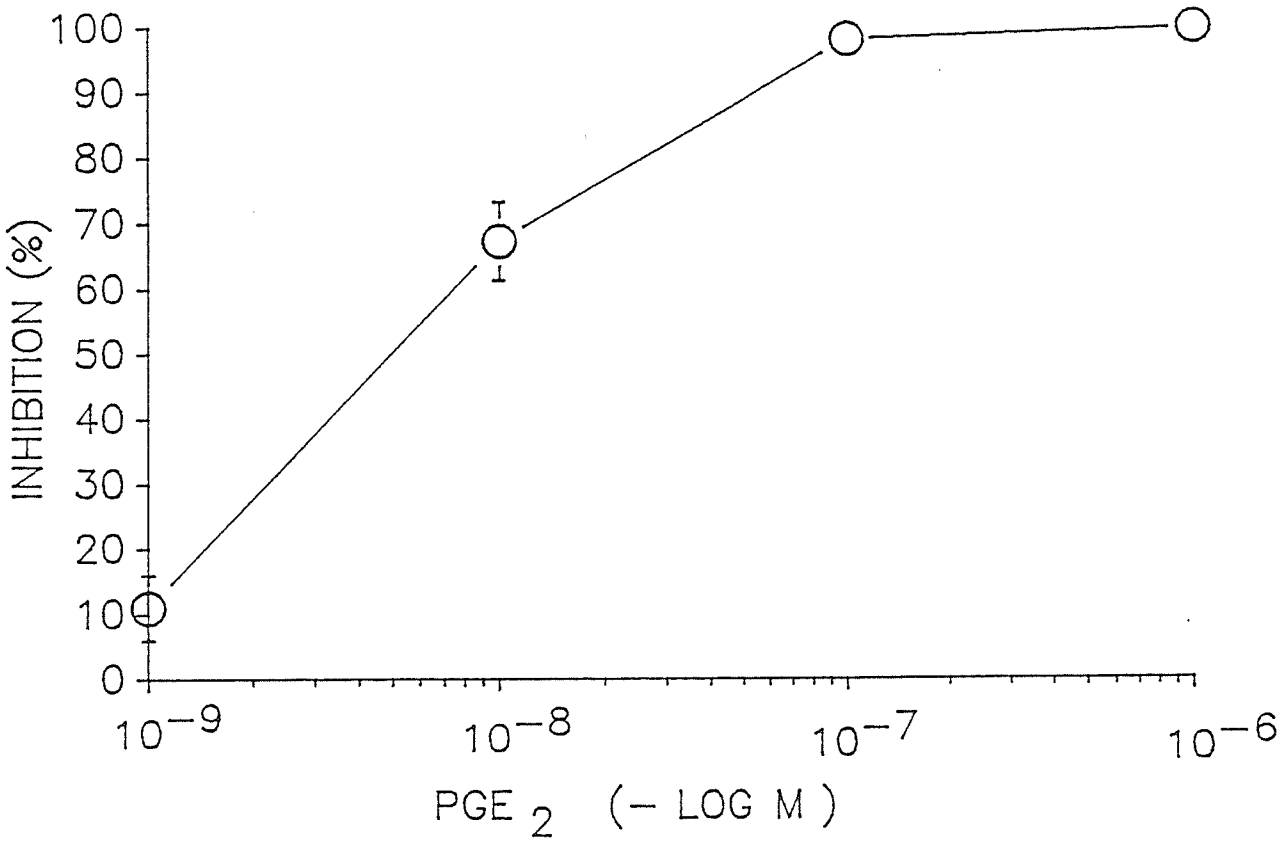


Figure 11. The effect of 127mM potassium on resting tension in equine airway smooth muscle. The resultant contraction is phasic: an initial spike followed by a relaxation to a stable elevated value.

Panel B

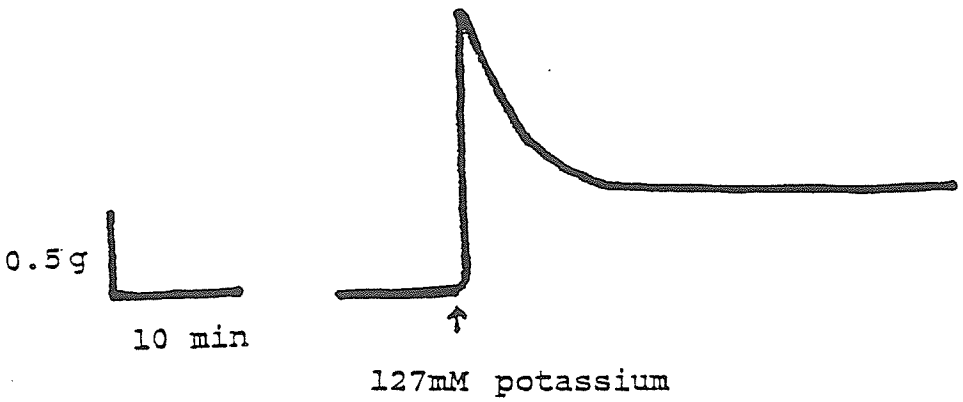


Figure 12. Effect of indomethacin (10^{-5}M) on ETYA ($3.3x10^{-5}$M)-pretreated muscles. Responses are expressed as a percentage of the maximal tension developed in control untreated muscles. Both panels are representative of seven experiments.

PANEL A.

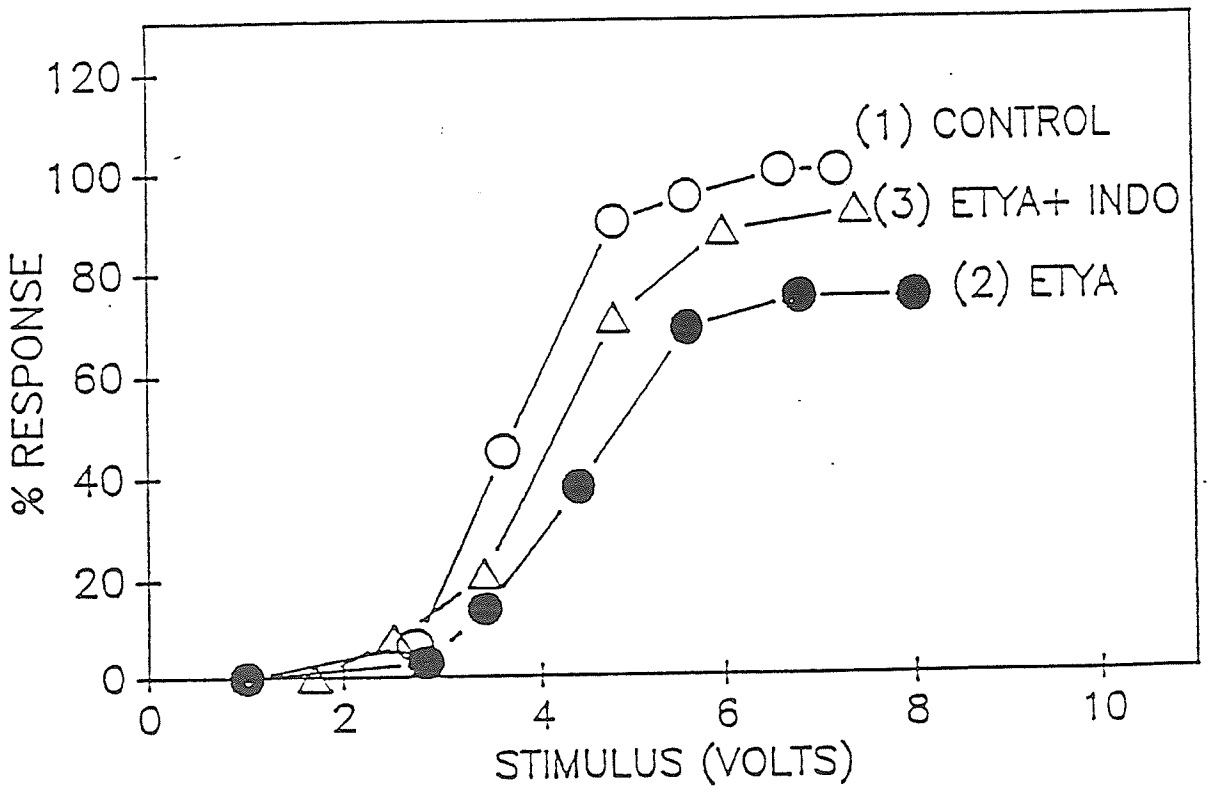
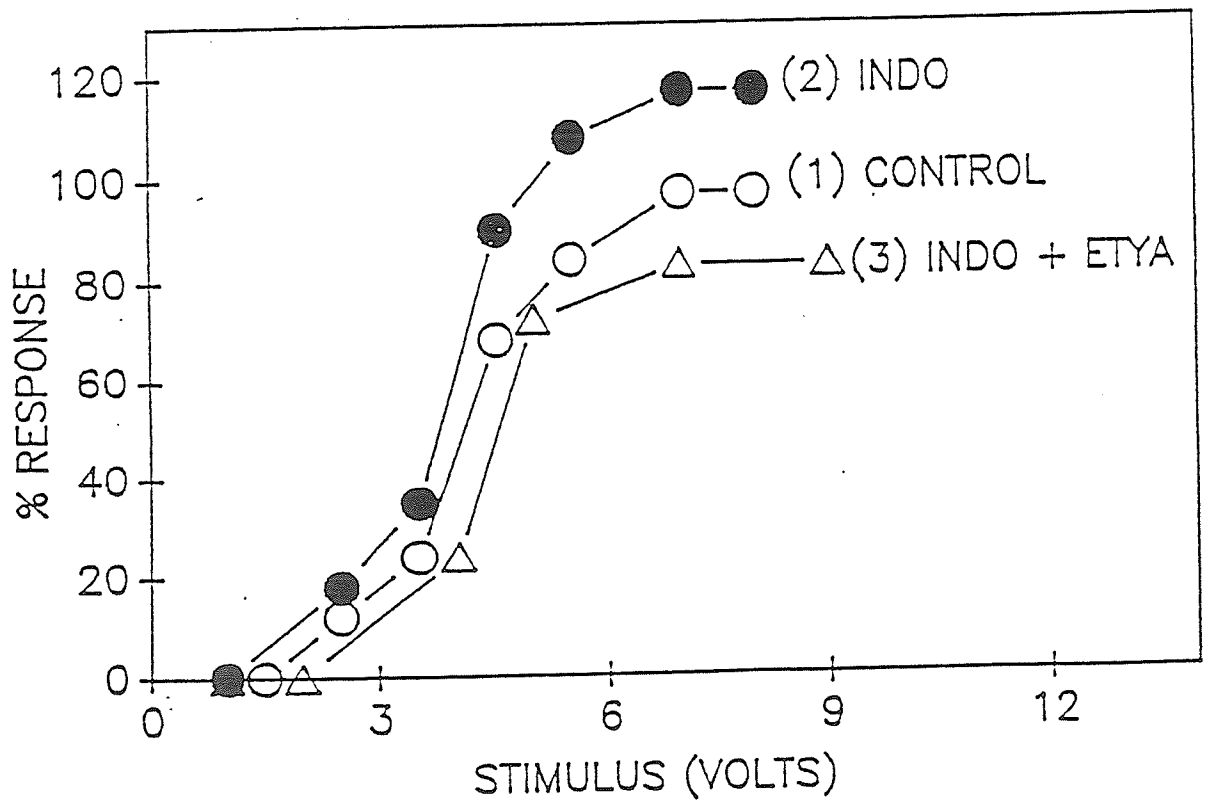


Figure 12. Effect of ETYA (3.3×10^{-5} M) on responses to electrical field stimulation in indomethacin (10^{-5} M)-pretreated muscles. Responses are expressed as a percentage of the maximal tension developed in control untreated muscles.

PANEL B



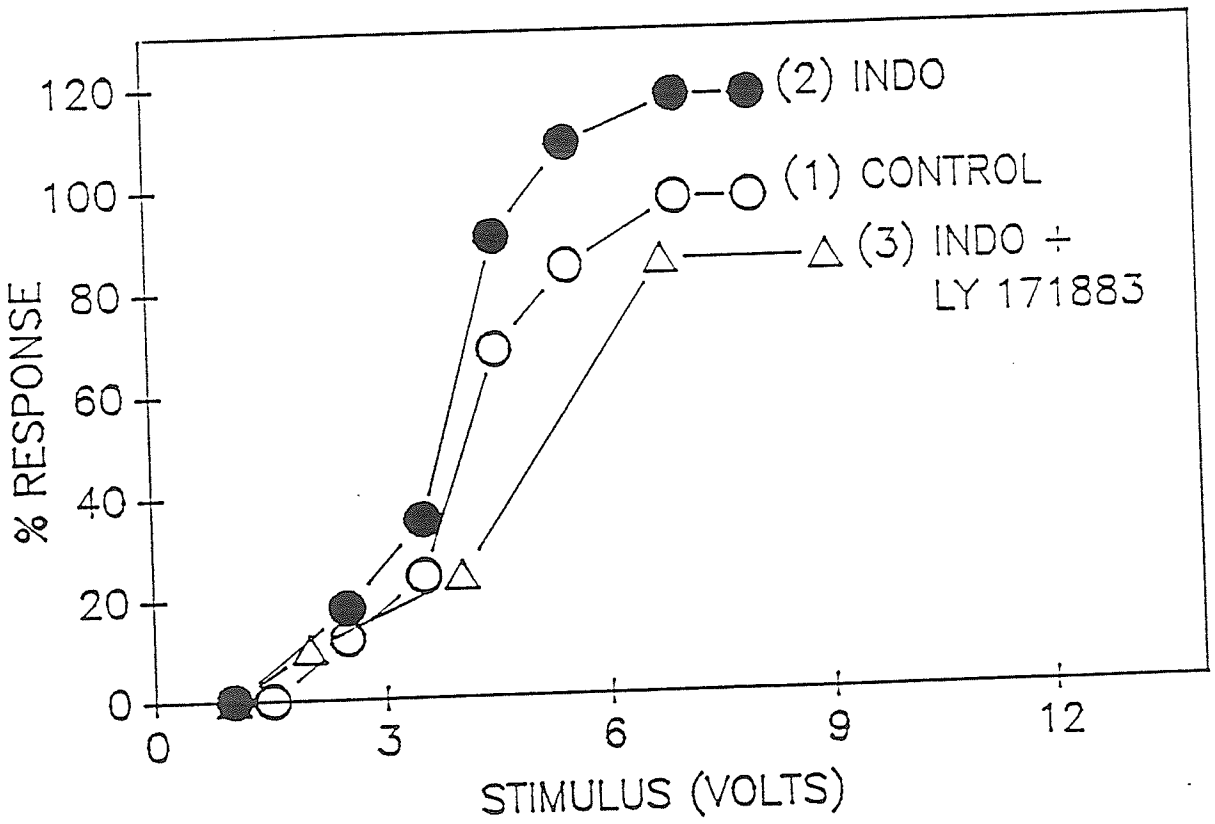
could be both inhibiting the lipoxygenase pathway and rerouting substrates to the cyclooxygenase pathway. The subsequent addition of INDO (Figure 12, panel A) shifted the S-R left to a V_{50} of 83 ± 8% (Table 2). The change in MR was not significant. Thus to establish that ETYA was indeed inhibiting the lipoxygenase pathway, and that the inhibition of these metabolites would affect neural and myogenic responses, we performed S-R relations in INDO-pretreated muscles.

Figure 12, panel B also shows that in the presence of INDO the S-R curve was shifted leftward and upward. The V_{50} was 68% ± 8% and the MR 141% ± 14% (Table 2). INDO also caused 15% increase in baseline tension (not shown, but consistent with that shown in figure 4).

The third curve was performed in the presence of ETYA ($3.3x10^{-5}M$) on INDO-pretreated muscles. ETYA caused a shift of the S-R curve rightward and downward. The V_{50} increased from 68% ± 8% to 115% ± 5% and MR decreased from 141% ± 14% to 97% ± 9%. This figure demonstrates that the effects of INDO are ETYA-sensitive ($p<0.05$).

To confirm that LY171883 can also blunt INDO-induced S-R relations, the former was added to INDO-pretreated muscles (figure 13). This and the previous results confirm that lipoxygenase metabolites do participate in INDO-induced contractile responses. The relevant data are summarized in Table 2.

Figure 13. Effect of LY 171883 ($3x10^{-5}M$) on the stimulus-response relationship in indomethacin ($10^{-5}M$) pretreated muscles. Responses are expressed as a percentage of the maximal tension developed in the control untreated muscles. This figure is representative of 4 experiments.



D. Effect of altered arachidonic acid metabolism on basal neurotransmitter release

Figures 14 through 17 represent the effect on basal release of neurotransmitter with treatment of INDO, PGE_2, ETYA and LTD_4.

All the muscles have been loaded with ^{14}C-choline to estimate the basal efflux of Ach. Figure 14 depicts the effect of INDO ($10^{-5}M$) on basal efflux. By the 4^{th} and 5^{th} sample, a stable rate of efflux was achieved. The mean of these two rate coefficients was taken and assigned a value of 1 for purposes of normalization. All subsequent effluxes were plotted as a fraction of 1. INDO enhances the normalized basal efflux from $0.69 \pm .04$ to $0.79 \pm .05$ (Table 3). This finding is consistent with the notion that cyclooxygenase metabolites affect basal tone in the preparation by inhibition of cholinergic neurotransmitter release.

In order to examine the direct effects of cyclooxygenase metabolites on EASM, PGE_2 ($10^{-6}M$) was added to INDO-pretreated muscles, and the basal efflux was examined. In this experimental paradigm, the control muscles were INDO pretreated. The mean efflux of the 6^{th} and 7^{th} sample were normalized to a value of 1 and, as previously stated, all subsequent data were expressed as a fraction of this value. Figure 15 shows that in INDO-treated control muscles, the efflux was relatively stable over a 20 minute period. In a corresponding set of muscles, the exogenous addition of PGE_2

Figure 14. Effects of indomethacin (10^{-5}M) on rate coefficient (R.C.) of basal efflux for EASM previously incubated with ^{14}C-choline. This figure represents the mean of nine experiments.

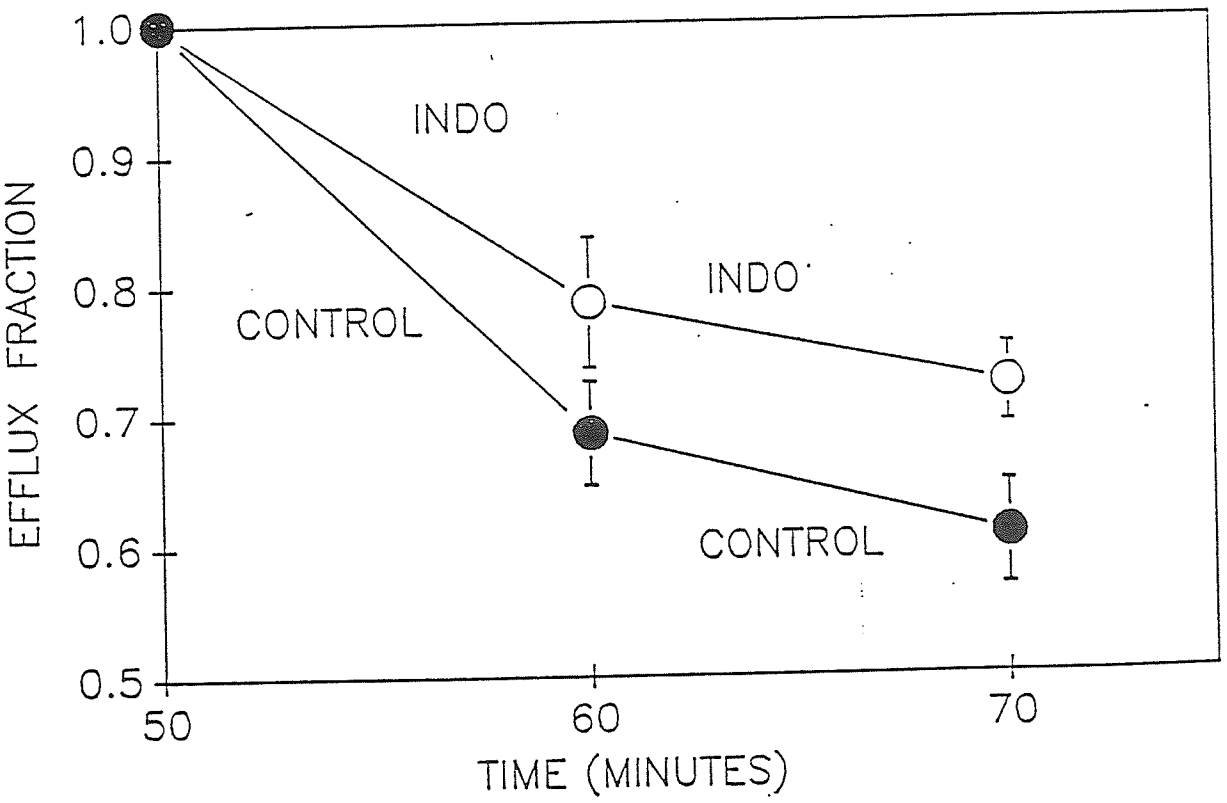
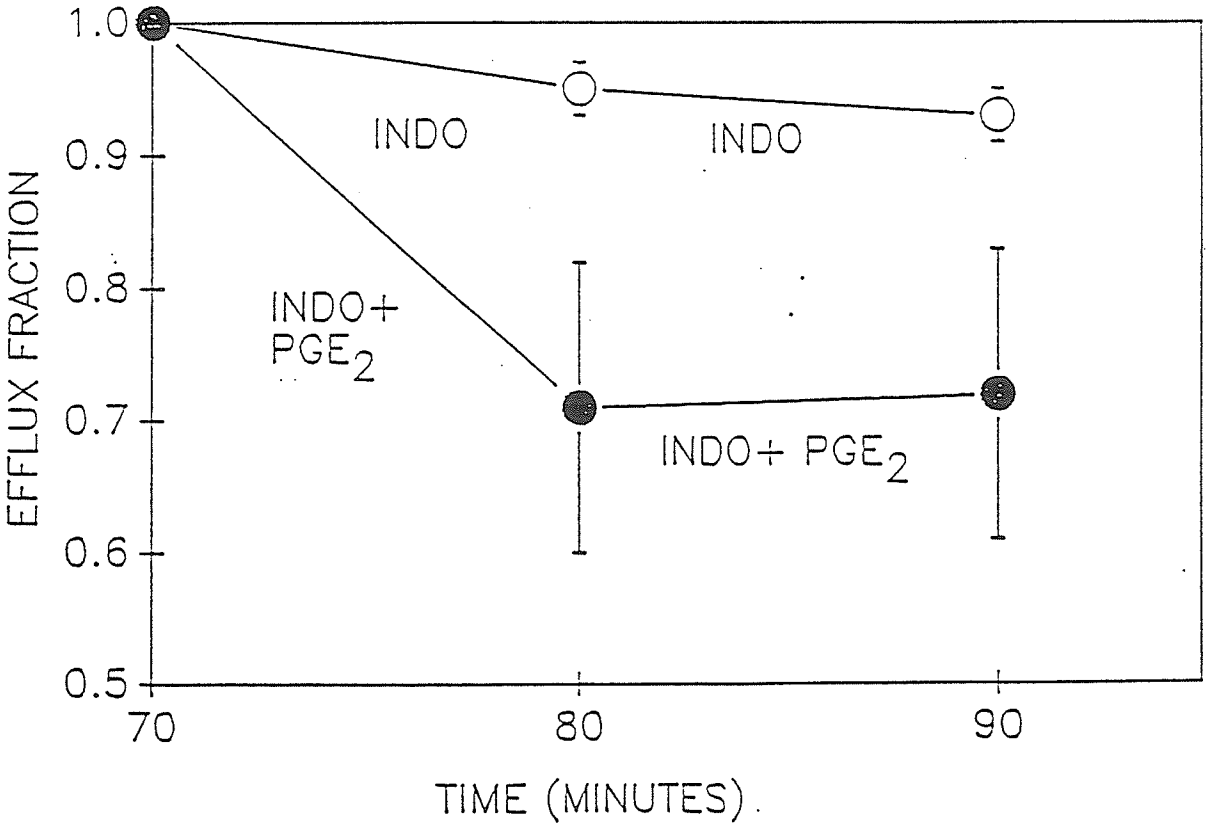


Figure 15. Effects of PGE_2 (10^{-6}M) on rate coefficient of basal efflux in indomethacin (10^{-5}M) pre-treated EASM (n=7). Tissues were previously loaded with ^{14}C-choline. Control tissues had only indomethacin treatment.



markedly inhibited ($p<.05$) basal efflux from 0.95 ± 0.02 to 0.72 ± 0.05 (Table 3). This finding confirms an inhibitory effect of PGE_2 on basal neurotransmitter release.

The effect of INDO may result not only from an inhibition of cyclooxygenase metabolites, but also from the rerouting of substrates through the competing lipoxygenase pathway as previously stated). Thus, the effect of ETYA (3.3×10^{-5} M) on basal release of label was examined. The results (figure 16) demonstrate that the inhibition of the lipoxygenase pathway produces a decrease in efflux fraction from 0.69 ± 0.04 to 0.59 ± 0.06 (Table 3). This suggests that lipoxygenase metabolites may also be participating in the modulation of neurotransmitter release.

In order to assess this possibility, the effect of LTD_4 (6×10^{-7}M) on basal release in ETYA pre-treated muscles was examined (figure 17). The control muscles were pretreated with INDO (10^{-5}M) and ETYA (3.3×10^{-5}M) respectively. In a corresponding set of identically treated muscles, exogenous LTD_4 was added and this caused a prompt enhancement ($p<.05$) of release from 0.72 ± 0.19 to 1.41 ± 0.21. This finding confirms that lipoxygenase metabolites can increase basal neurotransmitter release. All relevant data is summarized in Table 3.

TABLE 3. The effect on basal release of acetylcholine with treatment of INDO, ETYA, LTD_4 and PGE_2 .

Treatments	[a] Efflux fraction	
Control	0.69 (+/-) 0.04	(9)
INDO (10^{-5}M)	0.79 (+/-) 0.05 *	(9)
Control (INDO)	0.95 (+/-) 0.02	(7)
INDO+ PGE_2 (10^{-6}M)	0.72 (+/-) 0.11 *	(7)
Control	0.69 (+/-) 0.04	(5)
ETYA ($3.3x10^{-5}$M)	0.59 (+/-) 0.06	(5)
Control (ETYA)	0.72 (+/-) 0.19	(4)
ETYA+ LTD_4 ($6x10^{-7}$M)	0.41 (+/-) 0.21 *	(4)

LEGEND:
Mean (+/-) the standard error of the mean. The number in parenthesis represents the number of replications of experiments.
Statistical significance (*) is established when $p<.05$ using Student's T-test.
[a] Calculation of the efflux fraction is given in the methods.

Figure 16. Effects of ETYA (3.3×10^{-5}M) on rate cofficient of basal efflux for EASM (n=5) previously incubated with ^{14}C-choline.

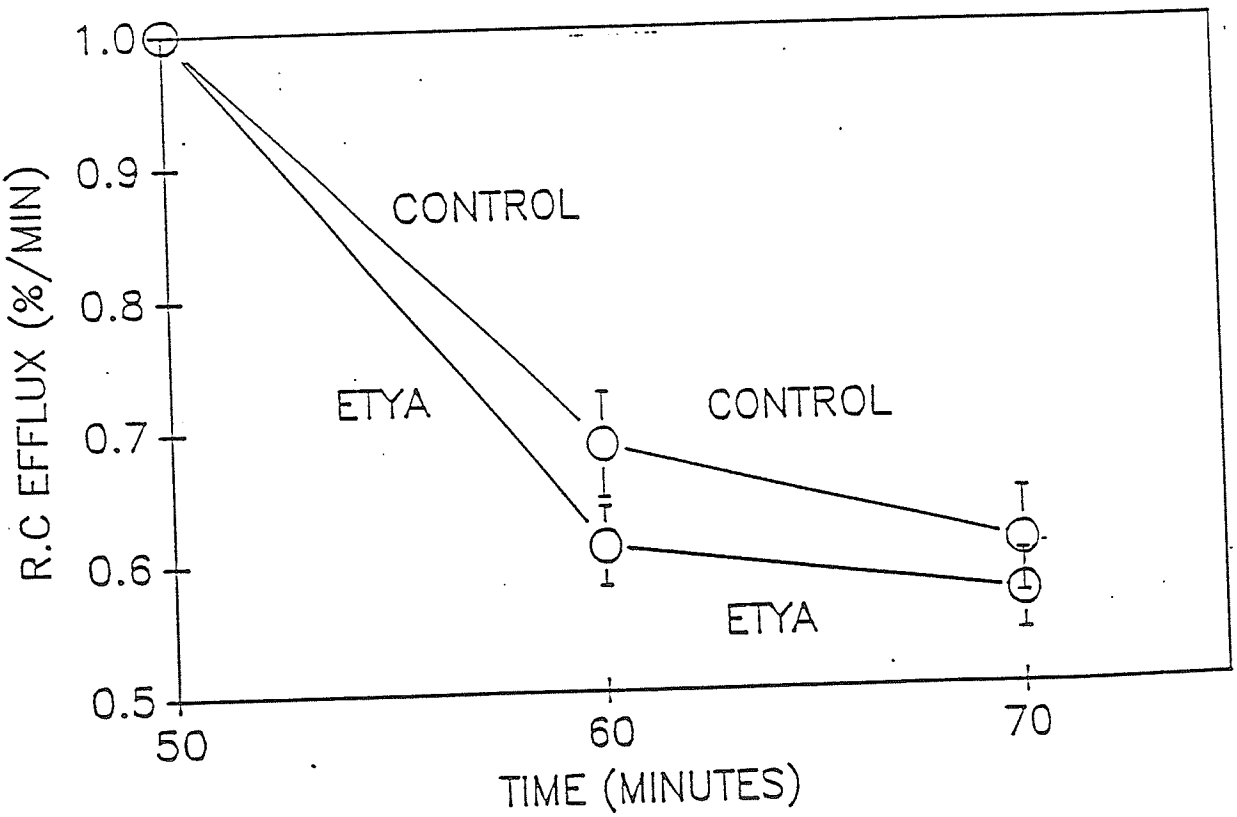
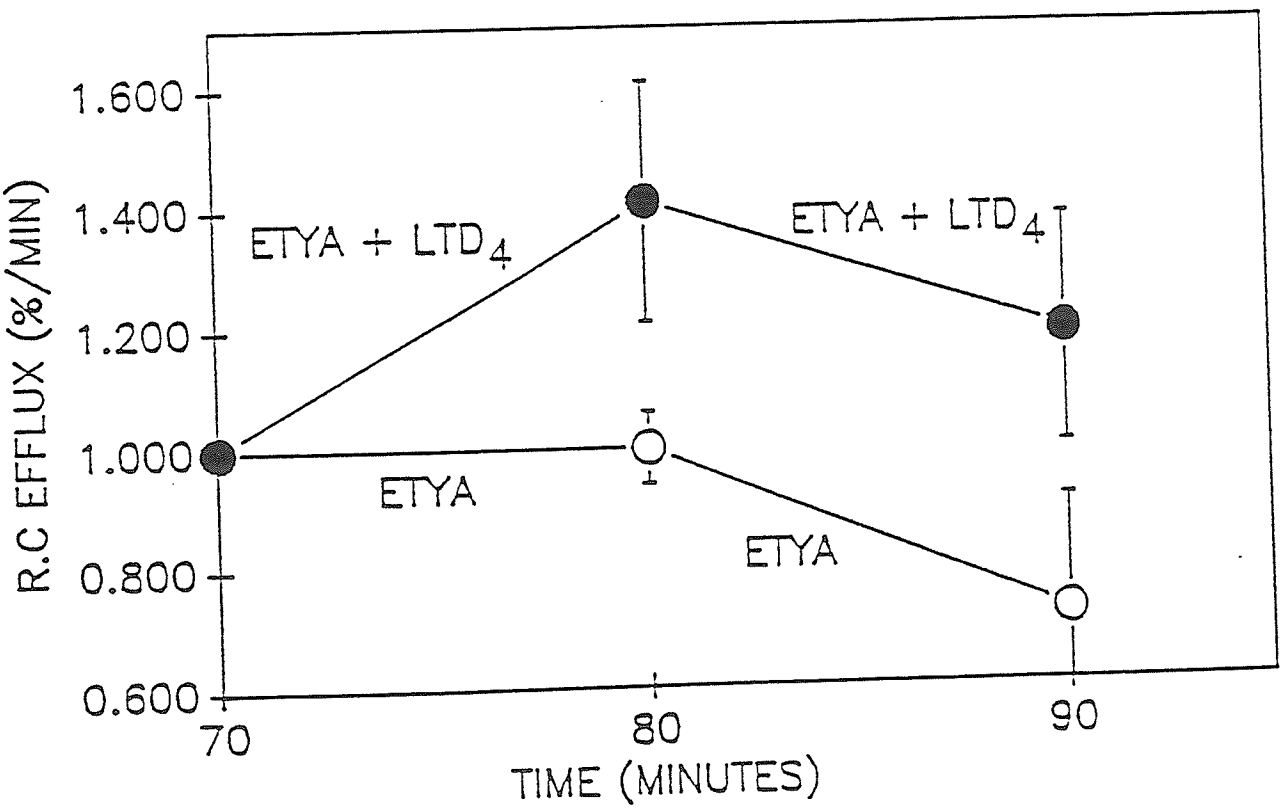


Figure 17. Effects of LTD_4 ($6 \times 10^{-7}M$) on rate cofficient of basal efflux in ETYA ($3.3 \times 10^{-5}M$) pre-treated EASM (n=4). Tissues were loaded with ^{14}C-choline prior to efflux. Control tissues received only ETYA treatment.



E. Effects of altered arachidonic acid metabolism on electrical field stimulated neurotransmitter release.

Tracheal smooth muscle is under vagal control which does not influence basal release but produces active neurotransmitter release. In order to assess the importance of arachidonic acid metabolites on active release of neurotransmitter from endogenous neural elements, the effects of INDO, PGE_2, ETYA and LTC_4 on electrical field stimulated (EFS) release was measured.

In this experimental paradigm, all drug additions are cumulative as shown in figures 18 and 19. Figure 18 shows the effect of INDO (10^{-5}M) and PGE_2 (10^{-6}M) on EFS-induced release. INDO caused a dramatic increase (approximately 2.5 fold) in efflux, an effect that was inhibited by PGE_2. This offers direct evidence confirming the hypothesis that PGE_2 may modulate tone in EASM by inhibition of pre-junctional cholinergic neurotransmitter release.

To further examine the contribution of the lipoxygenase pathway to the control of tone, ETYA (3.3×10^{-5}M) and LTC_4 (6×10^{-7}M) were added to INDO (10^{-5}M)-pretreated muscles (figure 19). ETYA inhibited the active efflux 2.5 fold, near to original control ratio. Serine-borate (0.5 M) was added prior to application of LTC_4 to prevent the conversion of LTC_4 to LTD_4. Serine borate had no effect on active release, confirming that ETYA had effectively blocked the lipoxygenase pathway. If this were not so, any LTC_4 leaking

Figure 18. Effects of indomethacin (10^{-5}M) and PGE_2 (10^{-6}M) on rate coefficient of electrical field stimulated (EFS) efflux in EASM (n=7) loaded with ^{3}H-choline. Values are expressed as a ratio of test efflux : control efflux. Details of calculations are given in the methods.

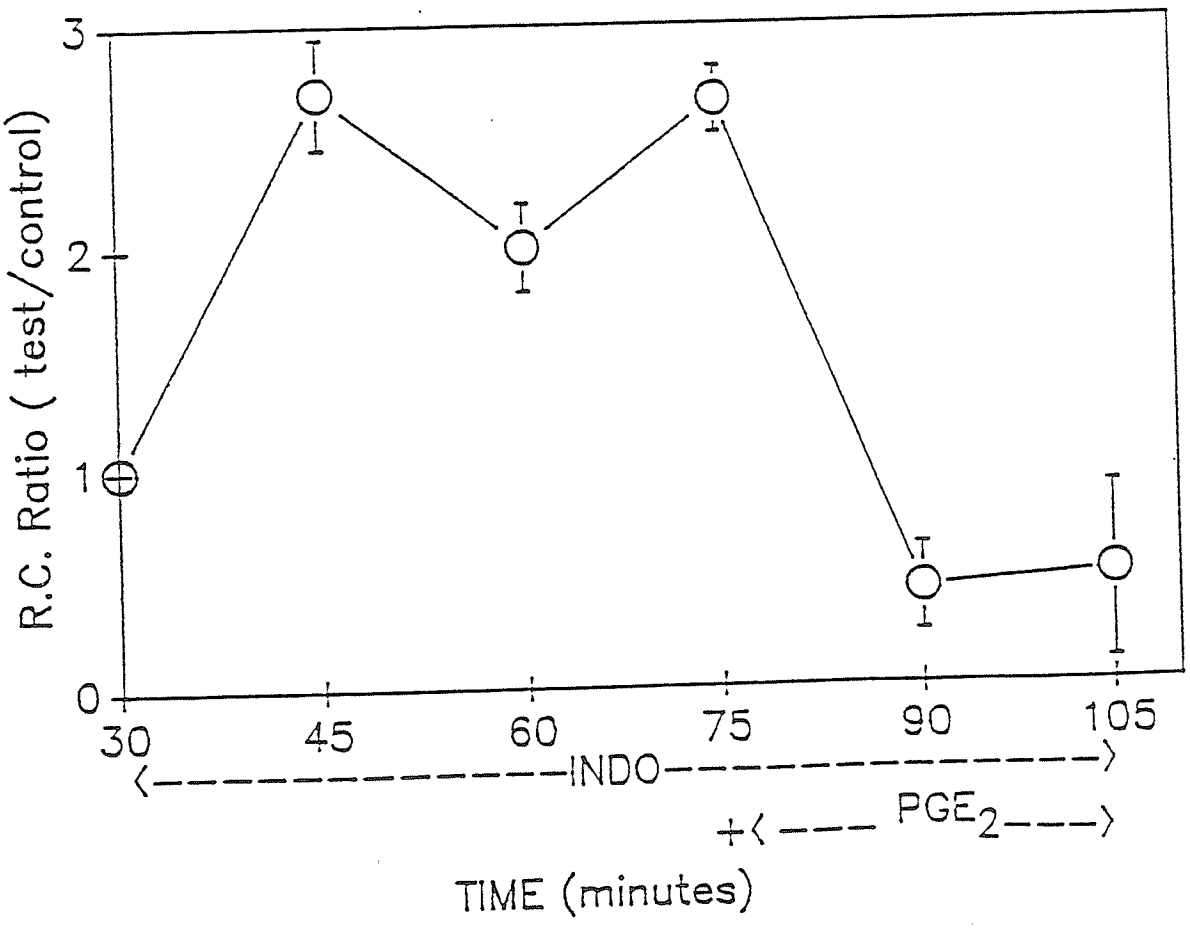
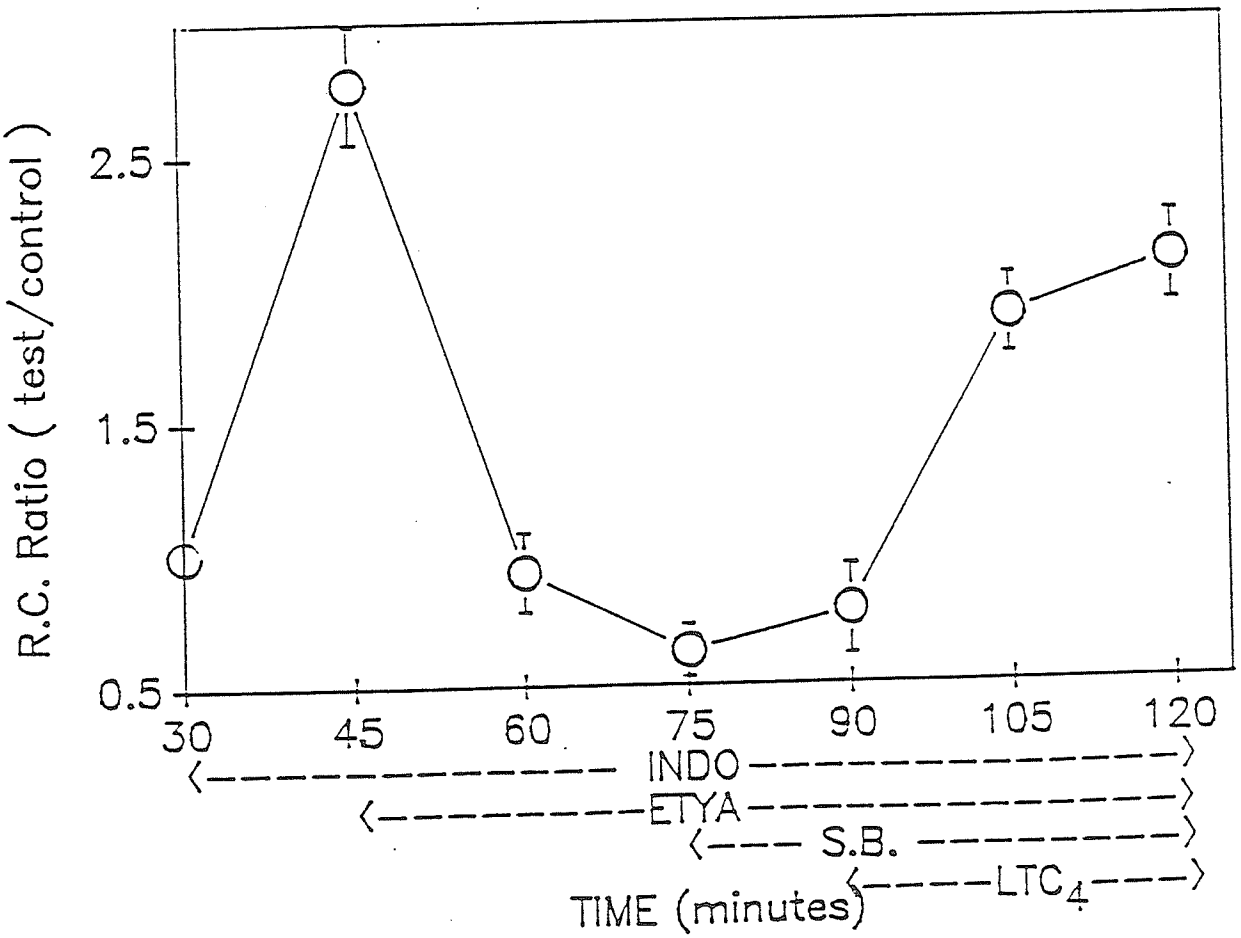


Figure 19. Effects of cumulative addition of indomethacin (10^{-5}M), ETYA (3.3 x 10^{-5}M), serine borate (0.5M) and LTC_4 (6 x 10^{-7}M) on rate coefficient of EFS efflux in EASM (n=8). Tissues were previously loaded with ^{3}H-choline. Values are expressed as a ratio of test efflux : control efflux. Details of calculations are given in the methods.



through should be unmasked with serine borate. Finally, the exogenous addition of LTC_4 increased active efflux 2 fold, demonstrating the capacity of lipoxygenase metabolites to regulate active neurotransmitter release. All relevant data are summarized in Table 4.

BLE 4. The effect on electrical field stimulated release acetylcholine with treatment of INDO, ETYA, S.B., LTC_4 and PGE_2 .

Treatment	[a] Efflux fraction	
Control	0.43 (+/-) 0.21	(8)
INDO (10^{-5}M)	1.1 (+/-) 0.35 *	(8)
Control	0.56 (+/-) 0.17	(8)
INDO+ ETYA ($3.3x10^{-5}$M)	0.37 (+/-) 0.17 *	(8)
Control	0.45 (+/-) 0.1	(8)
INDO+ ETYA+ S.B.(0.5M)	0.35 (+/-) 0.3 *	(8)
Control	0.51 (+/-) 0.14	(7)
INDO+ ETYA+ S.B.+ LTC_4 ($6x10^{-7}$)	1.07 (+/-) 0.3 *	(7)
Control	0.94 (+/-) 0.25	(7)
INDO+ PGE_2 (10^{-6}M)	0.4 (+/-) 0.23 *	(7)

LEGEND:
Mean (+/-) the standard error of the mean. The number in parenthesis represents the number of replications of experimements. Statistical significance (*) is established when $p<.05$ using Student's T-test.
[a] Calculations of the efflux fraction are stated in the methods.

DISCUSSION

A) Effects of INDO on neural and myogenic components in equine tracheal smooth muscle (TSM)

The purpose of these experiments was to investigate the effects of alterations in the balance of arachidonic acid metabolism between the cyclooxygenase and the lipoxygenase pathways on smooth muscle responsiveness and cholinergic neurotransmission of equine TSM.

INDO, a potent inhibitor of cyclooxygenase, produced actions which are consistent with (1) direct effects on smooth muscle by decreasing levels of generally inhibitory cyclooxygenase products, (2) inhibitory effects of cyclooxygenase products on cholinergic neurotransmitter release, (3) direct activation of the smooth muscle through increased levels of stimulatory lipoxygenase metabolites and 4) stimulatory effects of lipoxygenase products on cholinergic neurotransmitter release.

Our observation that INDO potentiated the response of equine TSM to electrical field stimulation (EFS) and shifted the S-R curve leftward and upward indicates potentiation of the neurogenic and, possibly, myogenic components. Since the cyclooxygenase pathway which is inhibited by INDO yields metabolites whose actions are primarily inhibitory on airway smooth muscle, these findings are consistent with the effects of INDO in the smooth muscle of human airways (Walters et al., 1982) and canine trachea (Yamaguchi et al., 1976) where it also potentiates responses.

The inhibition of guinea pig trachea by INDO, in contrast to its effects in equine TSM, deserves comment. Since PGF_{2a} (Orehek et al., 1973), thromboxane A_2, or low levels of PGE_2 (Burka et al., 1980) all have contractile effects in the guinea pig trachea, the net effect of INDO is to inhibit their production and allow relaxation.

On the other hand, INDO may also affect the lipoxygenase pathway. The observation that pretreatment with ATR and LY171883, a LTC_4/D_4 receptor antagonist (Aharony et al., 1987), significantly decreases the effect of INDO on basal tone suggests that modulation of both cholinergic and LT-sensitive components are involved in the response.

With reference to the effects of INDO on basal tone, we noted that this is increased by INDO and decreased by PGE_2 (IC_{50} $5x10^{-9}$ M) suggesting that inhibitory products of cyclooxygenase, which could be PGE_1, PGE_2, or PGI_2 are involved in the regulation of tone in equine airway smooth muscle and that they predominate functionally (vis a vis TXA_2 and PGF_{2a}).

In the canine trachea, Tesarowski and Kroeger (1981) have shown that INDO, which has little effect on the basal tone, potentiates responses to some stimulatory agents including histamine. Anderson et al. (1980) demonstrated the same potentiating effect of INDO in canine TSM. Exogenous addition of arachidonic acid caused marked relaxation of canine trachealis contracted with histamine and serotonin. This relaxation was INDO-sensitive, reflecting a

functionally important role of inhibitory PG's in this tissue. In contrast to canine TSM, equine TSM appears to possess a functionally dominant lipoxygenase pathway (Tesarowski, 1987). Evidence for this statement includes the observations that arachidonic acid induces a contractile response in equine TSM which is inhibited by FPL 55712, a LT receptor antagonist (Tesarowski, 1987).

In order to determine whether the effects of INDO result in part from increased ACh release from endogenous neural elements (Walters et al., 1984), the effect of ATR on INDO-pretreated equine TSM and radiolabelled release was examined. The observation that the INDO-induced increase in basal tone was partially ATR sensitive suggested that INDO-induced contractions in airways might indeed be mediated in part by acetylcholine (ACh) release. This was later confirmed with labelled release experiments (vidae infra), in which INDO enhanced both basal and electrically-induced efflux.

In control and INDO-pretreated muscles, ATR produced a rightward shift of the stimulus response (S-R) curve. With respect to the decrease seen at the lower stimulus strengths, our findings are consistent with those in canine TSM where tetrodotoxin, a selective sodium channel blocker, nearly abolishes responses to EFS (Bergen and Kroeger, 1986). Exposure of equine TSM to TTX, in ATR-pretreated muscles, produced an S-R relation superimposable on that of ATR treatment alone. Conversely, addition of TTX to un-

treated muscles produced a rightward shift of the S-R curve. Subsequent addition of ATR to TTX-pretreated muscles had no further effects. These findings suggest that neurotransmitters other than ACh do not play a role in contractile responses seen in equine TSM. In addition, HBr, a ganglionic blocker, had no effects on EFS-stimulated responses, confirming that ganglia are not involved.

With respect to the responses to the stronger stimuli, in addition to the cholinergic neural elements identified above, and a less excitable D-600 sensitive myogenic component was seen. As a consequence, the specific stimulus parameters are thought to determine the mode by which the muscle is activated. In the following discussion ATR-sensitive responses to low voltage are therefore designated as neural in origin whereas the ATR-insensitive component at higher voltages are considered to be myogenic. Pharmacological dissection of the responses to EFS thus permits evaluation of altered arachidonic acid metabolism on the two respective components.

B) Effects of INDO on cyclooxygenase-modulated neurotransmitter release.

The potent influences of prostaglandins on tone in the smooth muscle of the airways, blood vessels, gastrointestinal and reproductive tracts have been well described (see review by Bergstrom et al., 1968). Orehek and co-workers (1975) demonstrated that prostalgandins were released from

resting guinea pig trachea after mechanical stimulation of the tracheal mucosa. The epithelium has been proposed as a source of prostanoids that control the level of baseline tone in guinea-pig trachea (Orehek et al., 1973). Burka (1981) documented that the major prostanoid produced depends on the species and tissue in question. Sensitized guinea pig lung parenchyma releases considerable quantities of TXA_2 and PGI_2 when challenged with specific antigen (Boot et al., 1978), whereas the major prostanoid released from the guinea pig trachea is PGE_2 (Burka et al., 1981).

The first area of investigation had to do with the control of ACh release by PGE_2. The observation that INDO potentiated ATR-sensitive responses to EFS indicates a role for cyclooxygenase and/or lipoxygenase metabolites in the regulation of cholinergic transmitter release in a manner consistent with that proposed by Walters et al. (1985) who suggested that INDO may inhibit PGE_2-induced regulation of pre-junctional ACh release. An effect of these metabolites on the excitability of the smooth muscle membrane was indicated by the potentiation of responses to EFS in ATR-pretreated muscles (present findings). The preliminary findings suggested a regulatory role of PG's and/or LT's in the neurotransmitter release. The regulation of neurotransmitter release by both cyclooxygenase and lipoxygenase metabolites was later confirmed with labelled-release experiments (vidae infra).

To examine whether PGE_2, a prominent metabolite of the cyclooxygenase pathway produces effects which are consistent with the inhibitory roles discussed above, its effects on the neural and myogenic components of the S-R relationship and on labelled-release were investigated. Exogenous PGE_2 (10^{-8}M) significantly inhibited the neural component and, at higher concentrations (10^{-7}M), it inhibited both the neural and myogenic components. Addition of PGE_2 (10^{-7} M) to ATR-pretreated muscles produced a further decrease in response to stimulation at higher voltages (myogenic) but failed to shift the S-R relation rightward. These findings confirm the involvement of cyclooxygenase metabolites in regulation of both neural and myogenic responses. In the labelled-release experiments, PGE_2 significantly inhibited both basal and electrical field stimulated release.

With reference to the neural component, the above results are consistent with those obtained by Walters et al. (1985) in canine TSM. They demonstrated an inhibitory effect by low concentrations of PGE_2 on responses to EFS in canine TSM at low stimulus strengths. Increasing the concentration of PGE_2 inhibited responses at all stimulus intensities. These results support the role of PGE_2 in the regulation of airway contractility. As was discussed above, EFS of canine TSM occurs almost exclusively via the cholinergic neural component and therefore the effect on the myogenic component is not evaluated in that model.

Anderson and co-workers (1983) suggested that PGE_2 affected calcium mobilization caused by histamine treated canine tracheal smooth muscle and suppressed the contractile response to this agonist. Thus INDO, by inhibiting PGE_2 production, enhance the contraction of the muscle directly. The effects of PGE_2 on calcium mobilization have been reported for a variety of cell type (Emmons et al., 1979; Fassina et al., 1969). Indeed it has been suggested that in adrenergic nerve terminals, PGE_2 may function as a presynaptic regulator of neurotransmitter release by blocking calcium influx (Hedqvist, 1974).

C. Effects of INDO via increasing lipoxygenase metabolites

The possibility that the actions of INDO are also partially mediated by an increase in lipoxygenase metabolism was also investigated. The studies in equine TSM reveal a different relationship between the metabolism of arachidonic acid and the regulation of tone compared with canine TSM. The latter requires an increase in tone before the effects of arachidonic acid or its metabolites can be demonstrated (Anderson et al., 1980). In contrast, unstimulated equine TSM responds to the addition of arachidonic acid and/or INDO with a contraction (Tesarowski, 1987, present study). Morris et al. (1979) reported that NDGA, an inhibitor of lipoxygenase, and D-600, a voltage-gated calcium channel blocker, both attenuated the responses to INDO. Piriprost, a putative lipoxygenase inhibitor and possibly LT end-organ

antagonist (Bach et al., 1982), shifted the dose-response curve to INDO rightward without affecting maximal response (Tesarowski, 1987). These data support and strengthen the argument that inhibition of cyclooxygenase redirects substrates to favour the increased production of lipoxygenase metabolites.

Our preliminary observations showed that the INDO-induced increase in basal tone of equine TSM was partially sensitive to ETYA or LY171883. Since ETYA is an arachidonic acid analog which is an inhibitor of cyclooxygenase and lipoxygenase (Hamberg and Samuelsson, 1974), it was added after INDO-pretreatment to eliminate any interference from cyclooxygenase-related effects. ETYA has previously been shown to inhibit SRS-A formation by RBL-1 cells (Jakschik et al., 1978). In INDO-pretreated muscles, ETYA still caused a decrease in responses to EFS. It therefore seemed possible that a component of the effects produced by INDO might reflect increased levels of stimulatory lipoxygenase metabolites. In evaluating this possibility in electrically stimulated muscles our results showed that in INDO-pretreated muscles ETYA shifted the S-R curve rightward and downward, significantly inhibiting the myogenic component and to a lesser extent the cholinergic neural component. The effects of lipoxygenase metabolites on cholinergic neurotransmitter release was further investigated (vidae infra) and revealed that these metabolites, like cyclooxygenase metabolites, may also regulate neurotransmitter release. However lipoxygenase

metabolites, unlike cyclooxygenase metabolites, increased the labelled efflux.

The redirection of the metabolites of arachidonic acid to favour increased production of the bronchoconstrictor lipoxygenase metabolites has been proposed as a mechanism of the action of INDO on guinea-pig tracheal smooth muscle, human lung, rabbit trachea, and bovine lung (Walker, 1973; Adcock and Garland, 1980; Burka and Paterson, 1980; Watanabe-Kohno and Parker, 1980). Engineer and coworkers (1978) demonstrated that cyclooxygenase inhibitors enhance SRS-A release. Burka (1980) showed that guinea pig trachea treated with INDO can be further relaxed with ETYA, and the LTD_4 receptor antagonists L-649,923 and LY-163,443 (Burka, 1988) suggesting a functional antagonism between products of the lipoxygenase and cyclooxygenase pathways of arachidonic acid metabolism.

Tesarowski (1987) further quantified the response to INDO in equine TSM by analyzing the bath solution following cyclooxygenase inhibition by reverse phase high-pressure liquid chromatography. His results indicated that in the presence of INDO, a large quantity of LTE_4 could be detected. LTE_4, although not as potent as LTC_4 or LTD_4, is an important mediator of bronchoconstriction (Piper and Samhoun, 1987).

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The observation that LY171883 and ETYA (data not shown) had no effect on resting tension but both inhibited an INDO-

induced contraction indicated the necessity of active tone before effects of the drugs can be seen.

D. Effects of altered arachidonic acid metabolism on basal and electrical field stimulated neurotransmitter release

The above results obtained from the INDO S-R relations argue for an increased release of ACh through inhibition of cyclooxygenase metabolites and/or increased production of lipoxygenase metabolites. To examine the involvement of cyclooxygenase and lipoxygenase metabolites in these responses, labelled-release experiments were conducted.

Equine TSM strips treated with INDO showed significant increases in basal release of neurotransmitter. Exogenous addition of PGE_2 inhibited this release. These results substantiate the findings of Walters et al. (1984). They found that the contractile responses of canine TSM to EFS-stimulation diminished over a 2 hour period of time. Measured PGE_2 increased in the bath, an effect that was prevented by the addition of INDO. The latter not only prevented this inhibition but also markedly increased response to EFS-stimulation. In contrast, incubation of muscle alone, treatment with INDO or PGE_2, had little effect on cholinergic neurotransmission at the prejunctional site. The present experimental findings offer direct evidence of the modulation of pre-junctional neurotransmitter release by cyclooxygenase metabolites.

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The effects of lipoxygenase metabolites on cholinergic neurotransmitter release from airways, has not, to our knowledge, been previously investigated. Our results demonstrate that ETYA, a cyclooxygenase and lipoxygenase inhibitor, reduces choline efflux. This effect could be interpreted as the redirection of substrates favouring the cyclooxygenase pathway and therefore the inhibition of choline efflux. However, the exogenous addition of LTD_4, together with evidence obtained from the EFS-stimulated efflux experiments (vidae infra), demonstrated that lipoxygenase metabolites also participate in the regulation of neurotransmitter release. Unlike Tesarowski's results (1987) these results do not establish which pathway functionally predominates in this tissue.

To obtain a better understanding of the workings of altered arachidonic acid metabolism in the <u>in vivo</u> conditions, efflux experiments under EFS-stimulation conditions were examined. The tracheal smooth muscle is under the influence of the vagal system. The latter does not regulate the basal release of neurotransmitter, but does control the active release. Thus, it is important to examine the muscle in conditions that can be extrapolated to <u>in vivo</u> states.

To ensure that the release of the label seen in effect represents neurogenic release, muscles were incubated with TTX. The latter, being a neural sodium channel blocker, inhibits action potential-stimulated release of neurotransmitter from all nerves in the preparation. Thus, measuring

EFS-stimulated efflux, after the addition of TTX, would reveal any non-neurogenic-mediated efflux. The results (not shown) indicate that the majority of the EFS-induced efflux is indeed abolished with the addition of TTX. The small amount of residual efflux could represent release from elements other than the nerves. These results demonstrate that the EFS-induced release of label represents efflux predominantly from the neurogenic elements.

Muscles were firstly incubated with ATR, a muscarinic antagonist, to ensure that all results are interpreted as pre-junctional influences. INDO increases EFS-stimulated choline efflux, an effect that is inhibited with PGE_2. Our present results strongly indicate that PGE_2 inhibits pre-junctional release of choline, an effect that is reversed with INDO. Once again this is consistent with the hypothesis put forth by Walters et al.(1984). The latter showed that a role of PGE_2 in the regulation of airway contraction in canine TSM <u>in vitro</u> is via the constant synthesis of this metabolite. The latter inhibits the contractile responses to EFS and, at higher concentrations, contractions induced by ACh.

Other effects of INDO include the rerouting of substrates to the competing lipoxygenase pathway as discussed previously. This hypothesis was tested in further experiments. ETYA addition, after INDO, caused a dramatic decrease in EFS-stimulated choline efflux. This effect can no longer be attributed to the redirection of substrates to

the cyclooxygenase pathway as INDO was already present in the preparation. Thus, lipoxygenase metabolites may also participate in the control of neurotransmitter release, and these metabolites are produced with the addition of INDO. Exogenous addition of LTC_4, in the presence of serine-borate, enhanced EFS-induced label efflux and clearly demonstrated that lipoxygenase metabolites indeed participate in the pre-junctional modulation of choline efflux.

The redirection of substrates was hypothesized by Walker (1973). To date, no evidence had been obtained on the participation of lipoxygenase metabolites in the control of cholinergic neurotransmission. These studies provide such evidence and allow for a functional "ying-yang" relation between the two pathways.

In conclusion, our data are consistent with a functionally important role of cyclooxygenase- and lipoxygenase- metabolites in the modulation of airway smooth muscle tone. INDO may exert its effects by modulating the production and, therefore, the direct effects of potent cyclooxygenase- and lipoxygenase- associated metabolites and indirectly by enhancing ACh release from neural stores. Detailed analysis of the interactions of the pathways of arachidonic acid metabolism and the functional balance of the respective metabolites may provide insights into the regulation of airway smooth muscle tone as well, perhaps, of the aspirin-sensitivity of some asthmatics.

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