NEUROGENIC CONTROL OF CANINE BRONCHIAL

SMOOTH MUSCLE: COMPARISON OF THE NORMAL

AND EXPERIMENTALLY-INDUCED HYPERRESPONSIVE STATES

By

LUKE JEFFREY JANSSEN, B.Sc., M.Sc.

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AUTHOR: Luke Jeffrey Janssen, B.Sc. (McMaster University)
M.Sc. (McMaster University)

SUPERVISOR: Dr. E.E. Daniel

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ABSTRACT

Asthma is a disease characterized by airway hyperresponsiveness (AH) to various spasmogens. Ozone-inhalation causes transient AH of human and canine airway smooth muscle (ASM) and has therefore been used as a model of AH. The mechanism(s) underlying ozone-induced or asthma-related AH are not completely understood, but there is a growing body of evidence that there is a causal relationship between airway inflammation and AH.

In these studies, the mechanisms regulating canine bronchial smooth muscle (3rd to 5th order) activity were investigated using intracellular microelectrodes (to monitor electrical activity) and muscle baths (to monitor mechanical activity). The effects of ozone-inhalation and inflammatory mediators on regulation of ASM tone were also investigated.

Excitation of ASM (membrane depolarization and contraction) was elicited by activation of muscarinic receptors or thromboxane receptors; the former were found to be of the M_3-subtype. The data indicated that pharmacomechanical (rather than electromechanical) coupling was involved.

Inhibition of ASM was found to be initiated by activation of ß-adrenoceptors; these were found to be predominantly of the $ß_1$-subtype, though there was evidence that some $ß_2$-adrenoceptors were also involved. There was evidence of physiological antagonism between cholinergic receptor-mediated excitation and adrenoceptor-mediated inhibition due to reciprocal effects on adenylate cyclase.

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Release of acetylcholine from the cholinergic nerve endings was antagonized by activation of muscarinic receptors (M_1-subtype) or adrenoceptors (both β_1- and β_2- subtypes), and potentiated by activation of thromboxane receptors; these receptor populations were presumed to be located on the nerve endings. There was indirect evidence that neurotransmitter-release was also antagonized by a cyclo-oxygenase metabolite of arachidonic acid (presumably PGE_2).

Ozone-inhalation significantly enhanced contractions in response to electric stimulation, but did not significantly a'ter those to exogenously-added cholinergic agonist or to KCl. This AH was prevented by indomethacin (cyclo-oxygenase inhibitor) or by L-670,596 (thromoboxane receptor antagonist). It was concluded that the mechanism underlying ozone-induced AH involved decreased prejunctional inhibition (mediated by PGE_2) and/or increased prejunctional excitation (mediated by TxA_2).

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LIST OF ABBREVIATIONS

AA . . . . arachidonic acid

AC . . . . adenylate cyclase

Ach . . . . . . acetylcholine

ADP . . . . . . adenosine diphosphate

AF-DX . . . . . AF-DX 116

AH . . . . airway hyperresponsiveness

AP . . . . action potential

ASM . . . . . . airway smooth muscle

ATP . . . . . . adenosine triphosphate

Bch . . . . . . bethanechol

Ca^{++}_i . . . . intracellular calcium ion

cAMP . . . . . cyclic adenosine-3',5'-monophosphate

Cch . . . . . . carbachol

cGMP . . . . . cyclic guanosine-3',5'-monophosphate

CGRP . . . . . calcitonin gene related peptide

DAG . . . . . . diacylglycerol

DMSO . . . . . dimethylsulfoxide

EC_x . . . . . concentration eliciting x% maximal excitation

EDRF . . . . . endothelium-derived relaxing factor

EJP . . . . . . excitatory junction potential

EpDRF . . . . epithelium-derived relaxing factor

FS . . . . [electrical] field stimulation

GC . . . . guanylate cyclase

HETE . . . . . hydroxyeicosatetraenoic acid

hexa . . . . . . hexahydrosiladifenidol

IC_{50} . . . . concentration eliciting 50% inhibition

IDM . . . . . . indomethacin

IP_3 . . . . . inositol trisphosphate

Iso . . . . . . isoproterenol

Lt . . . . leukotriene

MLCK . . . . . myosin light chain kinase

NANC . . . . . non-cholinergic, non-adrenergic

NE . . . . norepinephrine

NMJ . . . . . . neuromuscular junction

Oxo . . . . . . oxotremorine

PAF . . . . . . platelet activating factor

PG . . . . . . prostaglandin

PHI . . . . . peptide histidine isoleucine

PI . . . . . . phosphoinositol

Pir . . . . . pirenzepine

PK . . . . . . protein kinase

pps . . . . . pulses per second

R_m . . . . . . [cell] membrane resistance

Sal . . . . . salbutamol

SAV . . . . . small agranular vesicle

SEM . . . . . standard error of the means

SGV . . . . . small granular vesicle

SR . . . . . . sarcoplasmic reticulum

TTX . . . . . tetrodotoxin

TxA_2 . . . . . thromboxane A_2

VIP . . . . . vasoactive intestinal peptide

V_m . . . . . . [cell] membrane potential

LIST OF DRUGS USED

drug		site of action	selectivity
AF-DX 116	antagonist	muscarinic receptors	$M_2 > M_3 > M_1$
atropine	antagonist	muscarinic receptors	$M_1 = M_2 = M_3$
Bch	agonist	muscarinic receptors	$M_2 = M_3 >> M_1$
Cch	agonist	muscarinic receptors	$M_1 = M_2 = M_3$
forskolin	agonist	adenylate cyclase	
Hexa	antagonist	muscarinic receptors	$M_3 > M_2 > M_1$
histamine	agonist	histamine receptors	
ICI 89,406	antagonist	ß-adrenoceptors	$\beta_1 >> \beta_2$
ICI 118,551	antagonist	ß-adrenoceptors	$\beta_2 >> \beta_1$
IDM	antagonist	cyclo-oxygenase	
Iso	agonist	ß-adrenoceptors	$\beta_1 = \beta_2$
L-670,596	antagonist	thromboxane receptors	
McN A343	agonist	muscarinic receptors	$M_1 >> M_2 = M_3$
nitrendipine	antagonist	calcium channels	voltage-operated
norepinephrine	agonist	adrenoceptors	$\alpha = \beta_1 = \beta_2$
oxotremorime	agonist	muscarinic receptors	$M_2 = M_3 >> M_1$
phentolamine	antagonist	adrenoceptors	$\alpha >> \beta$
pirenzepine	antagonist	muscarinic receptors	$M_1 >> M_2 = M_3$
potassium chloride	agonist	membrane	depolarizes
propranolol	antagonist	ß-adrenoceptors	$\beta_1 = \beta_1$
salbutamol	agonist	ß-adrenoceptors	$\beta_2 >> \beta_1$
timolol	antagonist	ß-adrenoceptors	$\beta_1 = \beta_2$
U46619	agonist	thromboxane receptors	

INTRODUCTION

(1) ASM Morphology

The overall design of the respiratory system of vertebrates can be compared to that of an inverted tree, with large passages progressively dividing into ever more numerous and smaller passages. The largest of these is the trachea, which divides into the left and right bronchi. The latter, in turn, divide into a number of second order bronchi, each of which conduct air to one of the lobes of lung (six in total in the case of the canine species). The second order bronchi within each lobe then branch into third order, fourth order, etc., until the tissue becomes a tangled web of microscopic airways with sac-like terminations, referred to as alveoli, where gas exchange with the blood occurs. The innervation to the airways has a similar branching structure, which mirrors that of the airways. Finally, the arterial and venous systems invade the lungs at the same point where the second order bronchi enter each lobe, and then continue to follow the branching network of airway passages. In the airways used in this project, the pulmonary artery and the pulmonary vein were typically found immediately juxtaposed to the airway (and on opposite sides), which greatly facilitated dissection and excision of the tissue.

It is important to keep in mind this overall structure, since there are regional differences with respect to structure and

function which will be referred to throughout this thesis; in addition, special attention will be focussed on these differences in section 3 and in the discussion of the data.

A more complete description of the various cellular components of ASM is given below. A thorough description of each is beyond the scope of this thesis. However, there are certain aspects which warrant description due to their relevance to this project.

(1.1) Smooth muscle The central focus of this project is the sheet of smooth muscle cells which regulate the flow of air throughout the airways. This sheet completely encircles the lumen of the smaller airways, much like the smooth muscle cells of the vasculature, but forms only a band running up and down the length (dorsal aspect) of the trachea and first order bronchi (de Jongste, 1987a). In both cases, however, the individual smooth muscle cells are aligned in the same direction, with the long axis of the cells perpendicular to the axis of the airway lumen.

Airway smooth muscle (ASM) cells are spindle-shaped, with a mean length of 800 μm (de Jongste, 1987a; Gabella, 1989). The cells are mechanically coupled by desmosomes or tight junctions, and by the intercellular matrix (fibers of collagen, elastin, and eleunin-oxytalin) (de Jongste, 1987a; Gabella, 1989). Gap junctions also link the smooth muscle cells, although their function is not only to provide structural integrity; these also allow communication of metabolic products between the cells (metabolic coupling) and, more importantly, electrical continuity

(electrical coupling; de Jongste, 1987a). In human and canine airways, gap junctions are abundant in the tracheal smooth muscle; these decrease in number in human (but not canine) ASM as one progresses down the airway (Kannan and Daniel, 1980; Daniel *et al.*, 1986).

The cell membrane is covered with small invaginations referred to as caveolae, whose function is still debated (de Jongste, 1987a). The membrane is also studded with receptors, ion channels, ion pumps, and ion exchangers, the significance of which are more fully described in section 2.

With respect to intracellular organelles, ASM cells possess a centrally-located, cigar-shaped nucleus, few mitochondria, and sparse amounts of sarcoplasmic reticulum (relative to skeletal muscle cells; de Jongste, 1987a).

The interior of the cell is laced with fibers of myosin and actin which are the most important components of the cells (from a functional perspective). Both are large polypeptides: myosin consists of two heavy chains (200 kDaltons) and two types of light chains (17 and 20 kDaltons each), while actin consists of two chains (42 kDaltons) wrapped in a helix (de Jongste, 1987a). Both have been found in all mammalian cells studied to date (de Lanerolle, 1989), although the physiological role typically attributed to them is as the primary contractile elements in cardiac, skeletal, and smooth muscles. When given the appropriate stimulus (usually increased levels of Ca^{++}), actin and myosin interact with each other to produce contraction of the cell; this

is more fully described in sections 2.1 and 2.2. In tracheal smooth muscle, each myosin filament has 12-14 filaments of actin in its immediate proximity, in contrast to the ratio of 6:1 typical of skeletal muscle (de Jongste, 1987a). ASM myosin content has been shown to decrease significantly as one progresses down the airways (from trachea to 4th or 5th order bronchi). The actin filaments are firmly anchored to the cell membrane (attached to structures referred to as dense bands) and to similar attachment sites in the cytoplasm (dense bodies; de Jongste, 1987a).

(1.2) Innervation Vagal nerve fibers originating in the CNS extend to hundreds of parasympathetic ganglia found in the wall of the larger airways; these ganglia are linked by the interganglionic nerve trunk, thus forming a neural plexus within the wall of the airways (Coburn, 1989; Gabella, 1989; Said, 1989). The sympathetic nervous system, on the other hand, sends fibers to the superior cervical ganglia and stellate ganglia. Postganglionic fibers then project from the sympathetic and parasympathetic ganglia and terminate on airway smooth muscle cells, secretory cells, and nerves (Gabella, 1989). Unlike some other smooth muscle systems, such as the vas deferens, there is an unusually large separation between the nerve varicosities and the smooth muscle (Gabella, 1987; Daniel *et al.*, 1986): for example, these have been shown to be separated by 140 nm or more in the canine species (Daniel *et al.*, 1986). This large gap at the NMJ has important functional implications which will be described subsequently.

There are also sensory nerves which originate in the epithelial and adventitial layers of ASM and which project via the vagus nerve or the dorsal root ganglia to the ganglia and thus allow integration of the sensory input into the effector output or on to higher centers of the CNS (Said, 1989).

Numerous studies have shown that both the density and the type of innervation changes from region to region in ASM (it should also be pointed out that both these parameters vary from species to species). For example, the density of the innervation in human and canine ASM was found to be less than 1 axon per 100 muscle cell profiles in trachealis, but 18 axons per 100 muscle cells in 4th-7th order bronchi (Daniel *et al.*, 1986; Daniel, 1988). Coburn has shown that catecholamine-containing neurons may be rich in one segment of human ASM and absent in another (Coburn, 1989).

The nature of these different nerves (i.e., the neurotransmitters released) has been characterized in many different ways. Electronmicroscopy has been used to identify cholinergic, adrenergic, and peptidergic fibers on the basis of the presence of small granular vesicles, small agranular vesicles, and large agranular vesicles, respectively; however, there is often debate when this approach is used to identify the nerve types. Histochemical approaches have also been developed, including acetylcholinesterase histochemistry to identify cholinergic fibers, catecholamine fluorescence staining for adrenergic fibers, and immunohistochemistry for neuropeptide-releasing nerves. Autoradiographic techniques have been used to identify adrenergic

fibers following the uptake of tritiated norepinephrine or epinephrine. Finally, pharmacological approaches have been used extensively to identify cholinergic nerve-mediated responses on the basis of sensitivity to atropine. Adrenergic nerve-mediated responses, on the other hand, are identified on the basis of sensitivity to phentolamine, propranolol, or selective neurotoxins such as 6-hydroxydopamine. Those responses not affected by these agents are referred to as non-cholinergic, non-adrenergic (NANC) and are usually assumed to be mediated by peptidergic nerves.

In all species studied, whether mammal, reptile, amphibian, or bird, the primary excitatory efferent input is cholinergic in nature (Gabella, 1987; Coburn, 1989; Barnes, 1989). Other nerve types contributing to excitation of smooth muscle (but may nonetheless be sensory fibers) include those that release the neuropeptides substance P, neurokinins A and B, bombesin, and calcitonin gene related peptide (CGRP; Gabella, 1987; Barnes, 1989; Said, 1989). The adrenergic nervous system also seems to play a minor excitatory role in species such as rat, rabbit, and human (Gabella, 1987).

The nature of the primary inhibitory efferent input, on the other hand, varies from species to species. In many cases - including the canine, guinea pig, and human species - the inhibitory fibers are predominantly adrenergic (Gabella, 1987). NANC fibers also play a significant inhibitory role in some of these same species (eg., human and guinea pig), and constitute the

primary inhibitory input in others such as the feline species; the neurotransmitter released by these NANC motor fibers is believed by many to be vasoactive intestinal peptide (VIP) and/or peptide histidine isoleucine (PHI; Lazarus *et al.*, 1986; Lundberg and Saria, 1987; Barnes, 1989; Said, 1989; Widdicombe, 1989).

The sensory innervation seems to typically employ neuropeptides, including tachykinins such as subtance P, neurokinins A and B, neuropeptide Y, VIP, PHI, neuropeptide K, and CGRP (Lundberg and Saria, 1987; Said, 1989; Widdicombe, 1989).

(1.3) Epithelium Overlying the surface of the airway lumen is a layer of epithelial cells, ciliated cells, and mucous cells. There are regional differences in the epithelial layer: pseudostratified columnar epithelial cells are found in the larger airways, while those in the smaller airways are simple cuboidal cells (Jacobs and Kaliner, 1989). The epithelial layer forms a barrier between the external environment (inspired air) and the corpus proper. The impermeability of the epithelium (i.e., the integrity of this barrier) is maintained by tight and intermediate junctions (zonula occludens and zonula adherens, respectively; Jacobs and Kaliner, 1989). Interspersed among the epithelial cells are goblet cells which, when stimulated by the innervation, secrete mucous to prevent drying out of the tissue as well as to trap inhaled dirt, bacteria, and viruses.

(1.4) Inflammatory cells A number of inflammatory cells can also

be found at various loci in ASM. Some are found in ASM under normal conditions (mast cells and alveolar macrophages), while others (neutrophils and eosinophils) migrate in from the blood under the influence of various chemotactic factors following inhalation of ozone (Fabbri *et al.*, 1984; Seltzer *et al.*, 1986; Sibille and Reynolds, 1990), LTB$_4$ (O'Byrne *et al.*, 1985; Sibille and Reynolds, 1990), or allergen (Chung, 1985b). All can synthesize and secrete various inflammatory mediators (various arachidonic acid metabolites, histamine, PAF, reactive oxygen metabolites, etc.) which have potent effects on ASM function and/or other inflammatory cells (Jacoby, 1989; Jacobs and Kaliner, 1989; Sibille and Reynolds, 1990). Finally, platelets are also a source of the inflammatory mediators listed above and can also release them following the same stimuli. The importance of these cells will be discussed in section 6.5.

(2) ASM Physiology

(2.1) Actomyosin ATPase activity Airway function, from the perspective of this thesis, is simply contraction and relaxation of bronchial smooth muscle. These, in turn, are simply manifestations of the reversible interaction between actin and myosin.

Actin activates a Mg^{++}-ATPase activity of myosin following the appropriate stimulus; in the process of metabolizing a molecule of ATP to ADP and inorganic phosphate, myosin moves a microscopic

distance along a strand of actin (de Jongste, 1987a; de Lanerolle, 1989). Thus, muscle cells can control the degree of contraction or relaxation, respectively, by up- or down- regulating the actin-activated Mg^{++}-ATPase activity of myosin (subsequently referred to as actomyosin ATPase activity).

(2.2) Phosphorylation of myosin   Unlike skeletal muscle actin, smooth muscle actin does not spontaneously increase actomyosin ATPase activity (Sellers et al., 1981; de Lanerolle, 1989). Phosphorylation of the 20 kDalton subunit (light chain) of myosin, however, increases such activity 11-fold (Sobieszek, 1977; Sellers et al., 1981).   Conversely, dephosphorylation of phosphorylated myosin is accompanied by a decrease in actomyosin ATPase activity and relaxation (Haeberle, 1985; de Lanerrolle, 1989).   Thus, phosphorylation of myosin is the primary and crucial step in regulating smooth muscle contraction.

It should be mentioned, however, that there is controversy regarding whether such phosphorylation is merely the "switch" which initiates contraction or is also necessary to maintain contractile force, since some have shown that steady-state phosphorylation correlates with maintained force (de Lanerolle et al., 1982; Haeberle et al., 1985; Gerthoffer, 1986) while others have shown that contractile force can be maintained even though myosin is dephosphorylated to pre-contraction levels (Dillon et al., 1981). The latter observations led to a modification of the "cross-bridge theory" accounting for how actin and myosin interact to produce

contractile force, this being referred to as the "latch-bridge theory" (Dillon *et al.*, 1981; de Jongste, 1987a; de Lanerolle, 1989); the key differences between the two mechanisms is that the latch bridges are activated several seconds after activation of the cross bridges, and are much more efficient (consume less energy for a given sustained contraction) than the cross bridges (de Jongste, 1987a; de Lanerolle, 1989).

At least three kinases have been identified which phosphorylate myosin light chain. The first of these to be identified was Ca^{++}/calmodulin-activated myosin light chain kinase (MLCK; Dabrowska *et al.*, 1978; Adelstein and Klee, 1981). When intracellular levels of Ca^{++} (Ca_i^{++}) exceed $\approx 10^{-6}$M, Ca^{++} binds to calmodulin; up to four Ca^{++} ions bind to each calmodulin molecule (de Jongste, 1987a). When calmodulin is saturated with Ca^{++}, it interacts with and activates MLCK, thus bringing about contraction (de Jongste, 1987a). Regulation of Ca_i^{++} is therefore a crucial step in regulating smooth muscle contraction, and are described in the next section.

Protein kinase C (PK-C) can also phosphorylate myosin following activation by phosphoinositide metabolites (de Jongste, 1987a; Coburn and Baba, 1989). Although this enzyme phosphorylates myosin to the same degree as MLCK (i.e., 2 molecules of PO_4 per molecule of myosin; Sellers *et al.*, 1981; Nishikawa *et al.*, 1983), it does so at a different site on the light chain than does MLCK (i.e., up to 4 molecules of PO_4 can be covalently attached to each myosin molecule; de Lanerolle, 1989; Nishikawa, 1983).

Finally, myosin is also a substrate of cAMP-dependent protein kinase (Noiman, 1980; Nishikawa *et al.*, 1984); phosphorylation by this kinase occurs at such a slow rate, however, that some believe that this mechanism of myosin phosphorylation is physiologically unimportant (Walsh *et al.*, 1981; de Lanerolle, 1989).

(2.3) Excitation-contraction coupling Since the phosphorylation-state of myosin determines the contractile state of the smooth muscle cell, it is important to describe the mechanisms which alter the activity of the kinases and phosphatases described above; for the sake of brevity, this will be restricted to those mechanisms known or believed to be operative in ASM.

Excitation and contraction in ASM is coupled in two ways – pharmacomechanically and electromechanically – as described below. In both cases, however, changes in Ca_i^{++} play a major role.

Ca_i^{++} is normally maintained at a level at which MLCK is relatively inactive (i.e., $\approx 10^{-8}$-10^{-7}M; de Jongste, 1987a). This low level is maintained by mechanisms which extrude Ca_i^{++} from the cell and those which sequester Ca_i^{++} into enclosed pools (away from the contractile elements).

Ca^{++}-extrusion is mediated by ionic exchange mechanisms, which use the Na^+- electrochemical gradient to drive Ca_i^{++} out against its electrochemical gradient (at rest, the intracellular and extracellular concentrations of Ca^{++} are 10^{-8} and 10^{-3}M, respectively; de Jongste, 1987a). In addition, some suggest there are Ca^{++} pumps on the cell membrane through which the ß-agonists can

produce relaxation (see section 2.4).

The sarcoplasmic reticulum (SR) is widely believed to be the most important of the intracellular organelles into which Ca_i^{++} is sequestered (Rasmussen *et al.*, 1987; de Jongste, 1987a; Twort and van Breeman, 1988; Coburn and Baba, 1989). Ion pumps are found in the membrane boundaries of the SR which continuously pump Ca_i^{++} against its electrochemical gradient into the SR lumen; the concentration of Ca^{++} within the lumen has been estimated to be approximately $10^{-4}M$. On the other hand, Ca_i^{++} is increased by its release from intracellular stores or by the influx of extracellular Ca^{++} through Ca^{++}-channels on the cell membrane, as described below.

Pharmacomechanical coupling   PK-C activity has been found to be coupled to muscarinic receptor activation through phosphoinositide metabolism (Kikkawa *et al.*, 1983; Baron *et al.*, 1984; Grandordy *et al.*, 1986; Rasmussen *et al.*, 1987; Baba *et al.*, 1989; Coburn and Baba, 1989). In particular, binding of muscarinic agonists to the muscarinic receptor induces a conformational change in the receptor leading to activation of phospholipase C, which then metabolizes inositol phospholipids in the membrane, yielding inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). PK-C is activated by DAG directly, while MLCK is activated by IP_3 indirectly by IP_3-induced release of Ca^{++} from the SR (Silver and Stull, 1985; Hashimoto *et al.*, 1985; de Jongste, 1987a; Twort and van Breeman, 1988). The fact that cholinergically mediated contractions are predominantly mediated by these mechanisms, rather than influx of extracellular Ca^{++}, is attested to by the finding

that such contractions in this tissue are insensitive to removal of extracellular Ca^{++} or interference of transmembrane Ca^{++} fluxes except when repeated stimulation leads to depletion of intracellular Ca^{++} stores (Farley and Miles, 1977; Coburn, 1979; Small and Foster, 1987; Coburn and Baba, 1989).

Other spasmogens which elicit contractions by inducing phosphoinositide metabolism include substance P (Grandordy *et al.*, 1988; Barnes, 1989), histamine (Grandordy *et al.*, 1987; Barnes, 1989), and in some species the leukotrienes C_4 and D_4 (Barnes, 1989).

Finally, Ca_i^{++} can also be increased by the opening of [voltage-independent] receptor-operated channels, which may also in turn release Ca^{++} from the SR (i.e., Ca^{++}-induced Ca^{++}-release; de Jongste, 1987a). Caffeine and related substances can also release Ca^{++} from intracellular stores (de Jonste, 1987a).

Electromechanical coupling MLCK can also be activated by extracellular Ca^{++} which enters the cells through various voltage-dependent Ca^{++}-channels; since the influx of Ca^{++} is determined in part by the membrane potential, this coupling is referred to as electromechanical coupling.

The voltage-dependent calcium channels have been differentiated on the basis of activation/inactivation characteristics and sensitivity to various Ca^{++}-channel antagonists into L- and T- types. L-type channels are activated over the range −20 to +40mV (and not inactivated) and are inhibited by the dihydropyridine Ca^{++} antagonists, while the T-type channels are

rapidly activated over the range -40 to -20mV (and rapidly inactivated) and are insensitive to the dihydropyridines (Coburn and Baba, 1989; Kotlikoff, 1989). The relative contributions of the T- and L- type channels in altering Ca_i^{++} levels during ASM excitation is currently a subject of much debate (Coburn and Baba, 1989; Kotlikoff, 1989).

Since the membrane potential is the prime link in electromechanical coupling, a great deal of energy is expended by the cell in maintaining and regulating the membrane potential. Membrane potential in ASM is maintained at approximately 50-70mV (negative inside; Suzuki et al., 1976; Ito and Tajima 1981; Inoue and Ito, 1986; de Jongste, 1987a; Small and Foster, 1987; Tomita, 1989) and is a sum total of the Na^+-, K^+-, Cl^--, and Ca^{++}-electrochemical gradients, which are in turn products of the cumulative effects of various ATPases (Na^+/K^+ and Ca^{++}/Mg^{++}), ion exchangers (Na^+/H^+, Na^+/Ca^{++}, and Cl^-/HCO_3^-), and the opened/closed states of the channels for each of these ions. Muscarinic receptor activation leads to the opening of non-specific cation channels (Baba et al., 1988) and possibly also Cl^--channels (Byrne and Large, 1988), which in both cases leads to membrane potential depolarization (and thus opening of the voltage-dependent Ca^{++}-channels). In addition, there are K^+-channels which are closed by binding of muscarinic agonists to muscarinic receptors; this results in a decreased K^+-conductance (referred to as the M-current) and depolarization (Coburn and Baba, 1989; Kotlikoff, 1989). There are also voltage-dependent K^+-channels in the

membrane which open during depolarization; in this case, however, hyperpolarization results, which returns the membrane potential toward its initial state. Thus, activation of muscarinic receptors produces a transient depolarization followed by a transient repolarization (de Jongste, 1987a); this electrical response is referred to as an excitatory junction potential (EJP). Unlike other smooth muscle systems in which such EJPs depolarize the membrane to a point where action potentials (APs) are elicited, ASM is very resistant to such AP-like spiking (Suzuki *et al.*, 1976; Coburn 1979; Small and Foster, 1987; Coburn and Baba, 1989; Kotlikoff, 1989). When they do appear, however, the depolarizing phase of the AP in guinea pig ASM seems to be secondary to Ca^{++} influx and not Na^+ influx, since they are not affected by TTX or Na^+-removal, but are antagonized by Ca^{++}-channel blockers or Ca^{++}-removal (Small and Foster, 1987). The suppression of APs, and the stability of the membrane potential itself, are largely owing to the hyperpolarizing effect of the K^+-channels. The effects of these voltage-gated K^+-channels are so profound that "it is practically impossible to depolarize the plasma membrane by >20mV, even with large cathodal current injection" (Coburn and Baba, 1989), an effect referred to as rectification. This effect, of course, can be eliminated using K^+-channel blockers (which prevent K^+-efflux) or high concentrations of K^+ (which eliminate the K^+-electrochemical gradient (Suzuki *et al.*, 1976; Small and Foster, 1987; Takuwa *et al.*, 1987; Himpens and Somlyo, 1988). There are at least two different types of such rectifying outward K^+-

currents: a Ca^{++}-activated current (McCann and Welsh, 1986; Small and Foster, 1987; Kotlikoff, 1989) and Ca^{++}-independent current (Kotlikoff, 1987 and 1989).

(2.4) Relaxation coupling mechanisms   Generally speaking, the mechanisms underlying relaxations are merely the opposite of the mechanisms described above which underly contraction. Thus, in the same way that increases in Ca_i^{++} are important in contraction, mechanisms which decrease Ca_i^{++} play a major role in relaxation. Closure of Ca^{++}-channels on the membrane surface or on the SR will necessarily lead to smaller or slower increases in Ca_i^{++}. Phosphorylation and subsequent inactivation (closure) of Ca^{++}-channels could be one way to directly antagonize such influx. Alternatively, hyperpolarization of the membrane would decrease the probability of opening of voltage-dependent Ca^{++}-channels and thus indirectly decrease the influx.   Elimination of Ca_i^{++} can be enhanced by increasing the activity of Ca^{++}-pumps on the membrane surface or the SR.   Enhancing the rate of Na^+/Ca^{++} exchange by allowing the influx of Na^+ will achieve the same endpoint.

Of course, contractions which are not elicited by Ca_i^{++} will not be altered by the mechanisms listed above.   Since such contractions are secondary to the activation of MLCK, however, mechanisms which antagonize MLCK will lead to relaxation. Phosphorylation of the various kinases can decrease their activity, thus producing relaxations (Miller et al., 1983).   Alternatively, there are a number of phosphatases which reverse the biochemical

and physiological effects of the kinases on actomyosin ATPase activity. One of these is protein phosphatase 1, which is regulated by a protein referred to as inhibitor 1: phosphorylation of inhibitor 1 by cAMP-dependent protein kinase leads to inhibition of this phosphatase (Aitken *et al.*, 1982). Calcineurin is another of these phosphatases (Klee and Krinks, 1978), which seems to be activated by Ca^{++}/calmodulin (Stewart *et al.*, 1983; Aitken *et al.*, 1984). Finally, it was found that myosin can be phosphorylated by both MLCK and PK-C (such that a total of 4 molecules of PO_4 are attached to each myosin molecule), but that phosphorylation by PK-C of myosin already phosphorylated by MLCK leads to a <u>decrease</u> in actomyosin ATPase activity (Nishikawa *et al.*, 1983; de Lanerolle, 1989). This PK-C-mediated antagonism of MLCK would provide a possible site for intracellular integration of several inputs.

ß-adrenoceptors, adenosine $[A_2]$-receptors, PGE_2-receptors, and VIP-receptors have all been shown to be coupled to AC (Katsuki and Murad, 1976; Scheid *et al.*, 1979; Rinard *et al.*, 1979 and 1983; Torphy *et al.*, 1982 and 1985; Lazarus *et al.*, 1986; Small and Foster, 1987; Barnes, 1989; de Lanerolle, 1989; Madison *et al.*, 1989; Said, 1989), and thus mediate relaxation by some cAMP-dependent mechanism, most likely one involving cAMP-dependent protein kinase (PK-A). It has been shown that PK-A phosphorylates MLCK at two different sites, resulting in a decreased affinity of MLCK for the Ca^{++}-calmodulin complex (Conti and Adelstein, 1981; Nishikawa *et al.*, 1984), and that activation of ß-adrenoceptors and/or PK-A leads to decreased activity of MLCK (Adelstein *et al.*,

1978; Conti and Adelstein, 1981; Miller et $al.$, 1983; Obara and de Lanerolle, 1989). However, some have shown cAMP-dependent relaxations can occur without any changes in MLCK activity (Miller et $al.$ 1983; Gunst and Bandyopadhyay, 1989). In these cases, increases in intracellular cAMP may lead to closure of Ca^{++}-channels or enhancement of Ca^{++}-pump activity (Kumar, 1978; Bülbring and Den Hertog, 1980; Twort and van Breeman, 1988; Coburn and Baba, 1989; Gunst and Bandyopadhyay, 1989). In smooth muscle obtained from toad stomach (Scheid et $al.$, 1979; Scheid and Fay, 1984) and rabbit trachea (Schramm and Grunstein, 1989), ß-adrenergic relaxations were postulated to involve cAMP-dependent enhancement of Na^{+}/K^{+} pump activity and subsequent enhancement of Na^{+}/Ca^{++}-exchange, although evidence to refute this has since been obtained (Scheid, 1987). ß-agonists may not always act by decreasing Ca_{i}^{++}, since they have been shown to raise Ca_{i}^{++} in bovine tracheal smooth muscle (Febbel et $al.$, 1988) and ferret portal vein (Morgan and Morgan, 1984); in the former study, this was found to result from enhancement of transmembrane influx through channels which were also opened by carbachol and which were sensitive to dihydropyridine calcium channel blockers or 8-bromo-cGMP (Felbel et $al.$, 1988).

Atrial natriuretic peptide-receptors, on the other hand, are coupled to guanylate cyclase (Said, 1989). In addition, elevation of cAMP levels (eg., using ß-agonists) was also shown to stimulate cGMP-dependent kinase (Torphy et $al.$, 1982). Artificially increasing intracellular cGMP levels using nitroso compounds or 8-

bromo-cGMP led to relaxation of ASM (Katsuki and Murad, 1976; Torphy *et al.*, 1985) and vascular smooth muscle (Rapoport, 1986). Unlike PK-A, the target for cGMP-dependent protein kinase seems not to be MLCK, since it can only phosphoryate MLCK at one site and phosphorylation at this site does not alter the activity of MLCK (Nishikawa *et al.*, 1984). On the other hand, nitroso compounds or cGMP-dependent protein kinase (in activated form) were shown to lead to a decrease in Ca_i^{++} (Felbel *et al.*, 1988). cGMP was also shown to antagonize the effects of the channels [Ca^{++}?] mediating TEA-induced APs in canine trachealis (Richards *et al.*, 1986). It may be that cGMP antagonizes PI metabolism, thus inhibiting contractions which utilize this transduction pathway (Coburn and Baba, 1989), as it seems to do in vascular smooth muscle (Rapoport, 1986). Unlike cAMP-dependent relaxant mechanisms, cGMP-dependent relaxations in canine ASM were not sensitive to cholinergic receptor activation (Torphy *et al.*, 1985).

(2.5) Myogenic versus neurogenic control of ASM Certain regulatory mechanisms which modulate ASM activity are contained entirely within the smooth muscle cells, while others originate in the efferent nerves; these are referred to as myogenic and neurogenic control mechanisms, respectively. Generally speaking, the two differ in the duration and specificity of their effects: neurogenic control is typical of rapid or continuously varying responses and is usually associated with localized responses, whereas myogenic responses are usually slower, more prolonged, and widespread. For

example, tissues such as human and canine ASM usually do not demonstrate spontaneous activity, but become active when the nerves of these tissues are stimulated. Thus the control of these tissues is predominantly neurogenic in origin. Other tissues, such as guinea pig ASM, demonstrate considerable contractile activity in the absence of any exogenous stimuli and are thus largely myogenically regulated. An example of a system in which both mechanisms are important is rat uterine smooth muscle in which it has been shown that the tissue is modestly innervated and has few gap junctions before parturition, at which point the innervation disappears and gap junctions become ubiquitous; thus the tissue rapidly (within days) switches from being largely neurogenically controlled to being largely myogenically controlled in anticipation of parturition (Garfield, 1985). Human ASM also shows a similar dichotomy in that the larger airways have been shown to be sparsely innervated with numerous gap junctions, while the smaller airways are densely innervated and have few gap junctions. Similarly, the density of the innervation in canine ASM increases dramatically as one progresses down the tracheobronchial tree (although gap junctions are numerous throughout). These differences suggest myogenic control predominates in the trachealis, while neurogenic control is more important in the bronchi.

Neurogenic control of ASM arises at several levels, all of which are channeled through the ganglia in the wall of the airways. Ganglionic neurons integrate the signals sent to them by the vagal (parasympathetic) and sympathetic innervations, as well as from

various sensory receptors in the airways (pulmonary stretch receptors, irritant receptors, C-fiber endings; Lundberg and Saria, 1987; Said, 1989; Widdicombe, 1989), and convey the integrated signal throughout the neural plexus (via the interganglionic nerve trunk,) and to the effector cells (smooth muscle, vasculature, and epithelium; Lundberg and Saria, 1987; Coburn, 1989; Said, 1989). The neurons within the ganglia of rabbit, cat, and ferret airways have been found to fall into discrete groupings which are differentiated on the basis of the electrical response generated by the neurons (some generate action potentials, while others apparently do not; some "spike" during inspiration, while others do so during expiration; Coburn, 1989). These differences are beyond the scope of this project. However, there is one detail which has great relevance to this study: some of these intraganglionic neurons have been found to project for long distances within the interganglionic nerve trunk, and to influence the activity of neurons in other ganglia. Thus stimulation of one part of the plexus elicites similar changes in the nerve cells throughout the preparation (Coburn, 1989). As a result, ASM behaves in a syncitial fashion in contrast to the regionally distinct behaviour typical of gastrointestinal smooth muscle or vascular smooth muscle (de Jongste, 1987a; Gabella, 1987). The unusually large separation between the nerve varicosities and the smooth muscle together with the presence of numerous gap junctions (Daniel *et al.*, 1986) also contributes to this syncitial nature of neural control of ASM, since any given nerve ending can thus

influence a larger number of muscle cells than if the separation were smaller.

(3) Regional differences: rationale for this study

Countless studies have already been carried out looking at all aspects of the physiology of ASM. Thus, this study might appear to be redundant. However, the vast majority of the studies were carried out using tracheal smooth muscle, which has since been found to be markedly different from bronchial smooth muscle in many respects. I have already described how this tissue is much more densely innervated than tracheal smooth muscle, with a richer supply of adrenergic fibers, while the tracheal smooth muscle has a greater number of gap junctions (section 1.1). I have also explained how these differences might suggest the two tissues are regulated in very different fashions: i.e., a greater degree of neurogenic regulation in bronchial smooth muscle than in the trachealis (section 2.5). The epithelial layer in the larger airways was shown to be different from that in the smaller airways both in terms of structure (section 1.3) and function (see section 6.4 below); this may be important, considering the important role played by the epithelium in ASM physiology and the pathophysiology of asthma (section 6.4). Regional differences in sensitivity to various pharmacological agents have been reported frequently (Moore et al., 1986; Fujiwara et al., 1988; Mapp et al., 1989). Thus, tracheal and bronchial smooth muscles appear to function and be regulated in very different ways.

Although much has been learned regarding regulation of tracheal smooth muscle activity, there are crucial gaps in our understanding of the regulation of bronchial smooth muscle activity. This is particularly relevant considering the fact that it has been shown that constriction of the trachea is less important in determining peripheral resistance to airflow than constriction of the bronchi, particularly the larger order bronchi (Pride, 1971). This may in part be a product of the differences in gross morphology: the smooth muscle cells in the bronchi completely encircle the airway, while they form only a sheet on the dorsal aspect of the trachealis (section 1.1); thus, constriction in the former results in a uniform narrowing of the lumen while contraction in the latter only results in a pinching off of the lumen from one side.

For all of these reasons, it should no longer be assumed that tracheal smooth muscle is representative of ASM as a whole (i.e., that changes in the activity of tracheal smooth muscle observed in a particular study will be representative of changes in whole airway activity); thus, additional studies of basic ASM physiology using bronchial smooth muscle as a model are essential.

(4) Electrophysiological studies of ASM

An important aspect in understanding the physiology and pathophysiology of ASM is the electrical activity of the tissues, since such activity is often the prelude to changes in mechanical activity. As already described in section 2.3, canine ASM

maintains a stable membrane potential of 60-70mV (inside negative) and manifests EJPs and sometimes APs in response to stimulation of the nerves. As a result of the development of intracellular microelectrode electrophysiological techniques, it is now possible to routinely monitor electrical activity in individual smooth muscle cells. This is accomplished by inserting a microelectrode into the cell and placing a ground electrode close to but still outside the cell. The electrode used in such studies is typically a glass micropipette heated to the point at which the glass can be rapidly drawn out into a very fine filament (outer diameter of tip is typically <0.5 μm); the pipette is then filled with 3M KCl to act as conducter for the electrical signals being measured. This produces a high resistance electrode (tip resistance of 30-80MΩ) which is sensitive to small changes in voltage differences (or ionic currents) across the membrane. Of course, this high sensitivity can also be counterproductive, as electronic interference can be induced electrostatically from the building power supply; fluorescent lights are a particularly strong source of such interference (which manifests as "60 cycle noise"), but can arise from any electrical appliance. Additional interference may be introduced by radio and television transmissions, or high frequency "noise" generated by computers. The signal may also be obscured by small changes produced by diffusion of the KCl from the microelectrode (tip potential or junction potential). To circumvent these problems, a great deal of effort is often required to "ground out" all potential sources of interference and to shield

the apparatus from external sources.

Having eliminated all sources of electronic interference, the next obstacle is to eliminate sources of physical or vibrational interference. It is obvious that in a preparation in which one inserts a microelectrode held by a mass of several kilograms into a single cell (less than a millimeter long) and which is part of a sheet of tissue capable of moving several millimeters in response to electrical stimulation, physical movement can be greatly detrimental. Again, a great deal of effort is expended in attempting to minimize unwanted movement in all parts of the apparatus (using platforms filled with sand and/or air cushions) as well as learning how to stretch and pin down the tissue in such a way as to immobilize it without damaging it or altering its normal physiology (eg., excessive stretching can lead to depolarization and spasm).

Once stable and "clean" (i.e., noise-free) recordings are possible, studies can be carried out in which changes in membrane potential are monitored in response to electrical field stimulation (FS) or pharmacological stimulation. The objects of interest include the baseline potential difference and resistance across the circuit (membrane potential and membrane resistance, respectively), as well as the transient changes in potential and resistance associated with nerve-mediated EJPs or APs.

Intracellular microelectrode electrophysiology is particularly suited to differentiating between presynaptic/prejunctional versus postsynaptic/postjunctional effects of pharmacological agents. For

example, a drug-induced decrease in the magnitude of FS-elicited contractions could be explained by a drug-induced decrease in the release of neurotransmitter (a prejunctional effect) or by a direct (postjunctional) inhibitory effect of the drug on the smooth muscle. Differentiation between the two can be accomplished by observing the effect of the drug on exogenously added neurotransmitter: if this is also decreased similarly, the effect was postjunctionally directed, whereas if this is unaffected the effect was directed at a prejunctional site. In the case of β-agonist induced inhibition in ASM, however, both postjunctional and prejunctional inhibitory mechanisms are employed, making this approach too ambiguous. Electrophysiological studies, however, can easily distinguish between the two types of inhibition, since the former manifests itself as a change in membrane potential (V_m) and membrane resistance (R_m), while the latter manifests as a decrease in the magnitudes of FS-elicited EJPs. Since the size of the EJP is directly proportional to the amount of neurotransmitter released by the nerve, the degree of prejunctional inhibition is directly proportional to the changes observed in the EJPs.

There are a number of caveats which must be considered before making such conclusions, however. First, EJP peak height can also be affected by changes in R_m (a postjunctional effect), since Ohm's law dictates that (membrane) potential and (membrane) resistance are directly correlated. For this reason, the conclusion that an inhibitory effect involves a prejunctional mechanism based on the observation that EJP heights are decreased must be accompanied by

the observation that membrane resistance is unaffected. Changes in R_m can be measured directly by passing a current of known magnitude (eg., a square pulse of 500 nA) across the membrane and measuring the potential change produced (again, based on the principle of Ohm's law). In ASM, however, the presence of many low resistance contacts between cells (i.e., gap junctions) makes this approach ineffective. Changes in R_m can also be monitored indirectly by monitoring the rate of rise or the rate of decay of individual EJPs. If the drug in question alters R_m, the rate of rise (depolarization) and rate of decay (repolarization) will no longer follow the same exponential rate measured before addition of the drug. Thus, demonstration of a decrease in EJP peak height with no change in the dynamic characteristics of the EJP necessarily leads to the conclusion that the effect is directed at a prejunctional site.

Second, FS often elicits a mechanical response which may lead to the ejection of the microelectrode from the cell. It is conceivable that the EJPs measured in different cells are not identical, even under identical conditions. Thus, the finding that the EJP measured in one cell before adding a drug is larger than the EJP measured in another cell after adding the drug may not necessarily allow the conclusion that inhibition has really occurred. This may be a problem in studies using tissues in which the various cells can differ as described. In canine ASM, however, the cells are tightly coupled by gap junctions; this is manifested in the relatively large space constant of the tissue and by the

usual failure to measure a potential change when current is injected into a single cell (Daniel, personal communication). In addition, the unusually large separation between the nerve varicosities and the motor endplates together with the dense innervation, implies that nervous control is a much more diffuse phenomenon in canine ASM than in many other tissues. As a result of all the factors outlined above (the ubiquity of gap junctions; dense innervation; large gap at the NMJ), responses in any given cell are comparable, if not identical, to responses in other nearby cells. This was predicted theoretically as well as observed empiracally in these studies. Thus, it is inconsequential in the studies presented here when impalement of a cell during a study is lost but is re-established shortly thereafter (in the same or a different cell).

(5) Characterization of receptors

Many studies have been carried out to characterize the receptor subtypes involved in various ASM responses. Such studies essentially involve the use of selective or specific agonists and antagonists to modulate (elicit, potentiate, antagonize, etc.) a given response. Since the objectives of these studies include a characterization of the receptors which mediate the effects of cholinergic and adrenergic innervations, the specific approach taken in characterizing such receptors is given below.

(5.1) Muscarinic receptors Originally, cholinergic receptors were

classified into two groups - muscarinic and nicotinic - the former being sensitive to the antagonist atropine (Dale, 1921). Recently, however, it has been realized that muscarinic receptors can be sub-classified into at least four subgroups on the basis of pharmacological properties and their protein structures, and that these in turn may possibly be further sub-classified (Birdsall and Hulme, 1985; Birdsall et al., 1986; Eglen and Whiting, 1986; Waelbroeck et al., 1987). Characterization of muscarinic receptor subtypes is difficult due to the lack of highly selective or specific agonists and antagonists for each subtype. At present, such classification is based primarily on the relative sensitivities of receptors to a number of antagonists.

Muscarinic receptors were first divided into M_1- and M_2-subtypes on the basis that some were highly sensitive to the antagonist pirenzepine (M_1-receptors) but others were much less so (M_2-receptors) (Hammer et al., 1980; Birdsall and Hulme, 1985; Doods et al., 1987; Waelbroeck et al., 1987). The agonist McNeil A343 was also shown to act preferentially on M_1-receptors (Birdsall and Hulme, 1985; Clague et al., 1985; Birdsall et al., 1986; Choo et al., 1986; Mutschler et al., 1987), while the agonists bethanechol (Bch) and oxotremorine (Oxo) showed selectivity for M_2- (i.e., non-M_1-) receptors (Birdsall and Hulme, 1985; Clague et al., 1985; Noronha-Blob et al., 1988; Baumgold and White, 1989).

M_2-receptors were subsequently shown to be a heterogenous population of receptors which could be distinguished on the basis of sensitivity to the antagonist AF-DX 116 (Birdsall et al., 1986;

Giachetti *et al.*, 1986; Doods *et al.*, 1987; Waelbroeck *et al.*, 1987). These two subgroups of M_2-receptors have been referred to as the M_{2A}- and M_{2B}-, M_{2a}- and M_{2B}-, or M_2- and M_3- subtypes, respectively; these subtypes have also been referred to as cardiac- and smooth muscle- subtypes, respectively, after the tissues in which they were first studied (Waelbroecke *et al.*, 1987; Birdsall *et al.*, 1988). The discovery of an antagonist selective for M_3-receptors, hexahydrosiladifenidol (Birdsall *et al.*, 1988; Roffel *et al.*, 1988; Bognar *et al.*, 1989), has facilitated characterization of these non-M_1-receptor subtypes. The agonists Bch and Oxo do not distinguish between M_2- and M_3- receptors (Clague *et al.*, 1985; Gardner *et al.*, 1988).

Batink *et al.* (1987) have reported their finding of a third non-M_1-receptor in exocrine glands, having even lower sensitivity to AF-DX 116 than the other two subtypes, and subsequently proposed yet another schema for naming muscarinic receptors: this proposal would have non-M_1-receptors being subdivided into M_2- (cardiac), M_3- (smooth muscle), and M_4- (exocrine or glandular) subtypes.

There is also now evidence of at least two subtypes of M_1-receptors; these were first shown to be differentially sensitive to the M_3-selective antagonists hexahydrodifenidol and hexahydrosiladifenidol (Lambrecht *et al.*, 1988; Mutschler *et al.*, 1987) and subsequently to the M_1-selective agonists McN A343 (Eltze *et al.*, 1988). These subtypes are referred to as M_{1a}- and M_{1B}- or as ganglionic- and hippocampal-, subtypes (Mutschler *et al.*, 1987; Eltze *et al.*, 1988; Lambrecht *et al.*, 1988).

Muscarinic receptors have been shown to mediate their effects through coupling to adenylate cyclase, guanylate cyclase, PI metabolism, and various ion channels (Birdsall and Hulme, 1985; Harden, 1989). Attempts have been made to demonstrate a correlation between receptor differentiation on the basis of sensitivity to the various agonists and antagonists and differentiation on the basis of coupling mechanisms (i.e., to show that muscarinic receptors of a given subtype are typically found coupled to one of these mechanisms); however, there is no evidence in the literature that such a simplistic view can be taken (Harden, 1989).

(5.2) Adrenergic receptors Decades ago, the sympathetic nervous system was recognized to mediate the effects of the sympathetic nervous system through receptors, referred to as adrenoceptors (Dale, 1906). It was later found that catecholamines elicited varying effects when used at different concentrations (Berger and Dale, 1910) and that the potency orders of various derivatives of epinephrine were the same for one set of physiological responses but different from the potency orders characteristic of another set of responses (Ahlquist, 1948). The former set of responses were found to be sensitive to dibenamine, phenoxybenzamine, tolazine, and various ergot alkaloids, while the latter were sensitive to dichloroisoproterenol. As a result, adrenoceptors were subdivided into two types: α- and β- receptors. Since then, a still expanding array of pharmacological agents has been identified which

differentiate between the two types; these include phentolamine, (α-antagonist), isoproterenol (β-agonist), and propranolol, timolol, pindolol (β-antagonists; Agrawal, 1984).

It was subsequently discovered that α-adrenoceptors could be further divided into α_1- and α_2- subtypes, based on sensitivity to selective agonists like methoxamine (α_1), BHT 920 (α_2), or clonidine (α_2), or antagonists like prazosin (α_1), clozapine (α_1), yohimbine (α_2), and rauwolscine (α_2; Agrawal, 1984).

Likewise, β-adrenoceptors were shown to be a heterogenous population of receptors, which are now referred to as β_1- and β_2-receptors (Lands et al., 1967). Although it was recognized that there are two subtypes of β-receptors, these were not easily distinguished due to the lack of highly selective (or specific) agonists or antagonists, or of agents whose selectivity was misunderstood (O'Donnell, 1985). For example, norepinephrine (NE) was shown to be somewhat more selective for β_1-receptors than β_2-receptors, but only by a factor of four (i.e., the separation between concentration-response curves representing the effects of NE at β_1-receptors and β_2-receptors, respectively, would be only 0.6 log units). Recently, more selective agents have been identified or synthesized including the β_1-agonist prenalterol; the β_2-agonists salbutamol, fenoterol, and procaterol; the β_1-antagonist ICI 89,406; and the β_2-antagonist ICI 118,551 (Bilski et al., 1983; Hedberg et al., 1985; O'Donnell, 1985).

(5.3) Schild plot analysis   Agonists and antagonists which are

specific for a particular receptor subtype are extremely useful in such studies, as there is little room for debate in these cases: such agents will have effects <u>only</u> if the particular receptor subtype is present. Unfortunately, agents with such receptor specificity are rarely available, and for this reason other agents must be used which are only selective between receptor subtypes; the difficulty in interpreting the effects of such agents is inversely proportional to the degree of selectivity. However, it is sometimes difficult to make conclusions based only on the effects of a single selective agent, as this agent can act non-selectively at other receptors when used at sufficiently high concentrations. Thus, such studies must look at the effects of a number of agents with different selectivities and compare the "profile" of responses to those typical of the various receptor subtypes.

Another useful tool in pharmacological studies of receptors is the Schild plot analysis, which is briefly described below. This entire section is a condensation of the chapters written by O'Donnell (1985) and Kenakin (1987); for the sake of clarity and style, this will be the only reference to these authors as my source material.

In pharmacological studies in which the effects of an agonist (A) on a receptor (R) is competitively and reversibly blocked by an antagonist (B), there is an equilibrium which can be defined by the equations:

$$[A] + [R] \underset{k_{2A}}{\overset{k_{1A}}{\rightleftharpoons}} [AR] \qquad (I)$$

$$[B] + [R] \underset{k_{2B}}{\overset{k_{1B}}{\rightleftharpoons}} [BR] \qquad (II)$$
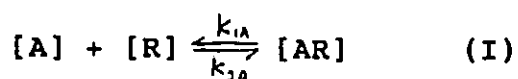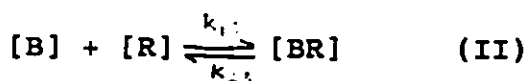
The total receptor population (R_T) in the system is defined by the equation:

$$[R_T] = [R] + [AR] + [BR] \qquad (III)$$

At equilibrium, the rate of association equals the rate of dissociation, and thus:

$$\frac{\delta[A]}{\delta t} = \frac{\delta[AR]}{\delta t} \qquad \text{or } k_{1A}[A] = k_{2A}[AR] \qquad (IV)$$

$$\frac{\delta[B]}{\delta t} = \frac{\delta[BR]}{\delta t} \qquad \text{or } k_{1B}[B] = K_{2B}[BR] \qquad (V)$$

Using equations (IV) and (V) to re-arrange equations (I) and (II), respectively, and substituting into equation (III), one obtains the equation

$$\frac{[AR]}{[R_T]} = \frac{[A]}{[A] + K_A\left(1 + \frac{[B]}{K_B}\right)} \qquad (VI)$$

$$\text{where } K_A = \frac{k_{2A}}{k_{1A}} \text{ and } K_B = \frac{k_{2B}}{k_{1B}}$$

Equation (VI) simply states that for any given concentration of agonist and antagonist, one can calculate the fraction of the total receptor population occupied by the agonist (i.e., $[AR]/[R_T]$) which directly determines the response to the agonist. For example, in the absence of antagonist (i.e., when $[B]=0$):

$$\frac{[AR]}{[R_T]} = \frac{[A]}{[A] + K_A} \qquad (VII)$$

In the presence of antagonist, however, $[AR]/[R_T]$ will be decreased (i.e., the effect of A is antagonized by B). Since the binding of both A and B to the receptor is competitive and reversible, the antagonism imposed by B can be overcome by increasing the concentration of A. Thus, there exists a concentration of A ($[A']$)

which will elicit the same response in the presence of [B] as would [A] when [B]=0. Under such conditions, equation (VI) yields:

$$\frac{[AR]}{[R_T]} = \frac{[A']}{[A'] + K_A\left(1 + \frac{[B]}{K_B}\right)} \qquad \text{(VIII)}$$

By combining equations (VII) and (VIII), one can define a concentration ratio (CR) which is given by the ratio of equieffective concentrations of agonist in the presence and absence of a given concentration of antagonist:

$$CR = \frac{[A']}{[A]} \approx \frac{[B]}{K_B} + 1 \qquad \text{(IX)}$$

or $\qquad \log(CR-1) = \log[B] - \log(K_B) \qquad$ (X)

Equation (X) forms the basis of the Schild plot analysis of agonist/antagonist competition, in which log(CR-1) is plotted against log[B] over a wide range of [B]. The expected outcome, if antagonism is competitive and reversible, is a linear plot with a slope of 1.0. The zero intercept on the abscissa is referred to as the pA_2 of the antagonist under the conditions of the particular experiment, and is an estimate of the pK_B of the antagonist (the two are not equivalent when the Schild plot is not linear and/or does not have unit slope). The pA_2 of the antagonists is used in characterizing the receptors responsible for mediating the particular response being measured. In general, when a single receptor class is being studied, the pA_2 ($-\log K_B$) can be considered to be a biological constant characterizing the receptor and the antagonist. It is uninfluenced by tissue variables such as receptor density and surprisingly consistent even between tissues.

There are a number of factors which can adversely affect the Schild plot analysis.

First, classical Schild analysis assumes that the receptor population under study is homogenous with respect to subtype. If there is more than one type of receptor sensitive to the agonist and antagonist being used, the interaction between agonist, antagonist, and receptor will be complex and the Schild plot produced will not conform to the expected outcome (i.e., linear with unit slope). This is a particular problem in studies of the subtype(s) of ß-adrenoceptors responsible for mediating ASM relaxation since it is often the case that both $ß_1$- and $ß_2$-receptors are present and both mediate the relaxant response (O'Donnell, 1985). Thus, when a selective antagonist is used with the intent of blocking one of the receptor subtypes, the agonist can still produce a portion (or all) of the relaxant effect by acting at the other receptor subtype. Under these circumstances, it is necessary to use agonists of opposite selectivities and demonstrate that the Schild plots obtained for a particular antagonist acting against both agonists are non-superimposable (i.e., yield different pA_2 values). On the other hand, this approach will yield plots for a given antagonist that are superimposable if the receptor population mediating the response is homogenous, even though agonists of very different selectivities are used.

Second, if there are other receptors present which are sensitive to the agonists being used and which mediate an opposing

response, the Schild plot analysis will be compromised. For example, in studies of the ß-receptors responsible for mediating ASM relaxation, there may be α-receptors which mediate contraction of the tissue, thus functionally antagonizing the relaxant response. For this reason, one must select agonists selective for the receptors in question (ß-receptors) and/or antagonists selective for the other receptor population (α-receptors). Similarly, it may be that activation of a completely different receptor system may also functionally antagonize the responses being quantified. Again using ASM as an example, it is known that activation of muscarinic receptors leads to functional inhibition of ß-receptor mediated relaxant responses through reciprocal effects on AC (the former inhibits, while the latter activates AC). Thus, the choice of type and/or concentration of spasmogen used to precontract the tissues can adversely affect the Schild plot analysis.

Third, there are various physiological processes which can alter the effective concentration of the agonist used. For example, there may be uptake mechanisms or degradative processes which moderate or contain (temporally and spatially) the response to the neurotransmitter. The Schild analysis assumes that an equilibrium exists between the receptors, agonist, and antagonist. If the agonist used can be influenced by these mechanisms (i.e., if it can be taken up or broken down), the effective concentration of agonist will be constantly changing (decreasing). The outcome typically manifests as plots with non-unit slopes. The same

adverse effect on the Schild analysis can be produced when insufficient time is given to allow for equilibration between receptors, agonist, and antagonist, or when the procedure involves the use of high concentrations of agonists (to overcome high concentrations of antagonist) which may lead to desensitization and/or inactivation of the receptors. Alternatively, the agonist or antagonist used (or even the precontracting agent used) may induce the release of neurotransmitter from nerve endings in the tissue.

In summary, then, Schild plot analysis is a useful tool in characterizing receptor-mediated responses; this approach can answer questions such as the subtypes of receptors involved in mediating a response (using pA_2 values which correspond to the subtypes), whether there are multiple subtypes mediating the same response to an agonist (yes, when antagonist plots using agonists selective for each are non-superimposable) and whether antagonism is competitive (yes, when slope of 1 is obtained). When the Schild plot obtained is not linear and/or does not have unit slope, one must consider that one or more of the following is the case:

- antagonism is not competitive
- a steady-state equilibrium does not exist between receptor, agonist, and antagonist (uptake, breakdown, insufficient equilibration period, desensitization of the receptor, release of endogenous neurotransmitter)
- the receptor population is not homogenous
- the drugs used functionally antagonize the response in the

study.

As such problems occur in these studies, they are considered in depth in the discussion.

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(6) Asthma and experimentally-induced hyperresponsiveness

Asthma is characterized primarily by wheezing, coughing, and breathlessness; in fact the word "asthma" comes from the Greek word for "panting" or "breathlessness" (Jacobs and Kaliner, 1989). These manifestations in turn result from airway narrowing caused by excessive bronchconstriction, hypersecretion and thickening of the mucous, and wall thickening due to hyperplasia and/or edema formation (Jacobs and Kaliner, 1989). Other manifestations include inflammation of the airways (eosinophilia; neutrophilia; release of inflammatory mediators), increased permeability of vascular endothelium and mucosa, damage to and desquammation of the airway epithelium; and airway hyperresponsiveness (AH) to various physical and pharmacological stimuli. Such stimuli include cold air, dust, exercise, environmental irritants, forced breathing, muscarinic agonists, histamine, ß-receptor antagonists, cyclo-oxygenase inhibitors, etc. (Hay, 1989; Simonsson *et al.*, 1967; Jacobs and Kaliner, 1989; Sibille and Reynolds, 1990). The various manifestations of asthma (and experimentally-induced AH, described below) are reversible; i.e., their severity can change frequently and unpredictably from minor to severe, and even revert back to the normal state.

Hyperresponsiveness can result from an increased sensitivity

to spasmogens such that hyperresponsive tissues are sensitive to the spasmogens at concentrations too low to elicit a response from control tissues; this is referred to as hypersensitivity. Alternatively, the hyperresponsive tissues may not be sensitive to concentrations which are sub-threshold for the controls, but instead produce larger responses for any given concentration of spasmogens than control tissues; this is referred to as hyperreactivity.

In order to gain some appreciation for the importance of asthma-related research, the following statistics have been culled from various sources. The prevalence of asthma in various populations has been reported to range from 0-25%; the rate attributed to the population of the U.S. is 3-6.6%, which is equivalent to $\approx$18 million people (Jacobs and Kaliner, 1989). The mortality rate is approximately 0.3 per 10,000 affected individuals (Jacobs and Kaliner, 1989). The disease also exacts a high cost in many other ways: "27 million patient visits, 6 million lost work-days, and 90.5 million days of restricted activity per year . . . . $362 million was spent for asthma medications in 1983" (Jacobs and Kaliner, 1989; note: these are U.S. statistics).

A number of inducers of asthma have been identified, including allergens, viral infections, and exposure to ozone; in addition, other agents have been identified which incite various symptoms of asthma, including SO_2, non-steroidal anti-inflammatory drugs, β-adrenergic antagonists, exercise, and forced breathing (Jacobs and Kaliner, 1989).

Numerous hypotheses have been proposed which attempt to account for the pathogenesis of asthma and AH; these have been summarized in great detail by Hay (1989) and are briefly outlined below.

(6.1) Altered activity of innervation to ASM   The concept that AH results from changes in the types of innervation which regulate bronchomotor tone was perhaps the first explanation for asthma (Alexander and Paddock, 1921).   Since then, a great deal of supportive evidence has been accumulated. Excess bronchomotor tone can be produced by excess excitatory input or diminished inhibitory input to the smooth muscle. Increased excitatory input can result from increased vagal activity or increased input from sensory nerves in the airways. For example, various inflammatory mediators can increase cholinergic neurotransmission prejunctionally, while sensory nerve activity can be increased by various stimuli to the epithelium or by infection or inflammation of the airways; Lundberg and Saria, 1987; Hay, 1989). Anticholinergics, such as atropine or ipratropium bromide, have therefore been used successfully to treat the symptoms of asthma and other airway disorders (Boushey et al., 1980; Svedmyr, 1983) although not as effectively as β-adrenoceptor agonists (Boushey et al., 1980), suggesting this may not be the primary defect in all cases. In such cases, it may be that there is a decrease in the inhibitory input supplied by the adrenergic innervation. For example, it has been shown that difficulty in breathing associated with nocturnal asthma is

inversely correlated with plasma levels of catecholamines (which can act directly on the smooth muscle or act prejunctionally to decrease the output of the cholinergic innervation; Barnes *et al.*, 1980). The latter would also explain why, in some individuals, β-antagonists can induce bronchoconstriction (Grieco and Pierson, 1971; Ind *et al.*, 1986). Decreased inhibitory input to the smooth muscle can also be secondary to a dysfunction associated with the inhibitory NANC innervation (Hay, 1989; Said, 1989), as suggested by the findings of de Jongste *et al.* (1987), who found the balance between cholinergic (excitatory) and NANC (inhibitory) nervous systems was altered in sensitized guinea pig tracheal smooth muscle compared to controls (de Jongste, 1987b) and in patients with asthma but not those with chronic obstructive lung disorders.

(6.2) Receptor-related pathology   Defects in ASM β-adrenoceptors (which mediate bronchodilation) were suggested by Szentivanyi to be the root problem (Szentivanyi, 1968); Szentivanyi later suggested this defect was accompanied by increased number or activity of α-adrenoceptors (which mediate bronchoconstriction; Szentivanyi, 1979/1980). Numerous studies have provided a voluminous mass of often conflicting data which both support and refute the proposal, often referred to as the Szentivanyi Hypothesis (Svedmyr, 1984; Barnes, 1989; Hay, 1989). For example, while some showed a strong correlation between the severity of the disease and a decrease in the number (Brooks *et al.*, 1979) and activity (Makino *et al.*, 1970) of β-receptors in asthmatics, others

showed no change (Galant *et al.*, 1980). Some explained the changes that were observed as being secondary to the medication used to treat the subjects in the studies (Galant *et al.*, 1980; Svedmyr, 1984). Similarly, the sensitivity of the receptors to β-agonists was shown to be both decreased (Barnes and Pride, 1983; Barnes, 1986 and 1989) and unchanged (Harvey and Tattersfield, 1982; Barnes, 1986; Goldie *et al.*, 1986). Alpha-agonists were shown to contract ASM in asthmatics but to have no effect on ASM from controls (Kneussl and Richardson, 1978; Black *et al.*, 1982; Barnes, 1989), while others showed these had no effect on ASM from either group (Goldie *et al.*, 1985). More importantly, α-adrenoceptor antagonists were found to be ineffective as a treatment for asthma (Barnes *et al.*, 1981; Svedmyr, 1983; Britton *et al.*, 1981; Barnes, 1989). Although the bulk of the evidence does implicate an important role for β-receptors in ASM physiology, the general consensus seems to be that the α-receptors are relatively unimportant with respect to ASM physiology or the pathophysiology underlying asthma (Hay, 1989).

A similar hypothesis to that above was proposed in which AH was attributed to increased activity of histaminergic H1-receptors (which mediate bronchoconstriction) and decreased activity of H2-receptors (which mediate bronchodilation and inhibition of mediator release; Chand 1980; Hay, 1989). However, there is little experimental support for this hypothesis. In particular, the number of H2-receptors in ASM from asthmatics was not different from that in normal ASM (Eiser, 1983) and H1-receptor antagonists

were ineffective in treating asthma (Karlin, 1972).

There is also a recent suggestion that asthma may be related to the loss of a "protective muscarinic receptor mechanism" (Ayala and Ahmed, 1989); i.e., loss of prejunctional inhibition of vagal (parasympathetic) activity mediated by muscarinic receptors is proposed to be the cause of excessive bronchoconstricition releated to asthma.

(6.3) Altered/defective smooth muscle physiology Changes in the normal physiological processes of the smooth muscle can also lead to manifestations of asthma, particularly bronchoconstriction. For example, sensitization of guinea pig tracheal smooth muscle was associated with membrane potential depolarization (McCaig and Souhrada, 1980; Souhadra and Souhadra, 1981; Small and Foster, 1987) followed by hyperpolarization (Small and Foster, 1987). This was attributed to an increased Na^+ influx followed by activation/enhancement of the electrogenic Na^+-pump (Small and Foster, 1987). Increased levels of Na_i^+ could lead to decreased Na^+/Ca^{++} exchange and thus net accumulation of Ca^{++} which could induce contractions directly or potentiate contractions to other stimuli. In addition, bronchial smooth muscle obtained from asthmatics demonstrated a greatly increased action potential-like spiking compared to smooth muscle from normals (Akaska et al., 1975; de Jongste, 1987a).

Alternatively, changes in those processes responsible for maintaining Ca_i^{++}-homeostasis (see section 2.3) ultimately resulting

in increased levels of Ca_i^{++}, could also account for excess smooth muscle activity (de Jongste, 1987b; Hay, 1989). This hypothesis, referred to as the "calcium hypothesis" (Hay, 1989), was first put forth by Middleton (1980). This may be explained by the findings of Rinard *et al.* (1979) that basal levels of cAMP were significantly lower in "asthmatic" compared to "non-asthmatic" dogs (although no change in isoproterenol-stimulated AC activity was noted). Similarly, ovalbumin-sensitized guinea pig trachealis was shown to have increased sensitivity to Ca^{++} (Weiss and Viswanath, 1979; Dhillon and Rodger, 1981), and verapamil was less effective against contractions elicited by KCl, histamine, or acetylcholine in the same preparation compared to controls (Perpina *et al.*, 1986).

(6.4) Epithelial damage or dysfunction   The observation that adverse changes in the epithelium (i.e., damage or loss) is typically coincident with asthma and other respiratory diseases (Laitinen *et al.*, 1985; Jacoby and Nadel, 1989; Hay, 1989) led some to suggest there was a causal relationship. The damage or loss of the epithelium itself could be caused by cytotoxins released by inflammatory cells, particularly eosinophil-derived major basic protein (Gleich *et al.*, 1973).

There are several hypotheses which attempt to account for how this loss of the epithelium results in AH.

One suggests the increased permeability of the epithelium allows greater access of various substances to the smooth muscle

cells, inflammatory cells, or nerves and which have potent effects on these tissues (eg., antigens, sensory-nerve-released neurotransmitters, inflammatory mediators, inhaled nicotine or smoke; Hogg, 1981).

Alternatively, loss of the epithelium could expose sensory nerves found in this layer. Irritation and excitation of the sensory nerve would lead to the release of neuropeptides (substance P? neurokinin A? CGRP?) which can then act on the smooth muscle and inflammatory cells to elicite the various signs and symptoms of asthma (Barnes, 1986b).

Another proposed mechanism was apparently modeled after the findings that the vascular endothelium regulates vasomotor tone through the release of various agents referred to as endothelium-derived relaxing factors (EDRFs) and endothelium-derived contracting factors. Thus, some reasoned that the epithelium synthesizes and releases an epithelium-derived relaxing factor (EpDRF) under normal conditions, and that the loss of the epithelium results in the loss of this inhibitory input (Flavahan et al., 1985). "Sandwich-type experiments", again modeled after experiments done to demonstrate the release of EDRF and action on vascular smooth muscle, have given support to this proposal (Tschirhart and Landry, 1986; Hay et al., 1987). There is experimental evidence which suggest EpDRF is an arachidonic acid metabolite (Flavahan et al., 1985; Hay et al., 1986), that it may act by altering electromechanical coupling (Gao and VanHoutte, 1988), and that its effects on the larger airways may differ from

that in the smaller airways (antagonism of contractions versus enhancement of relaxations, respectively; Stuart-Smith and VanHoutte, 1987).

Finally, damaged epithelial cells or cells exposed by loss of the epithelium may release chemotactic factors, thus inducing or enhancing the infiltration of inflammatory cells into the ASM and lumen (Jacoby and Nadel, 1989). Release of inflammatory mediators can then produce the various changes in smooth muscle, secretory cell, or nerve activities.

(6.5) Inflammatory response   A recurrent theme in the manifestations, causes, and underlying mechanisms summarized above (and in the models of asthma summarized below) is an inappropriate or exaggerated inflammatory response. Inflammatory mediators can elicit contractions of ASM, hypersecretion from secretory cells, enhancement of excitatory neurotransmission, and chemotaxis of additional inflammatory cells. Other mediators can have cytotoxic effects, resulting in widespread and non-selective damage to ASM tissues, culminating in loss of the epithelium, increased permeability of the venules, edema, etc..

Similarly, many stimuli which produce asthmatic symptoms and AH have stimulatory effects on the inflammatory response; these include antigens, viruses, toluene diisocyanate, and ozone.

A number of inflammatory mediators are the subject of intense research, as they are believed to play a major role in the underlying cause(s) of AH. These include prostaglandins D_2 and $F_{2\alpha}$

thromboxane A_2, leukotriene B_4, and platelet activating factor (O'Byrne *et al.*, 1984b and 1984c; O'Byrne *et al.*, 1985; Chung *et al.*, 1986; Cuss *et al.*, 1986; Fuller *et al.*, 1986). Each of these can mimic many, if not all, of the manifestations of AH and asthma (Aizawa *et al.*, 1985; Hargreave *et al.*, 1985; Page *et al.*, 1986; Holgate *et al.*, 1986a). These can be released from eosinophils, basophils, neutrophils, macrophages, and platelets which are found in the airways under non-pathological conditions or are recruited by various stimuli (Holgate *et al.*, 1986a and 1986b; Page *et al.*, 1986; Sibille and Reynolds, 1990). A pathological change in the inflammatory response would account for observations such as increased numbers of inflammatory cells in the airways of asthmatics (Dalquen, 1985; De Monchy, 1985), as well as increased levels of mediators in the airway tissues and body fluids of those with asthma or other forms of AH (Barnes *et al.*, 1982; Grandel *et al.*, 1983; Zakrzewsky *et al.*, 1984; Schönfeld *et al.*, 1985; Hay, 1989).

<u>(6.6) Experimental studies of asthma</u> Many other explanations have been proposed to explain the "cause" of asthma, which will not be presented here as the list is seemingly endless. It is hoped that the review provided demonstrates how much (and how little) has been learned about the pathophysiology underlying asthma, as well as the diversity in the areas of research directed at understanding what would appear on the surface to be a simple/singular disease. It is also hoped, however, that this review emphasizes the fact that

asthma is a complex disease. As a result, much remains to be learned about respiratory physiology, how this can be altered under pathological conditions, and how normal function can be restored.

This endeavour has greatly benefitted from various animal models, in which some of the physiological hallmarks of asthma are experimentally-induced. For example, the scientific literature abounds with reports that inhalation of ozone elicites some of the manifestations of asthma. These include AH, increased permeability and edema formation, hypersecretion of mucous, chemotaxis and infiltration of inflammatory cells, release of inflammatory mediators, and damage to or loss of the epithelium (Holtzmann et al., 1983a; O'Byrne et al., 1984b).

It is believed that these effects of ozone are all secondary to initiation of an inflammatory response, since the degree of ozone-induced AH was (1) directly proportional to the degree of infiltration of inflammatory cells and release of inflammatory mediators (Holtzmann et al., 1983a and 1983b; Fabbri et al., 1984); (2) prevented or reversed by drugs which antagonize the inflammatory response or the action of released mediators (Chung et al., 1986); and (3) prevented by depletion of neutrophils prior to ozone-inhalation (O'Byrne et al., 1984a).

Other models have been developed in which an inflammatory response is induced by inhalation of antigens in sensitized animals. The antigens used include Ascaris suum, Escherichia coli endotoxin, parainfluenza virus 3, and ovalbumin, while the species used include sheep, rabbits, guinea pigs, dogs, and horses (de

Jongste, 1987b; Wanner *et al.*, 1990). These models are similar to the ozone-model in many respects. Inhalation of antigen elicits bronchoconstriction, hyporesponsiveness to bronchorelaxants, hyperresponsiveness to spasmogens, mucous hypersecretion, eosinophilia, and neutrophilia (de Jongste, 1987b; Wanner *et al.*, 1990). One unique characteristic of these models, however, is that antigen challenge induces an immediate bronchoconstrictor effect followed by a subsequent bronchoconstrictor response and hyperresponsiveness 6-24 hours later (referred to as the early and late responses, respectively; Wanner *et al.*, 1990). In the sheep model, the early response was shown to be sensitive to TxA_2-receptor blockade, and the late response to leukotriene antagonists (Wanner *et al.*, 1990).

These models of asthma have proven invaluable to the development of our present understanding of airway physiology and the pathophysiology of asthma.

(7) Objectives

In this introduction, I have briefly reviewed the current understanding of asthma, particularly the impact it has on the human population (with respect to prevelance, morbidity, mortality, and costs to society) and the various hypotheses which attempt to explain the pathophysiology underlying the disease. I have also briefly reviewed the current understanding of ASM physiology. It is hoped that a better understanding of the latter may allow a better understanding of the former and thus lead to newer, more

effective, treatments for asthma.

Thus, this study will attempt to elucidate further the understanding of an animal model commonly used in studies of asthma (ozone-induced AH in the canine species). In particular, I will:

-investigate the roles of the cholinergic and adrenergic innervations in mediating excitation and inhibition, respectively, of isolated segments of canine bronchial smooth muscle (not trachea);

-identify the various mechanisms which modulate the activity or effects of the cholinergic and adrenergic innervations;

-demonstrate that *in vivo* inhalation of ozone induces AH of canine bronchial smooth muscle *in vitro*;

-determine whether ozone-induced AH involves changes in the effects of the cholinergic or adrenergic innervations or the mechanisms which modulate the activities of the same;

-suggest new approaches to the treatment of asthma or areas in which new or renewed interest on the part of researchers must be focussed in order to increase our understanding of ASM physiology and the pathophysiology of asthma.

MATERIALS AND METHODS

Dissection   In the majority of the experiments performed, tissues were obtained from adult mongrel dogs which were euthanized with sodium pentobarbital (100 mg/kg). In the remaining cases, tissues were obtained from adult mongrel dogs which were anesthetized using intravenous sodium pentobarbital (10 mg/kg), ventilated with ozone (or normal air) for 30 minutes, and euthanized with an intravenous injection of potassium chloride; this is further described below ("Ozone studies"). Whole pulmonary lobes were excised and kept in oxygenated Krebs-Ringers solution throughout the studies. The Krebs-Ringers solution consisted of the following (mM): NaCl 115.5; KCl 4.16; $CaCl_2$ 2.5; NaH_2PO_4 1.6; $MgSO_4$ 1.16; $NaHCO_3$ 21.9; D-glucose 11.1. In some experiments, the Krebs buffer also contained $10^{-5}M$ indomethacin, $10^{-7}M$ propranolol, or $10^{-8}M$ L-670,596 in order to block the effects of endogenous cyclo-oxygenase metabolism, catecholamines, and thromboxanes, respectively; inclusion of these antagonists will be noted in each case. Parenchymal tissue and vasculature overlying the ASM was dissected away, exposing the entire bronchiolar tree. The outer diameters of the tissues used in these studies ranged from 1-8 mm (3rd to 6th order). Similar results were obtained in all bronchi studied.

Temperature was 25°C during the dissection and 37°C during the studies. Throughout the dissection and study, the tissues were bubbled with $5\%CO_2$-$95\%O_2$.

Ozone studies   The *in vivo* preparations described in this section were prepared by my colleagues (G. Jones, J. Otis, and C. Lane); they are described here since the *in vitro* studies performed by myself would not be possible without their help.

The dogs were anesthetized with intravenous sodium pentobarbital (10 mg/kg); during the course of the *in vivo* study, this level of anesthetization was maintained by additional injections as required.

An endotracheal tube and an esophageal balloon catheter were inserted. The endotracheal tube was connected to a constant volume ventilator (Model 551, Harvard Apparatus, South Natick, MA) set at a tidal volume of 10 ml/kg and frequency of 30 per minute. The esophageal balloon catheter and a port at the proximal end of the endotracheal tube were connected to a differential pressure transducer and pressure amplifier (Hewlett Packard 267BC and 8805C, Waltham, Mass) to monitor transpulmonary pressure.

Airway responsiveness to aerosolized acetylcholine was assessed 30 minutes after induction of anesthesia. Airway responsiveness was assessed by obtaining dose-response curves to aerosolized acetylcholine. Acetylcholine aerosols were generated from a Bennett/twin nebulizer (Puritan Bennett, Los Angeles, CA) and delivered to the airways via the endotracheal tube at a constant airflow (0.1 l/sec) and a constant tidal volume (300 ml/breath). The mass median diameter of the particles was 2.5 µm (geometric standard deviation, GSD, 2.3).

Total pulmonary resistance (R_L) was determined by a method of

electrical subtraction (as described previously by Holtzmann *et al.* [1983a] and Walters *et al.* [1986]). To ensure a constant volume history, each dose was preceded by inflating the animal to a pressure of 30 cm H_2O. Baseline R_L was determined, then 5 inhalations of acetylcholine aerosol were delivered to the airways. Changes in R_L were monitored and the peak value determined. This sequence was repeated every 5 minutes with successively doubling concentrations of acetylcholine (concentration range: 0.07-40 mg/ml). Dose-response curves to acetylcholine were constructed by plotting the baseline value for R_L and the peak values after each concentration of acetylcholine aerosol delivered. From each curve, an acetylcholine provocative concentration (i.e., the concentration that increased R_L by 5.0 cm water 1^{-1} sec^{-1} above the baseline value) was calculated by interpolation as previously described by Holtzman *et al.* (1983a).

The dogs were then exposed to filtered air ("control dogs") or to ozone at a concentration of 3ppm (ozone-exposed dogs) for 30 minutes. Ozone was generated by passing 100% O_2 through a high intensity ozone generator (built by our engineering staff), and introduced into the ventilator output stream at a flow rate which produced a final concentration of 3 ppm, as measured by a photometric O_3 analyzer (Bendix 8002, Lewisburg, WVa).

Airway responsiveness to acetylcholine was again assessed, and the dogs were euthanized by intravenous injection of potassium chloride. Lobes of lung were excised from which ring segments were dissected and immediately placed into normal Krebs or Krebs

containing 10^{-5}M IDM or 10^{-8}M L-670,596 for study in the muscle baths; as a result, the time of exposure to these antagonists before the experiments were begun was 30-60 minutes. Tissues were washed with and maintained in these same Krebs solutions throughout the studies. These studies took, on average, 8-9 hours to perform (from anaesthetization to conclusion of the study).

<u>Organ bath studies</u> Ring segments approximately 5 mm in length were excised and surrounding parenchymal and vascular tissue was removed, after which they were vertically mounted in 3 ml organ baths using platinum hooks inserted through the lumen. Care was taken to avoid damaging the epithelium. While one of the two platinum hooks allowed attachment to a Grass FT.03 force displacement transducer using silk thread (Ethicon 4-0), the other hook served both as the anchor and as one of the poles of the field stimulation (FS) source (see figure 1). The other pole for field stimulation was a platinum pole not in physical contact with the tissue; the two poles were 2.0 cm apart.

A preload tension of approximately 1.25 (± 0.25) grams was imposed on the tissues and maintained throughout the studies; this preload had been previously determined to yield maximal responses. The tissues were then left to equilibrate for 2 to 2.5 hours during which FS-voltage giving maximal responses was determined (usually 60 volts). Isometric tension was recorded on a polygraph (Beckman R611). During this equilibration period, as well as the

periods of time between experimental assays, the tissues were washed every 20 minutes or more frequently.

Microelectrode studies Ring segments approximately 5 mm in length were excised from the lobe of lung. All parenchymal and vascular tissue was removed and the ring segment was opened by cutting perpendicularly to the axis of the smooth muscle bundles; care was taken not to damage the epithelium. The tissues were then carefully pinned out, epithelial face upward, in a chamber having a bath volume of 5 ml. The tissues were constantly perfused by Krebs-Ringers solution at a rate of 3 ml/minute. Field stimulation was achieved through a pore-type silver electrode which touched the tissue without damaging it, as well as a silver ground electrode located at a site remote from the tissue (see fig. 1). In this way, the tissues and the arrangement for field stimulation used in the organ bath and microelectrode studies were essentially identical.

Conventional microelectrodes (tip resistance of 30-80 MΩ when filled with 3M KCl) were pulled from boro-silicate capillary tubes. Impalements of the smooth muscle were made from the epithelial surface of the tissue. Membrane potential changes were observed on a dual beam oscilloscope (Tektronix D13; 5A22N differential amplifier; 5B12N dual time base) and recorded on 1/4" magnetic tape using a Hewlett-Packard instrumentation recorder.

Field Stimulation Field stimulation (FS) was provided by a

Grass S88 stimulator. Pulse rates ranged from 0.1 to 20 pps (with pulse durations of 0.3 or 0.5 milliseconds). The voltage used was that which gave maximal responses (generally 50-60 volts). Excitatory FS-induced responses had previously been shown to be sensitive to atropine and to TTX (Inoue and Ito, 1986).

Statistical Analysis

All responses reported herein are expressed as mean±standard error.

Contractions and excitatory junction potentials elicited by field stimulation at a given frequency were standardized by defining the response obtained before the addition of any agonist or antagonist as 100% (i.e., the control response), and all subsequent responses to the same frequency scaled appropriately. Tonic contractions elicited by exogenously added muscarinic agonists were standardized by exposing the tissues to 10^{-4}M Cch at the end of the experiment; this Cch-response was defined as 100% and all other responses scaled appropriately.

In those studies in which the effects of ozone-inhalation on contractility were measured, contractions to field stimulation or to Cch were standardized by defining as tension produced (expressed in mN) per mg tissue (dry weight).

The magnitudes of all relaxations elicited (whether by exogenously-added catecholamines or by field stimulation) were standardized by expressing them as a proportion of contracted tone, 0% corresponding to the tone existing before contraction and 100%

corresponding to the stable response to the contracting agent. This approach was possible since the tissues did not demonstrate spontaneous contractile activity.

When muscarinic or adrenergic agents were used to inhibit contractile responses to field stimulation, basal tension was defined as 0% and the magnitude of the reproducible FS-response elicited at the beginning of each study was defined as 100%. Responses to any given frequency of field stimulation were standardized in this way as a proportion (%) of the control responses to the same frequency.

Mean IC_{50}, EC_{10}, or EC_{50} values were determined by averaging the values obtained for each tissue studied. Schild plots were generated by plotting log (CR-1) against the concentration of antagonist used, where CR was the concentration ratio of the agonist (e.g., agonist-IC_{50} in the presence of antagonist over the agonist-IC_{50} in the absence of antagonist; see Introduction, section 5.3). Linear regression of the plots (least squares method) yielded the respective slopes and pA_2 values.

A two-tailed Student's t-test for unpaired data, or ANOVA when more appropriate, was used in making comparisons between means.

<u>Drugs Used</u> The following drugs were used in these studies: AF-DX 116 (11-2[[2-(diethylamino)methyl]-1-piperidinyl] acetyl-5,11-dihydro-6H-pyrido-[2,3-b][1,4]-benzodiazepine-6-one); atropine (Sigma); bethanecol chloride (2-[aminocarbonyl]oxy-N,N,N-trimethyl-1-propanaminium chloride; Research Biochemicals Inc.); carbamyl-

choline (2-[(aminocarbonyl)-oxy]-N,N,N,trimethylethanaminium chloride; Sigma); histamine (Sigma); indomethacin (Sigma); isoproterenol hydrochloride (Sigma); McNeil A343 (4-hydroxy-2-butyryltrimethylammonium chloride; Research Biochemicals Inc.); norepinephrine hydrochloride (Sigma); oxotremorine (Research Biochemicals Inc.); potassium chloride (Sigma); propranolol hydrochloride (Sigma); salbutamol hemisulfate (Sigma); U46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$; Sigma).

The selective ß-receptor antagonists ICI 89,406 [1-(2-cyanophenoxy)-3ß-(3-phenylureido)-ethylamino-2-propranolol] and ICI 118,551 [erythro-DL-1-(7-methylindan-4-yloxy)-3-isopropylamino-butan-2-ol hydrochloride], as well as timolol, were gifts from ICI, Alderly Edge, Great Britain. The thromboxane receptor antagonist L-670,596 ((-)6,8-difluoro-9-p-methylsulfonylbenzyl-1,2,3,4-tetrahydrocarbazol-1-yl-acetic acid) was a gift from Merck-Frosst Canada Ltd.. Phentolamine (rogitine mesylate) was a gift from CIBA-GEIGY Canada Ltd. Hexahydrosiladifenidol was a gift from Professors E. Mutchler and G. Lambrecht of the University of Frankfurt. Pirenzepine was a gift from Karl Thomae GMBH.

All drugs were solubilized in distilled water with the exceptions of AF-DX 116 (0.1 M HCl), ICI 89,406 (DMSO), indomethacin (2% Na_2CO_3, pH adjusted to 7.4 using conc. HCl), and U46619 (100% EtOH). DMSO and EtOH at the concentrations used in these studies did not have any effects of their own on the tissues.

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RESULTS

1. *Cholinergic control of ASM*

To determine the role of the cholinergic innervation in regulating activity of canine bronchial smooth muscle, the effects of various muscarinic agonists and antagonists on mechanical and electrical activity were investigated.

1.1. Effects of muscarinic agonists on mechanical responses

In order to study both prejunctional and postjunctional effects of the cholinergic innervation, the effects of muscarinic agonists on FS-induced contractions as well as on basal tone were investigated. Tissues were electrically stimulated every 5 minutes (10 pulses alternating between 1 and 10 pps) in the presence of various concentrations of the agents. Results of these studies are illustrated in figure 2 and summarized in figures 3 and 4. In addition, the effects of non-cholinergic spasmogens (KCl or histamine) on FS-induced contractions were also investigated in the same manner (figure 5) in order to elucidate whether the effects observed using cholinergic agonists required activation of muscarinic receptors *per se* or simply excitation of the smooth muscle.

Exogenously added carbachol (Cch) produced long-lasting and stable contractions over the concentration range $3 \times 10^{-9} - 10^{-6}$M (figures 2 and 3; n=5). The contractions produced by 10^{-6}M Cch were 94.1±3.3% of the defined maximum response (i.e., response elicited by 10^{-4}M Cch). In addition, Cch at relatively low concentrations

$(3 \times 10^{-9} - 10^{-8} M)$ potentiated contractions to FS (figures 2 and 4); this potentiation was significant only after $3 \times 10^{-9} M$ Cch ($P < 0.05$). Higher concentrations decreased the size of the FS-contractions.

McNeil A343 (McN) produced tonic contractions over the concentration range $10^{-6} - 10^{-4} M$, and inhibited FS-contractions when used over the concentration range $10^{-7} - 10^{-5} M$ (figures 2, 3 and 4; n=6). As was the case with Cch, the tonic contractions produced by McN developed slowly (relative to FS-contractions) and were stable; $10^{-4} M$ McN produced contractions equivalent to 62.4±4.2% of the response to $10^{-4} M$ Cch.

Bethanecol (Bch) had an excitatory effect on basal tension over the concentration range $3 \times 10^{-7} - 3 \times 10^{-5} M$, ultimately eliciting 85.5±2.6% of the maximal Cch response (figures 2 and 3; n=6). Contractions elicited by FS at both 1 and 10 pps were significantly potentiated ($P < 0.05$) by Bch over the concentration range $10^{-8} - 3 \times 10^{-7} M$, attaining mean maximum magnitudes of 144.2±23.0% and 111.2±6.2%, respectively in response to $10^{-7} M$ Bch (figures 2 and 4). Higher concentrations led to greatly decreased magnitudes of the FS-responses.

Oxotremorine (Oxo) potentiated FS-contractions when applied at $10^{-9} M$, except when tonic contractions were also produced, thus apparently masking a portion of the FS-response (as in figure 2). The net effect of $10^{-9} M$ Oxo on contractions elicited by 1 pps and 10 pps was an increase to 110±12.7% and 103±10.5%, respectively (n=6); this potentiation was not significant. Higher concentrations ($3 \times 10^{-9} - 10^{-7} M$) produced powerful tonic contractions

which masked any FS-responses which might otherwise have been observed. The mean tonic contraction elicited by 10^{-7}M Oxo was 83.2±1.8% of the mean maximal Cch response.

Similarly, KCl at 20mM enhanced contractions in response to stimulation at 1 and 10 pps FS significantly to 248.6±70.0% and 159.2±32.8% of control while having little or no effect on basal tone (figure 5); higher concentrations of KCl (40mM) caused these to be significantly decreased in size (to 50.9±18.2% and 43.9±11.9% of control) in parallel with the increased tonic activity (n=5).

Histamine potentiated FS-contractions when applied at sub-threshold concentrations (figure 5; n=5): responses to 1 pps stimulation frequency were increased in magnitude to 134.9±14.0% and 147.3±24.5% of control by 10^{-6} and 3×10^{-6}M histamine, respectively, while responses to 10 pps stimulation frequency were enhanced to 111.5±5.8 and 113.3±8.0%, respectively. Application of higher concentrations (10^{-5}M) often resulted in a decrease in the size of the FS-responses (responses to 1 or 10 pps were decreased to 96.0±34.9 and 62.1±13.2%, respectively) which also paralleled the increase in tonic contractions.

1.2. Effects of muscarinic agonists on electrical activity

The effects of various muscarinic agonists on V_m and on the magnitude of EJPs elicited by FS were investigated. Tissues were exposed to the muscarinic agents at various concentrations (increased every 20 minutes or longer) while monitoring V_m and excitatory responses to FS; FS was a single pulse or 5 pulses at

1 pps (when possible). Examples of these studies are provided in figure 6, and the data are summarized in table 1.

McN had a dose-dependent inhibitory effect, reducing the amplitudes of the FS-EJPs when applied over the concentration range 10^{-7}-10^{-5}M; the highest concentration used could completely eliminate the EJPs. V_m was not significantly altered by McN at concentrations below 10^{-5}M, while 10^{-5}M led to membrane depolarization.

Bch had excitatory effects on the EJPs and on V_m. At 10^{-7}M, Bch considerably increased the magnitude of the EJPs following single pulse FS without significantly affecting V_m. At 10^{-6}M, Bch had a time-dependent effect on V_m and on the magnitudes of the EJPs: over the initial period of a few minutes (in some cases, up to 7 minutes), membrane potential was depolarized only slightly while FS-EJPs were progressively potentiated until membrane potential depolarization became significant. Thereafter, the EJPs were considerably decreased in size. In two out of the five tissues tested, FS-EJPs became FS-induced oscillatory responses (as shown in figure 6); these were not included in the determination of the mean EJP magnitude listed in table 1.

Oxo at 10^{-9}M had little effect on the magnitude of EJPs and depolarized V_m slightly but not significantly. However, 10^{-8}M Oxo further depolarized the membrane potential more than 20 mV above the resting state and progressively decreased the magnitude of the FS-EJPs in all tissues used; these eventually became oscillatory or "slow wave-like" responses to FS. In two of the tissues, the

decrease in the size of the EJPs caused by the higher concentration of Oxo was preceded by a short period during which the EJPs were further enhanced, similar to the effects of 10^{-6}M Bch.

1.3. Characterization of the receptors mediating tonic contractions

The muscarinic receptors which mediate contraction in response to exogenously added muscarinic agonists were characterized using pirenzepine (Pir, M_1-selective), AF-DX 116 (AF-DX; M_2-selective), and hexahydrosiladifenidol (Hexa; M_3-selective). As Oxo was the most potent of the agonists used in this study, and since it is known to interact non-selectively with both M_2 and M_3 receptors (Clague et al., 1985; Gordon et al., 1988), this agent was used as the agonist.

Pir had little or no effect against Oxo-induced contractions when used at concentrations below 3×10^{-7}M (figure 7). Higher concentrations inhibited these contractions in a dose-dependent fashion: the Oxo concentration-response curve was displaced 0.4, 1.2, and 1.6 log units to the right by 3×10^{-7}, 10^{-6}, and 3×10^{-6}M Pir, respectively.

Similarly, AF-DX produced shifts in the Oxo concentration-response curves of less than one half log unit when used at concentrations below 10^{-6}M, while 10^{-6} and 10^{-5}M displaced the curve 1.0 and 1.8 log units, respectively (figure 7).

Hexa, on the other hand, potently antagonized the Oxo-induced contractions (figure 7); the Oxo concentration-response curve was

displaced to the right 0.3, 1.1, 2.0, and 3.3 log units by Hexa at concentrations of 3×10^{-8}, 3×10^{-7}, 10^{-6}, and 10^{-5}M, respectively.

Schild plot analysis of this data (figure 8) yielded linear plots with slopes not significantly different from 1.0 ($p < 0.05$). The pA_2 values for the antagonists were determined from linear regression analysis of these data: these were 6.61 (Pir), 6.81 (AF-DX), and 7.40 (Hexa).

1.4. Effects of selective muscarinic antagonists on FS-responses

The muscarinic receptors mediating inhibition of Ach release were further investigated by observing the effects of selective antagonists on EJPs (figure 9) and on FS-induced contractions (figure 10).

During electrical stimulation with 10 pulses delivered at 0.5 pps, ten EJPs were observed (P_1 to P_{10}) which decreased in magnitude with each successive stimulation (figure 9; n=7). This pattern appeared to be unchanged in 10^{-9}M Pir; similarly, the maximum overall response and V_m were unchanged by this concentration of Pir. At 10^{-8}M, however, Pir altered the pattern such that P_2 and P_3 were significantly larger than P_1. Although the remaining pulses (P_4 to P_{10}) then decreased in height, P_6 to P_{10} were not significantly smaller than P_1. The maximum overall response was also significantly increased to $119 \pm 12.9\%$ of control. These effects of 10^{-8}M Pir were not accompanied by a change in V_m nor a change in the rate of the initial exponential phase of repolarization ($t_{1/2} \approx 119.2 \pm 0.002$ms; n=5) after an EJP (measured for

P_3 and P_6 every 25 milliseconds after the EJP reached a maximum, and continued over a 500 millisecond period during which repolarization was exponential). When the concentration of Pir was increased to 10^{-7}M, the relative change in the pattern of responses to the 10 pulses was more pronounced than was the case for 10^{-8}M Pir. In this case, the magnitudes of P_1 to P_4 were progressively increased, after which P_5 to P_{10} were progressively decreased in magnitude; P_2 to P_{10} were all larger than P_1, although the differences were not significant for P_8 to P_{10}. The maximum overall response to the pulse train was significantly decreased by 10^{-7}M Pir to 49±9.5% of control, while the membrane potential was not significantly altered. Pir also influenced mechanical responses (figure 10): contractile responses to 10 pps were significantly potentiated by 10^{-9}M and 10^{-8}M Pir ($p < 0.01$) while those to 1 pps were significantly potentiated only by 10^{-8}M Pir ($p < 0.1$). Responses to both frequencies were significantly inhibited by 10^{-7}M Pir ($p < 0.001$).

Hexa and AF-DX, on the other hand, had no potentiating effect at all on mechanical responses (figure 10); instead, FS-contractions were dose-dependently decreased by these antagonists. In both cases, the antagonists were more effective against the lower of the two FS frequencies used.

2. Adrenergic control of ASM

To determine the role of the adrenergic innervation in regulating activity of canine bronchial smooth muscle, the effects of various adrenergic agonists and antagonists on mechanical and

electrical activity were investigated.

2.1. Effects of ß-agonists on carbachol-induced contractions

To investigate the relaxant effects of the adrenergic innervation, tissues were precontracted with concentrations of carbachol yielding submaximal contractions and given 2⌐ 30 minutes to allow precontracted tone to stabilize; precontracted tone was then stable for several hours. Relaxations of precontracted tone were elicited by exogenous addition of catecholamines, the concentrations of which were successively increased in a cumulative manner. A sample tracing is given in figure 11; the results of this study are summarized in figure 12.

NE relaxed tissues precontracted with 3×10^{-7}M Cch. The mean negative log IC_{50} for NE-induced reversal of such Cch-contractions was found to be 5.42 ± 0.14 (n=5); 3×10^{-5}M NE completely relaxed the precontracted tissues.

Like NE, Iso could completely relax tissues precontracted with 3×10^{-7}M Cch; the mean negative log IC_{50} was found to be 6.54 ± 0.15 (n=5). When the tissues were precontracted with a lower concentration of Cch (10^{-7}M), the potency and efficacy of Iso were enhanced slightly: the magnitude of relaxations elicited by 10^{-6}M Iso were increased from 89.5% to 104% and the mean negative log IC_{50} increased from 6.54 ± 0.15 to 6.67 ± 0.18 (n=5).

Sal also relaxed Cch-precontracted tissues. In this case, however, the potency and efficacy were extremely sensitive to the concentration of precontracting agent: Sal could elicite maximal relaxations of 105.5 ± 3.0% (n=3), 79.2 ± 6.5% (n=5), and 28.1 ± 2.3%

(n=6) when 2×10^{-8}, 10^{-7}, and 3×10^{-7}M Cch were used to precontract the tissues. The mean negative log IC_{50} for Sal could be calculated only in the first case and was 7.98 ± 0.0..

2.2. Selective antagonism of NE-induced relaxations

The effects of the selective antagonists on NE-relaxations in 3×10^{-7}M Cch-precontracted tissues was investigated using phentolamine (α-selective), ICI 89,406 (β_1-selective) and ICI 118,551 (β_2-selective). The antagonists were applied at the same time as the precontracting agent; unless otherwise noted, the β-antagonists did not influence the development or magnitude of precontracted tone.

ICI 118,551 had little or no effect on NE-induced relaxations when used at concentrations of 10^{-7}M or less; greater concentrations of the antagonist (10^{-6}-3×10^{-5} M), however, were highly effective (see figure 13 and table 2). ICI 89,406 also antagonized the relaxations in a dose-dependent fashion, though much lower concentrations were required to mediate the same degree of antagonism as ICI 118,551 (figure 13 and table 2). Schild plot analysis of this data (figure 14) produced plots with slopes of 0.94 ± 0.04 and 1.21 ± 0.10 for ICI 89,406 and ICI 118,551, respectively; neither of these slopes were significantly different from 1.0. Extrapolation of the data gave pA_2 values for these antagonists of 7.70 and 6.33, respectively.

2.3. Selective antagonism of Sal-induced relaxations

The effects of the selective ß-antagonists on the Sal concentration-relaxation curve were also investigated. For these experminents, we used lower concentrations of precontracting agent ($2x10^{-8}$ and 10^{-7}M Cch, instead of $3x10^{-7}$M) in order to maximize the magnitude of relaxations elicited by Sal. In spite of this, there was still considerable variation in the concentration-response curves when 10^{-7}M Cch was used, since complete relaxations were not possible in many tissues. A contributing factor to this variance may be that Sal at millimolar concentrations sometimes produced contractile responses.

In tissues precontracted with 10^{-7}M Cch, ICI 89,406 had little effect on the Sal concentration-relaxation curve (figure 15); even at $3x10^{-6}$ and 10^{-5}M, the curves were displaced to the right less than one log unit. ICI 118,551, on the other hand, antagonized the relaxations when used at concentrations of 10^{-8}M and greater (figure 15); as the concentration of antagonist was increased, the magnitude of the maximum relaxation was decreased. Due to the latter effect, these data could not be subjected to Schild plot analysis.

This effect was not seen in tissues precontracted with $2x10^{-8}$M Cch (i.e., Sal could completely reverse precontracted tone in all cases, irrespective of the concentration of antagonist used). Under these conditions, ICI 118,551 displaced the Sal concentration-relaxation curve to the right 0.81, 1.57, and 2.41 log units when used at 10^{-8}M, 10^{-7}M, and 10^{-6}M respectively. Schild plot analysis of this data (figure 14) produced a plot with slope

0.84±0.07 (not significantly different from 1.0); extrapolation from the data yielded a pA_2 value of 8.91.

2.4. Selective antagonism of FS-induced relaxations

Relaxations to nerve-released catecholamines were also investigated by field stimulating the tissues following precontraction with McN, which should both precontract the tissues (due to non-selective activation of M_3-receptor on the smooth muscle) and eliminate contractile responses to FS (due to activation of prejunctional M_1-receptors on the cholinergic nerve endings; see section 1.2), thus unmasking the relaxant response to FS). Field stimulation used was 10 pulses at either 1 or 10 pps. ß-antagonists, when employed in these studies, were applied once precontracted tone had stabilized and relaxant responses to FS were reproducible; there was one exception to this, in which case the tissues had been exposed to the antagonists for four hours before the study and had been washed numerous times before the study.

Such relaxant responses were blocked by 10^{-7}M propranolol (n=7); ICI 118,551 was impotent against these relaxations except when used at 10^{-6}M, while ICI 89,406 depressed them at 10^{-8}M and eliminated them at 10^{-7}M (n=5 in both cases; figure 16). In the first animal in which this effect was studied, pretreatment with these antagonists occurred four hours prior to the observation, and was preceded by numerous washings, attesting to the long-lasting effects of these antagonists.

2.5. Inhibition of FS-contractions by β-agonists

To investigate the effects of β-agonists on FS-contractions, tissues were field stimulated every 5 minutes (10 pulses at either 1 or 10 pps) in the presence of increasing concentrations of β-agonists (concentration increased every 15 minutes or when its effect had maximized). Before addition of any agent, tissues were field stimulated as described until responses were reproducible (generally less than 20 minutes, or 5 stimulations).

NE antagonized the contractions elicited by FS in a dose-dependent fashion (figure 17 and 18). Contractions elicited by the lower stimulation frequency (1 pps) were more susceptible to NE-induced inhibition than those at the higher stimulation frequency (10 pps; $p<0.01$). When a stimulation frequency of 1 pps was used, NE was found to antagonize FS-contractions with an IC_{50} of 9.4×10^{-7}M, and completely inhibit the contractions at 10^{-5}M. When the stimulation frequency was 10 pps, the same effects were noted at 1.7×10^{-6}M and 10^{-5}M, respectively.

NE-induced inhibition of contractile responses to FS was mimicked by Iso and by Sal; the cumulative concentration-response curves for all these agents are given in figure 18 (see also table 2). All three agents were capable of mediating complete inhibition and thus are full agonists in this respect. The concentration-response curves showed a rank order of potency of Iso ≥ Sal >> NE.

In additional studies, forskolin was found to antagonize FS-contractions (decreased ≈75% when used at 10^{-7}M) without any apparent effect on contractions elicited by 10^{-7}M Cch (n=2). Cch-

contractions were still apparently unaltered by 10^{-6}M forskolin (FS-contractions were eliminated), but were antagonized by 10^{-5}M forskolin (decreased $\approx$50%).

2.6. Antagonism of ß-agonist-induced inhibition of FS-contractions

To determine the subtype(s) of adrenergic receptors involved in this apparent prejunctional effect of NE, tissues were incubated with receptor selective antagonists for 20 minutes before beginning the experiment. Such preincubation did not affect the magnitude of the responses to field stimulation with the exception of 10^{-5}M ICI 118,551 which depressed the control responses less than 10%; higher concentrations of ICI 118,551 markedly antagonized the FS responses. DMSO, at the concentrations used in this study (to dissolve ICI 89,406), had no effect on the mechanical responses.

While phentolamine had no effect on NE-mediated inhibition of contractile activity (n=12), this inhibition was antagonized by timolol (table 2). Similarly, the effect of Iso on mechanical responses was antagonized by propranolol (table 2).

Inhibition by NE of FS-induced twitch contractions was antagonized by either ICI 89,406 or ICI 118,551, though the former was more potent than the latter. The effects of various concentrations of these antagonists on the NE concentration-response curve are shown in figure 19 (see also table 3).

Since NE could be acting at both the $ß_1$ and the $ß_2$ receptors, and since antagonism at one receptor might be masked by action at the other receptor, various combinations of the selective

antagonists were tried. The combined use of both antagonists at a concentration of 10^{-8}M caused a shift of 0.6 log units in the concentration-response curve; alone at this concentration, neither antagonist had an appreciable effect against the NE-effect. Subsequently increasing the concentration of ICI 118,551 or ICI 89,406 to 10^{-7}M led to net displacements of 0.8 and 1.1 log units, respectively. It is interesting when comparing the data in figure 19 and table 3, that 10^{-7}M of either antagonist in the presence of 10^{-8}M of the other antagonist achieved an equivalent or larger displacement than 3×10^{-7}M of either antagonist alone.

The effects of the selective ß-antagonists on Sal-induced decreases in FS-responses were also investigated. ICI 89,406 had only a small effect against Sal when used at 10^{-6}M, with higher concentrations displacing the Sal concentration-response curve to the right in a concentration-dependent fashion (see figure 20 and table 4). ICI 118,551, on the other hand, was highly potent at concentrations below 10^{-6}M, displacing the curve to the right 0.3, 1.2, and 2.0 log units when used at 10^{-9}, 10^{-8}, and 10^{-7}M, respectively.

The results of the experiments investigating the effects of ICI 89,406 and ICI 118,551 on NE- (summarized in figure 19 and table 3) and Sal- (summarized in figure 20 and table 4) induced inhibition was subjected to Schild plot analysis (figure 21). The slopes and pA$_2$ values derived from these plots are summarized in table 5. The curves pertaining to NE and Sal in the presence of ICI 89,406 are non-superimposable; the same is true of the curves

pertaining to the agonists in the presence of ICI 118,551.

2.7. Effects of β-agonists and antagonists on electrical responses to field stimulation

Tissues were exposed to the β-agonists and/or antagonists at various concentrations (concentrations increased every 20 minutes or longer) while monitoring the effects on V_m and responses to FS.

The magnitudes of EJP peak heights in response to both single pulses and repetitive stimulation were decreased by 10^{-6}M NE (n=3) and almost eliminated by 10^{-5}M NE (n=6; figure 22). In 6 out of 7 tissues exposed to 10^{-5}M NE, hyperpolarization was not noted; in the 7th tissue, V_m hyperpolarized less than 5 mV.

Similarly, FS-induced EJPs were antagonized by Iso (10^{-9}-10^{-6}M; figure 23) without a major effect on V_m (n=13); in 6 tissues exposed to Iso at concentrations of 10^{-6}M or less, only small hyperpolarizations (less than 5 mV) were observed. On the other hand, membrane depolarizations elicited by 3×10^{-7}M Cch (producing a mean depolarization of 18.2 ± 1.1mV) were insignificantly repolarized by 3×10^{-8} and 10^{-7}M Iso (1.0 ± 1.2 and 2.8 ± 1.4mV, respectively; n=5); these concentrations of Iso were sufficient to produce ≈60% and ≈90% inhibition of FS-responses, respectively (figure 18). Increasing the concentration of isoproterenol to 3×10^{-6}M led to a reversal of Cch-depolarization of 14.2 ± 2.9mV (n=5).

Both the effects of Iso (on V_m and EJP peak height) were not affected by phentolamine (n=5), but were antagonized by propranolol (n=6; figure 23) or by timolol (n=2; figure 24B); the latter agent

also antagonized the effects of NE (n=5).

Sal (5×10^{-8}M) also suppressed electrical activity (n=3) without any observable effects on V_m (n=3); this effect was antagonized by 10^{-7}M timolol (figure 24A). High concentrations of Sal (up to 10^{-4}M) did not overcome this antagonism caused by timolol, but instead caused depolarization and membrane potential oscillations in the example shown.

Inhibition of electrical responses to FS, induced by 10^{-5}M NE, was not affected by the simultaneous addition of 10^{-8}M ICI 89,406 plus ICI 118,551. Subsequently increasing the concentration of ICI 89,406 to 10^{-7}M, however, not only restored the electrical responses to their initial levels, but actually potentiated them (n=3; figure 25). Similar observations were made when Iso served as the β-agonist (n=2).

2.8. Effects of antagonists on endogenously released NE

In order to investigate any possible effects of spontaneously released NE, the effects of various β-receptor antagonists on control responses were observed. Twitch contractions elicited by FS (10 pulses at 1 pps) were potentiated by exogenously-added 10^{-7}M propranolol to 112.1±4.1% of control (n=4). Similarly, electrical responses to both single and multiple pulse field stimulation were potentiated by propranolol ($>10^{-7}$M) in 7 out of 21 tissues tested, and by timolol (10^{-8}-10^{-6}M) in two out of five tissues. In one experiment, EJP peak heights were increased by 80%, 120%, and 110% by 10^{-8}, 10^{-7}, and 10^{-6}M timolol, respectively

(figure 26). Neither ICI 89,406 (10^{-7}-3×10^{-6}M) nor ICI 118,551 (3×10^{-7}M) alone demonstrated this potentiating effect (n=4 and n=3, respectively).

3. Influence of thromboxanes on ASM function

To determine the role of thromboxane A_2 in regulating the activity of canine bronchial smooth muscle, the effects of a thromboxane mimetic (U46619) and various antagonists on mechanical and electrical activity were investigated.

3.1. Effects of U46619 on mechanical activity

U46619 potentiated FS-induced twitch contractions over the concentration range 10^{-10}-10^{-8}M (figures 27 and 28. The maximum degree of potentiation observed was 113.4±7.7% (1 pps) and 110.8±6.6% (10 pps) when 10^{-8}M U46619 was used; these changes were statistically significant (P<0.05). This concentration of U46619 had no significant effect on the EC_{50} for Cch, which was 7.30±0.05 in control tissues and 7.41±0.06 in U46619-exposed tissues (n=5 for both; figure 29). Higher concentrations of U46619 initiated large tonic contractions accompanied by a decrease in the magnitude of FS-contractions (figures 27 and 28). FS-contractions were never eliminated, however, despite these large tonic contractions; the former were still greater than 50% of control when the latter were apparently maximal.

Maximum contractions to U46619 occurred at 10^{-5}M; these were 52.8±10.2% of the response elicited by 10^{-4}M Cch (figure 28). These contractions showed no phasic component (i.e., there were no slow

oscillations superimposed on the tonic contractions).

During U46619-induced contracture, FS-contractions were followed by transient but large relaxations which were sensitive to propranolol (figure 27). These relaxations were not investigated further.

3.2. Pharmacological manipulation of effects of U46619 on mechanical activity

U46619-induced potentiation of FS-contractions was unaffected by 10^{-9}M L-670,596 but potently antagonized by 10^{-8}M L-670,596; the concentration-response curve for U46619 was displaced two log units to the right by the latter (figure 30). This concentration of L-670,596 had no effect of its own on FS-contractions. Pretreatment of the tissues with the adrenergic antagonists phentolamine plus propranolol (both 10^{-7}M) also enhanced FS-responses, but had no effect on U46619-induced potentiation of contractile responses to FS (1 pps); i.e., these were still significantly enhanced by 10^{-8}M U46619 ($P<0.05$; n=5; figure 30).

U46619-induced contractions were not significantly altered by 3×10^{-8}M atropine or 10^{-9}M L-670,596 (figure 31); 10^{-8}M L-670,596, however, displaced the U46619 concentration-contraction curve by almost two log units and significantly decreased the maximal effect of U46619 from 52.8 ± 10.1% to 28.1 ± 9.8% ($P<0.01$; n=5; figure 31).

3.3. Effects of U46619 on electrical activity

U46619 depolarized the membrane potential and altered FS-

elicited EJPs in a manner dependent on concentration, time, and membrane potential, as illustrated in figures 32 and 33.

Membrane potential (V_m) was decreased by 10^{-9}M U46619 to 2.0 ± 0.5 mV above V_R; this change was not significant ($P<0.05$). Increasing the concentration to 10^{-8}M, however, produced a large depolarization of the membrane: after 25 minutes of equilibration, this was 21.3 ± 4.3 mV above resting membrane potential (V_R). Occasionally, small oscillations in V_m were noted (amplitude less than 3 mV); these were continuously present in 1 of the 8 tissues tested, but would appear for brief periods after FS in 3 other tissues. The membrane was further depolarized slightly by 10^{-7}M U46619 to 22.8 ± 3.5 mV above V_R; at this concentration, U46619 also enhanced the membrane potentia   scillations by increasing their amplitude (mean of 3.9 ± 1.1 mV) and/or increasing the proportion of tissues showing this activity (oscillations were noted in 11 out of the 12 tissues tested); these oscillations had a mean frequency of ≈1 Hz. Increasing the concentration of U46619 to 3×10^{-7}M did not have any apparent additional effect.

FS-elicited excitatory junction potentials (FS-EJPs) were potentiated by U46619 in a membrane potential-dependent fashion; i.e., the degree of potentiation showed an inverse relationship with the degree of membrane depolarization (figures 32 and 33). For example, 10 minutes after adding 10^{-8}M U46619, the membrane was depolarized by only 5.3 ± 1.1 mV and FS-EJPs (elicited by 5 pulses at 1 pps) were significantly potentiated to $242.0\pm36.4\%$ of control; at 25 minutes after addition of the agent, the membrane stabilized

at 21.3±4.3 mV above V_R and FS-EJPs were decreased to 106.5±23.8% of control. Similarly, further depolarization of the membrane (by higher concentrations of U46619) led to further decreases in the magnitude of the EJPs, while reversal of membrane depolarization (by L-670,596) led to reversal of this decrease (i.e., "potentiation" of FS-EJPs), as shown in figures 32 and 33. Membrane hyperpolarization in response to electrical stimulation was not noted in any of the tissues depolarized by U46619.

The dynamic characteristics of the EJPs were also altered by U46619. That is, FS-EJPs appeared as a single "spike" in the absence of the agent, but were followed by several oscillations in the presence of U46619 at concentrations in excess of 10^{-9}M. These oscillations were initially large (often greater than 10 mV) but quickly decreased in size over time, eventually becoming indistinguishable from the continuous membrane oscillations (or dissipating entirely in those tissues in which membrane oscillations were not present).

3.4. Pharmacological manipulation of effects of U46619 on electrical activity

The effects of U46619 on membrane potential were almost completely reversed by 10^{-7}M L-670,596 (n=5; figure 32); the membrane potential existing after exposure to U46619 plus L-670,596 was not significantly different from that existing before addition of these agents (i.e., control conditions).

The U46619-induced potentiation of FS-EJPs, on the other hand,

seemed to be resistant to 10^{-7}M L-670,596, since the EJPs were 241.4±80.6% of control even after exposure to the antagonist for 30 minutes or longer (n=5; figures 32 and 33).

The slow oscillations in V_m induced by 3×10^{-7}M U46619 were antagonized by 10^{-8}M nitrendipine (NT); i.e., spontaneous or FS-induced slow oscillations were not seen in any of the tissues exposed to 3×10^{-7}M U46619 following exposure to NT (n=4; figure 32). U46619-induced membrane depolarization was partially reversed (by 3.9±0.7 mV) by 10^{-8}M NT.

4. CONTROL OF BRONCHOMOTOR TONE UNDER INFLAMMATORY CONDITIONS

To determine whether *in vivo* inhalation of ozone produces hyperresponsiveness of canine bronchial smooth muscle *in vitro* (and if so, to determine the mechanisms involved), the effects of FS and various pharmacological agents in the activity of tissues exposed to ozone or to normal dry air were investigated.

4.1. Effects of ozone-inhalation on tissue weight

In the experiments described in this study, responses were standardized as mN tension produced per milligram tissue. It was necessary, therefore, to determine whether ozone-inhalation altered the physical characteristics of the tissue, particularly tissue weight. Tissue weights were standardized by relating to unit area (length x outer diameter when flattened). The mean wet weight and dry weight of the tissues were found to be 568±129 mN/mm^2 and 103±22 mN/mm^2 respectively in control tissues, compared to 552±118 mN/mm^2 and 103±21 mN/mm^2 respectively in ozone-treated tissues.

These differences between the wet weights or between the dry weights were not significant.

4.2. Effects of ozone-inhalation on contractile responses

To follow changes in bronchial smooth muscle responsiveness over time, ring segments were electrically stimulated every 5 minutes with a train of 5 pulses at various frequencies (see figure 34). The first and last of such trains were always 5 pulses at 20 pps. Other frequencies tested were 0.1, 1.0, and 10 pps. The order in which these frequencies were delivered was randomized to avoid problems in interpreting the data (such as development of prejunctional inhibition; depletion of neurotransmitter in nerve endings; receptor desensitization; facilitation of successive stimulations). Following these series of electrical stimulations, the tissues were exposed to 150mM KCl. Once the KCl-induced contractions reached maximum, the tissues were washed repeatedly and allowed to recover. Approximately 45 minutes elapsed between the delivery of the first train of electrical pulses and the repeated washing (i.e., conclusion of the KCl-induced contracture). This series of FS-induced and KCl-induced contractions (figure 34) was repeated at 120, 210, and 300 minutes after first mounting the tissues in the baths (which was in turn approximately 60 minutes after euthanasia). Fifteen minutes before each series, the bath fluid was replaced and the preload tension re-adjusted to the optimal tension (1.25±0.25 gram).

In the absence of IDM or L-670,596, the mean responses to

electrical stimulation at any given time period were greater in ozone-treated tissues compared to control tissues, even six hours after exposure to ozone; figure 35 indicates when these differences were significant. In addition, the mean magnitudes of the responses in all tissues decreased over time, though this trend was found to be not significant.

The mean responses to KCl, on the other hand, tended to increase over time in both groups, though this trend was also not significant. Although the mean response elicited from ozone-treated tissues at any given point in time was always greater than the corresponding mean of the controls, the differences were never significant.

Although standardizing the responses by expressing as grams tension per gram dry weight clearly (and significantly) reveals hyperresponsiveness, the statistical errors were large. Since ozone-inhalation did not significantly alter the responses to KCl, we attempted to eliminate one source of error (i.e., inter-tissue variability) by expressing the FS-contractions as a proportion of the KCl response for each tissue at each time period tested. This approach decreased the size of the error bars and increased the differences between ozone and control tissues (i.e., a greater proportion of the pairs were now significantly different). An example of this is given in figure 36.

To test whether ozone-inhalation altered bronchial smooth muscle responsiveness to exogenously-added muscarinic agonist, additional tissues were mounted in organ baths containing normal

Krebs (i.e., not containing IDM or L-670,596) and maintained in the same manner as described above for approximately 120 minutes; this equilibration period was chosen since hyperresponsiveness to FS was clearly demonstrated at this point in time in the experiments summarized above. There was no statistically significant difference in the Cch EC_{10} or EC_{50} or the magnitude of the mean maximum responses to Cch in ozone-treated tissues compared to the controls (figure 40), even though hyperresponsiveness to FS was demonstrated in these same tissues (figure 37 inset; $P<0.10$).

4.3. Effects of indomethacin on ozone-induced hyperresponsiveness

Pretreatment of the tissues with 10^{-5}M IDM rendered all differences in FS-responses between control tissues and ozone-treated tissues insignificant (figure 35). In contrast to the progressive decrease over time observed in the absence of IDM, the mean responses in the presence of IDM tended to increase over time; this trend was not statistically significant.

4.4. Effects of L-670,596 on ozone-induced hyperresponsiveness

In the presence of the thromboxane receptor antagonist, L-670,596, the responsiveness to FS in all tissues decreased dramatically (and significantly) over time; in fact, the rate of decrease in the size of the FS-responses in the presence of L-670,596 was so rapid that no response could be elicited by low-frequency (0.1 or 1.0 pps) field stimulation at 210 or 300 minutes. As a result, the differences in responsiveness between control and

ozone-exposed tissues were decreased (often becoming insignificant) compared to the differences in tissues maintained in normal Krebs (figure 35). This decrease over time was unlikely to be due to a direct effect of the antagonist on the smooth muscle (i.e., physiological antagonism), since KCl responses did not decrease in magnitude over time in the presence of L-670,596 (figure 35).

It is interesting to note that the slight (but non-significant) enhancement of KCl-responses in ozone-treated tissues was decreased or even reversed in the presence of this antagonist (figure 35).

4.5. Effects of ozone-inhalation on prejunctional mechanisms

In the experiments summarized in sections B.2-B.4 above, tissues were stimulated with 5 electrical pulses at various frequencies, the first and last of which were always at a frequency of 20 pps (denoted in figure 34 as S_1 and S_2, respectively). .As S_2 was always preceded by a number of FS-trains, the magnitude of the responses elicited by S_2 might be modified by physiological mechanisms such as the development of prejunctional inhibition. Thus, comparison of the ratio of S_2/S_1 may provide information with regards to any changes in prejunctional inhibition. When IDM was present, the ratio S_2/S_1 was not significantly different from one (and thus exposure to ozone did not significantly alter S_2/S_1). When IDM was not present, however, the S_2/S_1 ratios were consistently greater in ozone-treated tissues compared to the controls (table 6); the differences were significant at all time

periods except the first (P<0.1).

DISCUSSION

(1) EXCITATION OF CANINE BRONCHIAL SMOOTH MUSCLE

(1.1) Postjunctional M_3-receptors

Excitatory responses to FS in canine ASM are mediated predominantly by muscarinic cholinergic mechanisms; this is demonstrated in the data presented here and has been reported previously (Coburn, 1979; Kannan and Daniel, 1980; Murlas et al., 1982; Inoue and Ito, 1986; Brichant et al., 1989). There is uncertainty, however, regarding the subtype(s) of muscarinic receptors involved in these responses. The postjunctional (i.e., muscle) receptor has been reported to be of the M_2-subtype in bovine and rat ASM (Roffel et al., 1987; Roffel et al., 1988; Scott and McMahon, 1988) and apparently M_3-subtype in guinea pig and human ASM (Mak and Barnes, 1989).

The data obtained in this study indicate the excitatory effects of nerve released or exogenously added cholinergic agonists on smooth muscle do not involve M_1-receptors. The initial evidence of this was our finding that contractions induced by FS or Oxo, as well as FS-induced EJPs, were antagonized by Pir only at concentrations equal to or exceeding $10^{-7}M$; sensitivit) to Pir at these concentrations is indicative of a non-M_1-mediated mechanism (Hammer et al., 1980; Birdsall and Hulme, 1985; Doods et al., 1987; Waelbroeck et al., 1987; Scott and McMahon, 1988). Similarly, Oxo and Bch, which were used here as non-M_1-agonists (Claugue et al., 1985; Noronha-Blob et al., 1988; Baumgold and White, 1989),

were full and potent agonists; McN (M1-selective; Claugue *et al.*, 1985; Birdsall *et al.*, 1988; Eltze *et al.*, 1988; Barnes, 1989), on the other hand, was only a partial agonist and was effective only at concentrations ten-fold higher than were required for the prejunctional effect (M_1-mediated) also described in this study.

The high potency of Hexa indicates these non-M_1-receptors are predominantly, if not exclusively, of the M_3-subtype. Although AF-DX also antagonized the effects of the agonists, the concentrations required were higher than should be the case for an M_2-mediated effect based on the reported pA_2 values and $IC_{50}s$ of this antagonist (Giachetti *et al.*, 1986; Waelbroeck *et al.*, 1987). Schild plot analysis of the data yielded pA_2 values of 6.6 for Pir, 6.8 for AF-DX, and 7.4 for Hexa. Published pA_2 values for these antagonists are provided in table 7 and support the conclusion that M_3- receptors are involved. The data presented do not exclude the existence of a small number of M_2-receptors,

Table 7 Published pA_2 values for selective muscarinic agonists

	pA_2 values		
	M_1	M_2	M_3
Pir	8.1[10]	6.5[3]	6.2[7]
	8.2[9]	6.6[4]	6.8[4]
	8.3[3]	6.8[4]	6.9[2]
			7.0[8]
			7.1[3]
AF-DX	6.2[3]	7.3[3,6]	6.3[2,3]
	6.3[10]	7.4[11]	6.4[6,11]
			6.5[8]
Hexa	7.3[1]	6.2[5]	7.5[2]
	7.5[5]	6.5[5]	7.6[8]
	7.8[1]	6.7[8]	7.8[5]
	7.9[5]		8.0[5]

(1) Eltze *et al.*, 1988
(2) Roffel *et al.*, 1988
(3) Waelbroeck *et al.*, 1987
(4) Clague *et al.*, 1985
(5) Lambrecht *et al.*, 1989
(6) Giachetti *et al.*, 1986
(7) Choo *et al.*, 1986
(8) Bognar *et al.*, 1989
(9) Birdsall and Hulme, 1985
(10) Micheletti *et al.*, 1988
(11) Ladinsky *et al.*, 1988

however, since the pA$_2$ value obtained experimentally for AF-DX was intermediate between the predicted values for M$_2$- and M$_3$- receptors. The pA$_2$ value obtained for Hexa, on the other hand, unequivocally corresponded to that for M$_3$-receptors. Brichant *et al.* (1989) have also shown that M$_3$-receptors are involved in producing contractions of the tracheal smooth muscle of this species.

Beck *et al.* (1987) investigated the role of muscarinic receptors in increases in pulmonary resistance following vagus nerve stimulation in an *in vivo* canine model. In contrast to the conclusion arrived at in these studies, they concluded the presence of a postjunctional excitatory M$_1$-receptor, based on their finding that Pir antagonized the vagal response. However, at higher concentrations, Pir also acts at M$_2$- or M$_3$-receptors, as shown in this study and others (Doods *et al.*, 1987). Since Beck *et al.* (1987) did not compare the concentrations of Pir required to antagonize the bronchoconstriction with those required to antagonize a response known to be M$_1$-mediated, it was premature to conclude that antagonism by Pir confirmed the involvement of M$_1$-receptors.

(1.2)   Postjunctional thromboxane receptors

These studies clearly show that the thromboxane mimetic (U46619) had potent postjunctional excitatory effects on cholinergic neurotransmission in this tissue.

Activation of the postjunctional receptors (i.e., on the smooth muscle) led to membrane depolarization and powerful

contractions. These effects were shown to involve activation of thromboxane receptors, as they were sensitive to the thromboxane receptor antagonist (L-670,596). It was also shown that the thromboxane receptor was directly coupled to the effector mechanisms (in contrast to a mechanism involving muscarinic receptors), since the U46619-contractions were unaffected by antagonism of muscarinic receptors (using atropine).

Some postjunctional excitatory effects of thromboxane-mimetics have been reported previously for canine trachealis (Inoue and Ito, 1985; Munoz *et al.*, 1986; Tamaoki *et al.*, 1987; Serio and Daniel, 1988), but canine bronchial smooth muscle has not been studied. Extrapolation of tracheal data to bronchi can lead to wrong conclusions, since it has been shown that the two tissues are very different with respect to morphology. For example, bronchial smooth muscle is very densely innervated by both cholinergic and adrenergic fibers, while the trachealis is much less innervated (with very few adrenergic fibers; Daniel *et al.*, 1986; Daniel, 1988). These differences suggest the two smooth muscle systems are regulated in very different fashions. This suggestion is supported by the findings of many other studies, which have shown differences between the two with respect to sensitivity to various pharmacological agents. In fact, these studies further underscore the contention that the tracheal and bronchial smooth muscle systems are distinct (from the perspective of pharmacological/physiological studies), since the postjunctional effects in canine trachealis were only minor (maximal U46619-

contractions were ≈10% of the response to electrical stimulation [Inoue and Ito, 1985] or less [Serio and Daniel, 1988]), whereas U46619 was found in this study to have a major effect on canine bronchial smooth muscle (maximal U46619-contractions were greater than the FS-responses). The relevance of this difference will be further discussed in section 7.1.

(2) MODULATION OF EXCITATORY NEUROTRANSMISSION

(2.1) Prejunctional inhibitory M_1-receptors

Nerve-released Ach has been shown to prejunctionally inhibit cholinergic neurotransmission in canine, guinea pig, and human ASM (Fryer and Maclagan, 1984; Ito and Yoshitomi, 1988; Minnette and Barnes, 1988). Again, the subtype(s) of receptors mediating this effect in the canine airway has not been investigated. These receptors have been identified as being of the M_2-subtype in isolated strips of human and guinea pig ASM (Minnette and Barnes, 1988), although others have given evidence suggesting that *in vivo* presynaptic inhibition in the guinea pig is M_1-receptor-mediated (Faulkner *et al.*, 1986; Fryer and Maclagan, 1984). As for the canine airways, the finding that McN antagonized FS-responses at concentrations which were too low to elicit a response from the postjunctional receptors suggested that the prejunctional auto-inhibitory receptors were M_1 in nature. The lack of such an inhibitory effect of the agonists Bch and Oxo (except at high concentrations), as well as the potentiating effect on FS-contractions of the M_1-antagonist Pir at concentrations selective

for M_1-receptors, also support this conclusion. Similar observations were made with respect to the effects of these agonists and antagonists on measurements of Ach-release as reflected by the magnitude of EJPs elicited by FS; i.e., McN reduced while Pir enhanced EJPs at concentrations selective for M_1-receptors. Moreover, Pir prevented the inhibition of subsequent EJPs mediated by Ach released by earlier EJPs.

It has only recently been recognized that M_1-receptors are not a homogenous population of receptors (Lambrecht *et al.*, 1988). Since these subtypes were first studied using M_1-receptor preparations from hippocampal slices and from superior cervical ganglia, they have been referred to as hippocampal (M_{1a}) and ganglionic (M_{1B}) subtypes (Lambrecht *et al.*, 1988; Eltze *et al.*, 1988; Mutschler *et al.*, 1987). Surprisingly, this heterogeneity was first indicated by a dichotomous sensitivity to the M_3-selective antagonists hexahydrodifenidol (Lambrecht *et al.*, 1988) and hexahydrosiladifenidol (Mutschler *et al.*, 1987). Also, in a study of the structure-activity relationships of derivatives of McNeil A343, Eltze *et al.* (1988) claimed that McNeil A343 was a potent M_{1B}-agonist but had little or no efficacy at M_{1a}-receptors. If this is so for peripheral muscarinic receptors, the fact that McN potently antagonized cholinergic neurotransmission in this tissue indicates the prejunctional muscarinic receptors are of the ganglionic or M_{1B}-subtype.

(2.2) Prejunctional excitatory muscarinic receptors?

In addition to M_1-receptors which inhibit Ach-release, there was some evidence which suggested the presence of non-M_1-receptors which potentiate Ach-release. This possibility arises from the finding that Bch and Oxo (as well as Cch) potentiated FS-contractions and FS-EJPs when applied at sub-threshold concentrations (sub-threshold with respect to producing significant tonic contractions). These observations can be explained by (1) proposing the presence of prejunctional non-M_1-receptors which prolong or potentiate Ach-release; (2) attributing to these agonists some kind of cholinesterase-inhibiting activity; or (3) proposing that these agonists at sub-threshold concentrations activate or prime the smooth muscle cells, albeit insufficiently to actually produce a tonic contraction, thus enhancing the FS-responses. Since sub-threshold doses of histamine or of KCl also potentiated FS-responses, it would seem that explanations involving cholinergic mechanisms need not be evoked and that the third explanation (i.e., priming of smooth muscle cell activity) is most likely the case. A mechanism involving altered (decreased) release of NE can be ruled out, since activation of ß-adrenoceptors was prevented (using propranolol) and since activation of α-adrenoceptors does not seem to influence Ach-release in this tissue.

(2.3) Prejunctional inhibitory ß-adrenoceptors

The data clearly show an inhibitory effect of catecholamines on mechanical responses in this tissue. However, it is difficult

to conclude from these data alone that the inhibition is directed at a prejunctional or postjunctional site, or both. Such a conclusion can usually be made after demonstrating a lack of an effect on the response to exogenously-added neurotransmitter but a distinct effect on nerve-released neurotransmitter (elicited by electrical stimulation of the tissues). This is not the case for studies of this tissue which look only at mechanical responses, since functional ß-adrenoceptors are known to also exist postjunctionally (mediating relaxation; Kannan and Daniel, 1980; Inoue and Ito, 1986; Small and Foster, 1987); thus, a NE-induced decrease in nerve-mediated mechanical activity of the tissue may involve both postjunctional and prejunctional mechanisms.

This uncertainty can be resolved if attention is focussed on the electrical responses (EJPs) of the tissue. Since the magnitude of the EJP is dependent on the amount of Ach released by the nerve endings, the postjunctional effects of NE (hyperpolarization of the V_m) are easily distinguished from its prejunctional effects (decrease in the magnitude of the EJP). Since exogenously-added catecholamines could completely inhibit FS-EJPs without altering resting membrane potential or Cch-induced membrane depolarization (except when the concentration of catecholamine was more than 10-fold higher than that required to mediate 100% inhibition of FS-responses), it is possible to conclude that the catecholamines acted prejunctionally to depress cholinergic neurotransmission.

Although it might be argued that the ß-agonists could inhibit the electrical responses by altering the activity of ion channels

in smooth muscle (i.e., by acting through a postjunctional mechanism) without affecting V_m, Ito and co-workers have shown that another β_2-agonist (procaterol) could inhibit cholinergic neurotransmission in canine trachealis (reflected in the magnitude of FS-elicited EJPs) without showing effects on R_m (or V_m) until concentrations 10-100 fold higher were used; the same was shown to be true of Iso-induced inhibition, although the difference in the concentration required for the prejunctional and postjunctional effects was not as large (Ito and Tajima, 1982; Ito, 1988). Moreover, I found that forskolin antagonized FS-elicited contractions when used at 10^{-7}M, while contractions elicited by 10^{-7}M Cch were unaffected by 10^{-7} or 10^{-6}M forskolin, indicating that elevation of levels of intracellular cAMP could exert a prejunctional effect in the absence of any postjunctional effect. Since the effects of β-adrenoceptor agonists likely involve activation of AC, these data suggest that cAMP elevation in bronchial smooth muscle may not be as effective in antagonizing responses to cholinergic agonists as its elevation in bronchial nerves is in inhibiting Ach-release (see section 2.5). Thus, the data are consistent with the conclusion that inhibition of FS-responses by β-agonists involves a prejunctional mechanism. Moreover, no other explanation is consistent with the additional finding that β-adrenoceptor antagonists enhanced the amplitudes of FS-elicited EJPs in tissues which had not been previously exposed to exogenously-added adrenergic agonists.

While studies to date have found that the postjunctional

effects of catecholamines are directed through β-adrenoceptors (Kannan and Daniel, 1980; Inoue and Ito, 1986; Leff *et al.*, 1986), this is not necessarily true of the prejunctional effects. In the guinea pig and the human, for example, the prejunctional receptors have been found to be of the α_2-subtype (Grundström *et al.*, 1984; Grundström and Andersson, 1985). The findings presented here confirm those of others (Vermiere and VanHoutte, 1979; Danser *et al.*, 1987) that the prejunctional receptors in the canine ASM are of the β subtype: antagonists such as propranolol and timolol, but not phentolamine, antagonized the NE-related effects. Also, Iso used at concentrations which are selective for β-receptors mimicked the effects of NE.

Martin and Collier, on the other hand, have directly measured the release of Ach from canine trachea and found that exogenously-added NE, even at concentrations of 10^{-5}M, did not seem to alter the release of Ach (Martin and Collier, 1986). Although this would appear to contradict the findings of this study, Martin and Collier compared the effects of NE on Ach-release induced by volleys of electrical stimulation (600 pulses per volley) at varying frequencies. With such intense electrical stimulation of a tissue in a 2.5 ml bath volume, it is possible that catecholamines released from adrenergic fibers (Vermiere and VanHoutte, 1979; Danser *et al.*, 1987) could have accumulated to such levels that the prejunctional adrenoceptors were already maximally activated and thus exogenous addition of more NE would have no effect. Although they tested the effects of propranolol on contractile responses to

FS, they did not report the effects of this agent on "control" levels of Ach-release. This explanation could also account for their finding that Ach output remained constant at frequencies of 5-20 pps but was three times higher at 2 pps, since NE re-uptake mechanisms could possibly take up the nerve-released NE more completely at this lower frequency and thus allow a greater level of Ach release (uptake blockers were not used in their study).

Alternatively, tissue-related differences may also explain the apparent contradiction between these findings and those of Martin and Collier, since the tissues used were bronchial smooth muscle and tracheal smooth muscle, respectively. The trachealis has a very sparse adrenergic innervation while the bronchial smooth muscle is densely innervated by both cholinergic and adrenergic fibers (Daniel *et al.*, 1986; Daniel, 1988). These anatomical differences between the tissues may manifest as functional differences: i.e., they may imply that prejunctional modulation of neurotransmission is less important (or even non-existent) in trachealis compared to bronchial smooth muscle. Indeed, in studies of canine trachealis muscle in the sucrose gap, no evidence of adrenergic prejunctional control of EJPs was found (Daniel *et al.*, 1987).

It is now possible to further define beta adrenoceptors as being of the B_1 or B_2 subtype using the antagonists ICI 89,406 and ICI 118,551 which have pA_2 values of approximately 9 for the B_1 and B_2 receptors respectively, and pA_2 values of 7 for the other B-receptor subtype (Bilski *et al.*, 1983; Hedberg *et al.*, 1985). The

results presented in this report clearly show that both β_1 and β_2 receptors are involved in prejunctional inhibition of cholinergic neurotransmission.

Firstly, both the β_1- and the β_2-selective antagonists could antagonize this effect of NE on both the field stimulated twitch contractions and electrical responses. In addition, the combined use of the two selective antagonists together achieved a greater degree of inhibition of NE-related effects than a higher concentration of either antagonist alone. Similarly, non-selective antagonists sometimes potentiated control responses to FS, while this was never seen when tissues were exposed to only one or the other of the selective antagonists.

Secondly, Schild plot analysis of the data supports this conclusion in two ways: (a), the Schild plots pertaining to NE and to Sal in the presence of one or the other selective β-antagonist are non-superimposable, which is necessary and sufficient to conclude that heterogeneity exists in the β-receptor population (see section 5.3 of Introduction) and (b), the Schild plots pertaining to the β_2-selective agonist salbutamol had slopes of one, while those of the poorly selective agonist NE did not. Inhibitors of neuronal uptake and of NE catabolism were not used in these studies; both processes could have decreased the effective concentration of exogenously added NE at the receptor and affected estimation of the Schild plot slopes and pA_2 values of the antagonists.

Finally, the electrophysiological data presented suggest both

receptors play a role in prejunctional inhibition of Ach-release. The involvement of the β_2-receptor is established by the observation that Sal antagonized FS-EJPs at a concentration which is selective for the β_2-receptor. The involvement of the β_1-receptor, on the other hand, is established by the observation that simultaneous exposure to both β_1- and β_2- selective antagonists at relatively low concentration (10^{-8}M) did not alter the effect of NE on electrical responses, while subsequently increasing the concentration of the β_1-antagonist to 10^{-7}M greatly reduced the effect of NE on these responses; this same concentration in the absence of the β_2-antagonist had no effect.

This conclusion (both β_1 and β_2 receptors on the cholinergic nerve endings) contradicts in part the findings of Danser *et al.*, who concluded that the prejunctional receptors in the canine airways were not of the β_2 subtype but of the β_1 subtype (Danser *et al.*, 1987). However, their conclusion was based on the absence of an effect of 10^{-7}M ICI 118,551 on NE-mediated inhibition (and the elimination of the NE-effect using the β_1-selective antagonist metoprolol); the data and explanation given above shows how this kind of evidence is insufficient to rule out the co-existence of β_1- and β_2-receptors.

(2.4) Arachidonic acid metabolites

The finding that incubation of the tissues in indomethacin greatly enhanced the responses to electrical stimulation strongly suggests a cyclo-oxygenase metabolite can potently antagonize

cholinergic neurotransmission. This metabolite was not identified in these studies, but many previous studies using canine tracheal and bronchial smooth muscle have demonstrated potent prejunctional inhibition imposed by PGE_2 (Ito and Tajima, 1981a and 1981b; Daniel et al., 1987; Inoue et al., 1984; Inoue and Ito, 1986; Moore et al., 1986; Walters et al., 1984; Madison et al., 1989; Serio and Daniel, 1989).

U46619 (and therefore TxA_2) had a potent prejunctional excitatory effect which manifested as enhanced cholinergic neurotransmission (reflected in the enhanced contractions and EJPs elicited by FS). It might be argued that this was just another manifestation of a postjunctional effect of U46619, involving closure of delayed rectifying channels on the smooth muscle (which would limit the size of FS-EJPs when opened) or enhanced sensitivity of the muscarinic receptors on the smooth muscle to the neurotransmitter. However, this was shown to be a prejunctional effect since the EC_{50} of Cch was unaltered by U46619 used at a concentration which had the greatest potentiating effect on the FS-contractions and EJPs. This prejunctional effect was shown to involve activation of thromboxane receptors, as it was antagonized by the thromboxane receptor antagonist. Although cholinergic neurotransmission in this tissue is known to be inhibited by prejunctional ß-adrenoceptors (above), the prejunctional effect of the thromboxane-analogue did not involve an alteration of this adrenergic regulation, since the potentiation of contractile activity was not altered by α- or ß- adrenergic blockers

(phentolamine and propranolol).

(2.5) Role for adenylate cyclase?

These studies have shown that NE, Iso, Sal, McN, and PGE_2 are all potent prejunctional inhibitors of Ach-release in this tissue. ß-adrenoceptors and PGE_2-receptors have been shown to be coupled to activation of adenylate cyclase in many systems (Torphy et al., 1982 and 1985; Felder et al., 1989; Madison et al., 1989); similarly, M_1-receptors can be indirectly coupled to adenylate cyclase through phosphoinositide metabolism (Felder et al., 1989). In addition, forskolin (which activates AC) antagonized contractions elicited by electrical stimulation at a concentration much less than that required for direct relaxations (as much as 100-fold difference in the concentrations required). This would suggest that the release of synaptic vesicles in the cholinergic nerve endings can be modulated by AC. One mechanism that can be envisioned is that these pharmacologically distinct receptors all couple to a common AC through G_s-proteins to increase intraneuronal levels of cAMP and thus enhance the activity of some cAMP-dependent Ca^{++}-extrusion mechanism and lower Ca^{++}_i (since release of the vesicles is critically dependent on Ca^{++}_i). Alternatively, activation of PK-A within the nerve endings may lead to phosphorylation and subsequent inhibition/inactivation of one or more control systems required for vesicular release; this has been suggested to be the case in guinea pig mesenteric artery, where cAMP/PK-A, cGMP/PK-G, and DAG/PK-C are all implicated in

regulation (facilitation) of neurotransmitter release from perivascular nerve endings (Ishikawa and Sperelakis, 1989). The data presented do not allow conclusions to be made, but do suggest a role for AC in regulating cholinergic neurotransmission.

(3) INHIBITION OF CANINE BRONCHIAL SMOOTH MUSCLE

(3.1) Relaxations are predominantly β_1-adrenoceptor mediated

The present study confirms previous findings that NE has an inhibitory effect on ASM through a direct postjunctional mechanism (Suzuki *et al.*, 1976; Kannan and Daniel, 1980; Russell, 1984; Inoue and Ito, 1986). Furthermore, it is indicated that this effect involves primarily β_1-adrenoceptor stimulation for the following reasons.

First, the relaxant effect of exogenously-added NE on Cch-precontracted tissues, as well as that of endogenously-released NE on tissues precontracted with McN, were not affected by the β_2-selective antagonist (except at high [non-selective] concentrations) but were potently antagonized by the β_1-selective antagonist in both cases. Although the contractions produced by the two muscarinic agonists in these instances were quite different in magnitude (3×10^{-6}M McN and 3×10^{-7}M Cch elicit contractions $\approx25\%$ and $\approx75\%$, respectively, of the response to 10^{-4}M Cch) the relative potencies of the β-receptor antagonists were the same in both cases. This observation indicates that the degree of precontraction did not alter the relative contribution of the two β-receptor subpopulations (i.e., β_1 versus β_2) through which NE

elicited relaxation.

Second, Schild plot analysis of the effects of selective antagonism of NE-induced relaxations also clearly showed that NE acts predominantly at β_1-receptors, since the β_1-antagonist plot was displaced to the left of the β_2-antagonist plot by a full log unit. The separation between the two NE-related Schild plots was one instead of the two log unit difference expected when these antagonists are used against selective agonists. However, it should be remembered that the separation between the plots is directly proportional to the selectivity of the agonist used and that NE is only weakly selective for β_1-receptors (O'Donnell, 1985).

(3.2) Relaxations may be partially β_2-receptor mediated

Relaxations produced by Sal were much more sensitive to the degree of precontraction than were those elicited by NE or Iso. Whereas both NE and Iso were full agonists in tissues precontracted with 3×10^{-7}M Cch, Sal was only a partial agonist when 3×10^{-7}M (EC_{70}) or 10^{-7}M (EC_{85}) Cch was used. Under these conditions the effective concentrations of Sal were sufficiently high to activate β_1-receptors (as well as β_2-receptors; Yabucchi, 1977).

When low concentrations of Cch (i.e., 2×10^{-8}M or EC_{40}) were used, Sal reversed precontracted tone completely and at concentrations which were appropriate for selective action at β_2-receptors. These relaxant responses were competitively antagonized by the β_2-selective antagonist with a pA_2 of 8.9. Thus, a small population of functional β_2-receptors were likely present in

addition to the predominant population of B_1-receptors.

(3.3) Postjunctional versus non-junctional location of receptors mediating relaxations

The existence of two different populations of B-receptors on canine ASM, which produce relaxations through different mechanisms, has already been discussed by Barnes et al. (Barnes et al., 1983). One such population was claimed to be acted on by nerve-released NE and found to be of the B_1 subtype, while the other population was claimed to be acted on by catecholamines circulating in the blood and found to be of the B_2 subtype (Barnes et al., 1983). Earlier, Bryan et al. proposed the existence of two such receptor populations on guinea pig ASM, and referred to them as the "innervated" and "hormonal" receptors, respectively (Bryan et al., 1981). The B_1-receptors described by Barnes et al. correspond to the postjunctional receptors described in this study, since both were activated following field stimulation of precontracted tissues (using 5-HT and McNeil A343, respectively) and were sensitive to selective B_1-blockade (using practolol and ICI 89,406, respectively).

The second population of receptors described by these authors, which was said to mediate the response to exogenously added (or circulating) catecholamines, was found to be of the B_2-subtype. The data presented here are also suggestive of the existence of such a receptor population, but minimize their physiological importance in mediating the relaxant responses to exogenously-added

or blood-borne NE. This may be surprising, since B_2-receptors were found to outnumber B_1-receptors in canine airways by a ratio of 4:1 (Barnes et al., 1980; Barnes, 1988). However, it is not clear that the cellular locus of the receptors measured by the ligand binding techniques used was smooth muscle. They may be located on cells other than airway smooth muscle and be important in mediating prejunctional inhibition of cholinergic neurotransmission, or modulating the activity of mucous cells, epithelial cells, secretory cells, or vascular smooth muscle (Barnes, 1988).

(3.4) NE-release not modulated by adrenoceptors In certain other smooth muscle systems, B-adrenoceptors on adrenergic nerve endings enhance NE-release (i.e., are "auto-excitatory). I have found that both B_1- and B_2- agonists are potent inhibitors of FS-contractions while only B_1-agonists are potent inhibitors of Cch-contractions, and interpreted this as indicating inhibitory B_1- and B_2- receptors are present on cholinergic nerve endings.

It could be argued, however, that there are "auto-excitatory" B_2-receptors on the adrenergic nerve endings and that exogenously-added B_2-agonists elicit or enhance the release of NE which then acts on B_1-receptors on the smooth muscle (to antagonize any contractions) and on the cholinergic nerves (to inhibit Ach-release). However, this mechanism predicts that the B_2-antagonist should have potentiated FS-elicited EJPs and contractions when used at concentrations which selectively activate B_2-receptors (i.e., less than 10^{-7}M), as well as decreased the magnitude of FS-

elicited relaxations in McN-precontracted tissues. None of these effects were noted in this study. In addition, Schild plot analysis indicated that while the population of ß-receptors mediating relaxation was largely homogenous (almost exclusively B_1 in nature), the population of ß-receptors mediating inhibition of FS-contractions was clearly heterogenous (the plots obtained in the former case had unit slopes, while those in the latter case had slopes significantly different from 1.0 when NE was used); this is also indicated by the finding that ICI 89,406 had a pA_2 of 7.70 against the prejunctional effect of NE and a pA_2 of 8.4 against the postjunctional effect of NE. Finally, this mechanism does not account for the findings that Sal antagonized EJP peak heights but did not hyperpolarize the membrane, even when high doses were used, as it should if it were only enhancing the postjunctional B_1-receptor effect. For these reasons, a mechanism involving facilitatory B_2-adrenoceptors on the adrenergic nerve endings seems implausible. On the other hand, the mechanism involving inhibitory B_2- (and B_1-) adrenoceptors on the cholinergic nerve endings adequately accounts for all the observations made.

(4) VARIOUS ASPECTS OF ASM PHYSIOLOGY

(4.1) Physiological antagonism between adrenergic and cholinergic mechanisms

Muscarinic receptors, like ß-adrenoceptors, have been shown to be coupled to AC through G-proteins and to functionally antagonize catecholamine-activated adenylate cyclase activity and

thus cAMP-dependent PK in canine tracheal smooth muscle (Miller *et al.*, Torphy *et al.*, 1983 and 1985; Sankary *et al.*, 1988, Madison *et al.*, 1989). As well, Gunst *et al.* have recently shown that the magnitude of the relaxant response elicited by Iso in canine trachealis precontracted with cholinergic agonists is determined in part by the amount of muscarinic receptor reserve (i.e., receptor "spareness"); in other words, a larger receptor reserve can more effectively "buffer" the contraction against catecholaminergic inhibition (Gunst *et al.*, 1989). Both findings account for the slight increase in potency and efficacy of Iso when concentrations of Cch were decreased three-fold. This does not, however, account for why Sal-relaxations were so much more sensitive to the concentration of precontracting agent than NE- or Iso- relaxations.

There are several possible explanations for the lack of efficacy of the β_2-agonist to relax muscarinic agonist-induced contractions and the decrease in its efficacy when Cch concentrations were increased. Possibly there is an inadequate density of β_2-receptors or a low intrinsic efficacy for Sal. Alternatively, Sal may have a diminished ability to inhibit one of two modes of excitation-contraction coupling mechanisms utilized by Cch (see below), while β_1-agonists interfere with both. Contractions in this tissue can involve electromechanical coupling (depolarization-induced opening of Ca^{++}-channels and Ca^{++}-influx) and/or pharmacomechanical coupling (release of Ca^{++} from intracellular stores linked to phosphoinositide metabolism; Farley

and Miles, 1977; Coburn, 1979; Leff, 1988). It might be, then, that B_1-receptors are coupled through both pathways (i.e., can inhibit both the opening of Ca^{++}-channels and the release from intracellular stores), while B_2-receptors are coupled to only one pathway. Since pharmacomechanical coupling predominates over electromechanical coupling when low concentrations of cholinergic agonist are used (whereas both occur at higher concentrations of cholinergic agonist; Farley and Miles, 1977; Coburn, 1979; Leff, 1988), and since Sal-induced relaxations were easily overcome by increasing the concentration of precontracting agent, the explanation proposed above suggests that the B_2-receptors are coupled to mechanisms which can oppose effects associated with phosphoinositide metabolism but not effects associated with Ca^{++}-influx following membrane potential depolarization. Other possibilities exist, but the data presented do not provide strong evidence for or against any of them.

(4.2) Continuous or background release of NE?

It was found in these studies that ß-antagonists could potentiate responses to FS, including responses to single pulse field stimulation. There is considerable controversy regarding the existence/role of prejunctional inhibitory receptors (Kalsner, 1984). One of the arguments made is that blockade of the receptors should not influence the responses to single pulse field stimulation (the released neurotransmitter can not retro-actively affect its own release). One counter-explanation is that any

increase in the response caused by an antagonist is not due to dysinhibition, but perhaps to a change in some biochemical or physiological property of the tissue which alters its sensitivity to cholinergic agents. This explanation is unlikely to account for the findings presented here, since the antagonists did not affect V_m or the responses to cholinergic agonists in the muscle baths. Similarly, the only agents that produced this effect were ß-antagonists (propranolol, timolol, ICI 89,406 plus ICI 118,551, but not phentolamine); although propranolol and ICI 118,551 are reported to have membrane stabilizing properties (Bilski et al., 1983), timolol does not (Barnes, 1988). Thus, ß-antagonist-induced potentiation of FS-responses could reflect inhibition of "background" or spontaneous release of NE in some of the tissues. With a bath perfusion rate in the microelectrode studies of 3 ml/minute and a bath volume of 5 ml, it is unlikely that this phenomenon is due to an accumulation of NE released from previous stimulations. If NE is indeed continuously released at low levels in vivo, and thus continuously maintains a low level of inhibition of cholinergic neurotransmission as Barnes has suggested (Barnes, 1988), this might account for certain manifestations of airway hyperresponsiveness. Instead of an impaired or decreased ß-adrenoceptor function, as suggested by some, the problem may originate in a decrease in the continuous or "background" release of NE, leading to dysinhibition of the ASM and hyperresponsiveness. This could account for the controversy surrounding the role of the ß-receptor in airway hyperresponsiveness (Lulich et al., 1988;

Barnes, 1988), since the emphasis in studies and discussions of this question usually centers on the postjunctional effect of sympathomimetics on the smooth muscle, rather than their prejunctional effects (Lulich *et al.*, 1988; Barnes, 1988). Such a prejunctional mechanism could explain the development of bronchoconstriction in some asthmatics in response to ß-blockers (Grieco and Pierson, 1971; Ind *et al.*, 1986) as well as nocturnal asthma (since plasma levels of epinephrine are decreased at this time; Barnes *et al.*, 1980). In further support of this possibility, prejunctional ß$_2$-adrenoceptors which inhibit cholinergic neurotransmission have been identified in human trachea (Bai *et al.*, 1989) and bronchi (Rhoden *et al.*, 1989).

(4.3)   Electromechanical versus pharmacomechanical coupling

Excitation and contraction in canine tracheal smooth muscle have been shown to be largely pharmacomechanically coupled (Coburn, 1979; Small and Foster, 1987). The data presented here suggest that the same is true of bronchial smooth muscle. First, exposure of the tissues to ß-agonists at concentrations which elicited large relaxations had little or no effect on V_m; similarly, electrical stimulation of tissues exposed to 10^{-5}M McN elicited large reversal of precontracted tone but did not elicit any hyperpolarization of the membrane, even though this was depolarized by as much as 15-20 mV.   Second, although ß-agonists were potent inhibitors of contractions and EJPs elicited by electrical stimulation, the IC$_{50}$ in each case often differed; FS-contractions were often much more

sensitive to a given concentration of β-agonist than were the FS-EJPs. Third, U46619 increased the magnitude of EJPs by ≈150% but increased the magnitude of the corresponding contractile responses by only ≈20-25%. And lastly, several agents often elicited slow oscillations after they depolarized the membrane (Oxo, Cch, Bch, U46619, and high concentrations of Sal) but never produced such oscillations in contractile activity.

These findings are consistent with the suggestion that electromechanical coupling has little or no importance in excitation-contraction coupling in this tissue. This suggestion then raises the question: if electromechanical coupling is unimportant in this tissue, why is membrane depolarization elicited by cholinergic agonists? That is, why do cholinergic agonists apparently open Na^+ and/or Cl^- channels and close the K^+-channels responsible for the M-current or others, thus producing the EJP, when release of Ca_i^{++} following muscarinic receptor activated PI-metabolism is the functionally relevant correlate? (see Introduction, section 2.3). Although the magnitude of the EJPs are directly correlated with the magnitude of Ach-release, there is apparently no physiological consequence of such a correlation.

(4.5) Subtypes of thromboxane receptors?

L-670,596 completely reversed the U46619-effect on membrane potential but did not reverse U46619-induced potentiation of FS-EJPs. This could suggest that there are multiple subtypes of receptors activated by U46619, one of which is insensitive to L-

670,596 (i.e., it is a selective antagonist). This apparently selective action of the antagonist has not been reported previously and should be kept in mind when using this antagonist. Alternatively, U46619-induced potentiation could be an irreversible effect. The latter explanation would explain why U46619-induced potentiation was antagonized in tissues _pretreated_ with L-670,596 (the mechanical studies; figure 30) whereas U46619-induced potentiation was apparently unaltered when L-670,596 was added _after_ the U46619-effect had developed (electrophysiological studies; figure 33).

These studies also provide useful information regarding the thromboxane receptor antagonist itself. L-670,596 has been shown to be a potent and selective antagonist of the receptor at which U46619 and TxA_2 act (Ford-Hutchinson _et al._, 1989). However, the data presented here suggest it acts as a non-competitive antagonist. First, while $10^{-9}M$ L-670,596 had essentially no effect on U46619-induced contractions, $10^{-8}M$ L-670,596 caused a rightward shift in the concentration-response curve of almost 2 log units. A similar phenomenon was noted with respect to the prejunctional effect of U46619. According to the assumptions underlying Schild plot analyses of agonist-antagonist interactions with receptors, a competitive antagonist should produce a shift of not more than 1 log unit when the concentration of the antagonist is increased ten-fold (see section 5.3 of Introduction). Second, maximal contractile responses to U46619 were ≈55% and ≈30% of the maximal Cch-response in the absence and presence of $10^{-8}M$ L-670,596,

respectively; a competitive antagonist should not alter the maximal response of a tissue in this way. This non-competitive behaviour of this antagonist has also not been reported previously, and should be kept in mind when using this antagonist.

(5) OZONE-INDUCED HYPERRESPONSIVENESS

(5.1) Ozone-inhalation induces hyperresponsiveness

The data presented show ozone-inhalation (3 ppm for 30 minutes) induces hyperresponsiveness of isolated canine bronchial smooth muscle, resulting in an increase in the force of contraction elicited by electrical stimulation at various frequencies (0.1-10 pps). Although many studies have shown ozone-induced hyperresponsiveness in canine tracheal smooth muscle, I know of no studies which show enhanced responsiveness to FS of bronchial smooth muscle in this species or others.

Ozone-induced hyperresponsiveness was more dramatic in tissues from some dogs than in others; this is reflected in the larger standard errors associated with the responses in ozone-treated tissues compared to their control counterparts. Other groups using a similar method or using *Ascaris suum* antigen to induce hyperresponsiveness have also found the approach to be less effective in some dogs than in others (Rinard *et al.*, 1979; Holtzmann *et al.*, 1983b).

Since no change in the net response to a given concentration of KCl or to a given frequency of FS was found, it would seem that ozone-inhalation produces hyperreactivity of bronchial smooth

muscle (as opposed to hypersensitivity; see section 6 of Introduction). The results of these studies allow certain suggestions to be made regarding the mechanism(s) underlying ozone-induced hyperreactivity.

(5.2) Role of adrenergic or cholinergic mechanisms in ozone-induced hyperreactivity

Since KCl-contractions were not significantly altered by ozone-inhalation, it can be concluded that the mechanisms underlying depolarization-induced contractions were largely unaffected by ozone-inhalation. On the other hand, the fact that contractions elicited by electrical stimulation were significantly enhanced indicates mechanisms involved in cholinergic control of canine bronchial smooth muscle were affected, since it has been shown that excitatory responses elicited by FS in this tissue seem to be mediated by cholinergic mechanisms exclusively (Russel, 1978; Ito and Tajima, 1981a; Inoue and Ito, 1986; Small and Foster, 1987). Beckett and colleagues have also shown ozone-inhalation potentiates receptor-mediated responses in this tissue while having no effect on depolarization-related responses (Beckett *et al.*, 1988), and that ozone-induced hyperreactivity in humans could be prevented by atropine (Beckett *et al.*, 1985), and thus concluded that parasympathetic mechanism(s) are involved. These should be considered to include receptor-effector coupling mechanisms and prejunctional mechanisms regulating acetylcholine release.

(5.3)   Role of arachidonic acid metabolites in ozone-induced
hyperreactivity

As the sensitivity of the tissues to Cch was not altered by ozone-inhalation, the hyperresponsiveness apparently involved a prejunctional change. The finding that the S_2/S_1 ratio was increased by ozone for all time periods tested (significantly in 3 of the 4 cases) is also consistent with an ozone-induced change in prejunctional regulation of cholinergic neurotransmission. In canine ASM, three autacoids play important roles in this respect: prostaglandin E_2 (section 2.4), norepinephrine (section 2.3), and acetylcholine itself (section 2.1). The fact that the differences in responsiveness between normal and ozone-exposed tissues were eliminated by pretreatment with IDM in bronchial smooth muscle (this study) and tracheal smooth muscle (O'Byrne et al., 1984b) strongly suggest changes in cyclo-oxygenase metabolite activity are responsible for ozone-related hyperresponsiveness. At least two mechanisms can be postulated and are illustrated in figure 38.

First, it may be that ozone-inhalation decreases the synthesis of inhibitory arachidonic acid metabolites. PGE_2 is synthesized and released in large amounts by whole airway smooth muscle (Walters et al., 1984; Seltzer et al., 1986; Serio and Daniel, 1989), cultured tracheal epithelial cells (Leikauf et al., 1985), and alveolar macrophages (MacDermot et al., 1984; Sibille and Reynolds, 1990) from both human and canine airways, and is known to prejunctionally inhibit cholinergic neurotransmission in this tissue as well as have inhibitory effects on the smooth muscle

itself (Moore *et al.*, 1986; Daniel *et al.*, 1987; Shore *et al.*, 1987; Madison *et al.*, 1989); thus, it may be that decreased PGE_2-synthesis is responsible for ozone-related hyperreactivity. PGE_2-synthesis could be decreased if ozone interfered directly with arachidonic acid release or with its metabolism. Alternatively, desquamation of the epithelium, as typically occurs following ozone-inhalation (Empey *et al.*, 1976; Fabbri *et al.*, 1984), would remove a major source of PGE_2, thus leading to a decrease in the net synthesis of this autacoid. An ozone-induced decrease in PGE_2-synthesis would account for the observation that in the absence of IDM, ozone-treated tissues continued to produce contractions even 5 hours after beginning the experiment while control tissues quickly lost their ability to contract. Unfortunately, measurements have not been carried out on PGE_2-synthesis or release in ozone-treated canine ASM.

Second, ozone-inhalation may have altered arachidonate metabolism such that the synthesis of excitatory arachidonic acid metabolites such as TxA_2 was initiated or enhanced. This has been proposed by many to play an important role in hyperresponsiveness induced by inhalation of ozone (Aizawa *et al.*, 1985), antigen (Chung *et al.*, 1986), and LTB_4 (O'Byrne *et al.*, 1985). Thromboxane A_2 is synthesized and released by human airways (Seltzer *et al.*, 1986), neutrophils (Goldstein *et al.*, 1978), macrophages (MacDermot *et al.*, 1984; Sibille and Reynolds, 1990), and airway epithelial cells (Leikauf *et al.*, 1985). Neutrophils and macrophages have been found in the epithelial layer (O'Byrne *et al.*, 1984b; Seltzer

et al., 1986), and in bronchoalveolar lavage fluid (Holtzmann *et al.*, 1983b; Fabbri *et al.*, 1984; O'Byrne *et al.*, 1984b and 1985; Chung *et al.*, 1986; Seltzer *et al.*, 1986) following ozone inhalation. The ability of TxA_2 to potentiate parasympathetic mechanisms in this tissue is supported by the demonstration of such an effect on FS-elicited excitatory responses of the thromboxane mimetic U46619 in this study and others (Chung *et al.*, 1986; Tamaoki *et al.*, 1987a), or of a thromboxane-like product released by platelets or A23187-stimulated alveolar macrophages in other studies (Tamaoki *et al.*, 1987a and 1987c). A similar effect of U46619 on tracheal smooth muscle has also been shown (Serio and Daniel, 1988).

The data do not explain why L-670,596 antagonized ozone-induced hyperreactivity *in vitro* (this study), but did not alter hyperreactivity demonstrated *in vivo* (Jones *et al.*, 1990). It may be simply a problem of accessibility to the appropriate sites (i.e., excitatory receptors on the cholinergic nerve endings). That is, in the *in vivo* studies, the antagonist must first enter from the lumen, pass through the epithelium and several other cell layers; in the *in vitro* studies, the antagonist is present in the bathing medium surrounding the tissues and thus has direct exposure to inner and outer aspects of the bronchial smooth muscle.

The data presented here suggest that neither decreased PGE_2-synthesis alone nor increased TxA_2-synthesis alone are sufficient to account for ozone-induced hyperreactivity. If only a decreased synthesis of inhibitory arachidonate metabolites were responsible,

then responses to field stimulation in control tissues exposed to indomethacin should be equivalent to responses in ozone-treated tissues not exposed to indomethacin (the latter were significantly smaller than the former in the majority of the cases). Similarly, if TxA_2 were not involved, then responses to field stimulation in tissues exposed to the thromboxane receptor antagonist should be equivalent to those in tissues not exposed to any antagonists (the latter were found to be significantly smaller in many cases).

On the other hand, if TxA_2 were the only factor responsible then the receptor antagonist should have been as effective as indomethacin (it was largely ineffective at a concentration which potently antagonized the effects of U46619 in this tissue).

Thus, it seems that ozone-induced hyperreactivity involves both changes together. An alteration in AA metabolism or shunting of AA endoperoxide intermediates from one cyclo-oxygenase metabolic product to another following activation of inflammatory cells is not a novel concept. Tripp *et al.* (1985) demonstrated that activation of mouse peritoneal macrophages led to decreased prostaglandin endoperoxide E-isomerase activity (i.e., the enzyme that catalyzes the isomerization of PGG_2/PGH_2 to PGE_2) while thromboxane synthetase activity was unchanged or enhanced. Similarly, infusion of A23187 into isolated dog and rabbit lungs (a procedure which has many effects including activation of inflammatory cells) caused TxA_2 levels to be increased but not PGE_2 levels (Littner and Lott, 1988). Tate *et al.* (1984) showed that reactive oxygen metabolites stimulated thromboxane synthetase in

rabbit lung.

Other metabolites of cyclo-oxygenase besides PGE_2 or TxA_2 may also play important roles in ozone-induced hyperresponsiveness. PGD_2 has been shown to potentiate FS-responses in canine ASM (Tamaoki *et al.*, 1987b) and to enhance the responses to histamine or methacholine in human airway smooth muscle (Fuller *et al.*, 1986), and is known to be released by mast cells (Peters *et al.*, 1983), alveolar macrophages (MacDermot *et al.*, 1984), and airway epithelial cells (Eling *et al.*, 1986). Similarly, $PGF_{2\alpha}$ potentiates responsiveness of airway smooth muscle (O'Byrne *et al.*, 1984c) and is released by airway epithelial cells (Leikauf *et al.*, 1985), alveolar macrophages (MacDermot *et al.*, 1984), or whole airway smooth muscle (Seltzer *et al.*, 1986).

(6) IMPLICATIONS OF PRESENT STUDY FOR ASTHMA IN HUMANS

(6.1) Tracheal and bronchial smooth muscles are not comparable

Throughout this thesis, I have referred to the findings of other groups that tracheal and bronchial smooth muscles are similar only in some respects, but dissimilar in others; these were summarized in section 3 of the Introduction. The results presented here extend these findings. While the thromboxane-mimetic had only minor postjunctional effects in canine trachealis (Serio and Daniel, 1988), this agent had potent postjunctional effects in bronchial smooth muscle. This has particular significance when one considers the important role apparently played by TxA_2 in ozone-

induced hyperresponsiveness and the fact that the bronchi play an important physiological role in determining peripheral resistance to airflow. According to a review of Pride (1971), the proportion of total peripheral resistance to airflow in human lung determined by the bronchi and bronchioles is greater than that determined by the trachea. This suggests that previous studies which have used trachealis should not be extrapolated to airway smooth muscle physiology as a whole, and thus may have less relevance to systemic pulmonary function than has been previously assumed. In addition, the studies presented here clearly show bronchial smooth muscle can easily be studied using both electrophysiological and muscle bath techniques, and therefore there is no need to depend exclusively on trachealis in studies which are intended to be extrapolated to the whole animal situation (i.e., asthma-related research).

(6.2) Canine model is inappropriate for studies relating to human species?

The finding that catecholaminergic-relaxations in the canine bronchi are predominantly β_1-receptor mediated has particular relevance to studies which use this species as a model in investigations relating to asthma, since the most common approach to the treatment of asthma is inhalation of β_2-agonists (such as salbutamol or fenoterol), whereas this study has shcwn such agents to be relatively inefficacious in eliciting relaxation of canine bronchial smooth muscle. Similarly, intense research is currently being directed toward demonstrating a role for leukotrienes in

asthma-related bronchoconstriction in humans and the use of leukotriene antagonists in treating asthma; leukotrienes have not been shown to have major effects on canine ASM (Serio and Daniel, 1989). Finally, non-cholinergic non-adrenergic inhibitory nerves may play an important role in human airway physiology but are apparently absent (or at least functionally irrelevant) in canine airways.

Admittedly, the canine model has been extremely useful in the past, and many important concepts and applications have been derived from the use of this model. However, the discordances identified above would imply that the canine species is not the most appropriate model for studies of human airway physiology, and it is strongly recommended that this be kept in mind in such studies.

(6.3)   New   approaches   to   treating   asthma-related bronchoconstriction?

Characterization of receptor-mediated effects have the potential to lead to the development of new drugs in the treatment of diseases. In this case, knowledge of the subtypes of muscarinic and adrenergic receptors mediating bronchoconstriction, bronchodilation, and inhibition of Ach-release could lead to new treatments for airway-related diseases like asthma.

For example, atropine has been used in the past to block muscarinic receptors in ASM and thus treat asthma-related bronchoconstriction. As could be expected, this was associated

with undesirable side-effects due to the action of atropine at all muscarinic receptors, including those at sites other than the ASM. However, the postjunctional receptors in ASM could be blocked using a selective muscarinic antagonist, thus avoiding the side-effects associated with antagonism of the other muscarinic receptor subtypes.

Similarly, cholinergic neurotransmission could be modulated (decreased) using a selective muscarinic agonist or selective ß-adrenergic agonists (as is currently practiced); again, the use of any one of these will avoid the side-effects associated with activation of the other muscarinic or adrenergic receptors. In fact, the latter approach (the use of selective muscarinic or adrenergic agonists) may be superior to the former approach (use of selective muscarinic antagonists), since it is intuitively more efficacious to prevent the development of bronchoconstriction (by preventing Ach-release) than to reverse contractions after they have developed.

Alternatively, since the various subtypes of muscarinic receptors may be coupled to their effector mechanisms in different ways (i.e., M_1-receptors and IP_3-metabolism; M_2-receptors and AC activity; McKinney et al., 1989; Goyal, 1989; Birdsall et al., 1985), knowledge of the coupling mechanisms involved might allow the development and use of physiological antagonists which would not affect action at the muscarinic receptors per se but would uncouple the receptor from the response (e.g., inhibitors of phosphoinositide metabolism). This is particularly suited to

airway-related diseases, since the agents can be applied more or less directly to the site of action (via inhalation) while minimally affecting muscarinic receptor coupling mechanisms at other sites.

A somewhat speculative but potentially effective approach might involve the use of combinations of agents which would alter each of the mechanisms referred to above only partially but in such a way as to achieve potent (synergistic) inhibition of bronchoconstriction with little or no side-effects. For example, if selective ß-adrenergic, muscarinic, and prostanoid agonists were collectively used at doses which inhibited Ach-release only partially, in conjunction with selective antagonists which blocked both the postjunctional muscarinic receptors and intracellular transduction mechanisms partially, it might be possible to compromise the ability of the smooth muscle to contract while having few or no undesired effects at other sites.

(7) FUTURE RESEARCH

Although much has been learned from these studies, they leave much to be clarified. Many questions have been left unanswered, or have been raised by these findings. A number of particularly important areas of future study can now be identified, as follows.

Activation of thromboxane receptors was found to lead to enhancement of EJP peak height initially, followed by membrane depolarization and appearance of slow wave activity ultimately. Both effects could be attributable to an effect of TxA_2 on ion

channels; as these effects are at least qualitatively similar to the effects of K^+-channel blockers like TEA, the possible regulation of K^+-channel activity by TxA_2 should be investigated. This may provide yet another potential therapy for asthma-related bronchoconstriction, assuming TxA_2 is found to play a causative role in the latter. Two particularly interesting candidates for such studies are the novel ATP-dependent K^+-channel agonist BRL 34915 (cromakalim) and antagonist glybenclamide.

Although the cholinergic and adrenergic innervations play a central role in regulating canine ASM activity, there are other regulatory mechanisms which may be important. Human ASM has been shown to be modulated by various neuropeptides, including substance P, CGRP, VIP, and PHI (Gabella, 1987; Lundberg and Saria, 1987; Barnes, 1989; Said, 1989; Widdicombe, 1989). There are also a number of inflammatory mediators which were not investigated in this study but which may have important physiological effects; such mediators include histamine, platelet-activating factor, and the various interleukins. All of these have been shown to have both prejunctional and postjunctional effects in other systems. Thus, the effects of these agents should be studied in this system, especially considering the important role of the inflammatory response not only on the smooth muscle itself, but also on the efferent innervation modulating the activity of the muscle and the afferent innervation which can release the neuropeptides.

There is growing interest in the role of the epithelium in regulating airway smooth muscle function, from which have come the

proposed existences of various epithelium-derived relaxing factors or contracting factors. The techniques used in this study inflict minimal or no damage to the epithelium, allowing the study of the electrophysiological and contractile effects of such putative factors.

(8) CONCLUSIONS

The work summarized in this thesis showed that excitation (membrane potential depolarization and contraction) of large intraparenchymal airways of the canine lung results following activation of M_3-receptors, presumably on the smooth muscle and at the neuromuscular junction. Evidence was also obtained for the existence of thromboxane-receptors on the smooth muscle which when activated also lead to excitation of the smooth muscle; this excitatory effect was independent of activation of muscarinic receptors.

In addition, exogenously added catecholamines and those released by field stimulation caused relaxation of these airways. These relaxations occured through a mechanism which did not require hyperpolarization of the membrane and which involved postjunctional adrenoceptors of the β_1 subtype, although there was additional evidence suggesting the presence of a smaller (and/or poorly coupled) population of β_2-receptors.

The data also demonstrated that cholinergic neurotransmission in this tissue is modulated by many autacoids. Nerve-released NE and Ach acted through prejunctional receptors of the β_1- and β_2-

adrenergic, and M_{1B}-muscarinic subtypes to inhibit Ach-release. Cyclo-oxygenase metabolites also had potent effects on Ach-release: one such metabolite (PGE_2?) potently inhibited Ach-release, while a TxA_2-like metabolite potently potentiated this release. The TxA_2-receptor-mediated mechanism did not involve adrenergic mechanisms.

Ozone-inhalation effectively enhanced excitatory responses to electrical stimulation without altering responses to KCl or Cch. This enhancement of excitatory neurotransmission was found to be secondary to alteration of prejunctional control mediated by cyclo-oxygenase metabolites. The data were consistent with a mechanism involving both the loss of prejunctional inhibition (presumably imposed by PGE_2) and increased prejunctional excitation imposed by TxA_2.

From these findings, new foci in airway physiological research and new approaches to the treatment of asthma-related bronchoconstriction were discussed.

REFERENCES

Adelstein, R.S., M.A. Conti, and D.R. Hathaway. Phosphorylation of smooth muscle myosin light chain kinase by the catalytic subunit of adenosine 3':5'-monophosphate-dependent protein kinase. *J Biol Chem* 253:8347-8350, 1978.

Adelstein, R.S., and C.B. Klee. Purification and characterization of smooth muscle myosin light chain kinase. *J Biol Chem* 256:7501-7509, 1981.

Agrawal, D.K.. Alpha-adrenoceptors in vascular smooth muscle and their role in hypertension. Doctoral [Ph.D.] tnesis, McMaster University, 1984.

Ahlquist, R.P. A study of the adrenotropic receptors. *Am J Phys* 153:586-600, 1948.

Aitken, A., T. Bilham, and P. Cohen. Complete primary structure of protein phosphatase inhibitor-1 from rabbit skeletal muscle. *Eur J Biochem* 126:235-246, 1982

Aitken, A., C.B. Klee, and P. Cohen. The structure of the B subunit of calcineurin. *Eur J Biochem* 139:663-671, 1984

Aizawa, H., K.F. Chung, G.D. Leikauf, R. A. Bethel, P. M. O'Byrne, T. Hirose, and J. A. Nadel. Significance of thromboxane generation in ozone-induced airway hyperresponsiveness in dogs. *J Appl Physiol* 59:1918-1923, 1985.

Akaska, K., K. Konno, Y. Ono, S. Mue, C. Abe, M. Kumagai, and I. Ise. Electromyography study of bronchial smooth muscle in bronchial asthma. *Tokohu J Exp Med* 117:55, 1975.

Alexander, H.S., and R. Paddock. Bronchial asthma: response to pilocarpine and epinephrine. *Arch Intern Med* 27:184-191, 1921.

Allen, S.L., D.J. Beech, R.W. Foster, G.P. Morgan, and R.C. Small. Electrophysiological and other aspects of the relaxant action of isoprenaline in guinea pig isolated trachealis, *Br J Pharmacol* 86, 843-854, 14985.

Ayala, L.E., and T. Ahmed. Is there less of a protective muscarinic receptor mechanism in asthma? *Chest* 96:1285-1291, 1989.

Baba, K., C.B. Baron, and R.F. Coburn. Phorbol ester effects on coupling mechanisms during cholinergic contraction of swine tracheal smooth muscle. *J Physiol* 412:23-42, 1989.

Bai, T.R., T. Lam, and F.Y.F. Prasad. Effects of adrenergic agonists and adenosine on cholinergic neurotransmission in human

tracheal smooth muscle. *Pulmonary Pharmacol* 1:193-199, 1989.

Barger G., and H.H. Dale. Chemical structure and sympathomimetic action of amines. *J Physiol* 51:19-59, 1910.

Barnes, P.J. Airway receptors and asthma. *N Eng Regul Allerg Proc* 7:219-227, 1986a.

Barnes, P.J. Asthma as an axon reflex. *Lancet* 1:242-245, 1986b.

Barnes, P.J. The Airways: Neural Control in Health and Disease; in Lung Biology in Health and Disease, vol.33, pp 57-85; New York City; Dekker; 1988.

Barnes, P.J. Cell-surface receptors in airway smooth muscle. in Airway Smooth Muscle in Health and Disease (R.F. Coburn, ed), pp 77-97, chapt. 5; Plenum Press, New York; 1989.

Barnes, P.J.. Muscarinic receptor subtypes: implications for lung disease. *Thorax* 44:161-167, 1989.

Barnes, P., G. Fitzgerald, M. Brown, and C. Dollery. Nocturnal asthma and changes in circulating epinephrine, histamine, and cortisol. *New Eng J Med* 303:263-267, 1980.

Barnes, P.J., P.W. Ind, and C.T. Dollery. Inhaled prazosin in

asthma. *Thorax* 36:378-381, 1981.

Barnes, P.J., P.W. Ind, and M.J. Brown. Plasma histamine and catecholamines in stable asthmatic subjects. *Clin Sci* 62:661-665, 1982.

Barnes, P.J., and N.N. Pride. Dose-response curves to inhaled ß-adrenoceptor agonists in normal and asthmatic subjects. *Br J Clin Pharmacol* 15:677-682, 1983.

Barnes, P.J., J.A. Nadel, J.M. Roberts, and C.B. Basbaum. Muscarinic receptors in lung and trachea: autoradiographic localization using [^{3}H]-quinuclidinyl benzylate. *Eur J Pharmacol* 86:103-106, 1983a.

Barnes, P.J., J.A. Nadel, B-E. Skoogh, and J.M. Roberts. Characterization of beta adrenoceptor subtypes in canine airway smooth muscle by radioligand binding and physiological responses. *J Pharm Exp Ther* 225:456-461, 1983b.

Baron, C.B., M. Cunningham, J.F. Strauss III, and R.F. Coburn. Pharmacomechanical coupling in smooth muscle may involve phosphatidylinositol metabolism. *Proc Nat Acad Sci USA* 81:6899-6903, 1984.

Batink, H.D., D. Davidesko, H.N. Doods, K.J. van Charldorp, A. de

Jong, and P.A. van Zwieten. Subdivision of M_2-receptors into three subtypes. *Br J Pharmacol* 90:81P, 1987.

Baumgold, J., and T. White. Pharmacological differences between muscarinic receptors coupled to phosphoinositide turnover and those coupled to adenylate cyclase inhibition. *Biochem Pharmacol* 38:1605-1616, 1989.

Beck, K.C., J. Vettermann, N.A. Flavahan, and K. Rehder. Muscarinic M1 receptors mediate the increase in pulmonary resistance during vagus nerve stimulation in dogs. *Am Rev Respir Dis* 136:1135-1139, 1987.

Beckett, W.S., W.F. McDonnell, D.H. Horstman, and D.E. House. Role of the parasympathetic nervous system in acute lung response to ozone. *J Appl Physiol* 59:1879-1885, 1985.

Beckett, W.S., C. Black, C. Turner, E.W. Spannhake, and H.A. Menkes. *In vivo* exposure to ozone causes increased *in vitro* responses of small airways. *Am Rev Respir Dis* 137:1236-1238, 1988.

Beinfield, W.H., and J. Seifter. Contraction of dog trachealis muscle in vivo: role of α-adrenergic receptors. *J Appl Physiol* 48:329-336, 1980.

Bilski, A., S. Dorries, J.D. Fitzgerald, R. Jessup, H. Tucker, and

J. Wale. ICI 118,551, a potent B_2 adrenoceptor antagonist. *Br J Pharmacol* 69:292P-293P, 1980.

Bilski, A.J., S.E. Halliday, J.D. Fitzgerald, and J.L. Wale. The pharmacology of a B_2-selective adrenoceptor antagonist (ICI 118,551). *J Cardiovasc Pharmacol* 5:430-437, 1983.

Birdsall, N.J.M., and E.C. Hulme. Multiple muscarinic receptors: further problems in receptor classification. in Trends in Autonomic Pharmacology (S. Kalsner, ed), vol.3, chapter 2, pp17-34; London/Philadelphia, Taylor and Francis; 1985.

Birdsall, N.J.M., C.A.M. Curtis, P. Eveleigh, E.C. Hulme, E.K. Pedder, D. Poyner, and M. Wheatley. Muscarinic receptor subtypes and the selectivity of agonists and antagonists. *Pharmacol* 37 (suppl):22-31, 1988.

Blakeley, A.G.H., A. Mathie, and S.A. Petersen. Interactions between the effects of yohimbine, clonidine and $[Ca]_o$ in the electrical response of the mouse vas deferens. *Br J Pharmacol* 88:807-814, 1986.

Bognar I.T., B. Baumann, F. Dammann, B. Knoll, M. Meincke, S. Pallas, and H. Fuder. M_2 muscarinic receptors on the iris sphincter muscle differ from those on iris noradrenergic nerves. *Eur J Pharmacol* 163:263-274, 1989.

Boucher R.C., P.D. Pare, and J.C. Hogg. Relationship between airway hyperreactivity and hyperpermeability in *Ascaris*-sensitive monkeys. J Allerg Clin Immunol 64:197-201, 1979.

Boushey, H.A., M.J. Holtzmann, J.R. Skeller, and J.A. Nadel. State of the art: Bronchial hyperreactivity. *Am Rev Respir Dis* 121:389-413, 1980.

Brichant, J.F., D.O. Warner, S.J. Gunst, and K. Rehder. M_3 Muscarinic receptor subtype mediates the response of canine tracheal smooth muscle strips to acetylcholine. *Physiol* 32:180, 1989.

Britton, J., J. Ayers, and G.M. Cochrane. Effect of inhaled α-blocker on airflow obstruction in asthma. *J R Soc Med* 74:646-648, 1981.

Brooks, S.M., K. McGowan, I.L. Bernstein, P. Altenau, and J. Peagler. Relationship between numbers of beta adrenergic receptors in lymphocytes and disease severity in asthmatics. *J Allerg Clin Immunol* 63:401-406, 1979.

Bryan, L.J., J.J. Cole, S.R. O'Donnell, and J.C. Wanstall. A study designed to explore the hypothesis that Beta-1 adrenoceptors are "innervated" receptors and beta-2 adrenoceptors are "hormonal"

receptors, *J Pharmacol Exp Ther* 216, 395-400, 1981.

Bülbring, E. and A. Den Hertog. The action of isoprenaline on the smooth muscle of the guinea pig taenia coli. *J Physiol* 304:277-296, 1980.

Byrne, N.G., and W.A. Large. Action of noradrenaline on single smooth muscle cells freshly dispersed from rat anococcygeus muscle. *J Physiol* (Lond.) 389:513-520, 1988.

Carswell, H., and S.R. Nahorski. ß-adrenoceptor heterogeneity in guinea pig airways: comparison of functional and receptor labelling studies. *Br J Pharmacol* 79:965-971, 1983.

Chand, N., Is Airway hyperreactivity in asthma due to histamine H_2-receptor deficiency? *Med Hypoth* 6:1105-1112, 1980.

Choo, L.K., F. Mitchelson, and Y.M. Vong. Interaction of some muscarinic agonists and antagonists at the prejunctional muscarinic receptor in the rabbit ear artery preparation. *Br J Pharmacol* 87:733-740, 1986.

Chung, K.F., T.W. Evans, P.D. Graf, and J.A. Nadel. Modulation of cholinergic neurotransmission in canine airways by thromboxane mimetic U46619. *Eur J Pharmacol* 117:373-375, 1985a.

Chung, K.F., A.B. Becker, S.C. Lazarus, O.L. Frick, J.A. Nadel, and W.M. Gold. Agonist-induced airway hyperresponsiveness and pulmonary inflammation in allergic dogs. *J Appl Physiol* 58:1347-1353, 1985b.

Chung, K.F., H. Aizawa, A.B. Becker, O. Frick, W.M. Gold, and J.A. Nadel. Inhibition of antigen-induced airway hyperresponsiveness by a thromboxane synthetase-inhibitor (OKY-046) in allergic dogs. *Am Rev Respir Dis* 134:258-261, 1986.

Clague, R.U., R.M. Eglen, A.C. Strachan, and R.L. Whiting. Action of agonists and antagonists at muscarinic receptors present in ileum and atria in vitro. *Br J Pharmacol* 86:163-170, 1985.

Coburn, R.F. Electromechanical coupling in canine trachealis muscle: Acetylcholine contractions. *Am J Phys* 236:C177-C184, 1979.

Coburn, R.F. Integration of neural inputs in peripheral airway ganglia. in <u>Airway Smooth Muscle in Health and Disease</u> (R.F. Coburn, ed) pp17-34, chapt. 2, 1989.

Coburn, R.F., and K. Baba. Coupling mechanisms in airway smooth muscle. in <u>Airway Smooth Muscle in Health and Disease</u> (R.F. Coburn, ed) pp 183-197, chapt. 10, 1989.

Cockcroft D.W., R.E. Duffin, J. Dolovich, and F.E. Hargreave.

Allergen-induced increase in non-allergic broncial reactivity. *Clin Allerg* 7:503-513, 1977.

Conti, M.A., and R.S. Adelstein. The relationship between calmodulin binding and phosphorylation of smooth muscle kinase by the catalytic subunit of 3':5' cAMP-dependent protein kinase. *J Biol Chem* 256:3178-3181, 1981.

Dabrowska, R., J.M.F. Sherry, D.K. Aromatorio, and D.J. Hartshorne. Modulator protein as component of myosin light chain kinase from chicken gizzard. *Biochem* 17:253-258, 1978.

Dale, H.H.. On some physiological actions of ergot. *J Physiol* 34:163-206, 1906.

Dale, H.H.. The action of certan esters and ethers of choline, and their relation to muscarine. *J Pharmacol Exp Ther* 6:147-190, 1914.

Dalquen, P. Morphological findings in fatal anaphylactic and anaphylactoid reactions. in Asthma and bronchial hyperreactivity; H. Herzog and A.P. Perruchond, edrs), *Prog Resp Dis* 19:189-193, 1985.

Daniel, E.E. Ultrastructure of airway smooth muscle. in Mechanisms in Asthma: Pharmacology, Physiology, and Management (J. Wood, ed)

pp179-203; 1988.

Daniel, E.E., M. Kannan, C. Davis, and V. Posey-Daniel. Ultrastructural studies on the neuromuscular control of human tracheal and bronchial muscle. *Resp Physiol* 63:109-128, 1986.

Daniel, E.E., C. Davis, and V. Sharma. Effects of endogenous and exogenous prostaglandin in neurotransmission in canine trachea. *Can J Physiol Pharmacol* 65:1433-1441, 1987.

Danser, A.H.J., R. VanDen Ende, R.R. Lorenz, N.A. Flavahan, and P.M. VanHoutte. Prejunctional β_1-adrenoceptors inhibit cholinergic transmission in canine bronchi. *J Appl Physiol* 62:785-790, 1987.

de Jongste, J.C. Airway smooth muscle function: structural, electrophysiological, biochemical, and mechanical aspects. in Human airway smooth muscle, pp 13-22, ch. 1, 1987a.

de Jongste, J.C. Airway smooth muscle and airway hyperresponsiveness: aims of the studies. in Human airway smooth muscle, pp 49-56, ch. 5, 1987b.

de Jongste, J.C., H. Mons, I.L. Bonta, and K.F. Kerrebijn. Cholinergic and non-adrenergic inhibitory nerve-mediated responses of isolated human airways in COPD and asthma. *Am Rev Respir Dis*

135:A181 (abstr.), 1987.

de Lanerolle, P., J.R. Condit Jr., M. Tanenbaum, and R.S. Adelstein. Myosin phosphorylation, agonist concentration, and contraction of tracheal smooth muscle. *Nature* (Lond.) 298:871-872, 1982.

de Lanerolle, P.. Cellular control mechanisms in airway smooth muscle. in <u>Airway Smooth Muscle in Health and Disease</u> (R.F. Coburn, ed), pp 99-125, chapt. 6; Plenum Press, New York; 1989.

de Monchy, J.G.R., H.F. Kauffman, P. Venge, G.H. Koeter, H.M. Jansen, H.J. Sluiter, and K. DeVries. Broncho-alveolar eosinophilia during allergen-induced late asthmatic reactions. *Am Rev Respir Dis* 131:373-376, 1985.

Dhillon, D.S., and I.W. Rodger. Hyperreactivity of guinea pig isolated airway smooth muscle. *Br J Pharmacol* 79:180P (abstr.), 1981.

Dillon, P.F., M.O. Aksoy, S.P. Driska, and R.A. Murphy. Myosin phosphorylation and the cross-bridge cycle in arterial smooth muscle. *Science* 211:495-497, 1981.

Doods, H.N., M.-J. Mathy, D. Davidesko, K.J. van Charldorp, and A. de Jonge. Selectivity of muscarinic antagonists in radioligand and

in vivo experiments for the putative M_1, M_2, and M_3 receptors. *J Pharmacol Exp Ther* 242:257-262, 1987.

Eglen, R.M., and R.L. Whiting. Muscarinic receptor subtypes: a critique of the current classification and proposal for a working nomenclature. *J Aut Pharmacol* 5:323-345, 1986.

Eiser, N.M. Hyperreactivity - Its relationship to histamine receptors. *Eur J Resp Dis* 64(Supp 131):99-114, 1983.

Eling, T.E., R.M. Danilowicz, D.C. Henke, K. Sivarajah, J.R. Yankaskas, and R.C. Boucher. Arachidonic acid metabolism by canine tracheal epithelial cells. Product formation and relationship to chloride secretion. *J Biol Chem* 261:12841-12849, 1986.

Eltze, M.. Assessment of selectivity of muscarinic antagonists for M_1 and M_2 receptors in rabbit isolated vas deferens. *Pharmacol* 37 (Supp 1):40-47, 1988.

Eltze, M., G. Gmelin, J. Wess, C. Strohmann, R. Tacke, E. Mutschler, and G. Lambrecht. Presynaptic muscarinic receptors mediating inhibition of neurogenic contractions in rabbit vas deferens are of the ganglionic M_1-type. *Eur J Pharmacol* 158:233-242, 1988.

Empey, D.W., L.A. Laitinen, L. Jacobs, W.M. Gold, and J.A. Nadel.

Mechanisms of bronchial hyperreactivity in normal subjects after upper respiratory tract infection. *Am Rev Respir Dis* 113:131-139, 1976.

Fabbri, L.M., H. Aizawa, S.E. Alpert, E.H. Walters, P.M. O'Byrne, B.D. Gold, J.A. Nadel, and M.J. Holtzmann. Airway hyperresponsiveness and changes in cell counts in bronchoalveolar lavage after ozone exposure in dogs. *Am Rev Respir Dis* 129:288-291, 1984.

Farley, J.M., and P.R. Miles. Role of depolarization in acetylcholine-induced contraction of dog trachealis muscle. *J Pharmacol Exp Ther* 201:199-205, 1977.

Faulkner, D., A.D. Fryer, and J. Maclagan. Postganglionic muscarinic inhibitory receptors in pulmonary parasympathetic nerves in the guinea pig. *Br J Pharmacol* 88:181-187, 1986.

Felbel, J., B. Trockur, T. Ecker, W. Landgraf, and F. Hofman. Regulation of cytosolic calcium by cAMP and cGMP in freshly isolated smooth muscle cells from bovine trachea. *J Biol Chem* 263:16764-16761, 1988.

Felder, C.C., R.Y. Kantermann, A.L. Ma, and J. Axelrod. A transfected m1 muscarinic acetylcholine receptor stimulates adenylate cyclase via phosphatidylinositol hydrolysis. *J Biol Chem*

256:20356-20362, 1989.

Flavahan, N.A., L.L. Aarhus, T.J. Rimele, and P.M. VanHoutte. Respiratory epithelium inhibits bronchial smooth muscle tone. *J Appl Physiol* 58:834-838, 1985.

Fryer, A.D., and J. Maclagan. Muscarinic inhibitory receptors in pulmonary parasympathetic nerves in the guinea pig. *Br J Pharmacol* 83.973-978, 1984.

Fujiwara, T., T. Itoh, and H. Kuriyama. Regional differences in the mechanical properties of rabbit airway smooth muscle. *Br J Pharmacol* 94:389-396, 1988.

Fujiwara, T., K. Sumimoto, T. Itoh, H. Suzuki, and H. Kuriyama. Relaxing actions of procaterol, a β_2-adrenoceptor stimulant, on smooth muscle cells of the dog trachea. *Br J Pharmacol* 93:199-209, 1988.

Fuller, R.W., C.M.S. Dixon, C.T. Dollery, and P.J. Barnes. Prostaglandin D_2 potentiates airway responsiveness to histamine and methacholine. *Am Rev Respir Dis* 133:252-254, 1986.

Gabella, G. Structure of airway smooth muscle and its innervation. in <u>Airway Smooth Muscle in Health and Disease</u> (R.F. Coburn, ed), pp 1-16, chapt. 1; Plenum Press, New York; 1989.

Gabella, G. Innervation of airway smooth muscle: fine structure. *Ann Rev Physiol* 49:583-594, 1987.

Galant, S.P., L. Durisetti, S. Underwood, and P.A. Insel. Beta-adrenergic receptors of polymorphonuclear particulates in bronchial asthma. *J Clin Invest* 65:577-585, 1980.

Gao, Y., and P.M. VanHoutte. Removal of the epithelium potentiates acetylcholine in depolarizing canine bronchial smooth muscle. *J Appl Physiol* 65:2400-2405, 1988.

Gardner, A.L., S.A. Darroch, L.K. Choo, and F. Mitchelson. The effect of some selective agonists and antagonists on peripheral muscarinic receptors. *Trends Pharmacol Sc* (supp):40-43, 1988.

Garfield, R.E.. Cell-to-cell communication in smooth muscle. in Calcium and contractility (A.K. Grover and E.E. Daniel, ed), pp 143-173, chapt 6; Humana Press Inc., New Jersey; 1985.

Gerthoffer, W.T.. Calcium dependence of myosin phosphorylation and airway smooth muscle contraction and relaxation. *Am J Physiol* 250:C597-604, 1986.

Giachetti, A., R. Micheletti, and E. Montagna. Cardioselective profile of AF-DX 116, a muscarine M_2 receptor antagonist. *Life*

Sci 38:1663-1672, 1986.

Gleich, G.J., D.A. Loegering, and J.E. Maldonado. Identification of a major basic protein in guinea pig eosinophil granules. *J Exp Med* 137:1459-1471, 1973.

Goldie, R.G., J.W. Paterson, and J.L. Wale. A comparative study of ß-adrenoceptors in human and porcine lung parenchymal strips, *Br J Pharmacol* 76:523-526, 1982.

Goldie, R.G., D. Spina, P.J. Henry, K.M. Lulich, and J.W. Paterson. *In vitro* responsiveness of human asthmatic bronchus to carbachol, histamine, ß-adrenoceptor agonists and theophylline. *Br J Clin Pharmacol* 22:669-676, 1986.

Goldstein, I.M., C.L. Malmstein, H. Kindahl, H.B. Kaplan, O. Rådmark, B. Samuelsson, and G. Weissmann. Thromboxane generation by human peripheral blood polymorphonuclear leukocytes. *J Exp Med* 148:787-792, 1978.

Goyal, R.K. Identification, localization, and classification of muscarinic receptor subtypes in the gut. *Life Sci* 43:2209-2220, 1989.

Grandel, K.E., E. Schwartz, D. Greene, M.L. Wardlow, R.N. Pinckard, and R.S. Farr. A platelet-activating factor (PAF) extracted from

acidified human plasma. *Fed Proc* 42:1026 (abstr.), 1983.

Grandordy, B.M., F.M. Cuss, A.S. Sampson, J.B. Palmer, and P.J. Barnes. Phosphatidylinositol response to cholinergic agonists in airway smooth muscle: relationship to contraction and muscarinic receptor occupancy. *J Pharmacol Exp Ther* 238:273-279, 1986.

Grandordy, B.M., K. Rhoden, and P.J. Barnes. Histamine H1 receptors in human lung: correlation of receptor binding and function. *Am Rev Respir Dis* 135:A274, 1987.

Grandordy, B.M., K.J. Rhoden, N. Frossard, and P.J. Barnes. Tachykinin induced phosphoinositide breakdown in airway smooth muscle and epithelium: relationship to contraction. *Mol Pharmacol* 33:515-519, 1988.

Grieco, M.H., and R.N. Pierson Jr. Mechanism of bronchoconstriction due to beta-adrenergic blockade. *J Allergy Clin Immunol* 48:143-152, 1971.

Grundström, N., R.G.G. Andersson, and J.E.S. Wikberg. Inhibition of the excitatory non-adrenergic, non-cholinergic neurotransmission in the guinea pig tracheo-bronchial tree mediated by α_2-adrenoceptors. *Acta Pharm Tox* 54:8-14, 1984.

Grundström, N., and R.G.G. Andersson. Inhibition of the

cholinergic neurotransmission in human airways via prejunctional alpha-2-adrenoceptors. *Acta Physiol Scand* 125:513-517, 1985.

Gunst, S.J., and S. Bandyopadhyay. Contractile force and intracellular Ca^{2+} during relaxation in canine tracheal smooth muscle. *Am J Physiol* 257:C355-C364, 1989.

Gunst, S.J., J.Q. Stropp, and N.A. Flavahan. Muscarinic receptor reserve and ß-adrenergic sensitivity in tracheal smooth muscle. *J Appl Physiol* 67:1294-1298, 1989.

Haeberle, J.R., D.R. Hathaway, and A.A. DiPauli-Roach. Dephosphorylation of myosin by the cationic subunit of a type-2 phosphatase produces relaxation of a chemically skinned uterine smooth muscle. *J Biol Chem* 260:9965-9968, 1985.

Hammer, R., C.P. Berrie, N.J.M. Birdsall, A.S.V. Burgen, and E.C. Hulme. Pirenzepine distinguishes between different subclasses of muscarinic receptors. *Nature* 283:90-92, 1980.

Hammer, R., and A. Giacetti. Muscarinic receptor subtypes: M1 and M2 biochemical and functional characterization. *Life Sci* 31:2991-2998, 1982.

Harden, T.K. Muscarinic cholinergic receptor-mediated regulation of cyclic AMP metabolism. in The Muscarinic Receptors. (J.H.

Brown, ed.), pp. 221-257, chapt.6; Humana Press, New Jersey, 1989.

Hargreave, F.E., P.M. O'Byrne, and E.H. Ramsdale. Mediators, airway responsiveness, and asthma. *J Allerg Clin Immunol* 76:272-276, 1985.

Harvey, J.W., and A.E. Tattersfield. Airway response to salbutamol. Effect of regular salbutamol inhalations in normal, atopic, and asthmatic subjects. *Thorax* 37:280-287, 1982.

Hashimoto, T., M. Hirata, and Y. Itoh. A role for inositol 1,4,5-trisphosphate in the initiation of agonist-induced contraction of dog tracheal smooth muscle. *Br J Pharmacol* 86:191-199, 1985.

Hay, D.W.P., S.G. Farmer, D. Raeburn, V.A. Robinson, W.W. Fleming, and J.S. Fedan. Airway epithelium modulates the reactivity of guinea pig respiratory smooth muscle. *Eur J Pharmacol* 129:11-18, 1986.

Hay, D.W.P., R.M. Muccitelli, D.L. Horstemeyer, K.A. Wilson, and D. Raeburn. Demonstration of the release of an epithelium-derived inhibitory factor from a novel preparation of guinea pig trachea. *Eur J Pharmacol* 136:247-250, 1987.

Hay, D.W.P. Postulated mechanisms underlying airway hyperreactivity: relationship to supersensitivity. in <u>Airway Smooth</u>

Muscle in Health and Disease (R.F. Coburn, ed), pp 199-235, chapt. 11; Plenum Press, New York; 1989.

Heaslip, R.J., F.R. Giesa, T.J. Rimele, and D. Grimes. Demonstration of the β_2-adrenoceptor intrinsic efficacy of AY-28,925 using the $PgF_{2\alpha}$-contracted guinea-pig trachea, *Eur J Pharmacol* 137:179-184, 1987.

Hedberg, A., F. Kempf Jr., M.E. Josephson, and P.B. Molinoff. Coexistence of beta-1 and beta-2 adrenergic receptors on the human heart: effects of treatment with receptor antagonists or calcium entry blockers. *J Pharmacol Exp Ther* 234:561-568, 1985.

Hedberg, A., F. Kempf Jr., M.E. Josephson, and P.B. Molinoff. Coexistence of beta-1 and beta-2 adrenergic receptors in the human heart: treatment with receptor antagonists or calcium entry blockers. *J Pharmacol Exp Ther* 234:561-568, 1985.

Henry, P.J., and R.G. Goldie. β_1-adrenoceptors mediate smooth muscle relaxation in mouse isolated trachea. *Br J Pharmacol* 99:131-135, 1990.

Himpens, B., and A.P. Somlyo. Free calcium and force transients during depolarization and pharmacomechanical coupling in guinea pig smooth muscle. *J Physiol* (Lond.) 395:507-530.

Hogg, J.C.. Bronchial mucosal permeability and its relationship to airways hyperreactivity. *J Allerg Clin Immunol* 67:421-425, 1981.

Holgate, S.T., C.C. Hardy, C. Robinson, R.M. Agius, and P.H. Howarth. The mast cell as a primary effector cell in the pathogenesis of asthma. *J Allerg Clin Immunol* 77:274-282, 1986a.

Holgate, S.T., P.H. Howarth, R. Beasley, R. Agius, and M.K. Church. Cellular and biochemical events in the pathogenesis of asthma. *Eur J Resp Dis* 68(S144):37-76, 1986b.

Holtzmann, M.J., L.M. Fabbri, P.M. O'Byrne, B.D. Gold, H. Aizawa, E.H. Walters, S.E. Alpert, and J.A. Nadel. Importance of airway inflammation for hyperresponsiveness induced by ozone. *Am Rev Respir Dis* 127:686-690, 1983a.

Holtzmann, M.J., L.M. Fabbri, B.-E. Skoogh, P.M. O'Byrne, E.H. Walters, H. Aizawa, and J.A. Nadel. Time course of airway hyperresponsiveness induced by ozone in dogs. *J Appl Physiol* 65:1232-1236, 1983b.

Ind, P.W., C.M.S. Dixon, R.W. Fuller, and P.J. Barnes. Anticholinergic blockade of propranolol-induced bronchoconstriction. *Thorax* 41:718, 1986.

Inoue, T., Y. Ito, and K. Takeda. Prostaglandin-induced inhibition of aceylcholine release from neuronal elements of dog tracheal tissue. *J Physiol* (Lond.) 349:553-570, 1984.

Inoue, T., and Y. Ito. Characteristics of neuro-effector transmission in the smooth muscle layer of dog bronchiole and modifications by autacoids. *J Physiol* (Lond.) 370:551-565, 1986.

Ishikawa, S. and N. Sperelakis. Cyclic nucleotide regulation of neurotransmission in guinea pig mesenteric artery. *J Cardiovasc Pharmacol* 13:836-845, 1989.

Ito, Y.. Pre- and post-junctional actions of procaterol, a β_2-adrenoceptor stimulant, on dog tracheal tissue. *Br J Pharmacol* 95:268-274, 1988.

Ito, Y., and K. Tajima. Actions of indomethacin and prostaglandins on neuro-effector transmission in the dog trachea. *J Physiol* (Lond.) 319:379-392, 1981a.

Ito, Y., and K. Tajima. Spontaneous activity in the trachea of dogs treated with indomethacin: an experimental model for aspirin-related asthma. *Br J Pharmacol* 73:563-571, 1981b.

Ito, Y., and K. Tajima. Dual effects of catecholamines in pre-and postjunctional membranes in the dog trachea. *Br J Pharmacol*

75:433-440, 1982.

Ito, Y., and T. Yoshitomi. Autoregulation of acetylcholine release from vagus nerve terminals through activation of muscarinic receptors in the dog trachea. *Br J Pharmacol* 93:636-646, 1988.

Jacobs, R., and M. Kaliner. Current concepts of the pathophysiology of allergic asthma. in <u>Airway Smooth Muscle in Health and Disease</u> (R.F. Coburn, ed), pp 277-299, chapt. 14; Plenum Press, New York; 1989.

Jacoby, D.B. Airway epithelial metabolism and airway smooth muscle hyperresponsiveness. in <u>Airway Smooth Muscle in Health and Disease</u> (R.F. Coburn, ed), pp 237-266, chapt. 12; Plenum Press, New York; 1989.

Jones, G.L., C.G. Lane, and P.M. O'Byrne. The effect of thromboxane receptor antagonists on ozone-induced airway hyperresponsiveness in dogs. *J App Physiol*, in press, 1990.

Kalsner, S. Limitations of presynaptic theory: no support for feedback control of autonomic effectors. *Fed Proc* 43:1358-1364, 1984.

Kannan, M.S., and E.E. Daniel. Structural and functional study of control of canine tracheal smooth muscle. *Am J Physiol* 238:C27-

C33, 1980.

Karlin, J.M. The use of antihistamines in asthma. *Ann Allerg* 30:342-345, 1972.

Katsuki, S., and F. Murad. Regulation of adenosine cyclic 3',5'-monophosphate and guanosine cyclic 3',5'-monophosphate levels and contractility in bovine tracheal smooth muscle. *Mol Pharmacol* 13:330-341, 1976.

Keen, M., and S.R. Nahorski. Muscarinic acetylcholine receptors linked to the inhibition of adenylate cyclase activity in membranes from the rat striatum and myocardium can be distinguished on the basis of agonist efficacy. *Mol Pharmacol* 34:769-778, 1988.

Kenakin, T.P. Drug antagonism. in Pharmacologic analysis of drug-receptor interactions, pp 205-244, ch. 9, Raven Press, New York, 1987.

Klee, C.B., and M.H. Krinks. Purification of cyclic 3',5'-nucleotide phosphodiesterase inhibitory protein by affinity chromatography on activator protein coupled to sepharose. *Biochem* 17:120-126, 1978.

Kikkawa, U., Y. Takai, Y. Tanaka, R. Miyake, and Y. Nishizuka. Protein kinase C as a possible receptor protein of tumor-promoting

phorbol esters. *J Biol Chem* 258:11442-11445, 1983.

Kotlikoff, M.. Potassium currents in isolated airway smooth muscle cells. *Am Rev Respir Dis* 135:A273, 1987.

Kotlikoff, M.. Ion channels in airway smooth muscle. in <u>Airway Smooth Muscle in Health and Disease</u> (R.F. Coburn, ed), pp 169-182, chapt. 9; Plenum Press, New York; 1989.

Kumar, M.A.. The basis of beta adrenergic bronchodilation. *J Pharmacol Exp Ther* 206:528-534, 1978.

Ladinsky, H., E. Giraldo, E. Monferini, G.B. Schiavi, M.A. Viganò, L. DeConti, R. Micheletti, and R. Hammer. Muscarinic receptor heterogeneity in smooth muscle: binding and functional studies with AF-DX 116. *Trends Pharmacol Sc* 9(Supp):44-48, 1988.

Laitinen, L.A., M. Heino, A. Laitinen, T. Kava, and T. Hoahtela. Damage of the airway epithelium and bronchial reactivity in patients with asthma. *Am Rev Resp Dis* 131:599-606, 1985.

Lambrecht, G., U. Moser, M. Wagner, J. Wess, G. Gmelin, K. Raseiner, C. Strohmann, R. Tacke, and E. Mutschler. Pharmacological and electrophysiological evidence for muscarinic M_1 and M_2 receptor heterogeneity *Trends Pharmacol Sc* 9(Supp):82, 1988.

Lambrecht, G., R. Feifel, M. Wagner-Röder, C. Strohmann, H. Zilch, R. Tacke, M. Waelbroeck, J. Christophe, H. Boddeke, and E. Mutschler. Affinity profiles of hexahydrosiladifenidol analogues at muscarinic receptor subtypes. *Eur J Pharmacol* 168:71-80, 1989.

Lands, A.M., A. Arnold, J.P. McAuliff, F.P. Luduena, and T.G. Brown. Differentiation of receptor systems activated by sympathomimetic amines. *Nature* (Lond.) 214:597-598, 1967.

Lands, A.M., F.P. Luduena, and H.J. Buzzo. Differentiation of receptors responsive to isoproterenol. *Life Sci* 6:2241-2249, 1967.

Lazarus, S.C., C.B. Basbaum, P.J. Barnes, and W.M. Gold. cAMP immunohistochemistry provides evidence for functional VIP receptors in trachea. *Am J Physiol* 251:C115-C119, 1986.

Leff, A.R.. Endogenous regulation of bronchomotor tone. *Am Rev Resp Dis* 1378, 1198-1216, 1988.

Leff, A.R., and N.M. Munoz. Interrelationship between alpha- and beta-adrenergic agonists and histamine in canine airways. *J Allerg Clin Immunol* 68:300-309, 1981.

Leff, A.R., J. Tallet, N.M. Munoz, and N. Shoulberg. Physiological antagonism caused by adrenergic stimulation of canine tracheal

muscle. *J Appl Physiol* 60:216-224, 1986.

Leikauf G.D., I.F. Ukei, J.A. Nadel, and J.H. Widdicombe. Release of cyclooxygenase products from cultured epithelium derived from human and dog trachea. *Fed Proc* 44:1920, 1985.

Littner, M.R., and F.D. Lott. Cyclooxygenase products from endogenous arachidonic acid in isolated dog and rabbit lungs. *Am Rev Respir Dis* 138:383-388, 1988.

Lulich, K.M., R.G. Goldie, and J.W. Paterson. Beta-adrenoceptor function in asthmatic bronchial smooth muscle. *Gen Pharmacol* 19:307-311, 1988.

Lundberg, J.M., and A. Saria. Polypeptide-containing neurons in airway smooth muscle. *Ann Rev Physiol* 49:557-572, 1987.

MacDermot, J., C.R. Kelsey, K.A. Waddell, R. Richmond, R.K. Knight, P.J. Cole, C.T. Dollery, D.N. Landon, and I.A. Blair. Synthesis of leukotriene B_4 and prostanoids by human alveolar macrophages: analysis by gas chromatography/mass spectrometry. *Prostaglandins* 27:163-179, 1984.

Madison, J.M., C.A. Jones, R.M. Sankary, and J.K. Brown. Differential effects of prostaglandin E_2 on contraction of airway smooth muscle. *J Appl Physiol* 66:1397-1407, 1989.

Mak, J.C.W., and P.J. Barnes. Muscarinic receptor subtypes in human and guinea pig lung. *Eur J Pharmacol* 164:223-230, 1989.

Makino S., J.J. Oulette, C.E. Reed, and C.W. Fishel. Correlation between increased bronchial responses to acetylcholine and diminished metabolic and eosinopenic responses to epinephrine in asthma. *J Allerg* 46:178-189, 1970.

Mapp, C.E., P. Chitano, N. DeMarzo, P. DiBlasi, M. Saetta, A. DiStefano, V.M. Bosco, L. Allegra, and L.M. Fabbri. Responses to acetylcholine and myosin content of isolated canine airways. *J Appl Physiol* 67:1331-1335, 1989.

Martin, J.B., and B. Collier. Acetylcholine release from canine isolated airway is not modulated by NE. *J Appl Physiol* 61:1025-1030, 1986.

Mathé, A.A., E.Y. Tong, and P.W. Tisher. Norepinephrine release from the lung by sympathetic nerve stimulation inhibition by vagus and methacholine. *Life Sci* 20:1425-1430, 1977.

McCaig, D.J., and J.F. Souhadra. Alteration of electrophysiological properties of airway smooth muscle from sensitized guinea pigs. *Resp Physiol* 41:49-60, 1980.

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McCann, J.D., and M.J. Welsh. Calcium-activated potassium channels in canine airway smooth muscle. *J Physiol* (Lond.) 372:113-126, 1986.

McKinney, M., D. Anderson, and L. Vella-Rountree. Different agonist-receptor active conformations for rat brain M1 and M2 muscarinic receptors that are separately coupled to two biochemical effector systems. *Mol Pharmacol* 35:39-47, 1989.

Micheletti, R. Muscarinic inhibition of intestinal motility. *Trends Pharmacol Sc* 9(Supp):87, 1988.

Middletown, E. Jr. Antiasthmatic drug therapy and Ca^{++} ions: review of pathogenesis and role of calcium. *J Pharmaceut Sci* 69:243-251, 1980.

Miller, J.R., P.J. Silver, J.T. Stull. The role of myosin light chain kinase phosphorylation in beta-adrenergic relaxation of tracheal smooth muscle. *Mol Pharmacol* 24:235-242, 1983.

Minnette, P.A., and P.J. Barnes. Prejunctional inhibitory muscarinic receptors on cholinergic nerves in human and guinea pig airways. *J Appl Physiol* 64:2532-2537, 1988.

Moore, D.H., P.W. Abel, G.A. Rinard, A.M. Puckett, and J.B. Walter. Regional differences in relaxation of electrical field stimulation

in canine airway smooth muscle by verapamil, isoproterenol, and prostaglandin E_2. *J Pharmacol Exp Ther* 238:76-82, 1986.

Morgan, J.P. and K.G. Morgan. Alteration of cytoplasmic ionized calcium levels in smooth muscle by vasodilators in the ferret. *J Physiol* 357:539-551, 1984.

Murlas, C., J.A. Nadel, and J.M. Roberts. The muscarinic receptors of airway smooth muscle: their characterization in vitro. *J Appl Physiol* 52:1084-1091, 1982.

Mutschler, E., G. Gmelin, U. Moser, and J. Zaagsma. Structure-activity relationships of drugs acting selectively at different muscarinic receptors. in Pharmacology, (M.J. Rand and C. Raper, eds.) pp:67-75; International Congress Series No. 750; Elsevier Sc. Publishers, B.V., Amsterdam; 1987.

Nishikawa, M., H. Hidaka, and R.S. Adelstein. Phosphorylation of smooth muscle heavy meromyosin by calcium-activated phospholipid-dependent protein kinase. *J Biol Chem* 258:14069-14072, 1983.

Nishikawa, M., P. de Lanerolle, T.M. Lincoln, and R.S. Adelstein. Phosphorylation of mammalian myosin light chain kinases by the catalytic subunit of cyclic AMP-dependent protein kinase and by cyclic GMP-dependent protein kinase. *J Biol Chem* 259:8429-8436, 1984.

Noiman, E.S.. Phosphorylation of smooth muscle myosin light chains by cAMP-dependent protein kinase. *J Biol Chem* 255:11067-11070, 1980.

Noronha-Blob, L., B. Canning, D. Costello, and W.J. Kinnier. Selective agents for muscarinic receptors linked to phosphoinositide breakdown. *Eur J Pharmacol* 154:161-167, 1988.

Obara, K., and P. de Lanerolle. Isoproterenol attenuates myosin phosphorylation and contraction of tracheal muscle. *J Appl Physiol* 66:2017-2022, 1989.

O'Byrne, P.M., E.H. Walters, B.D. Gold, H.A. Aizawa, L.M. Fabbri, S.E. Alpert, J.A. Nadel, and M.J. Holtzmann. Neutrophil depletion inhibits airway hyperresponsiveness induced by ozone exposure. *Am Rev Respir Dis* 130:214-219, 1984a.

O'Byrne, P.M., E.H. Walters, H. Aizawa, L.M. Fabbri, M.J. Holtzmann, and J.A. Nadel. Indomethacin inhibits the airway hyperresponsiveness but not the neutrophil influx induced by ozone in dogs. *Am Rev Respir Dis* 130:220-224, 1984b.

O'Byrne, P.M., H. Aizawa, R.A. Bethel, K.F. Chung, J.A. Nadel, and M.J. Holtzmann. Prostaglandin $F_{2\alpha}$ increases responsiveness of pulmonary airways in dogs. *Prostaglandins* 28:537-543, 1984c.

O'Byrne, P.M., G.D. Leikauf, H. Aizawa, R.A. Bethel, I.F. Ukei, M.J. Holtzmann, and J.A. Nadel. Leukotriene B_4 induces airway hyperresponsiveness in dogs. *J Appl Physiol* 59:1941-1946, 1985.

O'Donnell, S.R. ß-adrenoceptor classification in the respiratory tract. in <u>Trends In Autonomic Pharmacology</u> (S. Kalsner, ed), vol.3, chapt. 1, pp 1-16; Taylor and Francis, London/Philadelphia; 1985.

Page, C.P., S. Sanjar, D. Alvemini, and J. Morley. Inflammatory mediators of asthma. *Eur J Resp Dis* 68(Supp 144):163-189, 1986.

Perpina, M., J. Cortijo, J. Esplugues, and E.J. Morcillo. Different ability of verapamil to inhibit agonist-induced contraction of lung parenchymal strips from control and sensitized guinea pigs. *Resp* 50:174-184, 1986.

Peters, S.P., D.W. MacGlashon Jr., E.S. Schulman, R.P. Schleimer, and L.M. Lichtenstein. The production of arachidonic acid (AA) metabolites by purified human lung mast cells (HMC). *Fed Proc* 42:1375, 1983.

Pride, N.B.. The assessment of airflow obstruction: role of measurements of airways resistance and of tests of forced expiration. *Br J Dis Chest* 65:135-169, 1971.

Rapoport, R.M.   Cyclic guanosine monophosphate inhibition of contraction may be mediated through inhibition of phosphoinositide hydrolysis in rat aorta.   *Circ Res* 58:407-410, 1986.

Rasmussen, H., Y. Takuwa, and S. Park.   Protein kinase C in the regulation of smooth muscle contraction.   *FASEB J* 1:177-185, 1987.

Rhoden, K.J., L.A. Meldrum, and P.J. Barnes.   Inhibition of cholinergic neurotransmission in human airways by β_2-adrenoceptors.   *J Appl Physiol* 65:700-705, 1988.

Richards, I.S., C. Murlas, J.M. Ousterhout, and N. Sperelakis.   8-bromo-cyclic GMP abolishes TEA-induced slow action potentials in canine trachealis muscle.   *Eur J Pharmacol* 128:299-302, 1986.

Rinard, G.A., A.R. Rubinfeld, L.L. Brunton, and S.E. Mayer.   Depressed cyclic AMP levels in airway smooth muscle from asthmatic dogs.   *Proc Nat Acad Sci* 76:1472-1476, 1979.

Rinard, G.A., A. Jensen, and A.M. Puckett.   Hydrocortisone and isoproterenol effects on trachealis cAMP and relaxation.   *J Appl Physiol* 55:1609-1613, 1983.

Roffel, A.F., W.G. in't Hout, R.A. de Zeeuw, and J. Zaagsma.   The M_2-selective antagonist AF-DX 116 shows high affinity for muscarine

receptors in bovine tracheal membranes. *Nauyn-Schmiedeberg's Arch Pharmacol* 335:593-595, 1987.

Roffel, A.F., C.R.S. Elzinga, R.G.M. van Amsterdam, R.A. de Zeeuw, and J. Zaagsma. Muscarinic M_2-receptors in bovine tracheal smooth muscle: discrepancies between binding and function. *Eur J Pharmacol* 153:73-82, 1988.

Rugg, E.L., D.B. Barnett, and S.R. Nahorski. Co-existence of $beta_1$ and $beta_2$ adrenoceptors in mammalian lung: evidence from direct binding studies. *Mol Pharmacol* 14:996-1005, 1978.

Russell, J.A.. Responses of isolated canine airways to electric stimulation and acetylcholine. *J Appl Physiol* 45:690-698, 1978.

Russell, J.A.. Noradrenergic inhibitory innervation of canine airways. *J Appl Physiol* 48:16-22, 1980.

Russel, J.A.. Differential inhibitory effect of isoproterenol on contraction of canine airways. *J Appl Physiol* 57:801-807, 1984.

Russell J.A., and S. Bartlett. Adrenergic neurotransmission in airways: inhibition by acetylcholine. *J Appl Physiol* 51:376-383, 1981.

Said, S.I.. Polypeptide-containing neurons and their function in

airway smooth muscle. in <u>Airway Smooth Muscle in Health and Disease</u> (R.F. Coburn, ed), pp 55-76, chapt. 4; Plenum Press, New York; 1989.

Sankary, R.M., C.A. Jones, J.M. Madison, and J.K. Brown. Muscarinic cholinergic inhibition of cyclic AMP accumulation in airway smooth muscle. *Am Rev Resp Dis* 138:145-150, 1988.

Scheid, C.R.. ß-adrenergic relaxation of smooth muscle: differences between cells and tissues. *Am J Physiol* 253:C369-C374, 1987.

Scheid, C.R., T.W. Honeyman, and F.S. Fay. Mechanism of ß-adrenergic relaxation of smooth muscle. *Nature* 277:32-36, 1979.

Scheid, C.R., and F.S. Fay. ß-adrenergic effects on transmembrane ^{45}Ca fluxes in isolated smooth muscle cells. *Am J Physiol* 246:C431-C438, 1984.

Schönfeld, W., M. Koller, J. Knoller, W. Konig, W. Muller, and H. VanDerHardtt. Leukotrienes and their metabolism in plasma and bronchial fluids. *J Allerg Clin Immunol* 75:126 (abstr.), 1985.

Schramm, C.M., and M.M. Grunstein. Mechanisms of protein kinase C regulation of airway contractility. *J Appl Physiol* 66:1935-1941, 1989.

Scott, T., and K.K. McMahon. Antagonists and agonists interactions with the muscarinic receptor of the rat large airways. *J Pharmacol Exp Ther* 247:136-142, 1988.

Sellers, J.R., M.D. Pato, and R.S. Adelstein. Reversible phosphorylation of smooth muscle myosin, heavy meromyosin, and platelet myosin. *J Biol Chem* 256:13137-13142, 1981.

Seltzer, J., B.G. Bigby, M. Stulbarg, M.J. Holtzmann, J.A. Nadel, I.F. Ukei, G.D. Leikauf, E.J. Goetzl, and H.A. Boushey. O_3-induced changes in bronchial reactivity to methacholine and airway inflammation in humans. *J Appl Physiol* 60:1321-1326, 1986.

Serio, R., and E.E. Daniel. Thromboxane effects on canine trachealis neuromuscular function. *J Appl Physiol* 64:1979-1988, 1988.

Serio, R., and E.E. Daniel. Eicosanoids and peripheral neurotransmission. *Prostaglandins, leukotrienes, and essential fatty acids: Reviews* 38:237-246, 1989.

Shore, S., B. Collier, and J.G. Martin. Effects of endogenous prostaglandins on acetylcholine release from dog trachealis muscle. *J Appl Physiol* 62:1837-1844, 1987.

Sibille Y, and H.Y. Reynolds. Macrophages and polymorphonuclear neutrophils in lung defense and injury. *Am Rev Resp Dis* 141:471-501, 1990.

Silva, D.G., and G. Ross. Ultrastructural and fluorescence histochemical studies on the innervation of the tracheo bronchial muscle of normal cats and cats treated with 6-hydroxydopamine. *J Ultrastruc Res* 47:310-328, 1974.

Silver, P.J., and J.T. Stull. Phosphorylation of myosin light chain and phosphorylase in tracheal smooth muscle in response to KCl and carbachol. *Mol Pharmacol* 25:267-274, 1985.

Simonsson, B.G., F.M. Jacobs, and J.A. Nadel. Role of the autonomic nervous system and the cough reflex in the increased responsiveness of airways in patients with obstructive airway disease. *J Clin Invest* 46:1812-1818, 1967.

Small R.C., and R.W. Foster. Electrophysiologic behavior of normal and sensitized airway smooth muscle. *Am Rev Resp Dis* 136:S7-S11, 1987.

Sobieszek, A. Vertebrate smooth muscle myosin. Enzymatic and structural properties. in The biochemistry of smooth muscle (N.L. Stephens, ed); pp 413-443; University Park Press, Baltimore; 1977.

Souhadra, M., and J.F. Souhadra. Reassessment of electrophysiological and contractile characteristics of sensitized airway smooth muscle. *Resp Physiol* 46:17-27, 1981.

Stewart, A.A., T.S. Ingebritsen, and P. Cohen. The protein phosphatase involved in cellular regulation: purification and properties of a Ca^{++}/calmoduiin-dependent protein phosphatase (2B) from rabbit skeletal muscle. *Eur J ⌐⌐chem* 132:289-295, 1983.

Stuart-Smith, K., and P.M. VanHoutte. Heterogeneity in the effects of epithelium removal in the canine bronchial tree. *J Appl Physicl* 63:2510-2515, 1987.

Suzuki, H., K. Morita, H. Kuriyama. Innervation and properties of the smooth muscle of the dog trachea. *Jap J Physiol* 26:303-320, 1976.

Svedmyr, N.. Airway hyperreactivity. Cholinergic and adrenergic receptors. *Eur J Resp Dis* 64(Supp 131):71-98, 1983.

Svedmyr, N.. Szentivanyi's hypothesis of asthma. *Eur J Resp Dis* 65 (Supp 36):59-65, 1984.

Szentivanyi, A.. The beta-adrenergic theory of the atopic abnormality in bronchial asthma. *J Allerg* 42:203-232, 1968.

Szentivanyi, A.. The conformational flexibility of adrenoceptors and the constitutional basis of atopy. *Triangle* 18:109-115, 1970.

Szentivanyi, A.. The radioligand binding approach in the study of lymphocytic adrenoceptors and the constitutional basis of atopy. *J Allerg Clin Immunol* 5:5-11, 1980.

Takuwa, Y., N. Takuwa, and H. Rasmussen. Measurement of cytosolic free Ca^{2+} concentration in bovine tracheal smooth muscle using aqueorin. *Am J Physiol* 253:C817-827, 1987.

Tamaoki, J., K. Sekizawa, M.L. Osborne, I.F. Ukei, P.D. Graf, and J.A. Nadel. Platelet aggregation increases cholinergic neurotransmission in canine airway. *J Appl Physiol* 62:2246-2251, 1987a.

Tamaoki, J., K. Sekizawa, P.D. Graf, and J.A. Nadel. Cholinergic neuromodulation by prostaglandin D_2 in canine airway smooth muscle. *J Appl Physiol* 63:1396-1400, 1987b.

Tamaoki, J., K. Sekizawa, I.F. Ukei, P.D. Graf, J.A. Nadel, and T.D. Bigby. Effects of macrophage stimulation on parasympathetic airway contraction in dogs. *Eur J Pharmacol* 138:421-425, 1987c.

Tate, R.M., H.G. Morris, W.R. Schroeder, and J.E. Repine. Oxygen metabolites stimulate thromboxane production and vasoconstriction

in isolated saline-perfused rabbit lungs. *J Clin Invest* 74:608-613, 1984.

Torphy, T.J., W.B. Freese, G.A. Rinard, L.L. Brunton, and S.E. Mayer. Cyclic nucleotide-dependent protein kinases in airway smooth muscle. *J Biol Chem* 257:11609-11616, 1982.

Torphy, T.J., G.A. Rinard, M.G. Rietow, and S.E. Mayer. Functional antagonism in canine tracheal smooth muscle: inhibition by methacholine of the mechanical and biochemical responses to isoproterenol, *J Pharmacol Exp Ther* 227:694-699, 1983.

Torphy, T.J., C. Zheng, S.M. Peterson, R.R. Fiscus, G.A. Rinard, and S.E. Mayer. Inhibitory effects of methacholine in drug-induced relaxation, cyclic AMP-accumulation, and cyclic AMP-dependent protein kinase action in canine tracheal smooth muscle. *J Pharmacol Exp Ther* 233:409-417, 1985.

Tripp, C.S., K.M. Leahy, and P. Needleham. Thromboxane synthase is preferentially conserved in activated mouse peritoneal macrophages. *J Clin Invest* 76:898-901, 1985.

Tschirhart, E., and Y. Landry. Airway epithelium releases a relaxant factor: demonstration with substance P. *Eur J Pharmacol* 132:103-104, 1986.

Twort, C.H.C., and C. vanBreeman. Human airway smooth muscle in tissue culture: control of the intracellular calcium store. *Am Rev Resp Dis* 137:A10 (abstr.), 1988.

Vermeire, P.A., and P.M. VanHoutte. Inhibitory effects of catecholamines in isolated canine bronchial smooth muscle. *J Appl Physiol* 46:787-791, 1979.

Waelbroeck, M., M. Gillard, P. Robberecht, and J. Christophe. Muscarinic receptor heterogeneity in rat central nervous system. 1. Binding of four selective antagonists to three muscarinic receptor subclasses: a comparison with M2 cardiac muscarinic receptors of the C type. *Mol Pharmacol* 32:91-99, 1987.

Walsh, M.P., A. Persechini, S. Hinkins, and D.J. Hartshorne. Is smooth muscle myosin a substrate for cAMP-dependent protein kinase? *FEBS Lettr* 126:107-110, 1981.

Walters, E.H., P.M. O'Byrne, L.M. Fabbri, P.D. Graf, M.J. Holtzmann, and J.A. Nadel. Control of neurotransmission by prostaglandins in canine trachealis smooth muscle. *J Appl Physiol* 57:129-134, 1984.

Walters, E.H., P.M. O'Byrne, P.D. Graf, L.M. Fabbri, and J.A. Nadel. The responsiveness of airway smooth muscle in vitro from dogs with airway hyper-responsiveness in vivo. *Clin Sci* 71:605-

611, 1986.

Wanner, A., W.M. Abraham, J.S. Douglas, J.M. Drazen, H.B. Richerson, and J.S. Ram. Models of airway hyperresponsiveness. *Am Rev Resp Dis* 141:253-257, 1990.

Weiss, E.B., and S.G. Viswanath. Calcium hypersensitivity in airway smooth muscle: isometric tension responses following anaphylaxis. *Resp* 38:266-272, 1979.

Widdicombe, J.G.. Nervous receptors in the tracheo-bronchial tree. in Airway Smooth Muscle in Health and Disease (R.F. Coburn, ed), pp 35-53, chapt. 3; Plenum Press, New York; 1989.

Yabuuchi, Y.. The ß-adrenoceptor stimulant properties of OPC-2009 in guinea pig isolated tracheal, right atrial and left atrial preparations, *Br J Pharmacol* 61:513-521, 1977.

Zakrzewski, J.T., N.C. Barnes, P.J. Piper, and J.F. Costello. Sputum leukocytes and prostanoids: possible synergistic mediators in cystic fibrosis, bronchiectasis, or chronic bronchitis. *Prostaglandins* 28:641 (abstr.), 1984.

Table 1 Effects of muscarinic agonists on membrane potential and on magnitude of EJPs.

agent	concentration (M)	depolarization from control membrane potential (mV)	EJP magnitude (% control)	
			single pulse[*]	multiple pulse[*]
	10^{-7}	1.6±1.8	69.1±14.3	66.5±14.7
McN (6)[§]	10^{-6}	2.4±1.8	7.7±3.6	31.6±11.6
	10^{-5}	12.7±1.8	0	1.3±0.9
	10^{-7}	-(0.9±0.6)	216.6±43.8	159.3±19.6
Bch (5)	10^{-6}; 3-5 min	3.0±1.6	559.4±97.3	192.3±1.1
	10^{-6}; ≈15 min	16.5±1.5	76.9±5.4	53.8±2.2
	10^{-9}	5.2±1.6	96.1±18.4	90.7±18.8
Oxo (6)	10^{-8}	20.8±1.4	(-)[◆]	(-)[◆]

* EJPs elicited by single or multiple (5 pulses at 1 pps) pulse FS

§ value of n

◆ in tissues treated with 10^{-8}M Oxo, FS elicited no response or led to enhancement of oscillations present before stimulation.

Table 2 Mean negative $logIC_{50}$ of agonists against FS-contractions[*] in the presence of various non-selective ß-antagonists.

Norepinephrine
    control 6.03 ± 0.16 (8)[§]
    $+10^{-7}$M timolol 5.17 ± 0.09 (3)

Iso
    control 7.72 ± 0.14 (4)
    $+10^{-8}$M propranolol 7.06 ± 0.31 (3)
    $+10^{-7}$M propranolol 6.63 ± 0.19 (3)

[*] field stimulation used was 10 pulses at 1 pps

[§] parenthesized numbers indicate the value of n

Table 3 Mean negative $logIC_{50}$ for NE against contractions elicited by FS[*] in the presence of ICI 89,406 and/or ICI 118,551[§].

[antagonist]	10^{-7} ICI 89,406 alone	10^{-7} ICI 89,406 (plus 10^{-8} M ICI 118,551)	10^{-7} ICI 118,551 alone	10^{-7} ICI 118,551 (plus 10^{-8} M ICI 89,406)
	NE-IC_{50} in the presence of:			
zero	6.03±0.16 (8)	6.03±0.16 (8)	6.03±0.16 (8)	6.03±0.16 (8)
10^{-8}	ND[*]	5.49±0.16 (3)	ND	5.49±0.16 (3)
10^{-7}	5.29±0.05 (1)	4.95±0.15 (3)	ND	5.32±0.11 (3)
$3x10^{-7}$	5.08±0.10 (6)	ND	5.42±0.11 (4)	ND
$3x10^{-6}$	4.43±0.14 (5)	ND	5.01±0.14 (4)	ND
10^{-5}	ND	ND	4.59±0.12 (4)	ND
$3x10^{-5}$	4.17±0.12 (5)	ND	ND	ND

[*] field stimulation used was 10 pulses at 1 pps.

[§] data from which these IC_{50}s were determined are presented in figure 19; numbers in parentheses indicate value of n.

[*] ND = not done

Table 4 Mean negative $\log IC_{50}$ for Sal against FS-contractions[♦] in the presence of ICI 89,406 or ICI 118,551[§].

[antagonist]	Sal-IC_{50} in the presence of:	
	ICI 89,406	ICI 118,551
zero	7.40±0.13 (6)	7.40±0.13 (6)
10^{-9}	ND[*]	7.12±0.14 (3)
10^{-8}	ND	6.22±0.09 (4)
10^{-7}	ND	5.43±0.12 (4)
10^{-6}	6.84±0.27 (3)	ND
3×10^{-6}	6.21±0.32 (3)	ND
3×10^{-5}	5.50±0.08 (4)	ND

[♦] field stimulation used was 10 pulses at 1 pps.

[§] data from which these IC_{50}s were determined are presented in figure 20; numbers in parentheses indicate value of n.

[*] ND = not done

Table 5 Schild plot slopes (m)[*] and extrapolated pA_2 values[*] for ICI 89,406 and ICI 118,551.

antagonist agonist	ICI 89,406	ICI 118,551
NE	m= 0.50±0.05 pA_2= 8.40	m= 0.62±0.07 pA_2= 7.21
Sal	m= 1.01±0.17[§] pA_2= 6.45	m= 1.06±0.09[§] pA_2= 8.95

[*] obtained from linear regression of data summarized in figure 21.

[§] slope not significantly different from 1.0 (P<0.001).

Table 6 Effects of ozone-inhalation on S_2/S_1 ratios[§] at various times after beginning the studies.

time (min)	CTL	OZ
10	1.15±0.05 (4)	1.18±0.06 (5) [-]
120	0.46±0.12 (4)	0.87±0.09 (5) [*]
210	0.29±0.14 (4)	0.70±0.06 (5) [*]
300	0.30±0.15 (4)	0.67±0.11 (5) [*]

§ see text for explanations of S_2/S_1 ratio
- not significantly different from CTL
* significantly different from CTL (P<0.01)
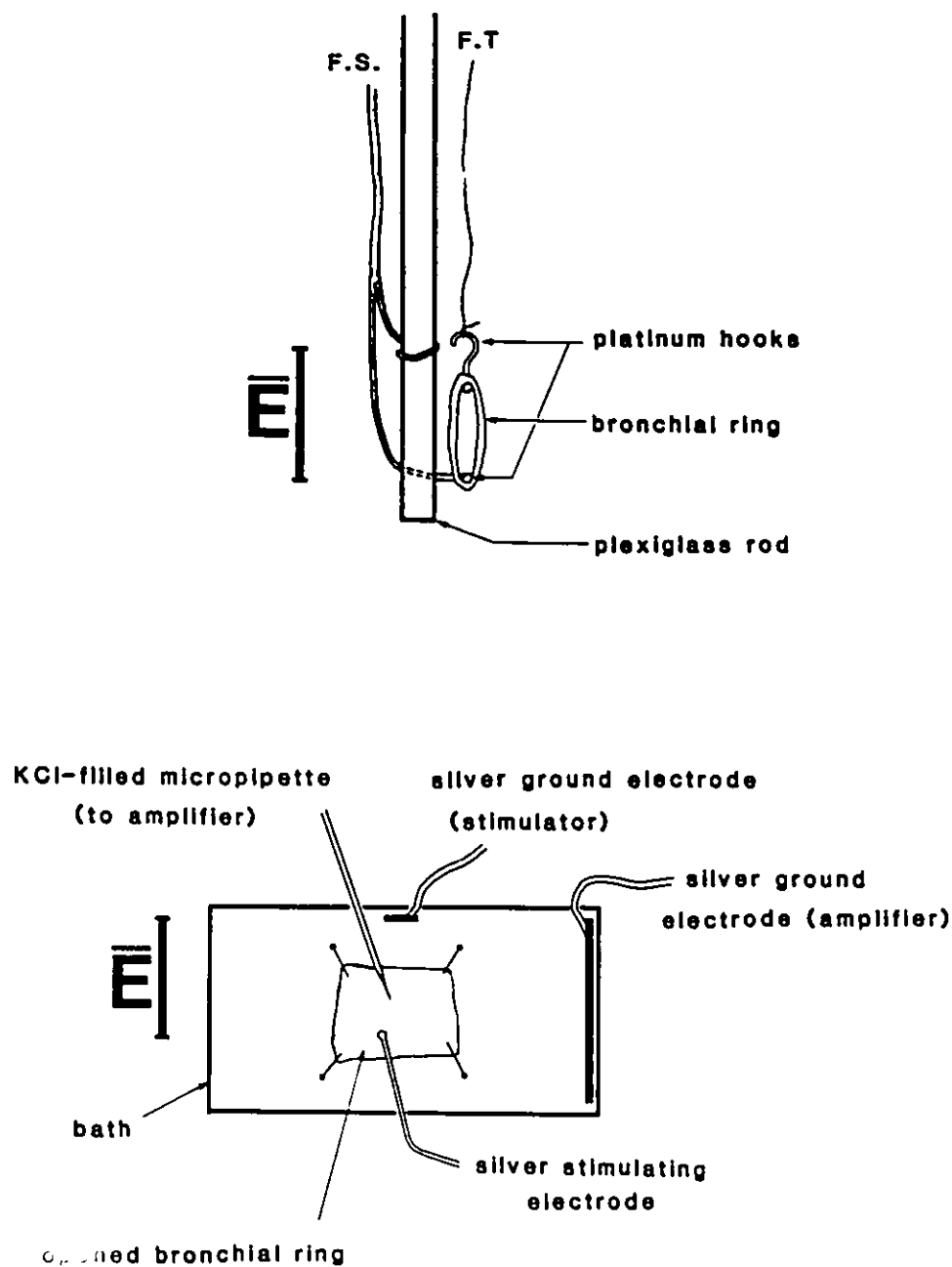
Figure 1 Design of apparati (drawn to scale) used to field stimulate bronchial smooth muscle and measure the electrical or mechanical responses. (A) Apparatus used in the muscle bath studies; the entire apparatus was immersed in a 3 ml organ bath containing warmed, bubbled Krebs buffer as described in the text. F.S. = field stimulation source; F.T. = force transducer. (B) Apparatus used in the electrophysiological studies. With respect to both apparati, the axis of the smooth muscle bundles lay in the same direction as the electrical field ($\bar{E}$) induced by the F.S..
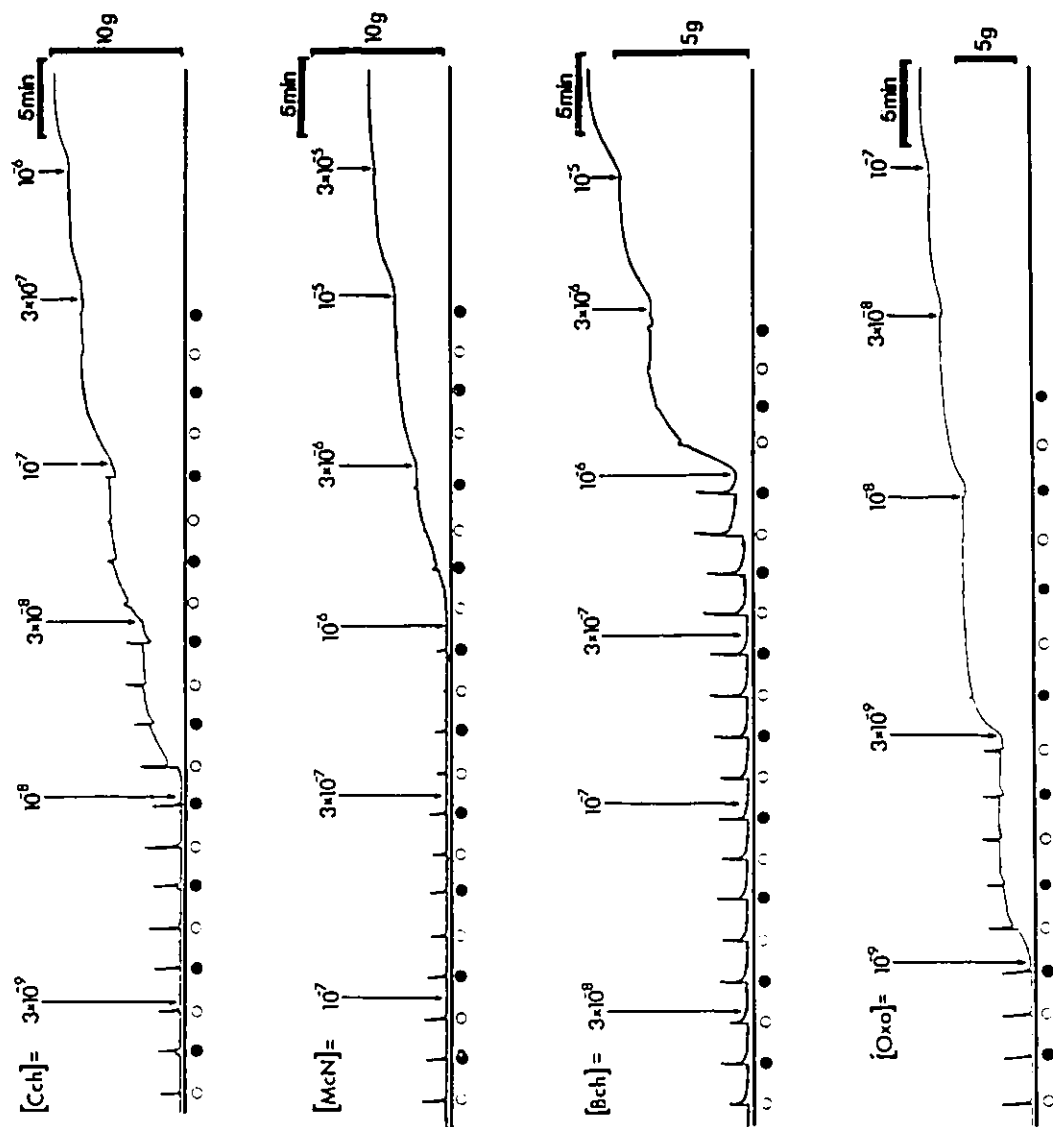
Figure 2 Tonic contractions and modulation of FS-contractions produced by cholinergic agonists. Tissues were electrically stimulated every five minutes (10 pulses at 1 [o] or 10 pps [•]), during which the concentrations of the agonists were progressively increased (approximately every 20-30 minutes). Agonists used were Cch ($3 \times 10^{-9} - 10^{-6}$M), McN ($10^{-7} - 3 \times 10^{-5}$M), Bch ($3 \times 10^{-8} - 10^{-5}$M), and Oxo ($10^{-9} - 10^{-7}$M).
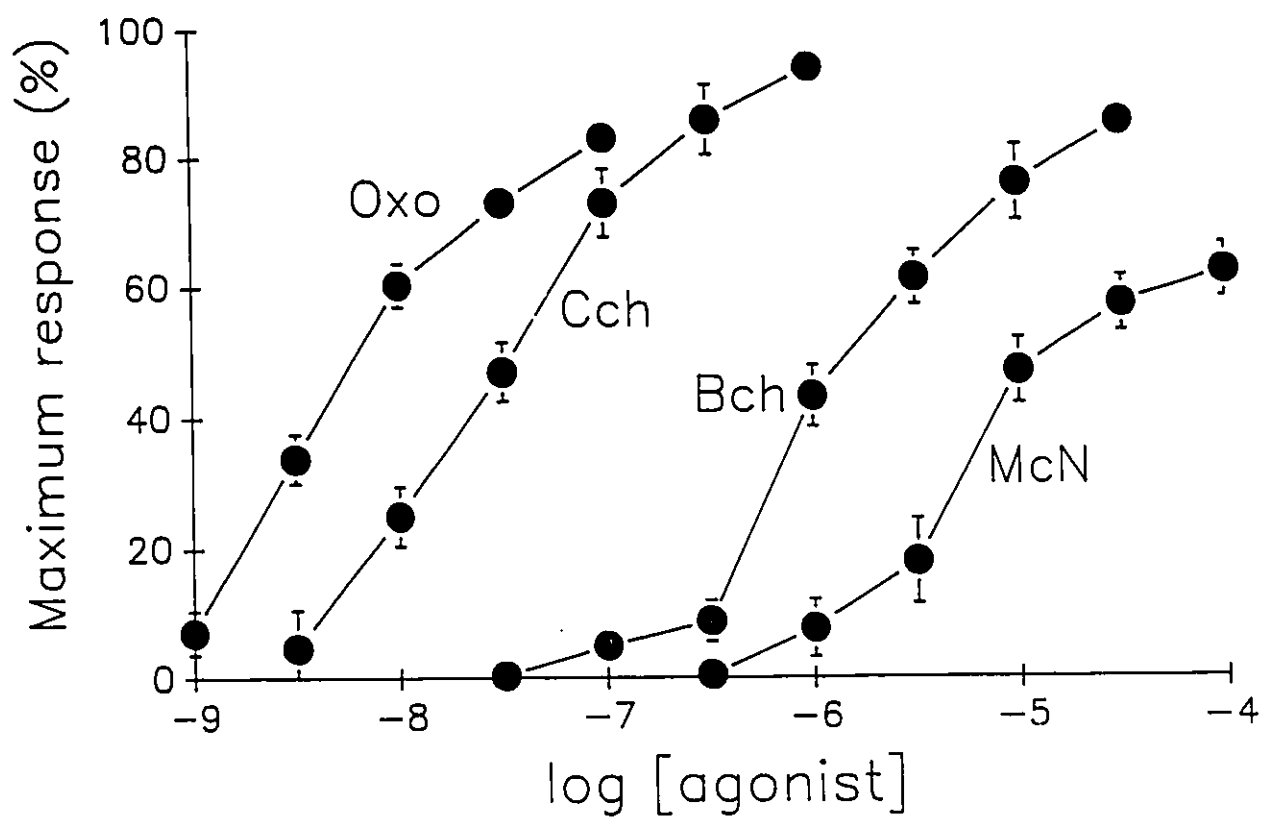
Figure 3 Concentration-response curves showing tonic contractions produced by the cholinergic agonists Cch (n=5), McN (n=6), Oxo (n=6), and Bch (n=6).
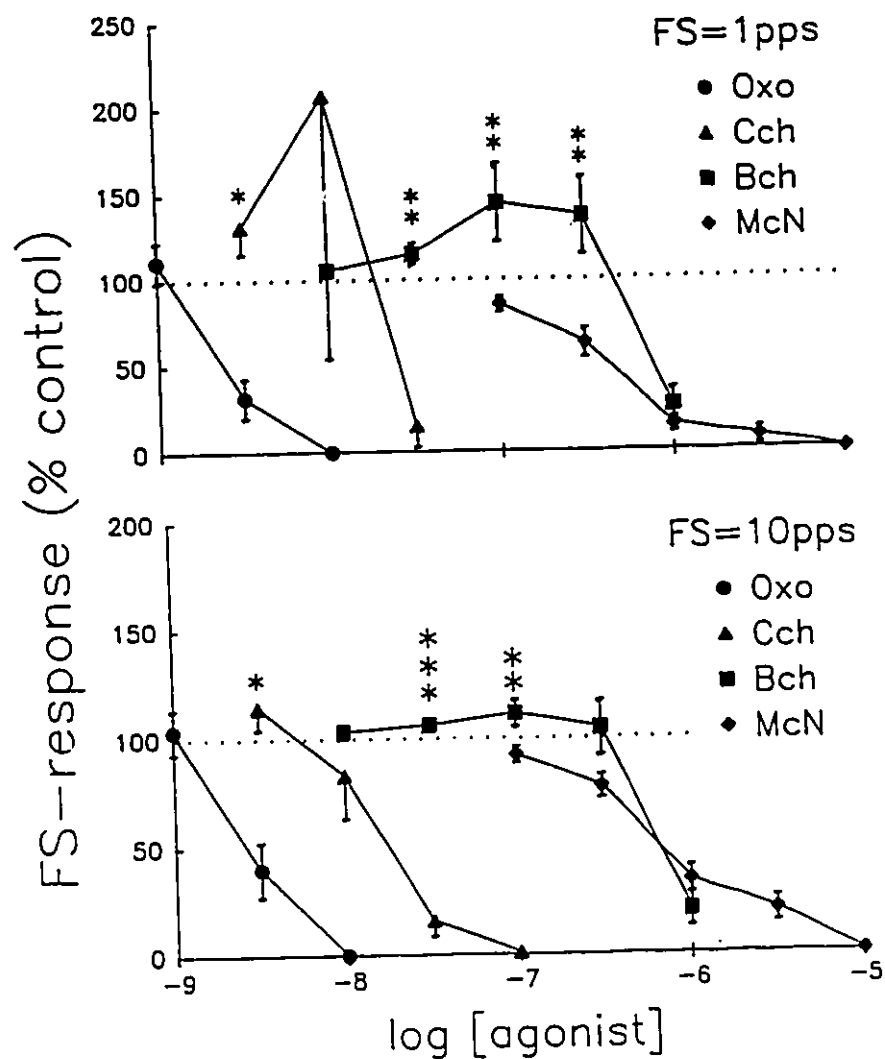
Figure 4 Concentration-response curves showing effects on FS-elicited twitch contractions of Cch (n=5), McN (n=6), Oxo (n=6), and Bch (n=6). Field stimulation used was ten pulses at 1 pps (above) or at 10 pps (below). Asterisks indicate mean responses were significantly greater than control (p<0.05).
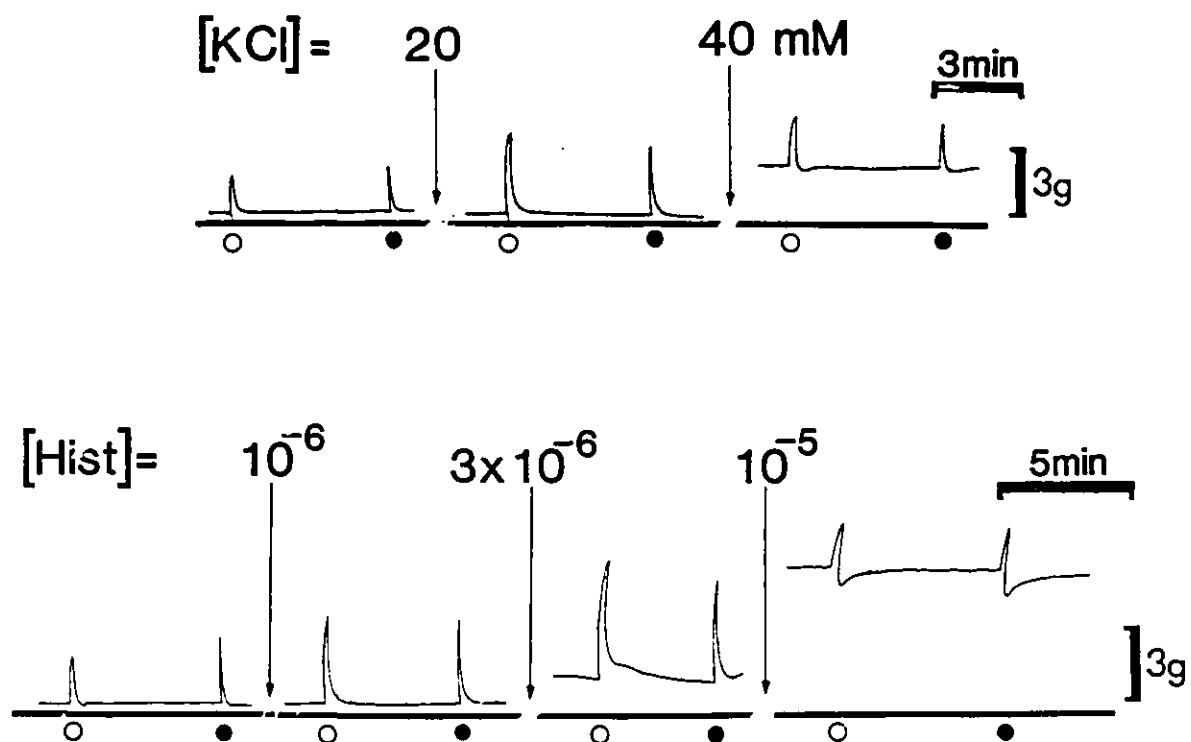
Figure 5 Sample tracings showing the effects of KCl (20 and 40 mM) or of histamine (10^{-6}-10^{-5}M) on FS-induced contractions. Tissues were electrically stimulated every 5 minutes with 10 pulses at 1 pps (o) or 10 pps (•).
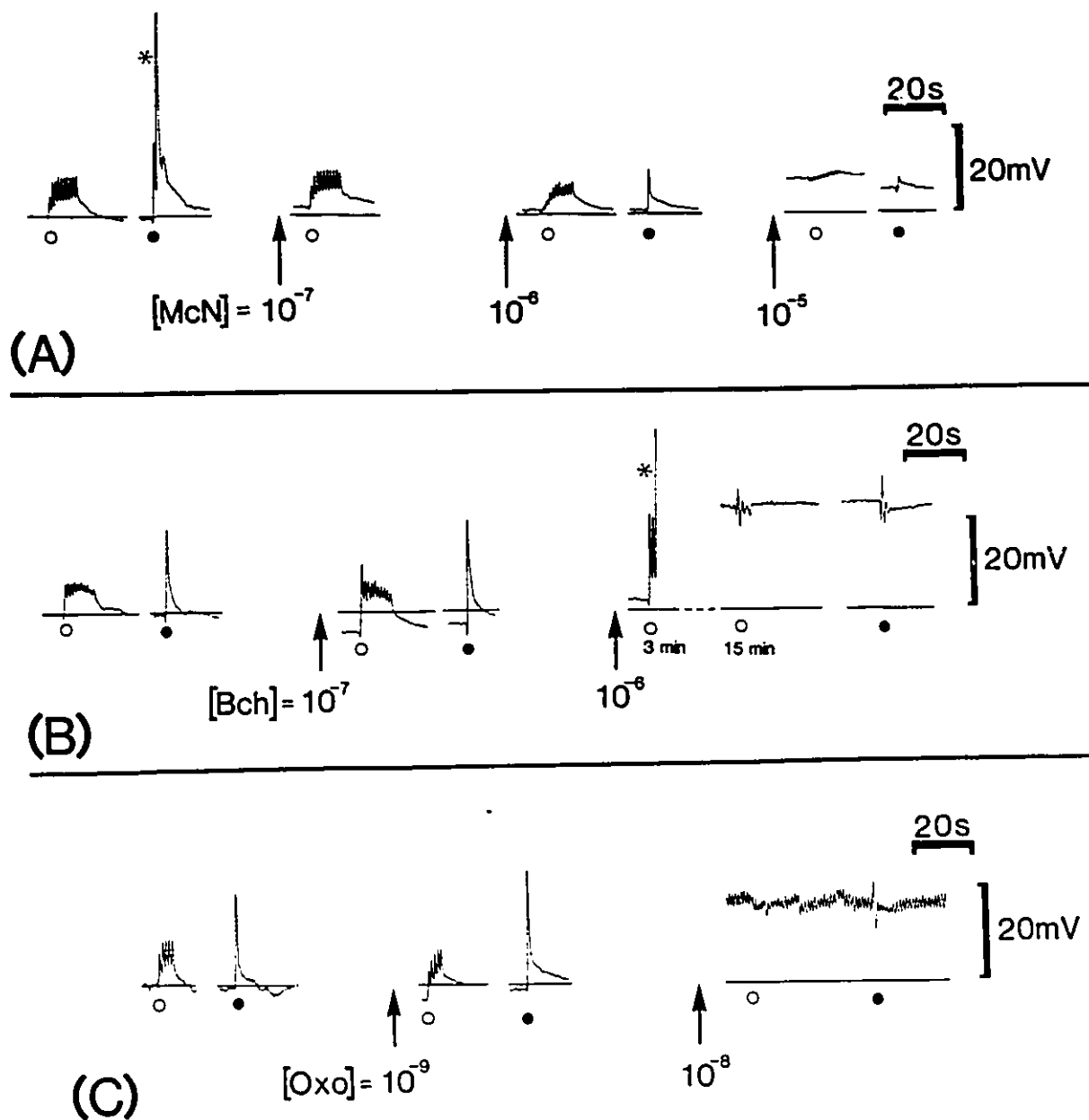
Figure 6 Tracings showing the effects of (A) McN, (B) Bch, and (C) Oxo on electrical responses elicted by FS (1 [o] or 10 [•] pps). Resting membrane potential (V_m) is indicated by the horizontal line. FS-induced contractions often led to ejection of the microelectrode from a cell or its movement into another cell (indicated by *); recordings made from any individual tissue were therefore derived from responses in a number of different cells of the tissue. Thus, some changes in V_m apparent in these figures may not be representative of the population mean; table 1 lists the mean effect of an agonist on V_m.
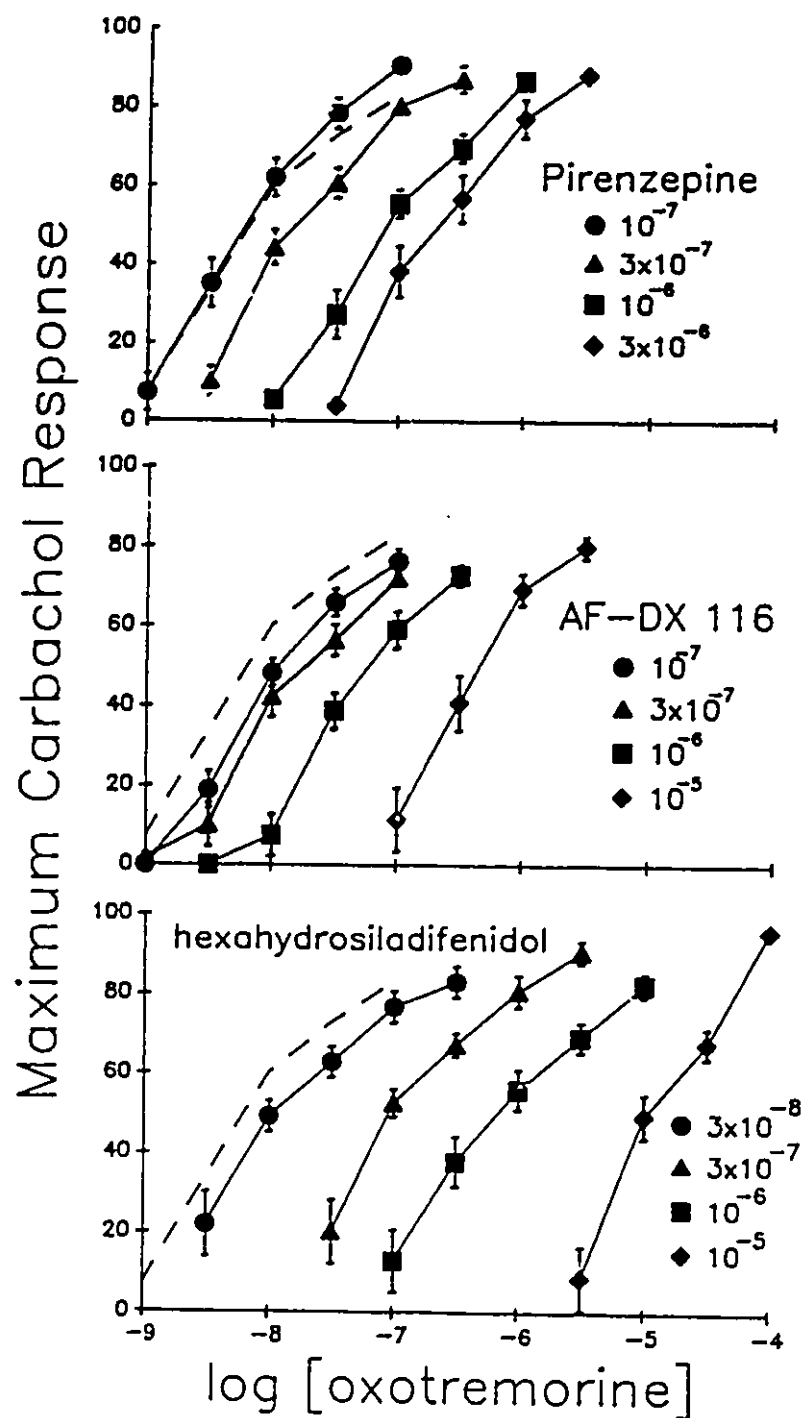
Figure 7 Effects of selective antagonists on Oxo-induced contractions. Antagonists used were Pir (top; 10^{-7}-$3x10^{-6}$M), AF-DX 116 (middle; 10^{-7}-10^{-5}M), and Hexa (bottom; $3x10^{-8}$-10^{-5}M); n=5 in all cases. The control concentration-response curve, shown in figure 3, is also given (dotted line).
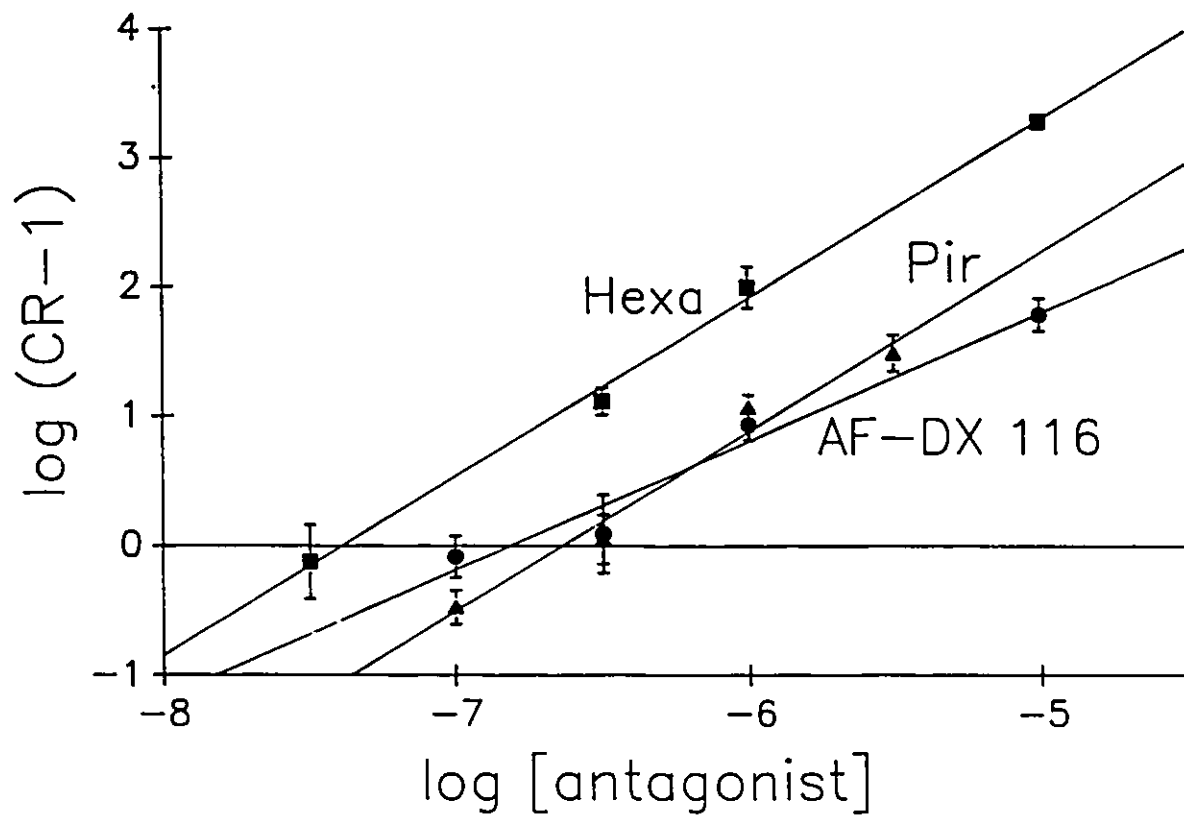
Figure 8 Schild plot analysis of effects of Pir, AF-DX 116 and Hexa on Oxo-induced tonic contractions (see figure 5). Linear regression of the data gave the lines of best fit shown.
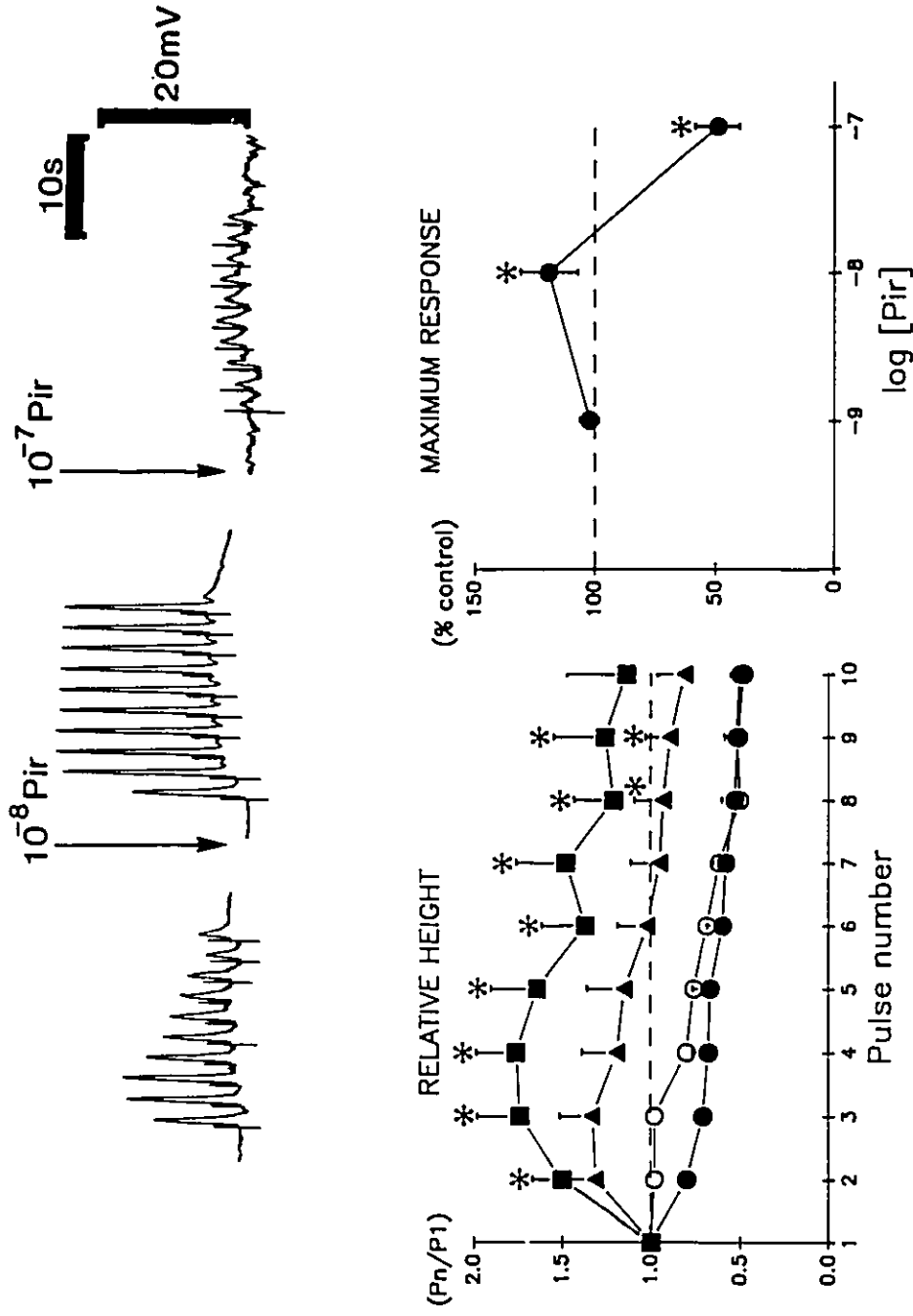
Figure 9   Effects of Pir on FS-elicited EJPs. EJPs were elicited by FS (10 pulses at 0.5 pps, delivered every 5 minutes) in the presence of increasing concentrations of Pir (increased every 25 minutes or longer; n=7). (A) Sample tracing.   (B) Effect of Pir on the size of each of the ten EJPs (expressed as a fraction of the first EJP).   (C) Effect of Pir on the overall maximum response (expressed as a % of the control response). Concentrations of Pir used were 10^{-9} [•], 10^{-8} [▲], and 10^{-7}M [■]; control responses are indicated by [○]; asterisks indicate the corresponding response was significantly different from control (p<0.05).
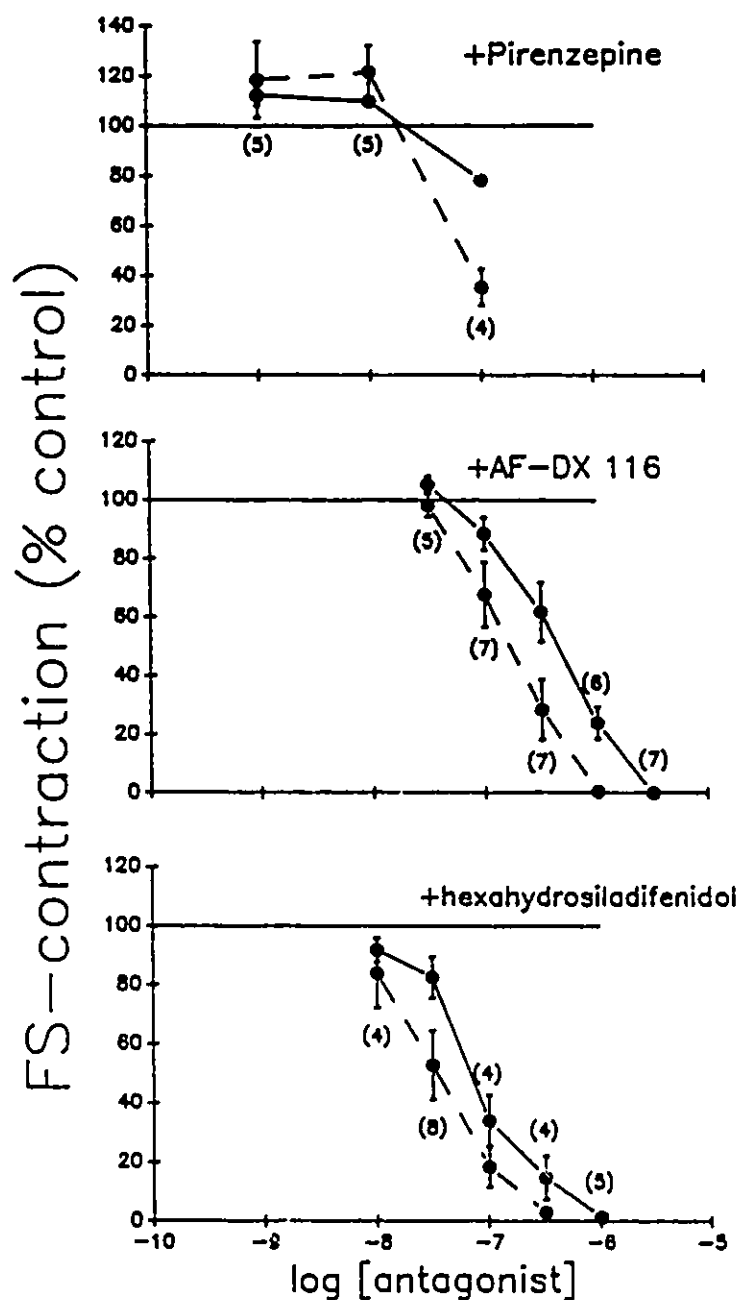
Figure 10 Effects of selective antagonists on FS-induced twitch contractions. Antagonists used were Pir (top), AF-DX 116 (middle), and Hexa (bottom). Numbers in parentheses indicate n for each concentration of antagonist used. Field stimulation used was 10 pulses at 1 pps (dashed line) or at 10 pps (solid line). 100% control is also indicated in the figure in order to facilitate comparison.
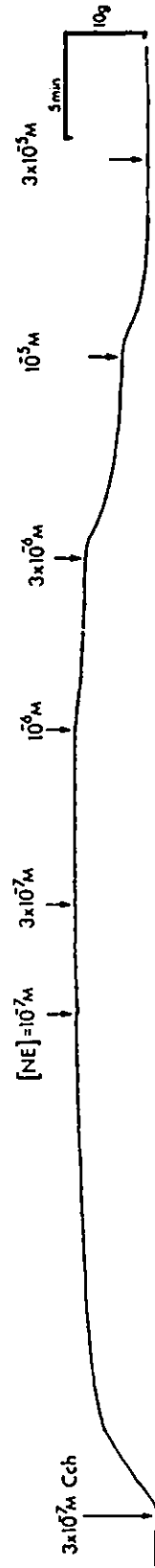
Figure 11. Inhibitory effect of NE (10^{-7} to 3×10^{-5}M) on tonic contraction induced by 3×10^{-7}M Cch.
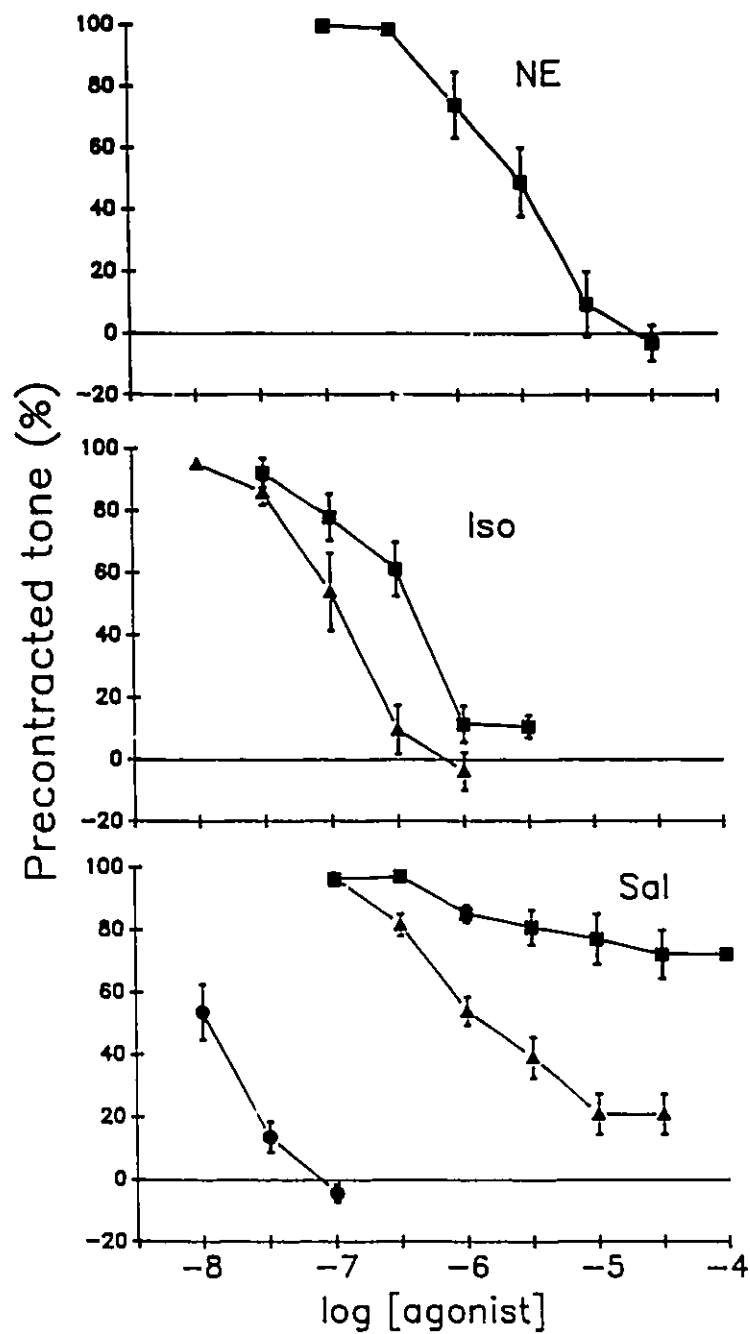
Figure 12. Concentration-relaxation curves for NE (top), Iso (middle), and Sal (bottom). Tissues were precontracted using 2×10^{-8} [•], 10^{-7} [▲], or 3×10^{-7}M [■] Cch. Tissues to which NE was added had been pretreated with 10^{-7}M phentolamine.
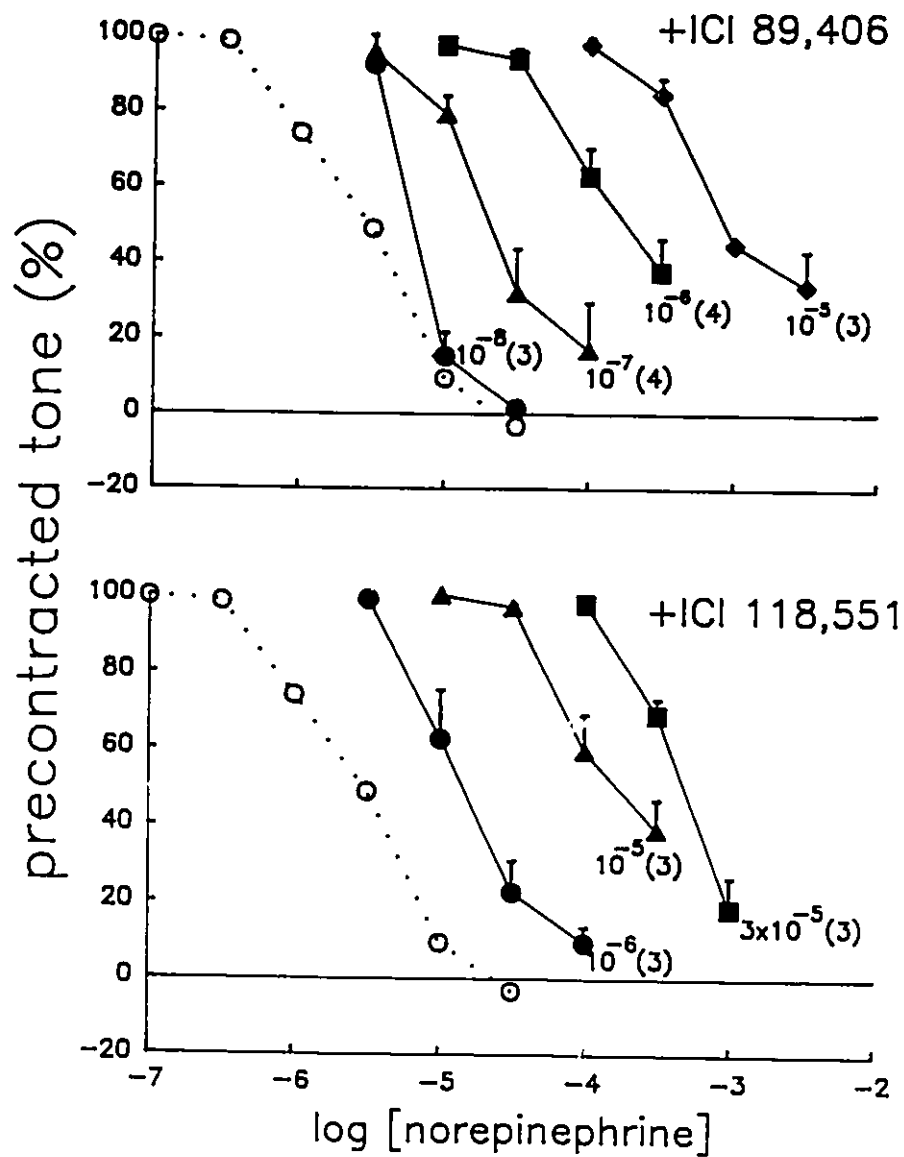
Figure 13. Selective antagonism of NE-relaxations in tissues precontracted with $3\times10^{-7}M$ Cch using ICI 89,406 (top) or ICI 118,551 (bottom). The control NE concentration-response curve shown in figure 12 is also shown (dotted line).
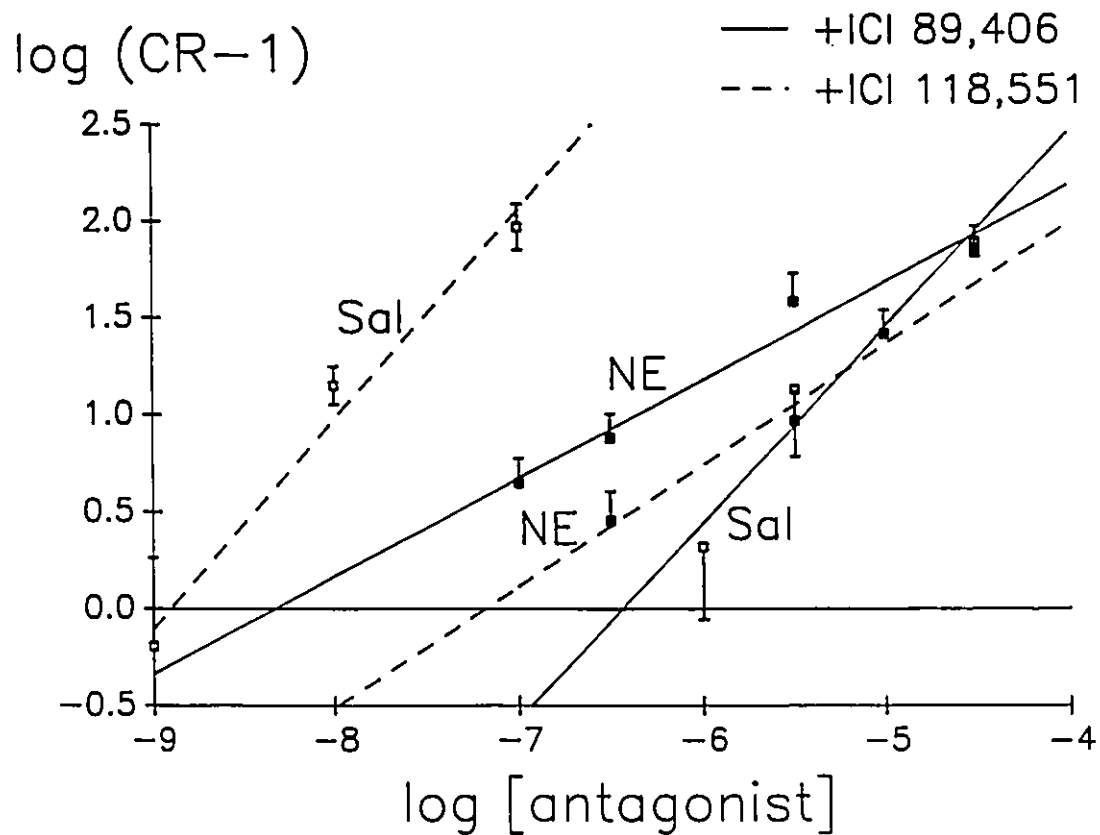
Figure 14. Schild plots for the selective ß-receptor antagonists ICI 89,406 (dashed lines) and ICI 118,551 (solid line) acting to inhibit relaxations induced by NE or Sal; concentrations of Cch used to precontract the tissues were 3×10^{-7}M and 2×10^{-8}M, respectively. Linear regression of the data gave the lines of best fit (dotted lines).

+ICI 89,406

○ control
● 3×10^{-6} (n=3)
▲ 10^{-5} (n=4)

+ICI 118,551

○ control
● 10^{-8} (n=3)
▲ 10^{-6} (n=4)
■ 3×10^{-6} (n=4)
◆ 10^{-5} (n=4)

+ICI 118,551

○ control
● 10^{-8} (n=5)
▲ 10^{-7} (n=5)
□ 10^{-6} (n=5)

Precontracted tone (%)

log [salbutamol]
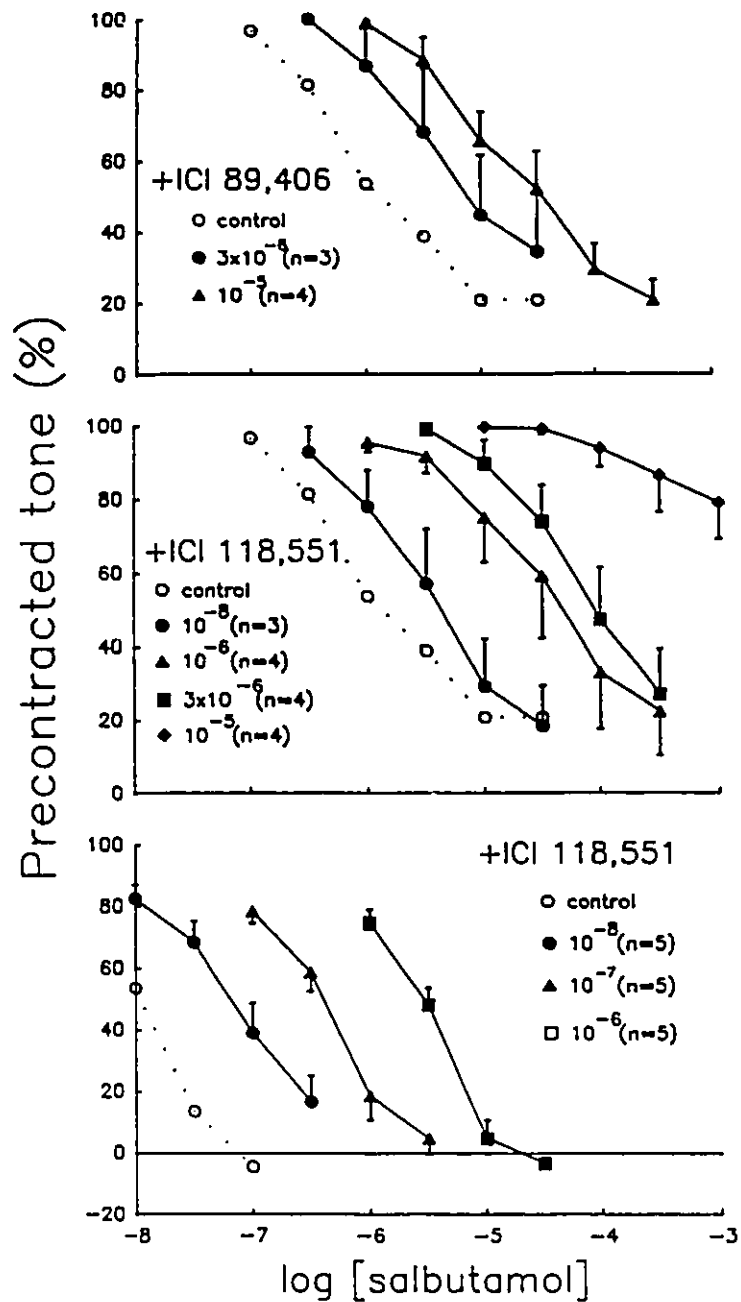
Figure 15. Selective antagonism of Sal-relaxations in tissues precontracted with 10^{-7}M Cch using ICI 89,406 (top) and ICI 118,551 (middle), or in tissues precontracted with 2×10^{-8}M Cch using ICI 118,551 (below). The control Sal concentration-relaxation curves shown in figure 12 are also given (dotted lines).
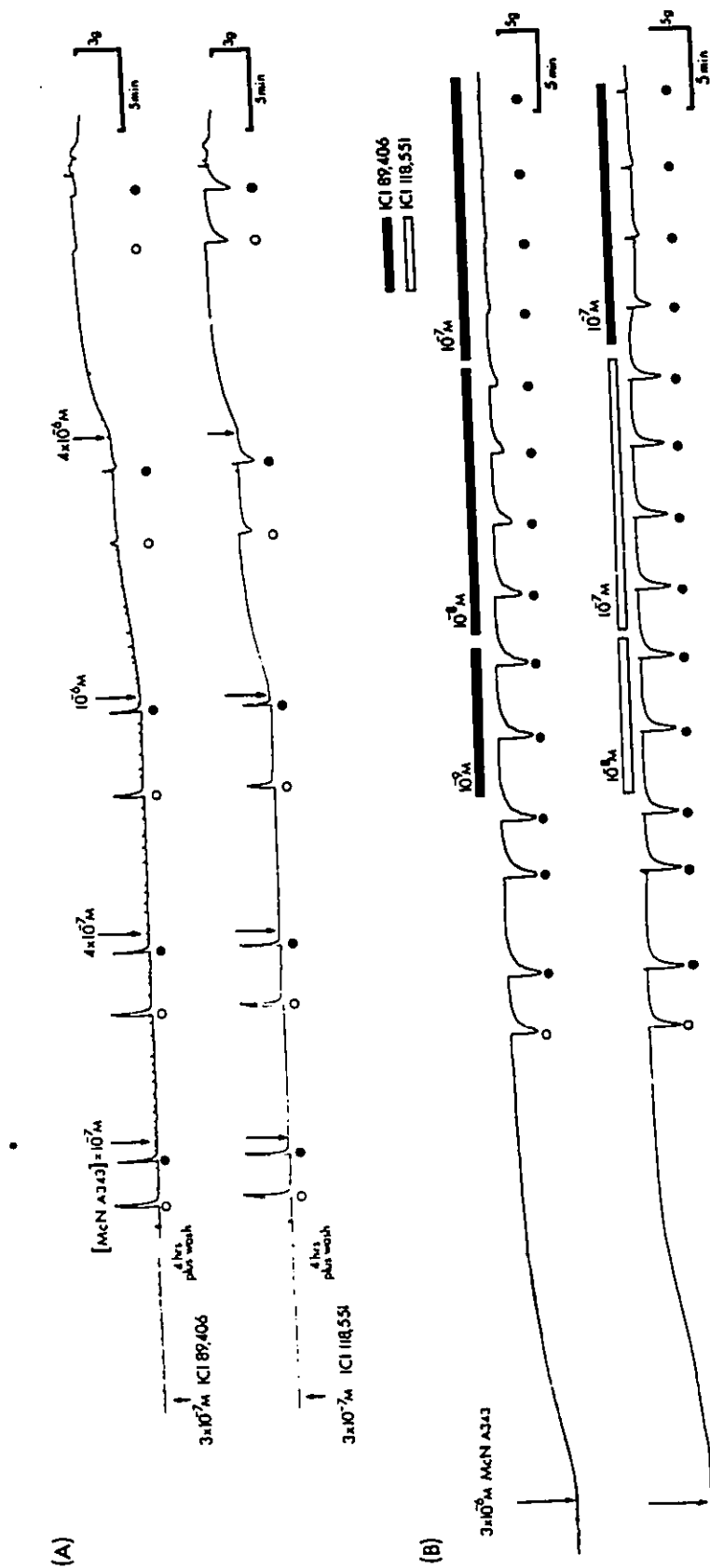
Figure 16   (A) Effects of the M_1-selective muscarinic receptor agonist McNeil A343 (10^{-7} to 4×10^{-6}M) on contractile responses to FS (10 pulses at 1 [o] or 10 pps [·]). Note that these tissues had been treated with 3×10^{-7}M ICI 89,406 (above) or ICI 118,551 (below) four hours previously, and that the physiologic solution had been replaced numerous times before these tracings were made.   (B) Effects of increasing concentrations of the β-receptor antagonists ICI 89,406 (10^{-9} to 10^{-7}M) or ICI 118,551 (10^{-8} and 10^{-7}M) on relaxant responses to FS (5 pulses at 10 pps, o; 10 pulses at 10 pps, ·) in tissues precontracted with 3×10^{-6}M McNeil A343.
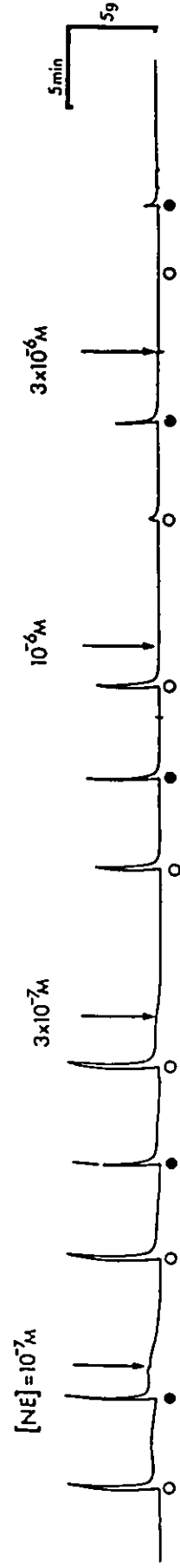
Figure 17 Inhibitory effect of NE (10^{-7}–3×10^{-6} M) on twitch contractions elicited by FS (10 pulses at 1 [○] or 10 pps [•]).
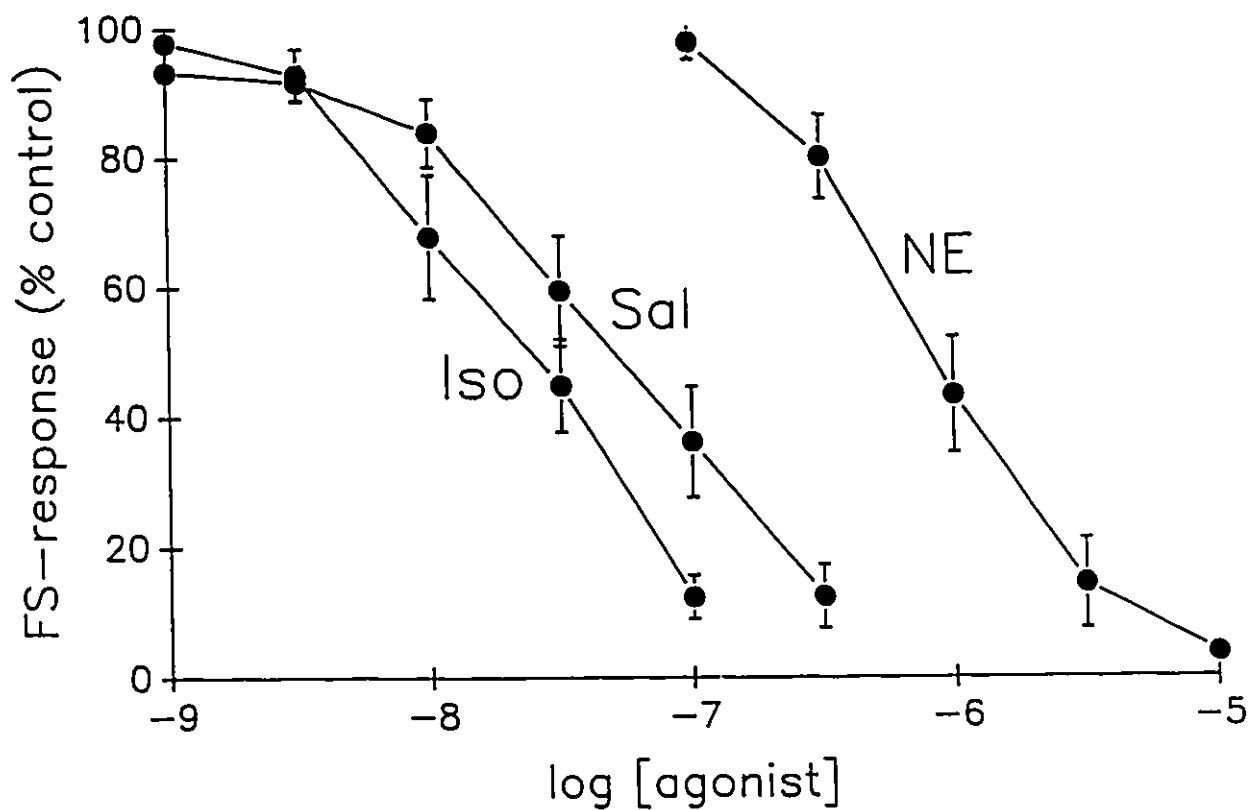
Figure 18 Concentration-response curves for inhibition of FS-contractions produced by NE (n=8), Iso (n=4), or Sal (n=6). Twitch contractions were elicited by FS (10 pulses at 1 pps). Tissues to which NE was added were pretreated with $10^{-7}M$ phentolamine.
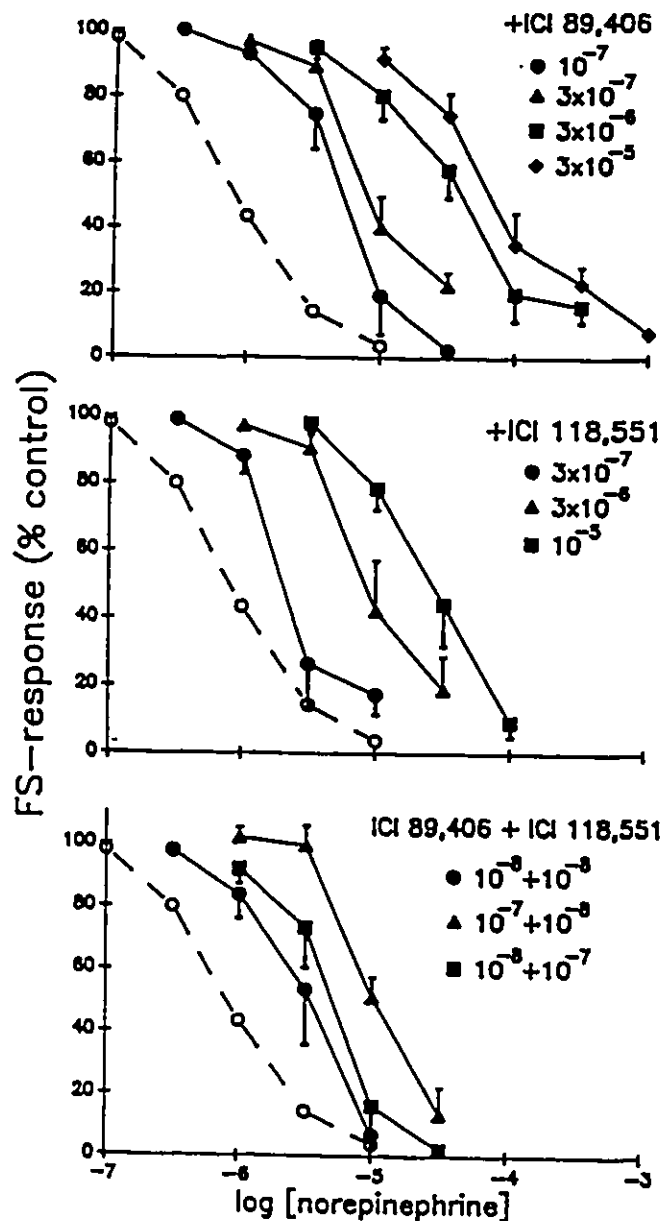
Figure 19 Selective antagonism of prejunctional inhibitory effect of NE. (Top) ICI 89,406 alone at 3×10^{-7}, 3×10^{-6}, and 3×10^{-5}M. (Middle) ICI 118,551 alone at 3×10^{-7}, 3×10^{-6}, and 10^{-5}M. (Bottom) ICI 89,406 plus ICI 118,551 in the following combinations: both 10^{-8}M [•]; 10^{-7}M and 10^{-8}M, respectively [■]; 10^{-8}M and 10^{-7}M, respectively [▲]. The control NE concentration-response curve (figure 18) is also shown (dashed line). See table 5 for values of n in each case.
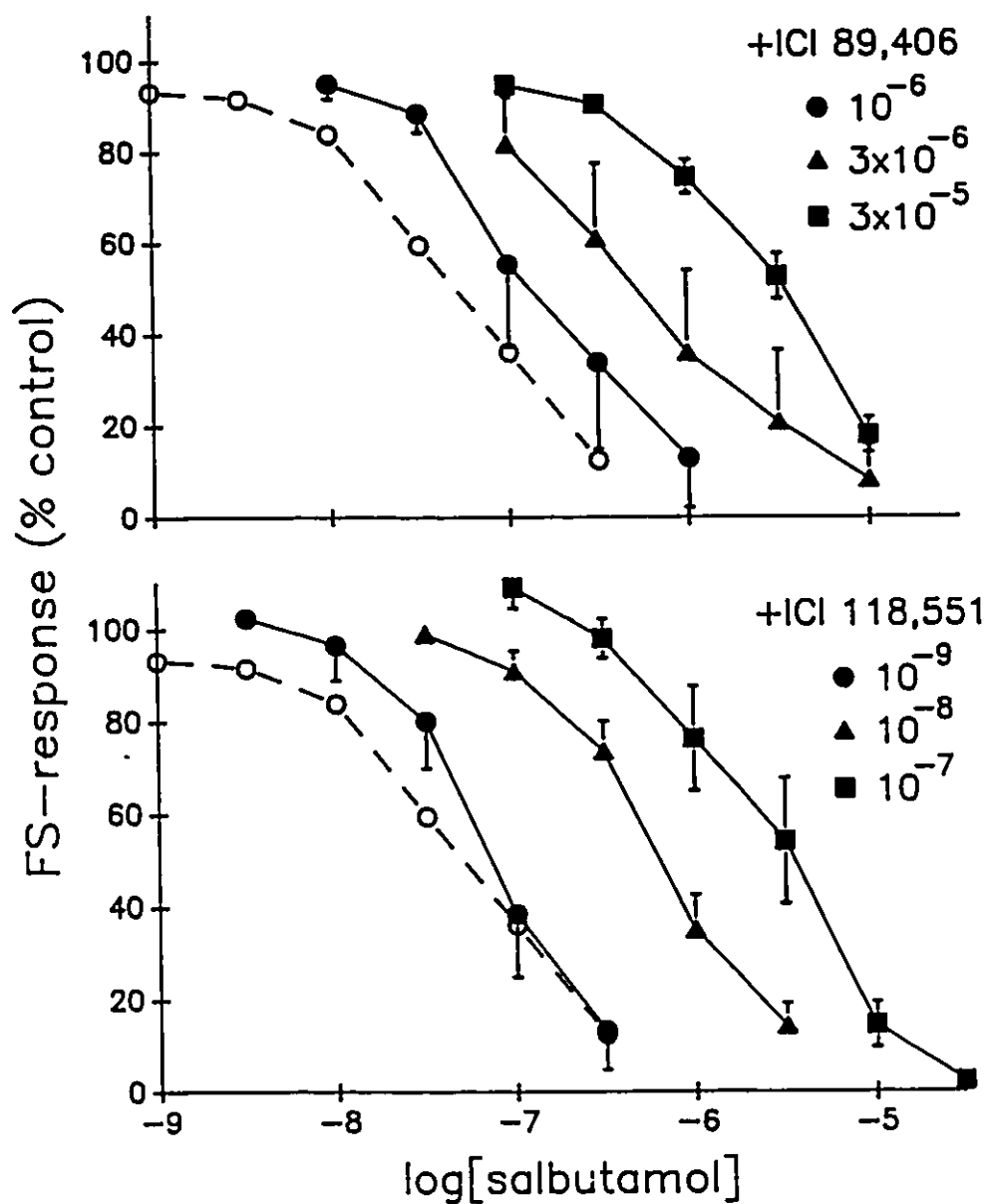
Figure 20 Concentration-response curves for Sal (showing inhibition of FS-contractions) in the presence of ICI 89,406 (10^{-6}, 3×10^{-6}, and 3×10^{-5}M, above) or ICI 118,551 (10^{-9}-10^{-7}M, below). The control Sal concentration-response curve (figure 18) is also shown (dotted line). See table 6 for values of n.
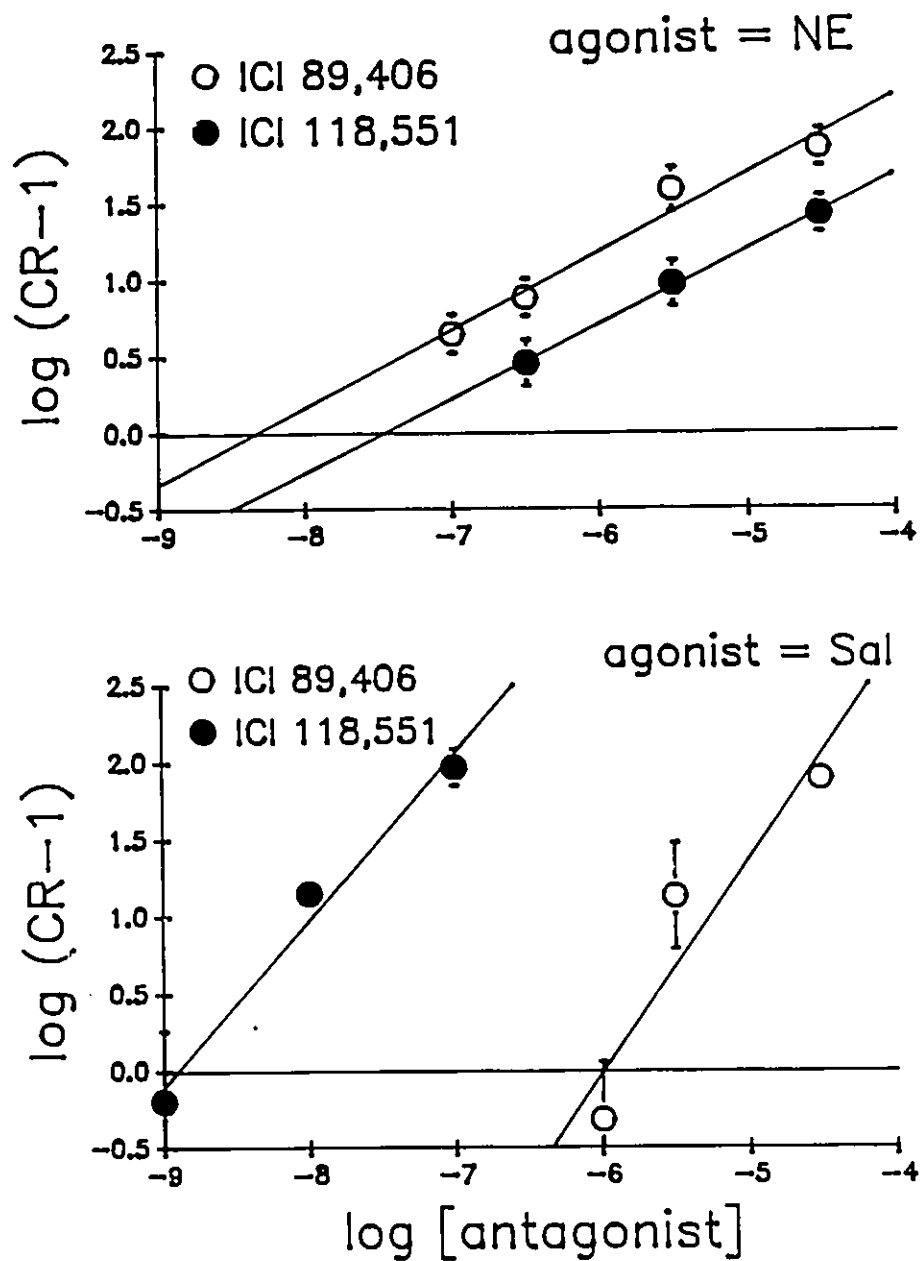
Figure 21 Schild plots for the selective ß-receptor antagonists ICI 89,406 and ICI 118,551 acting against NE-induced inhibition (above) or Sal-induced inhibition (below) of FS-contractions.
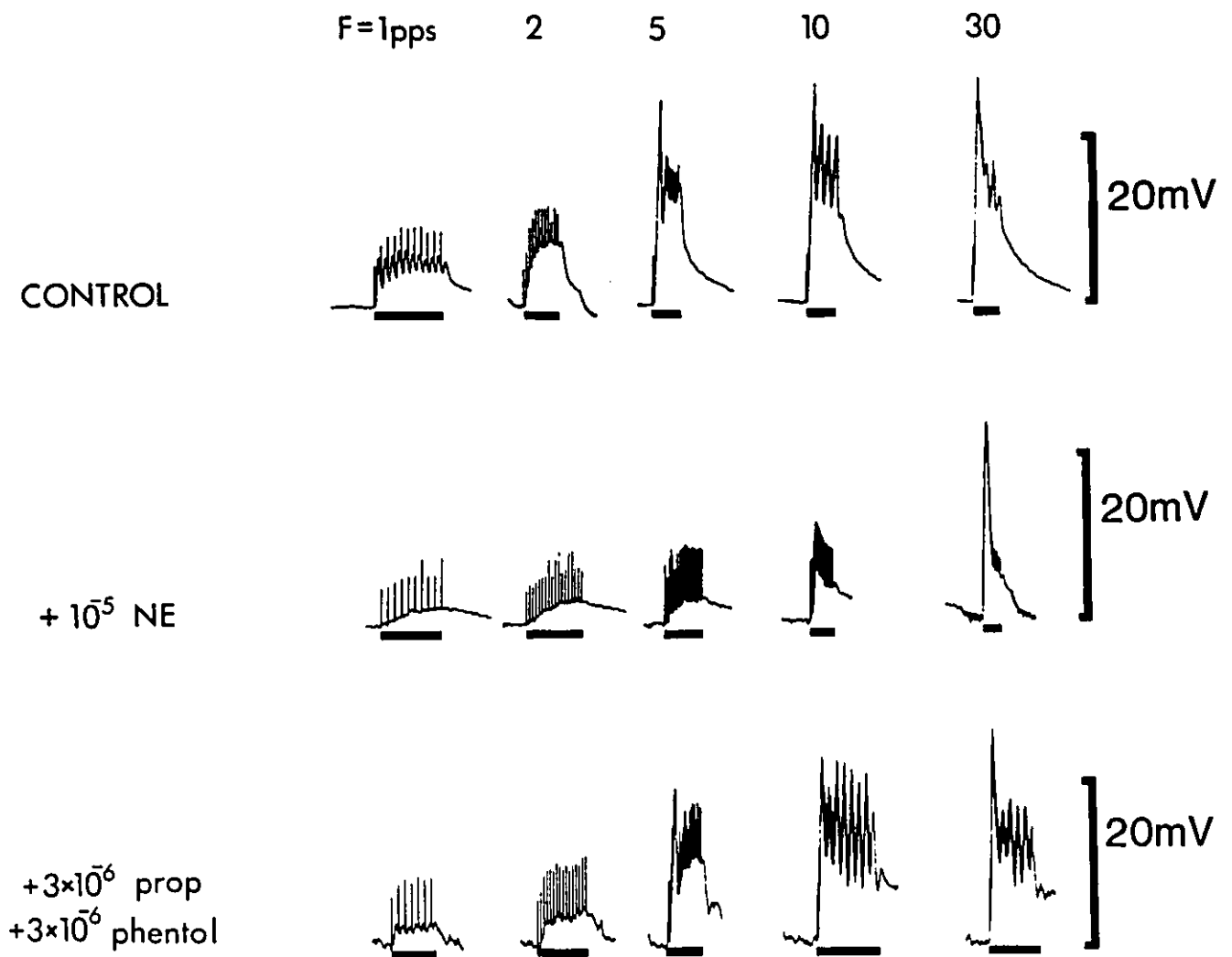
Figure 22 Electrical responses to FS [1-30 pps maintained until the response reached a plateau, indicated by the horizontal bars), and the effects of subsequent additions of norepinephrine (10^{-7}-10^{-5}M) and 10^{-5}M propranolol on the same. Before addition of propranolol, the tissues were exposed to 10^{-7}M phentolamine; this had no effect on NE-induced inhibition.
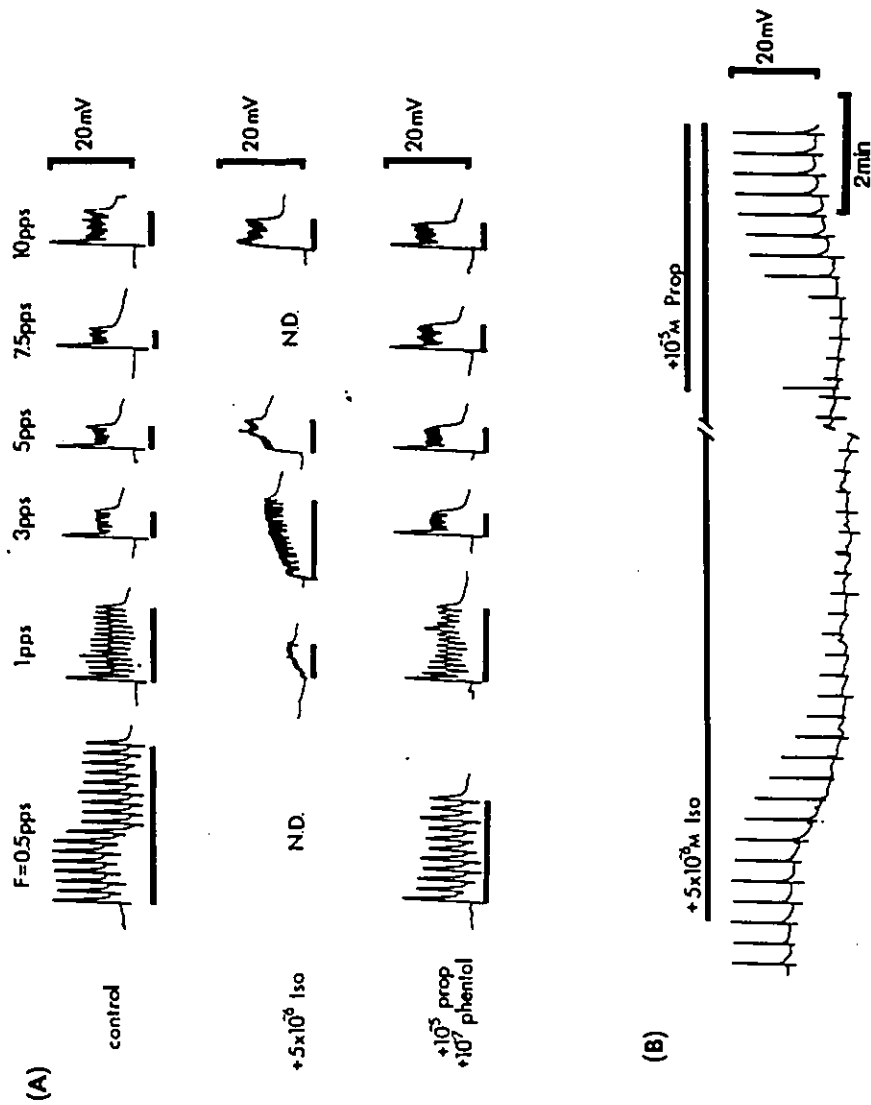
Figure 23 Electrical responses to FS [0.5 to 10 pps maintained for period indicated by horizontal bars (A), or 2 pulses at 20 pps given every 20 seconds (B)], and the effects of serial additions of 3×10^{-6}M Iso, 10^{-7}M phentolamine (no effect; therefore not shown), and 10^{-5}M propranolol on the same. A and B were derived from the same animal. N.D. = not done.
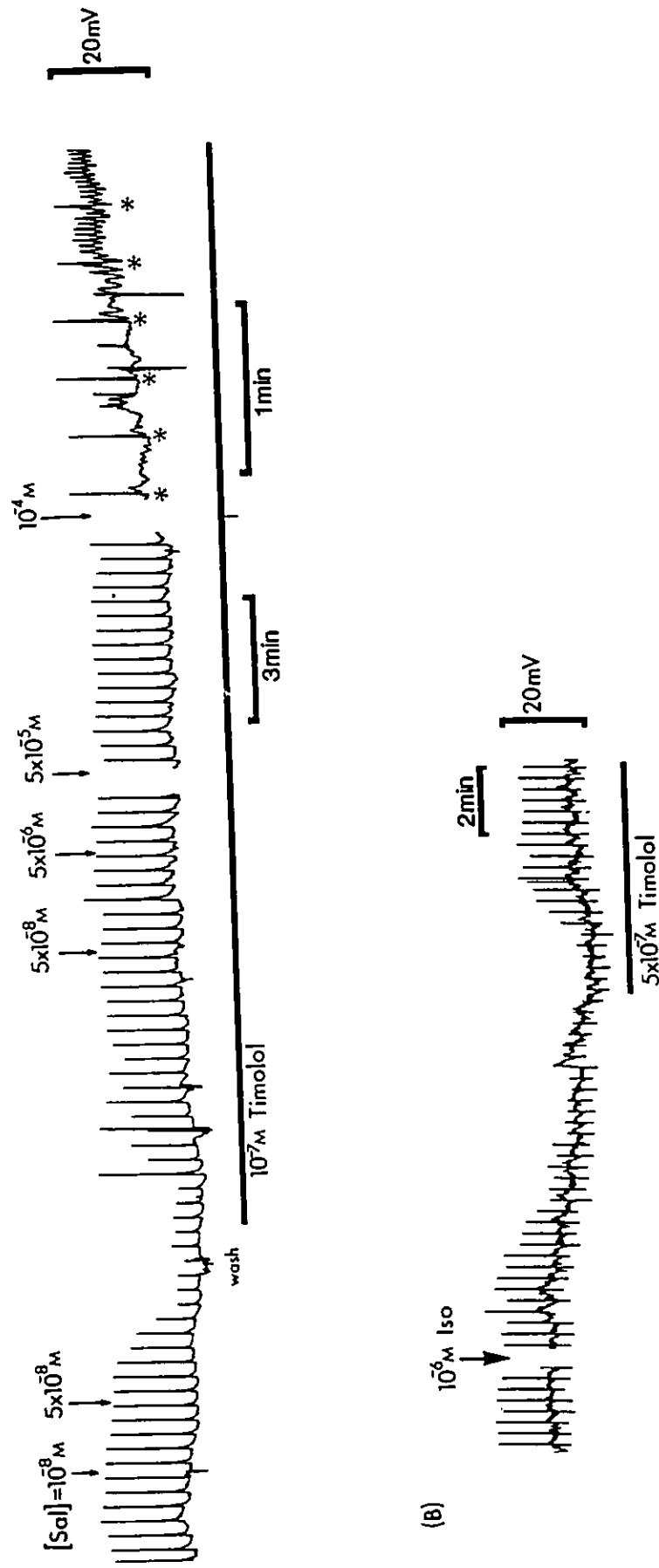
Figure 24 Inhibition of EJPs elicited by FS (single pulse every 20 seconds) by Sal (A) or Iso (B) and reversal of this inhibition by timolol. Note that the last part of the tracing in (A) which shows the effect of 10^{-4} M Sal has been expanded (single pulses are denoted by "*") to show how this concentration of Sal produced membrane depolarization and oscillations.
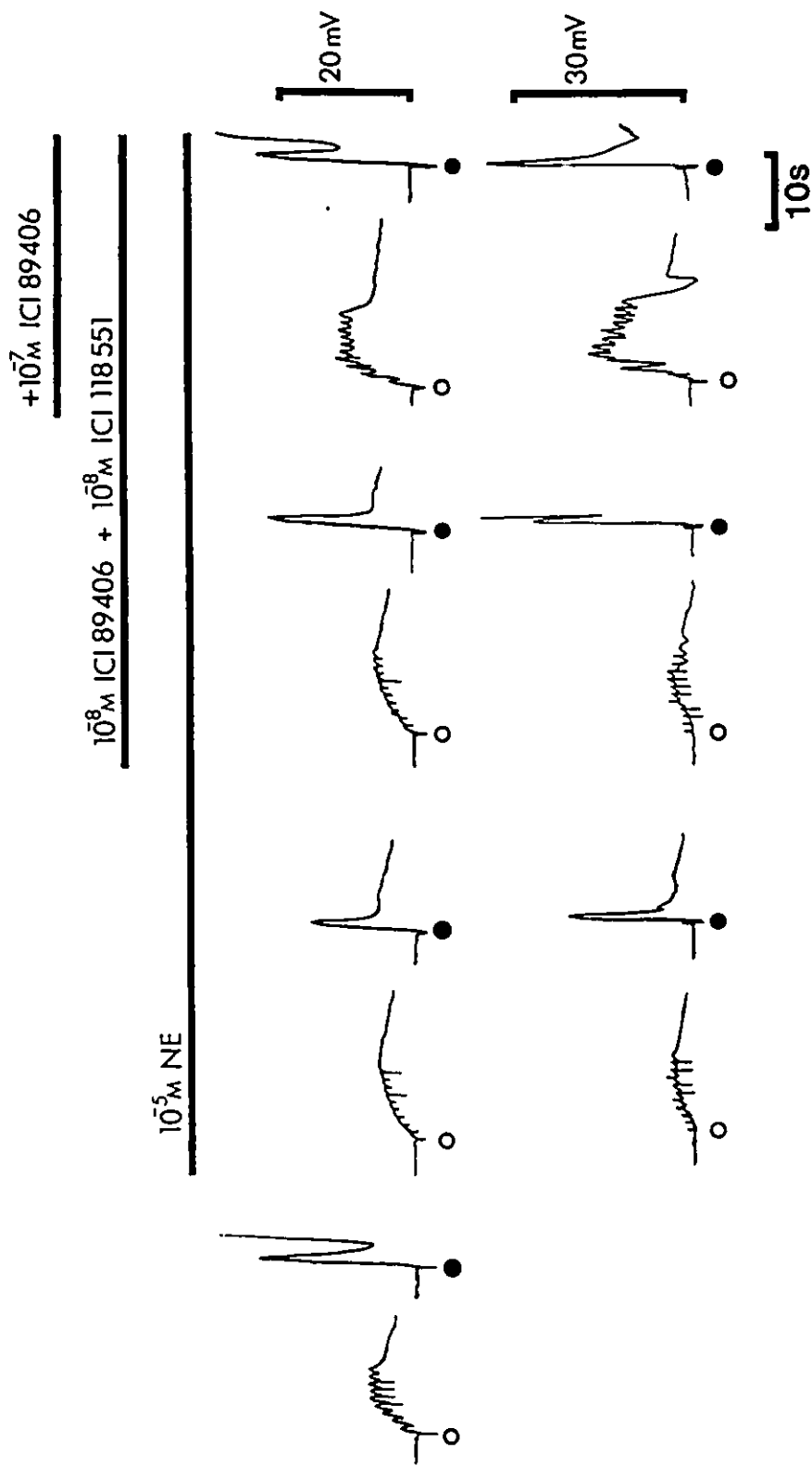
Figure 25   Effects of the combined addition of both ICI 89,406 and ICI 118,551 (both at 10^{-8}M), and of subsequently increasing the concentration of ICI 89,406 (to 10^{-7}M), on NE-induced inhibition of FS-EJPs in tissues from two animals.   In the second tissue, a complete control response was not obtained since even a single pulse led to ejection of the microelectrode from the cell (loss of microelectrode impalement in the cell is indicated in the figure by an asterisk [*]); instead, two examples of responses to single pulse field stimulation [▲] are given.   In all other cases, field stimulation was 10 pulses at 1 pps [○] or 10 pps [●].
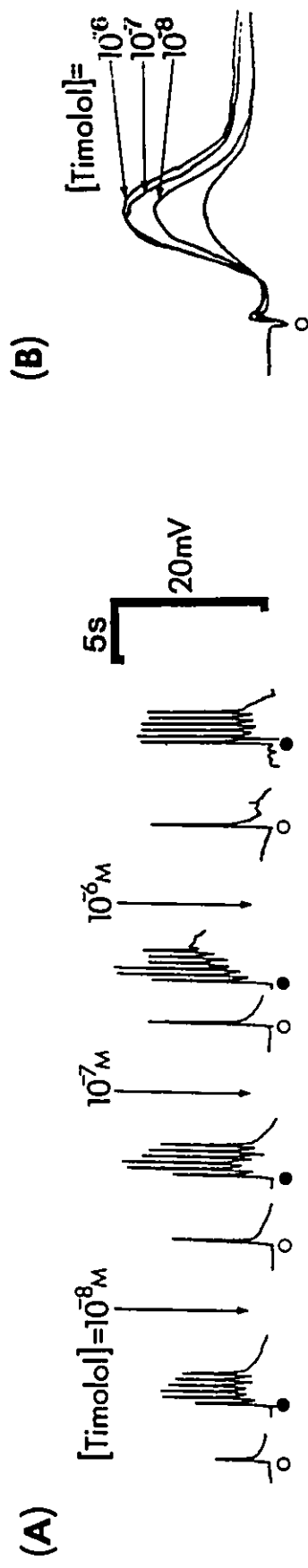
Figure 26 Sample tracing showing the effect of timolol (10^{-8} to 10^{-6}M) on electrical responses to FS (single pulse [o]; 6 pulses at 0.5 pps [•]). The responses to single pulse FS shown in (A) are magnified and superimposed upon each other in (B) to facilitate comparison.
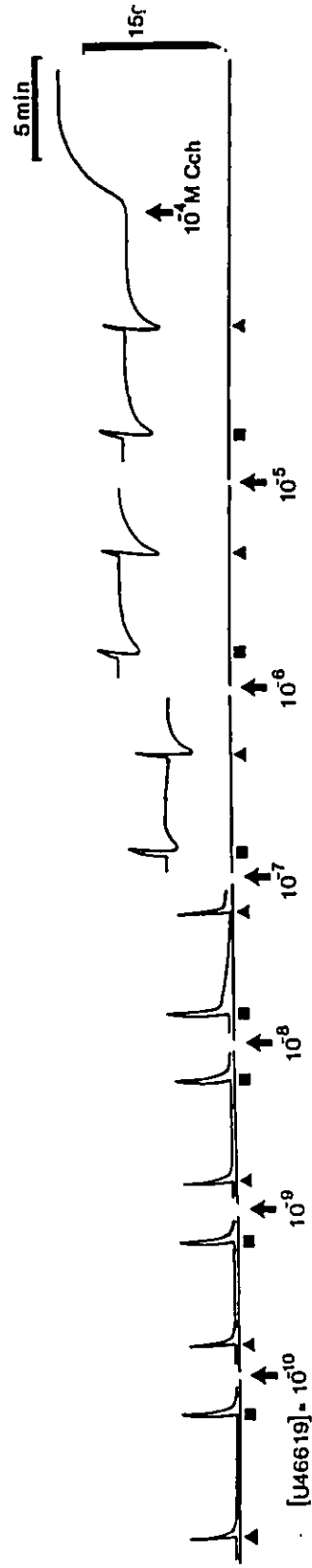
Figure 27 Effects of U46619 (10^{-10}–10^{-5}M) on resting tone and on twitch contractions to electrical stimulation (10 pulses at 1 [▲] or 10 pps [■]). The response to 10^{-4}M Cch is also shown.
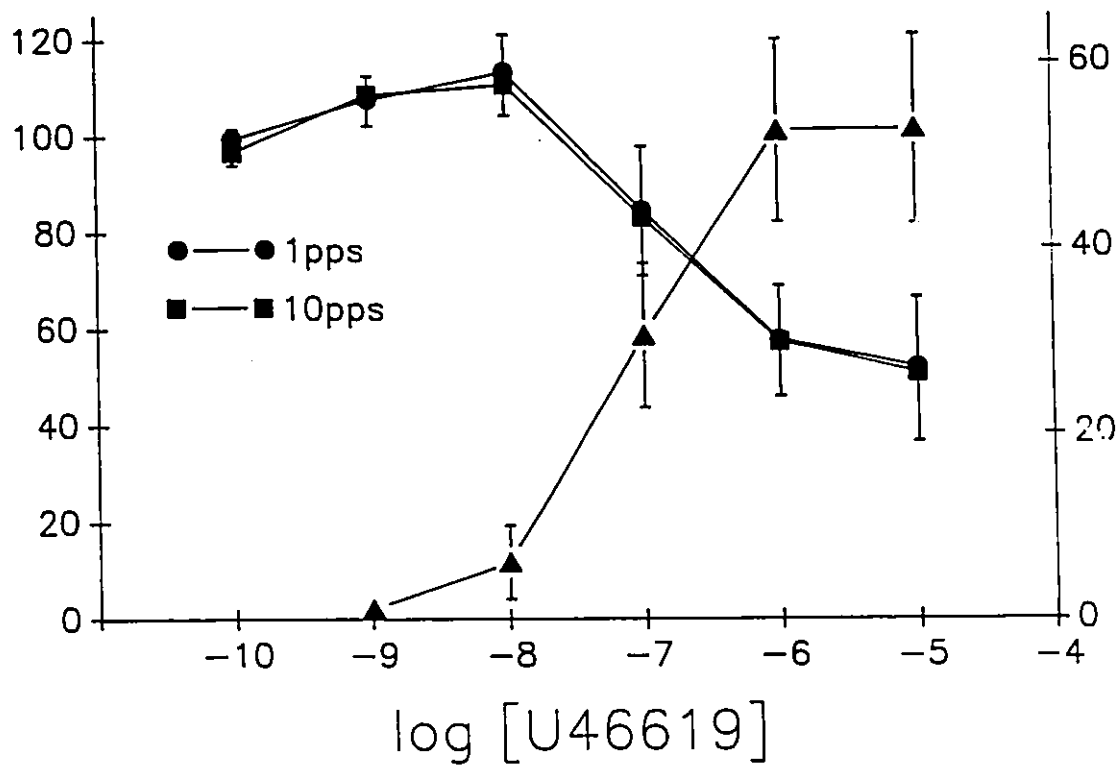
Figure 28 Tonic contractions generated by U46619 (▲) and effects of U46619 on twitch contractions elicited by FS at 1 pps (•) or 10 pps (■).
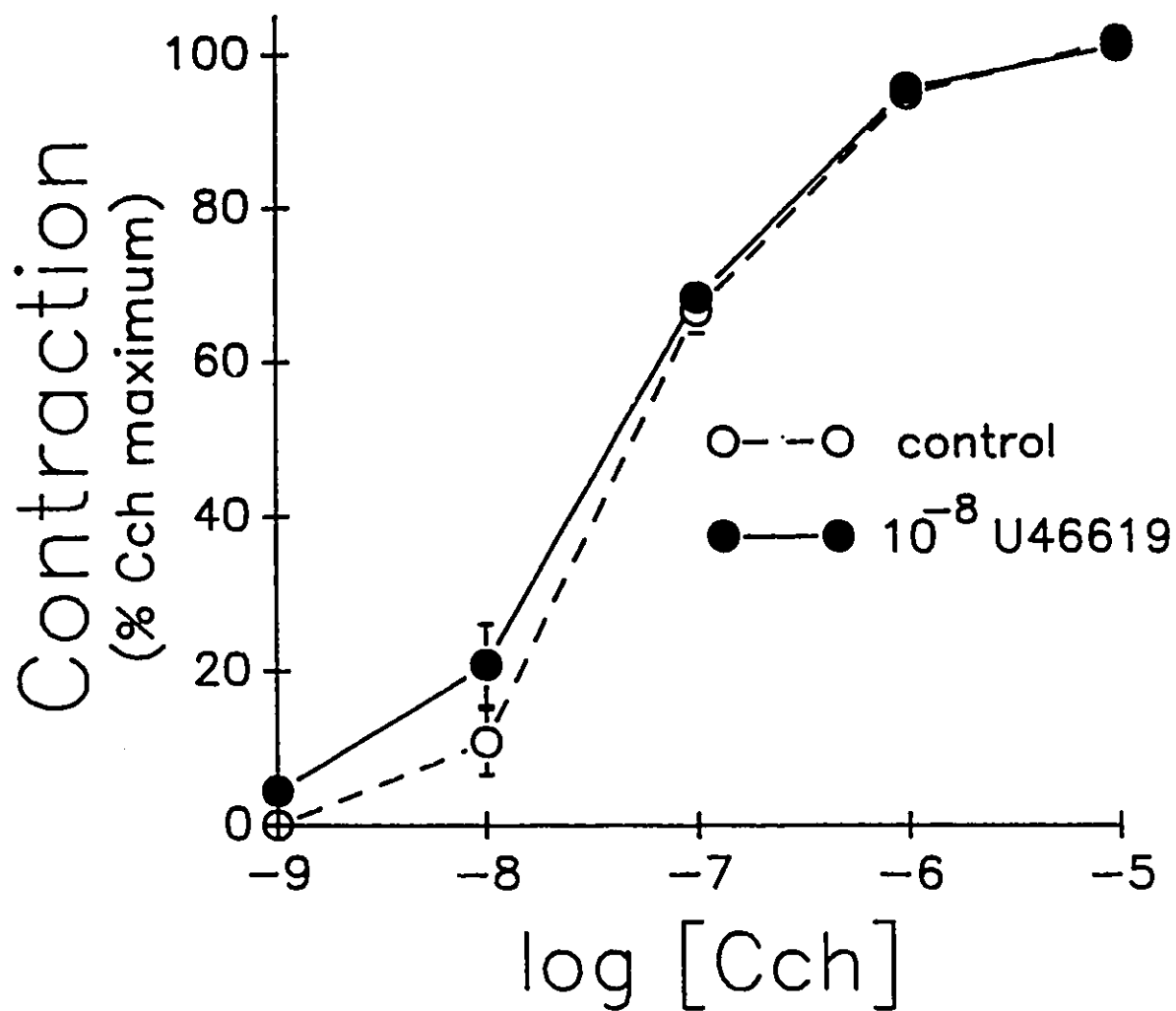
Figure 29 Tonic contractions generated by Cch (open circles and dotted line), and effect of 10^{-8}M U46619 on the same (filled circles and solid line). Responses were standardized as a proportion (%) of the response to 10^{-4}M Cch.
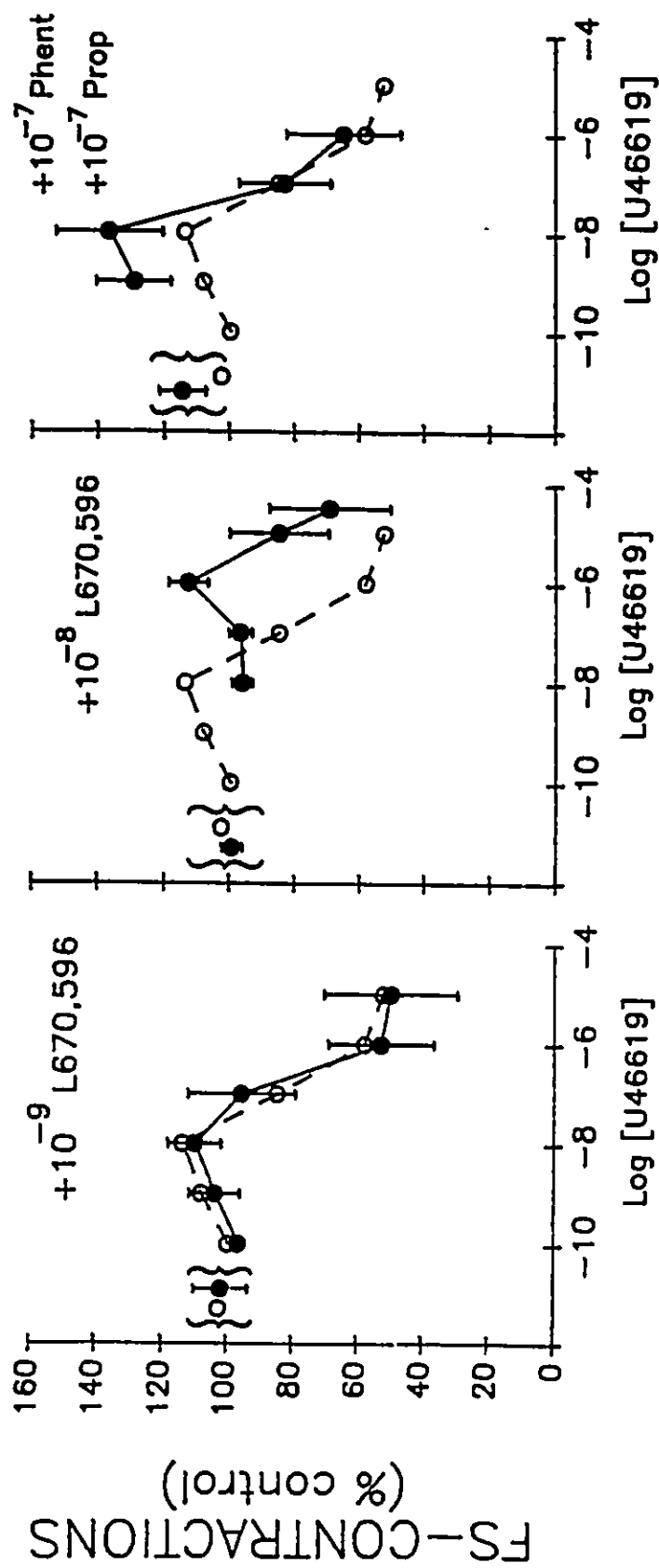
Figure 30 The effects of 10^{-9}M L-670,596 (left), 10^{-8}M L-670,596 (middle) or 10^{-7}M propranolol plus 10^{-7}M phentolamine (right) on U46619-induced modulation of FS-contractions (5 pulses at 1 pps); the control concentration-response curve shown in figure 28 is also shown (dotted line).
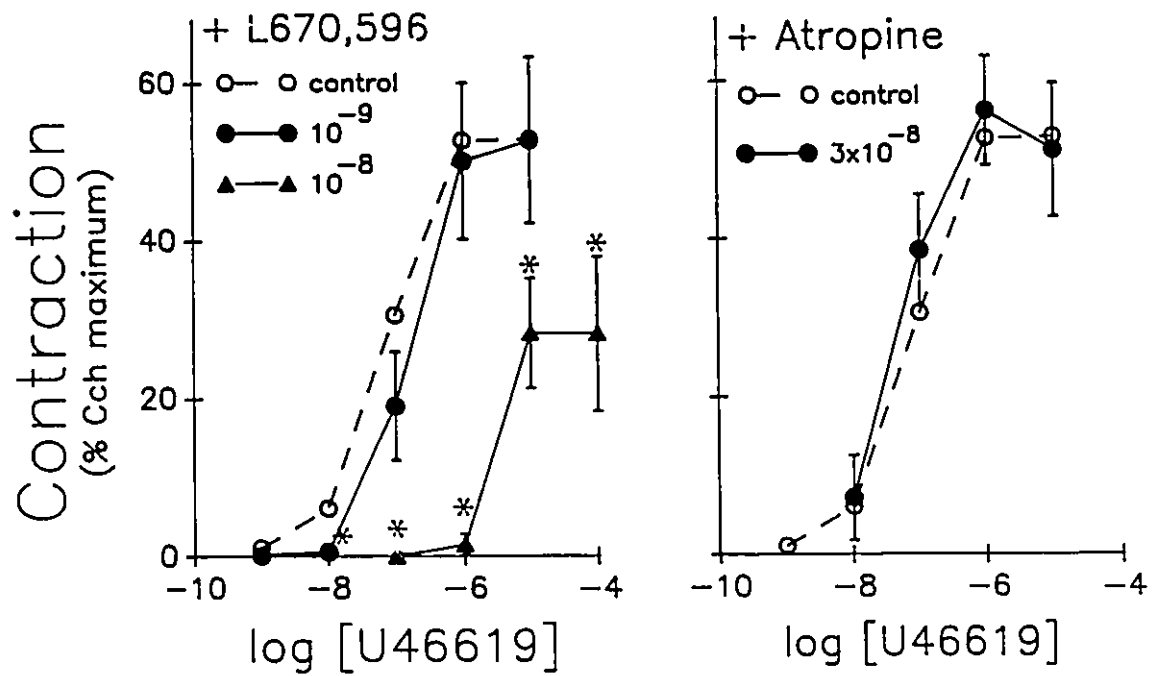
Figure 31   The effects of 10^{-9} or 10^{-8}M L-670,596 (left) or 3×10^{-8}M atropine (right) on U46619-induced contractions; the control concentration-response curve (figure 28) is also shown (dotted line). Asterisks indicate the corresponding response was significantly different from the control response (P<0.05).
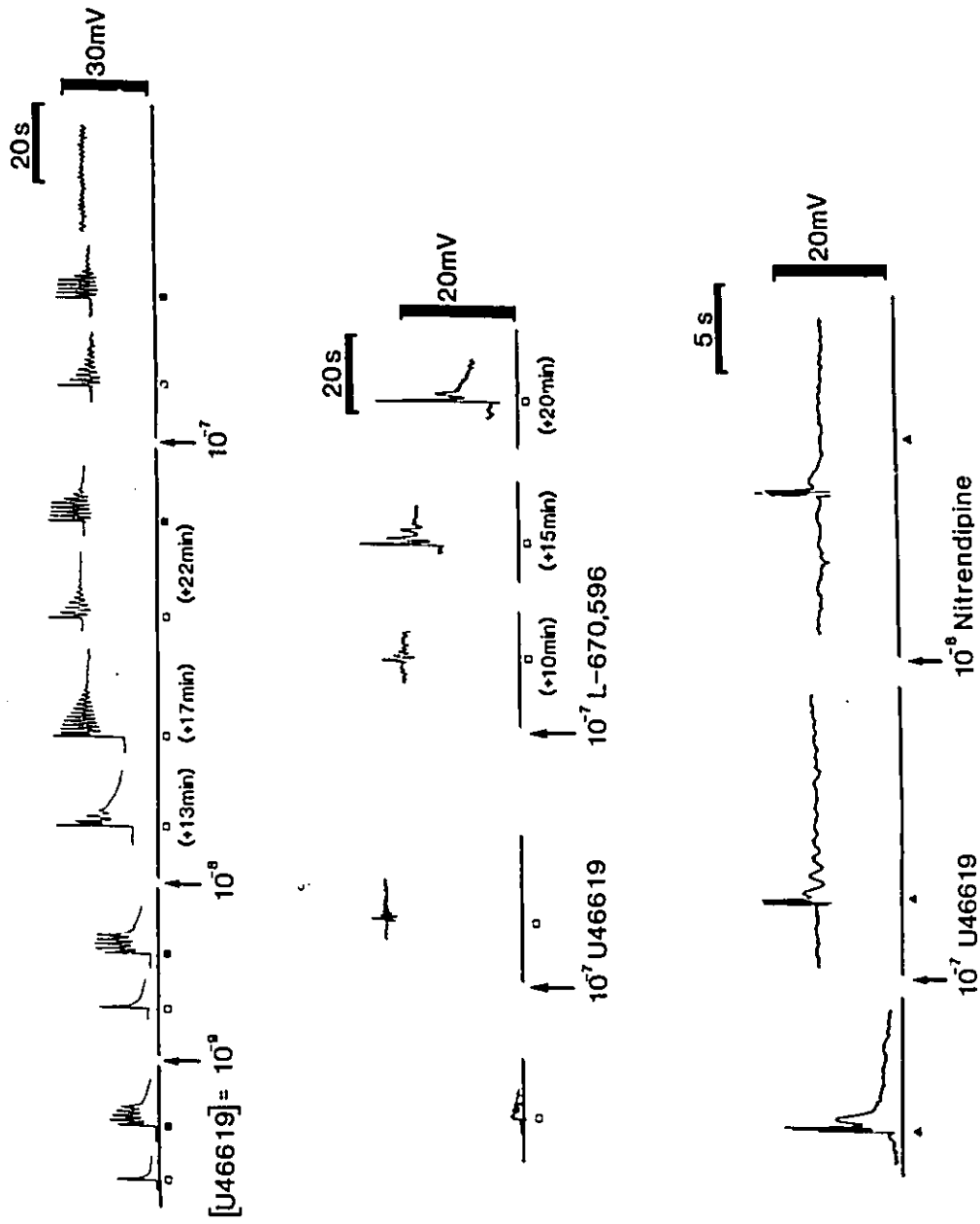
Figure 32 Effects of U46619 on electrical activity (top), and antagonism of the same by 10^{-7}M L-670,596 (middle) or 10^{-8}M nitrendipine (bottom). Resting membrane potential is indicated by the horizontal line. FS used was single pulse ( ), 5 pulses at 1.0 pps (■), or 3 pulses at 10.0 pps (▲).
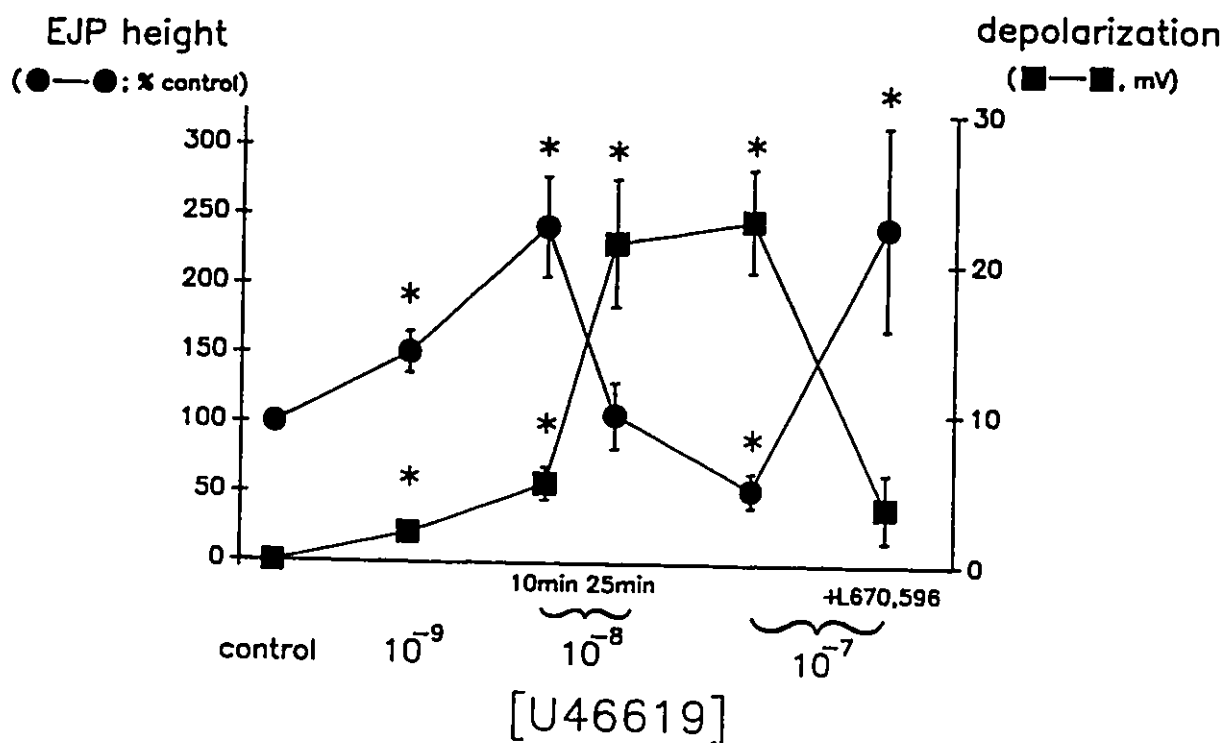
Figure 33 Effects of U46619 on FS-induced EJPs and on membrane depolarization. Note that in the case of 10^{-8}M U46619, both the initial and final effects are given (indicated by "10 min" and "25 min", respectively). The effects of 10^{-7}M L-670,596 on tissues exposed to 10^{-7}M U46619 are also shown. Asterisks indicate responses which are significantly different from control responses (P<0.05). Field stimulation used was 5 pulses at 1 pps.
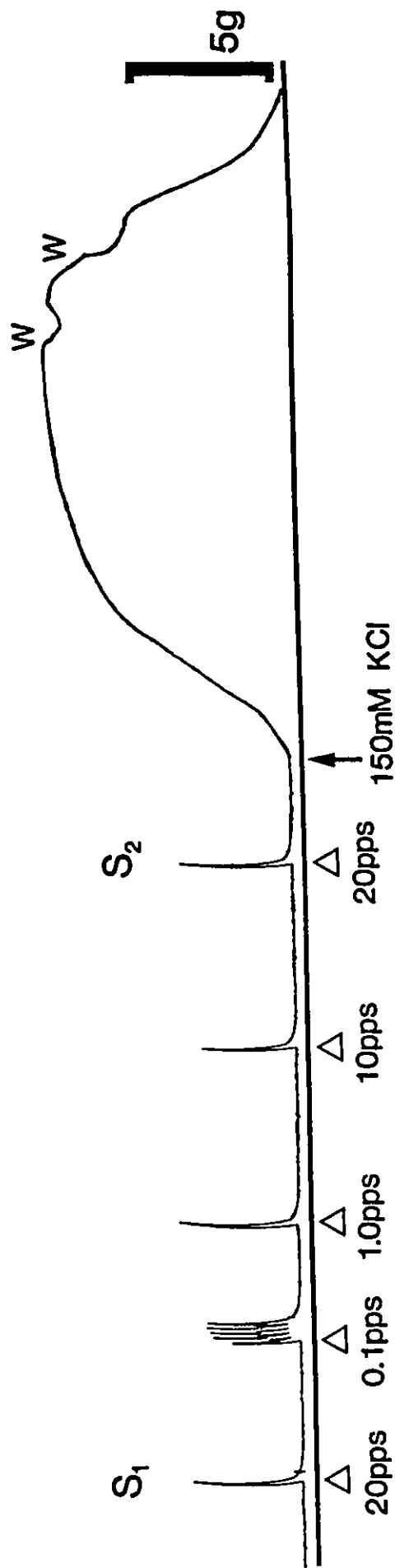
Figure 34 Tracing showing procedure used to assay ozone-induced hyperreactivity. Tissues were electrically stimulated approximately every 5 minutes with 5 pulses at various frequencies (0.1-20 pps), then exposed to 150 mM KCl. The first and last of the multiple pulse trains always had a pulse frequency of 20 pps (S_1 and S_2, respectively, as indicated). Tissues were washed (W) several times after the KCl response had maximized.
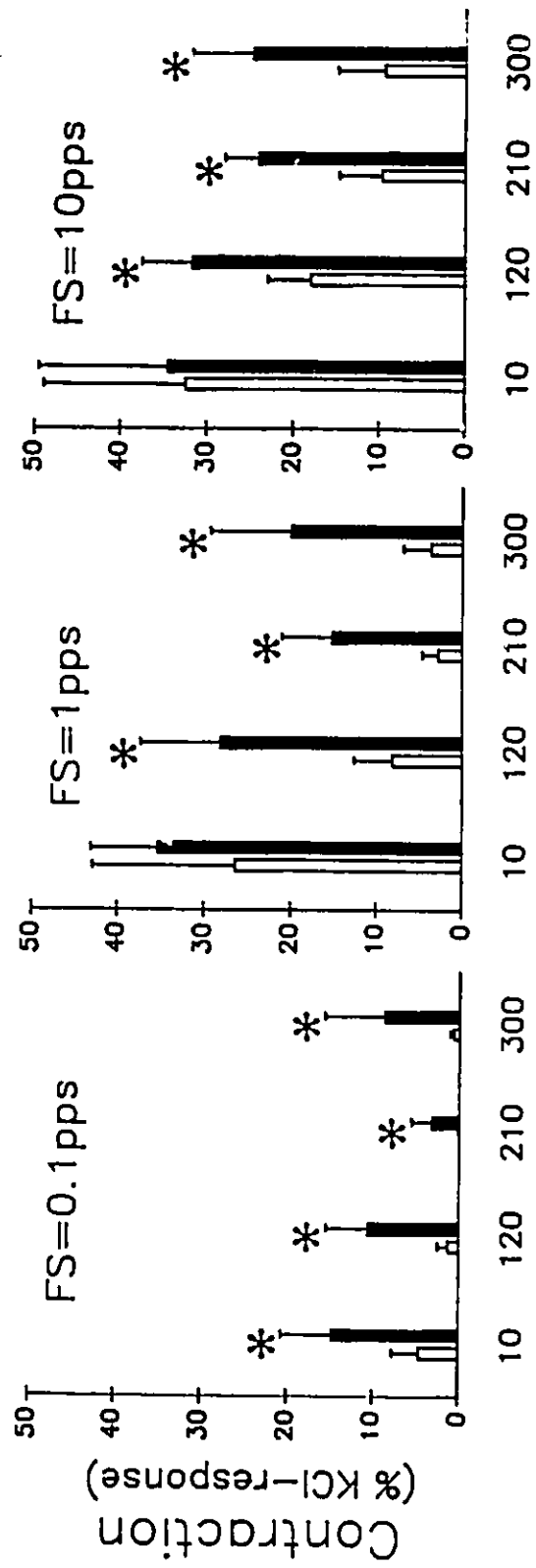
Figure 36 Ozone-induced hyperresponsiveness in canine bronchi. The data presented here are identical to those presented in figures 35-38 (in the absence of any antagonist); in this case, however, the data are standardized by expressing as a per cent of the response to KCl in each tissue at each time period tested. Times (minutes) after commencing the experiments are given at the bottom of the figures. Asterisks indicate when the differences between responses in control and ozone-exposed tissues were significant (P<0.1).
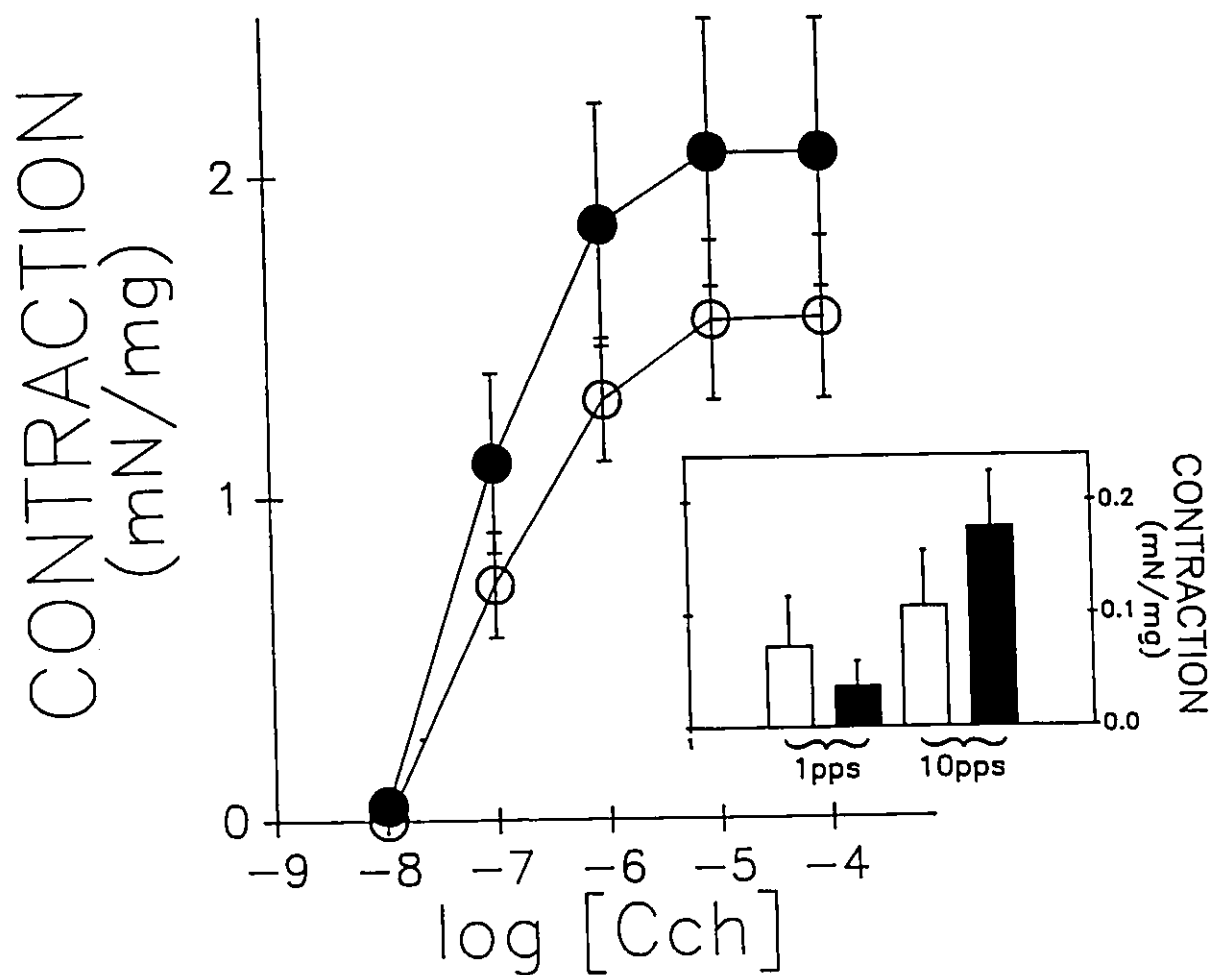
Figure 37 Sensitivity to Cch (10^8-10^{-4}M) and to FS (inset; 5 pulses at 1 or at 10 pps, as indicated) in control tissues (open circles or open bars) compared to ozone-treated tissues (closed circles or closed bars); n=4 for both groups of tissues. Statistically significant differences in sensitivity between the two groups are indicated by asterisks.
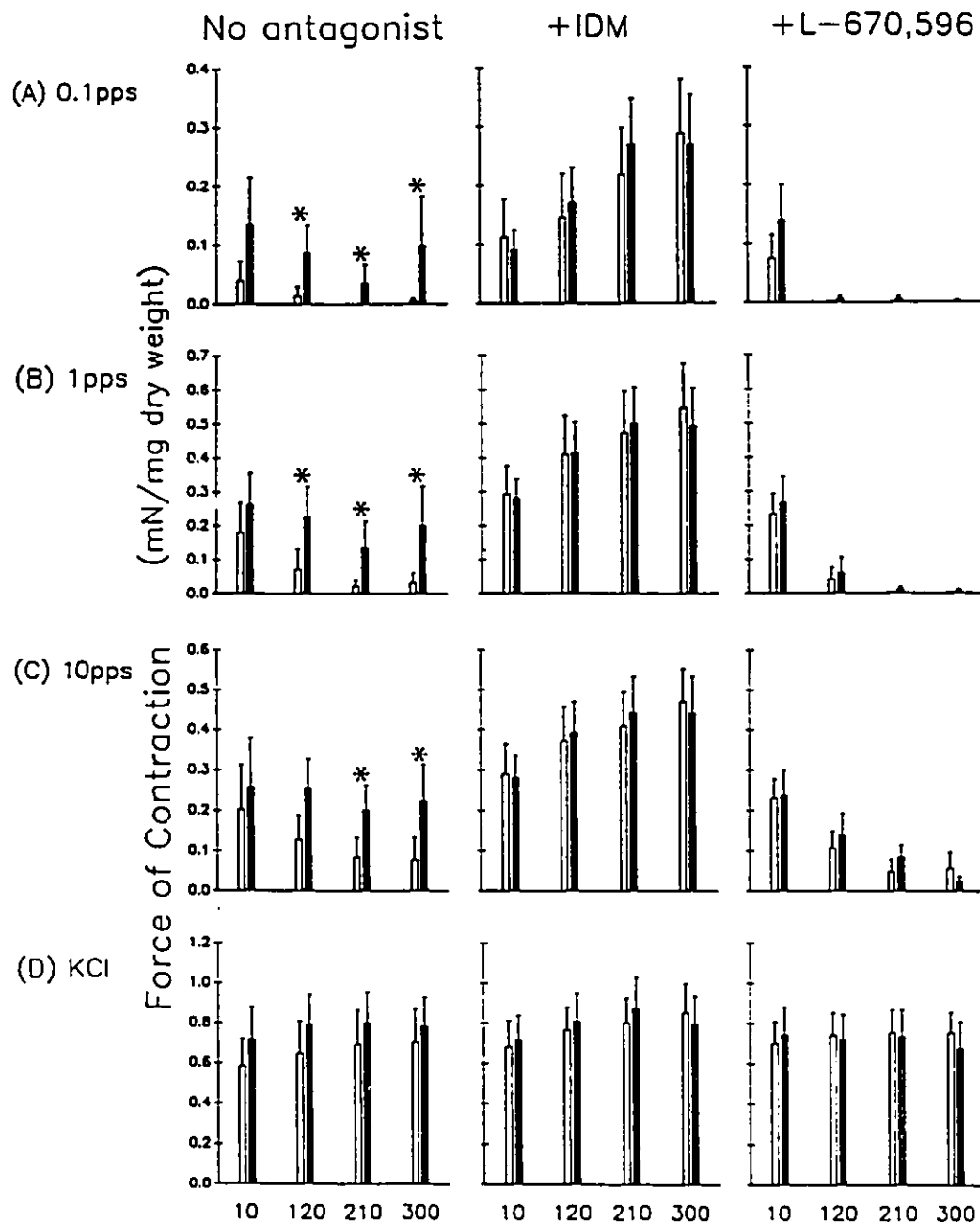
Figures 35   Ozone-induced hyperresponsiveness of canine bronchi.   Bars show
the mean responses to various stimuli (5 pulses at 0.1, 1.0, and 10 pps [A, B,
and C, respectively] or 150 mM KCl [D]) in normal tissues (open bars) and ozone-
exposed tissues (closed bars) at various times after commencing the experiments.
The tissues from which these responses were elicited were maintained in normal
Krebs (n=8; left); Krebs containing 10^{-5}M IDM (n=8; middle); or Krebs containing
10^{-8}M L-670,596 (n=5; right).   Times (minutes) after commencing the experiments
are given at the bottom of the figures.   Asterisks indicate when the differences
between responses in control and ozone-exposed tissues were significant (P<0.1).

Figure 38 Proposed mechanisms accounting for ozone-induced hyperresponsiveness. Contractions (CON) and relaxations (REL) are elicited by KCl, FS, PGE_2, or TxA_2, as shown. The contractile effect of FS is altered by ozone-induced changes in PGE_2 and TxA_2 synthesis, while responses to KCl are unaltered.